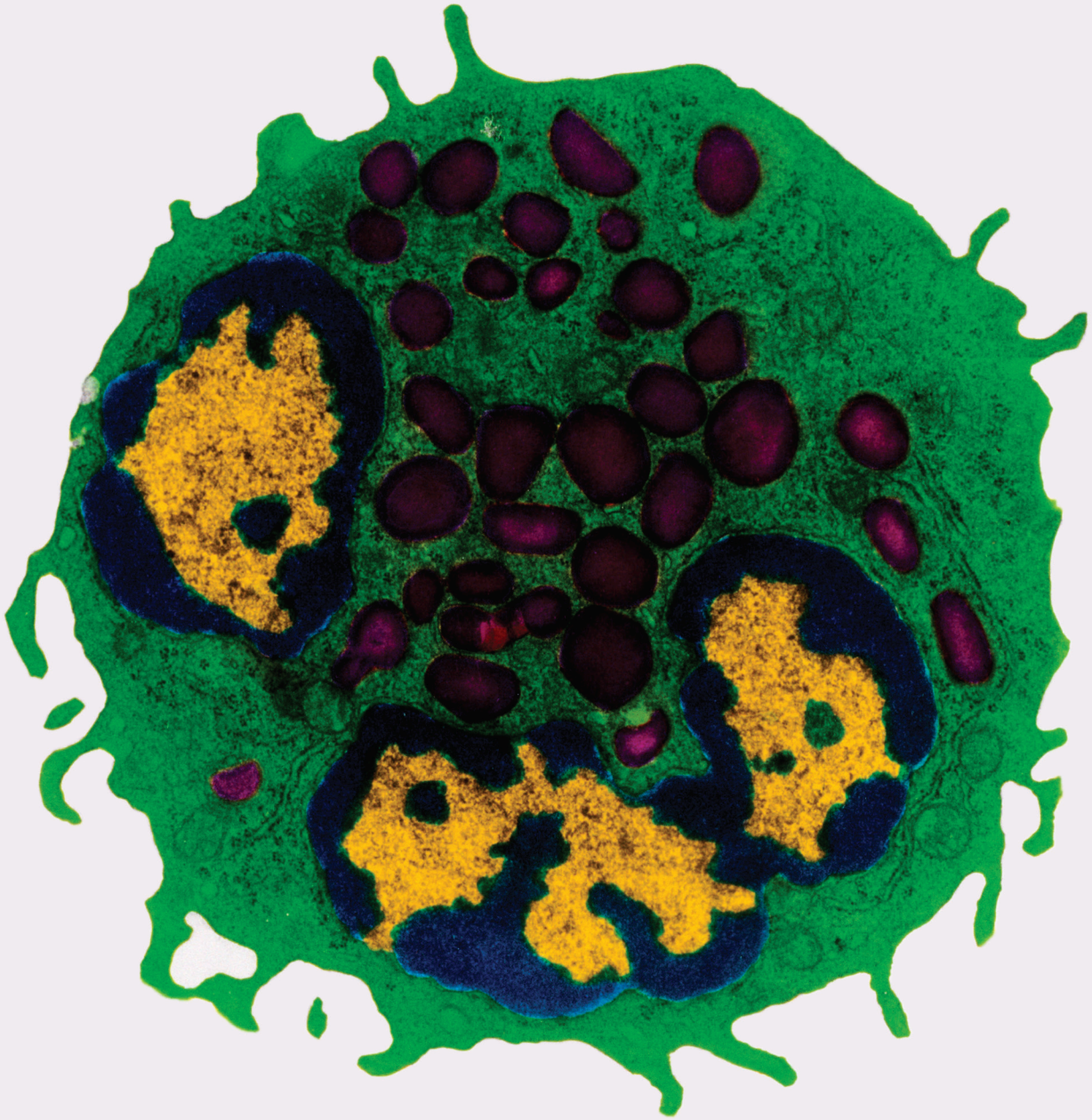
Mediators of Inflammation

Cross Talk Between Inflammation and Metabolic Disorders

Lead Guest Editor: Jie Chen
Guest Editors: Yan Yang and Kong Wen



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Editorial

Cross Talk between Inflammation and Metabolic Disorders

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Metabolic disorders are a number of diseases including atherosclerosis, diabetes, obesity, gout, rheumatoid arthritis (RA), osteoporosis, and osteopenia. Acute or chronic inflammatory processes often coexist with occurrence and development of these diseases, and to date, many studies have implied that metabolic diseases are associated with inflammation. Furthermore, in some studies, the related inflammatory cells and molecules are also confirmed. For example, type 2 diabetes has been wildly considered an inflammatory disease with inflammatory or anti-inflammatory cells including $CD11b^{+}CD11c^{+}$ myeloid cells and $CD3^{+}$T cells and cytokines such as IL-1β [1, 2]. RA has long been believed as a chronic inflammatory joint disease characterized by persistent synovitis, systemic inflammation, and autoantibodies, and reducing synovitis and systemic inflammation by disease-modifying antirheumatic drugs can effectively improve joint function [3]. Acute inflammation of gout is mediated principally by macrophages, neutrophils, and inflammatory cytokines including IL-1β in response to monosodium urate crystals [4]. Although the association between the inflammation and metabolic disorders has been continuously reported, the inflammatory mechanism and involved factors of the pathogenesis of these diseases still need to be further clarified. This special issue consists of a number of original research articles and review papers on the topic of cross talk between inflammation and metabolic disorders.

Cytokines, both inflammatory and anti-inflammatory, play vital roles in the process of acute or chronic inflammation [5, 6]. A research by Himmerich et al. focused on patients with anorexia nervosa and found that several cytokines including GM-CSF, MCP-4, and IL-4 were positively associated with all three parameters of body water (intracellular, extracellular, and total body water), whereas IFN-γ, IL-6, and IL-10 were negatively associated with all the parameters. This finding suggests an interaction between body water and the cytokine system, confirming the existence of the underlying mechanism related to anorexia nervosa-associated inflammatory processes.

Risk factors, including dietary, environmental, and habitual factors, are considered to be involved in the cause and progress of some metabolic diseases [7, 8]. Moreover, for some certain metabolic disorders, other coexisting metabolic diseases are also risk factors. For instance, except for dietary factors, obesity is also a risk factor for gout [9], indicating a deep relationship on the mechanism between the two or more diseases. Li et al. analyzed the factors that are related to the serum uric acid levels and found significant differences between gout with coronary heart disease patients and gout with cerebral infarction patients. They also discovered a history of smoking as a risk factor that affects the therapeutic effects of young gout with cerebral infarction patients. Wang et al. determined that a higher phosphorus value and higher alkaline phosphatase concentration and a longer use of glucocorticoids were risk factors for bone mass loss in kidney transplant recipients and implied that maintaining an appropriate weight and exercising appropriately may help to maintain bone mass.

During the process of inflammation, immune cells play vital roles in promoting or regulating the progress of

inflammatory response. Macrophages, comprising of proinflammatory M1 and anti-inflammatory M2 group, are reported to be associated with many inflammatory diseases such as obesity, asthma, and atherosclerosis [10]. In this special issue, Yang et al. reported that by regulating the polarization of macrophages toward M2, GLP-1R has a protective effect in the progression of coronary atherosclerosis. Wang et al. were also focused on M2 polarization and found a protective mechanism related to miR-6869-5p and protein tyrosine phosphatase receptor type O in gestational diabetes mellitus. Moreover, in a study by Su et al. focused on therapy of obese complicated with polymicrobial sepsis by exploring a mouse model, macrophages were found to be involved in the mechanism by which administrated glutamine alleviated inflammation and attenuated acute kidney injury. Except for cells, a big group of inflammatory molecules also exist and are involved in inflammation. Peng et al. summarized how CCN3, a member of the CCN proteins which is a family of extracellular matrix-associated proteins, acts in immune-related metabolic diseases and the related mechanisms.

Antivirus immune response upon infection results with an inflammatory process. Chronic cytomegalovirus (CMV) infection significantly irritates the immune system and has been found to be one of the main determinants of immune senescence in the elderly [11, 12]. Accordingly, Lin et al. investigated the relationship between CMV infection and leukocyte telomere length and found a close relevance between previous CMV infection and shorter leukocyte TL. Novel coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 is a deadly disease due to the status of hyperinflammation after infection [13]. Zheng et al. investigated the potential predictors of COVID-19 mortality and risk factors for hyperinflammation in COVID-19 and found uric acid levels to be a potential factor associated with inflammatory markers.

Collectively, all the original research and review articles in this special issue cover many important aspects in the area of interaction between inflammation and metabolic disorders, which may provide new strategies for the diagnosis and treatment of metabolic diseases in the clinic.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this Special Issue.

Authors' Contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Jie Chen
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Research Article

Factors Influencing the Serum Uric Acid in Gout with Cerebral Infarction

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Background. Although the relationship between gout and cardiovascular has been well demonstrated, there is little information about the difference between gout with cerebrovascular disease and cardiovascular disease. In this study, the differences between gout with cerebral infarction (gout+CI) and gout with coronary heart disease (gout+CHD) and related factors that affect serum uric acid (sUA) levels in gout+CI were investigated by a cross-sectional study. *Method*. The patients from Jiangxi Provincial People's Hospital with gout+CHD, gout+CI, and gout with coronary heart disease and cerebral infarction (gout+CHD+CI) between 2016 and 2020 were included in this study, and the medical record data were collected and analyzed. *Results*. We observed significant differences in age, drinking, hypertension, long-term use of diuretics and NSAIDs, sUA, CRE, and blood glucose in patients with gout+CHD and gout+CI. The sUA level was significantly positively correlated with smoking, CRE, and TG in the gout+CI group and was only positively correlated with CRE in the gout+CHD group and the gout+CHD+CI group ($p < 0.05$). Interestingly, the sUA level was only negatively correlated with the age and gender in the gout+CI group ($p < 0.05$). After excluding factors with no significant statistical effect, only age, gender, smoking, CRE, and TG were included in the multiple linear regression model. It suggested that smoking, CRE, and TG are positively correlated with the sUA level, while age was negatively correlated with the sUA level. *Conclusions*. There are many discrepancies in clinical characteristics between gout +CHD patients and gout+CI patients, especially that the factors that affect UA levels are significantly different. The data also suggested that uric acid-lowering therapy may need to be strengthened in the young gout+CI patients with a history of smoking.

1. Introduction

Gout is a chronic inflammatory disease, which was caused by abnormal purine metabolism and decreased uric acid excretion leading to increased levels of sUA. It is characterized by swollen, hot, and painful joints. However, gout is a systemic disease, usually complicated by cardiovascular diseases, hypertension, dyslipidemia, diabetes, and other diseases [1]. Hyperuricemia is associated with an increased risk of cardiovascular disease [2]. Patients with gout have a higher risk of coronary heart disease than those without gout. In addition, compared with gout patients who have received antigout treatment, gout patients who have not received antigout treatment have a significantly increased risk of coronary heart disease [3]. However, there are few data on the relationship between gout and cerebrovascular diseases. A previous study has shown that higher sUA levels are an independent risk factor for coronary heart disease. In addition, a positive correlation between the risk of coronary heart disease and the level of UA was observed [4]. It has been reported that different UA concentrations have different effects on brain injury. An appropriate concentration of UA can significantly improve cell viability and reduce cell apoptosis and has a neuroprotective effect, while a high concentration of UA reduces cell viability [5]. Although increased levels of sUA have been shown to be associated with increased risk of cerebral infarction (CI) [6, 7], there are few data on the influencing factors of sUA levels in patients with gout and CI. Therefore, in this study, we conducted a cross-sectional study on the gout+CHD group, the gout+CI group, and the gout

+CHD+CI patients to explore the characteristics of gout+CI patients and the factors that affect uric acid in these patients. The data showed significant differences in age, drinking, hypertension, long-term use of diuretics and NSAIDs, sUA, CRE, and blood glucose in patients with gout+CHD and gout+CI. In addition, there are many discrepancies in factors affecting sUA levels between gout+CHD patients and gout +CI patients, and the data also suggested that UA-lowering therapy may need to be strengthened in the young gout+CI patients with a history of smoking.

2. Materials and Method

2.1. Research Objects. All patients who were diagnosed with gout complicated with CHD, gout complicated with CI, or gout complicated with CHD and CI and then discharged from Jiangxi Provincial People's Hospital from January 1, 2016, to November 1, 2020, were included in the cross-sectional study. Criteria included a discharge diagnosis of gout+CHD, gout+CI, or gout+CHD+CI and age ≥ 18 years. Criteria excluded anemia, chronic tumor, myelodysplastic or lymphatic proliferative disease, primary renal inadequacy, secondary gout, chronic hepatic insufficiency, pregnancy or lactation in females, and patients with incomplete information. This study was approved by the ethics committee of Jiangxi Provincial People's Hospital. Patients' information was acquired and collected from the electronic medical record system of Jiangxi Provincial People's Hospital, without actual contact with any patients.

The main data sources for the diagnosis of gout, CHD, CI, and hypertension were acquired from the hospital electronic medical record, especially the discharged diagnosis and medical history, which includes the usage records of maintenance drugs (e.g., uric acid-lowering drugs and antihypertensive drugs) and CHD medicine treatment for secondary prevention, and previous medical records. We collected demographic characteristics, clinical characteristics, and laboratory parameters (e.g., age, gender, BMI, blood pressure, smoking, drinking, sUA levels, creatinine, total cholesterol, triglyceride, low-density lipoprotein, blood glucose, and long-term use of diuretics and nonsteroidal anti-inflammatory drugs) from the hospital's discharge record. Fasting venous blood was collected in the morning for all laboratory tests.

2.2. Diagnostic Criteria. Gout: patients who had a diagnosis of gout and had received drugs to lower sUA before admission, such as febuxostat, colchicine, or allopurinol according to the previous medical history, or patients who were newly diagnosed with gout during their hospitalization between 2016 and 2020, which met the 2015 ACR/EULAR classification criteria for gout [8].

CHD: patients who had a diagnosis of CHD and had received secondary prevention for CHD before admission according to the previous medical history or patients who were newly diagnosed with CHD on the basis of the typical clinical symptoms, electrocardiogram characteristics and myocardial necrosis markers, or coronary angiography, during their hospitalization from January 1, 2016, to November 1, 2020.

CI: patients who had a diagnosis of CI and had received lipid-lowering, antiplatelet aggregation and other relevant treatment before admission according to the previous medical history or patients who were newly diagnosed with CI on the basis of focal neurological defects and brain CT or MRI examinations during their hospitalization from January 1, 2016, to November 1, 2020.

Hypertension: patients who were previously diagnosed with hypertension and had received antihypertensive therapy or patients whose systolic pressure ≥ 140 mmHg and/or diastolic pressure ≥ 90 mmHg were found twice during their hospitalization from January 1, 2016, to November 1, 2020.

Long-term use of diuretics and nonsteroidal anti-inflammatory drugs (NSAIDS) was defined as taking the medicine for three months or more.

2.3. Statistical Analysis. The continuous data were calculated and expressed as median (min–max) or mean ± standard deviation; the classified variables were represented as the count and percentage. Statistically significant differences were determined using the chi-squared test, one-way analysis of variance, Kruskal-Wallis test, and Bonferroni's multiple comparison test. Pearson (point-biserial) correlation analysis was performed among sUA levels, demographic characteristics, and clinical and laboratory variables. Variables with $p < 0.05$ were involved in the multivariate linear regression model. All data were statistically analyzed using Statistical Package for the Social Sciences (SPSS) software version 21. Two-tailed $p < 0.05$ was considered statistically significant.

3. Results

3.1. Demographic and Laboratory Variables and Clinical Characteristics among the Three Groups. The information was collected from 3464 patients with gout from January 1, 2016, to November 1, 2020, which included 626 gout+CHD patients, 584 gout+CI patients, and 151 gout+CHD+CI patients. According to the inclusion and exclusion criteria, 753 discharged patients were finally included, including 353 cases of gout+CHD, 298 cases of gout+CI, and 102 cases of gout+CHD+CI. The demographic information, laboratory variables, and clinical characteristics of the subjects are listed in Table 1. Most of the patients were male (gout+CI+CHD: 84 (82.4), gout+CHD: 328 (92.9), and gout+CI: 270 (90.6)). The vast majority of patients are elderly patients (gout +CHD+CI: 76.5 ± 11.8, gout+CHD: 67.8 ± 11.7, and gout +CI: 70.4 ± 11.6). There are statistically significant differences in age, gender, BMI, drinking, long-term use of diuretics, long-term use of NSAIDs, sUA, CRE, TC, TG, LDL, and blood glucose among the gout+CHD group, the gout+CI group, and gout+CHD+CI group ($p < 0.05$), while no significant differences in other variables such as smoking and hypertension were observed ($p > 0.05$) (Table 1).

Next, we compared the characteristics that have significant differences between the three groups in pairs. As shown in Table 2, the gout+CHD and gout+CI+CHD groups, as well as the gout+CI and gout+CI+CHD groups, had

Table 1: Comparisons of variables in the gout+CI group, the gout+CHD group, and the gout+CI+CHD group.

Characteristics	Gout+CI+CHD ($n = 102$)	Gout+CHD ($n = 353$)	Gout+CI ($n = 298$)	p value
Age (year)	76.5 ± 11.8	67.8 ± 11.7	70.4 ± 11.6	<0.001[2]
Gender, male, n (%)	84 (82.4)	328 (92.9)	270 (90.6)	0.006[1]
BMI (kg/m^2)	23.9 ± 3.7	24.7 ± 3.6	24.1 ± 3.4	0.027[2]
Underweight (<18.5), n (%)	5 (4.9)	15 (4.3)	14 (4.7)	
Normal weight (18.5~23.9), n (%)	45 (44.1)	136 (38.5)	136 (45.6)	
Overweight (24.0~27.9), n (%)	41 (40.2)	147 (41.6)	110 (36.9)	
Obesity (≥28), n (%)	11 (10.8)	55 (15.6)	38 (12.8)	
Smoking, n (%)	35 (34.3)	162 (45.9)	128 (43.0)	0.115[1]
Drinking, n (%)	21 (20.6)	98 (27.8)	123 (58.7)	<0.001[1]
Hypertension, n (%)	49 (48.0)	155 (43.9)	158 (53.0)	0.068[1]
Long-term use of diuretics, n (%)	3 (2.9)	10 (2.8)	39 (13.1)	<0.001[1]
Long-term use of NSAIDs, n (%)	27 (26.5)	84 (23.8)	25 (8.4)	<0.001[1]
sUA (μmol/L)	454.1 ± 133.4	497.4 ± 132.8	465.5 ± 118.2	<0.001[2]
CRE (mmol/L)	94.5 (78.0-117.3)	99.0 (79.0-125.0)	91.5 (74.8-114.3)	0.016[3]
TC (mmol/L)	4.0 ± 1.2	4.2 (3.5-5.0)	4.3 (3.5-5.1)	0.003[3]
TG (mmol/L)	1.3 (0.9-1.9)	1.5 (1.1-2.0)	1.4 (1.0-1.9)	0.041[3]
LDL (mmol/L)	2.1 (1.4-2.9)	2.2 (1.8-2.9)	2.4 (1.9-3.0)	0.004[3]
Blood glucose (mmol/L)	5.7 (4.7-7.0)	5.8 (5.0-7.5)	5.5 (4.8-7.0)	0.011[3]

BMI: body mass index; sUA: serum uric acid; CRE: creatinine; TC: total cholesterol; TG: triglycerides; LDL: low-density lipoprotein; gout+CI+CHD: gout with cerebral infarction and coronary heart disease group; gout+CHD: gout with coronary heart disease group; gout+CI: gout with cerebral infarction group. Data with a normal distribution were represented as the mean ± standard deviation, and data with abnormal distribution were represented as median (interquartile range). The classified variables were represented as the count and percentage. [1]Chi-squared test, [2]one-way analysis of variance, and [3]Kruskal-Wallis test were used for the significance of difference between three groups.

Table 2: Comparison of classified variables within groups.

	Gender, male		Drinking		Smoking		Long-term use of diuretics		Long-term use of NSAIDs		Hypertension	
	χ^2	p value	χ^2	p value	χ^2	p value	χ^2	p value	χ^2	p value	χ^2	p value
Gout+CI vs. gout+CHD	1.157	0.282	13.159	<0.001	0.565	0.452	8.324	0.004	21.966	<0.001	5.373	0.02
Gout+CHD vs. gout+CI+CHD	10.322	0.001	2.109	0.146	4.321	0.038	0.003	0.954	0.307	0.58	0.546	0.46
Gout+CI vs. gout+CI+CHD	5.083	0.024	14.115	<0.001	2.349	0.125	24.412	<0.001	27.515	<0.001	0.755	0.385

p value of significance assessed by the chi^2 test (categorical variables) and Bonferroni correction.

statistically significant gender differences ($p < 0.05$); the gout +CI and gout+CHD groups, as well as the gout+CI and gout +CI+CHD groups, had statistically significant differences in drinking and long-term use of diuretics and NSAIDs ($p < 0.05$). Abnormal metabolism plays an important role in gout and cardiovascular and cerebrovascular diseases. Therefore, we also explored the differences in metabolic parameters between groups. There were statistically significant differences in TC and LDL between the gout+CI and gout+CI+CHD groups, as well as the gout+CHD and gout +CI+CHD groups ($p < 0.05$), while no considerable difference was observed between the gout+CI and gout+CHD groups. However, the CRE and blood glucose were markedly decreased in gout+CI patients when compared with gout +CHD patients ($p < 0.05$) (Table 3). The comparison between groups found that there was no difference in BMI between any two groups, but sUA was significantly lower in the gout+CI patients than in the gout+CHD group ($p < 0.05$) (Table 4). The above results suggest that there are significant differences in the clinical characteristics of gout +CI and gout+CHD. It is necessary to further explore the influencing factors of the sUA level in patients with gout+CI.

3.2. Correlation of sUA with Demographic Characteristics, Clinical Characteristics, and Biochemical Variables. The uric acid value of gout+CI was significantly lower than that of gout+CHD patients, but there was no statistical difference between the gout+CI and gout+CI+CHD groups. It is possible that CI has an effect on gout+CI+CHD patients. In order to explore the reason why the sUA level of the gout+CI patients is statistically lower than that of the gout+CHD patients, we conducted an analysis of factors related to the sUA level. As shown in Table 5, sUA levels in the gout+CI patients was negatively correlated with age and gender and

Table 3: Comparison of nonnormal distribution or unequal comparison of continuous variables within groups.

	TC	CRE	TG	LDL	Blood glucose
Gout+CI *vs.* gout+CHD, *p* value	0.671	0.013	0.097	0.614	0.016
Gout+CHD *vs.* gout+CI+CHD, *p* value	0.025	0.786	0.058	0.041	0.162
Gout+CI *vs.* gout+CI+CHD, *p* value	0.002	1	0.746	0.003	1

CRE: creatinine; TC: total cholesterol; TG: triglycerides; LDL: low-density lipoprotein. *p* value of significance assessed by Kruskal-Wallis' test (continuous variables) and Bonferroni correction.

Table 4: Comparison of normal distribution and equal comparison of continuous variables within groups.

	sUA	BMI	Age
Gout+CI vs. gout+CHD, *p* value	0.005	0.076	0.018
Gout+CHD vs. gout+CI+CHD, *p* value	0.008	0.107	<0.001
Gout+CI vs. gout+CI+CHD, *p* value	1	1	<0.001
F	7.306	3.579	21.884
p	<0.001	0.028	<0.001

sUA: serum uric acid; BMI: body mass index. *p* value of significance assessed by one-way analysis of variance (continuous variables) and Bonferroni correction.

positively correlated with smoking, CRE, and TG ($p < 0.05$). In contrast, the levels of sUA in the gout+CHD group and the gout+CI+CHD group were only positively correlated with CRE ($p < 0.05$). These results reveal that the influencing factors of UA in patients with gout+CI are significantly different from those of gout+CHD.

3.3. Multiple Linear Regression of sUA Factors Related to Gout +CI Patients. The above results show that multiple factors are significantly related to sUA in patients with gout+CI. We further used multiple linear regression to analyze the influencing factors of UA in patients with gout+CI. After excluding nonstatistically significant factors, age, gender, smoking, CRE, and TG were included in the multiple linear regression model, and Pearson correlation analysis was performed on the various factors of the sUA level. The multiple linear regression model was statistically significant ($F = 15.714$, $p < 0.001$). As is shown in Table 6, smoking ($B = 28.887, p = 0.026$), CRE ($B = 1.277, p \leq 0.001$), and TG ($B = 15.993, p = 0.005$) showed a positive correlation with sUA levels, while age ($B = -1.392, p = 0.013$) was negatively correlated with sUA levels. However, gender was not significantly associated with sUA levels ($p = 0.595$). Among the relevant factors, CRE had the most significant influence on sUA levels (beta = 0.380).

4. Discussion

Here, we conducted a cross-sectional study on the gout +CHD group, gout+CI group, and gout+CHD+CI patients to explore the characteristics of gout+CI patients and the factors that affect uric acid in these patients. The data showed significant differences in age, drinking, hypertension, long-term use of diuretics and NSAIDs, sUA, CRE, and blood glucose in patients with gout+CHD and gout+CI. The sUA level of the gout+CI group was lower than that of the gout+CHD group. In addition, there are differences in factors affecting uric acid levels between gout+CHD patients and gout+CI patients.

In the gout+CI group, there were significant correlations between age, smoking, CRE, and TG and sUA, while a negative correlation between age and sUA was observed, which may be because the population we included is mainly elderly men (70.4 ± 11.6). Young men are more likely to have an unhealthy lifestyle, including excessive alcohol, sugary drinks, and excessive intake of high-purine foods, such as meat and seafood. It has shown that unhealthy eating habits will gradually improve with age, which may lead to a decrease in sUA levels [9, 10], but this is contrary to some other studies [11, 12]. This may indicate that the younger the patient, the more timely sUA should be reduced. Women often have low sUA levels and rarely get gout. However, there is no significant correlation between gender and sUA level in our study, which may be because the gout women included in the group are all elderly women who have passed menopause and have lost estrogen protection. However, in the group comparison and Spearman correlation analysis, there is a statistical difference in gender, which is consistent with a previous study [13].

Among these related factors, CRE has the most significant impact on sUA levels. Two-thirds of sUA are excreted through the kidneys, and one-third is excreted through the intestines. sUA is filtered in the glomerulus and then reabsorbed and secreted in the renal tubules, but about 90% of the excreted UA is reabsorbed [14–16]. The renal excretion of sUA depends on specific transporters, including URAT1 (uric acid transporter 1), GLUT9 (glucose transporter 9), and BCRP (breast cancer resistance protein). Therefore, impaired renal function may lead to an increase in sUA, which may be a decrease in glomerular filtration or a decrease in renal tubular resecretion [17], accompanied by an increase in CRE. Adversely, It has been proved that the level of sUA is closely related to the progression of nephropathy [18, 19], which may be related to sUA causing kidney damage by stimulating the adrenaline-angiotensin system and promoting the proliferation of vascular smooth muscle cells [20, 21], as well as high uric acid. Symptoms of hyperemia can affect renal hemodynamics and cause glomerular perfusion disorders [22]. In addition, sUA crystals may damage the renal tubules through inflammasome [23], resulting in a decrease in the glomerular filtration rate and an increase in creatinine levels. In this study, we only collected data on patients with gout. Therefore, these patients may suffer a large amount of inflammatory burden and potential kidney damage, which is consistent with the results of this study.

Table 5: Correlation of sUA with demographic characteristics, clinical characteristics, and laboratory variables.

Variables	Gout+CI+CHD		Gout+CHD		Gout+CI	
	Pearson coefficient	p value	Pearson coefficient	p value	Pearson coefficient	p value
Age (year)	-0.094	0.350	-0.050	0.924	-0.127	0.028
Gender, male, n (%)	-0.162	0.103	-0.073	0.173	-0.126	0.029
BMI (kg/m^2)	0.019	0.850	0.064	0.233	0.068	0.243
Smoking, n (%)	0.133	0.182	0.033	0.537	0.209	<0.001
Drinking, n (%)	0.161	0.107	0.052	0.332	0.089	0.125
Hypertension, n (%)	-0.016	0.876	0.026	0.620	-0.075	0.198
Long-term use of diuretics, n (%)	-0.038	0.706	0.055	0.306	0.073	0.210
Long-term use of NSAIDs, n (%)	0.104	0.299	0.075	0.160	-0.060	0.300
CRE (mmol/L)	0.198	0.046	0.230	<0.001	0.369	<0.001
TC (mmol/L)	-0.058	0.564	-0.029	0.592	0.036	0.532
TG (mmol/L)	-0.037	0.709	0.037	0.489	0.169	0.003
LDL-C (mmol/L)	0.110	0.272	-0.035	0.509	-0.004	0.947
Blood glucose (mmol/L)	0.065	0.517	0.088	0.100	0.013	0.826

Spearman correlation analysis was used for the significance of difference.

Table 6: Multiple linear regression of factors related to sUA in the gout+CI group.

Variables	Unstandardized coefficient		Standardized coefficient	T	p	VIF
	B	SE	Beta			
Age	-1.392	0.557	-0.137	-2.501	0.013	1.114
Gender, male	11.595	21.803	0.029	0.532	0.595	1.077
Smoking	28.887	12.937	0.121	2.233	0.026	1.091
CRE	1.277	0.181	0.380	7.051	<0.001	1.075
TG	15.993	5.706	0.150	2.803	0.005	1.065

High levels of sUA may cause proinflammatory endocrine imbalance in adipose tissue [24]. Animal experiments have shown that high sUA caused by excessive intake of fructose-containing beverages can lead to the accumulation of triglycerides, and it has also been shown that sUA levels are positively correlated with TG [25, 26]. In addition, the relationship between TG and sUA levels has been demonstrated to be genetically related [27]. In this study, we also found that there is a positive correlation between sUA and TG in gout+CI patients, and the related mechanisms need to be further explored. Previous studies have found that after adjusting the confounding factors that affect the sUA level, the sUA level of the smoking group was significantly lower than that of the nonsmoking group [28]. However, it has been reported that female smokers have a positive correlation with sUA levels, while no significant correlation has been found between male smokers and sUA levels [13]. Conversely, another study demonstrated that male smokers were negatively correlated with sUA levels, while female smokers did not find a significant correlation with sUA levels [29]. The related mechanisms remain elusive, since sUA has not only prooxidative activity but also antioxidant effect [30, 31], which can act as an antioxidant against oxidative stress induced by smoking. Our results show that smoking is positively correlated with sUA levels, and the reasons for the different results may be related to the differences in race, diet, environmental factors, and genetics of the population included in each study.

5. Conclusions

Here, we found that the level of sUA in patients with gout +CI was significantly positively correlated with smoking, CRE, and TG and negatively correlated with age. These data may help us strengthen the monitoring and management of gout patients: for the smoking, high CRE, high TG, and younger gout patients, sooner sUA-lowering treatment should be started. Actually, smoking and triglyceride are both risk factors for CI. The main limitation of this study is that cross-sectional studies cannot provide evidence of causality. At the same time, this study has the following shortcomings: this study only included Chinese people, which limits our ability to extend the results to other races; this study did not include asymptomatic hyperuricemia patients, which limits the generality of our study; in addition, only a small number of women with gout are included, and the results may be controversial if they are grouped by gender. Therefore, further prospective research and basic research are needed to explore the relationship between gout and CI and the factors that affect the uric acid level of gout+CI.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors' Contributions

YL, HY, and YT reviewed the medical records, analyze the data, and wrote the first draft. YL and LD reviewed the literature and finalized the revised manuscript. All authors have read and approved the final manuscript. Yi Li, Hongyi Yang, and Yao Tian contributed equally to this work.

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Review Article

The Emerging Roles of CCN3 Protein in Immune-Related Diseases

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The CCN proteins are a family of extracellular matrix- (ECM-) associated proteins which currently consist of six secreted proteins (CCN1-6). CCN3 protein, also known as nephroblastoma overexpressed protein (NOV), is a member of the CCN family with multiple biological functions, implicated in major cellular processes such as cell growth, migration, and differentiation. Recently, CCN3 has emerged as a critical regulator in a variety of diseases, including immune-related diseases, including rheumatology arthritis, osteoarthritis, and systemic sclerosis. In this review, we will briefly introduce the structure and function of the CCN3 protein and summarize the roles of CCN3 in immune-related diseases, which is essential to understand the functions of the CCN3 in immune-related diseases.

1. Introduction

The name of the CCN family derived from the acronym of the first three discovered proteins, namely, cysteine-rich protein 61 (CYR61, CCN1), connective tissue growth factor (CTGF, CCN2), and nephroblastoma overexpressed (NOV, CCN3) [1–3]. The other three members, WISP1 (CCN4), WISP2 (CCN5), and WISP3 (CCN6), are considered Wnt-inducible secreted proteins, participating in the Wnt signaling pathway. All the CCN members (except for CCN5) share four conservative homologous domains following an N-terminal secretion signal-peptide: insulin growth factor-binding protein (IGFBP), von Willebrand factor type C (vWC), thrombospondin type 1 repeat (TSP-1), and carboxy-terminal knot domain (CT) [4]. CCN5 is the particular one that lacks the CT domain. The CCN members are matricellular proteins, and their main function is to facilitate the interaction between cells and extracellular matrix (ECM) rather than maintaining structural stability. As secreted proteins, CCNs have crucial roles in multiple biological processes through combination with heparan sulfate proteoglycan (HSPG), different types of integrators, and other noncanonical receptors [5–8]. CCNs promote the adhesion, mitosis, and migration of human fibroblasts through interaction with integrins $\alpha 6\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 5$, and HSPG which also play a critical role in the process of mediating fibroblast adhesion [4, 9, 10]. Moreover, it has been shown that adhesion of CCN1 to fibroblasts can induce apoptosis, while adhesion to endothelial cells can promote cell survival [11]. CCN2 can also bind to integrins $\alpha v\beta 3$ and HSPG, which induces the rat activated hepatic stellate cell adhesion [12]. Although CCN3 shares homologous structures with CCN1 and CCN2, it is quite different in some biological functions. For example, CCN3 is not necessary for embryonic development compared to CCN1 and CCN2 [13]. However, CCN3 can induce angiogenesis through the ligands $\alpha v\beta 3$ and $\alpha 5\beta 1$ [14].

CCN3/NOV (nephroblastoma overexpressed) was first isolated from the myeloblastosis-associated virus- (MAV-) induced nephroblastoma in day-old chicks [3]. In human early embryonic development, CCN3 is widely expressed in the derivative of all three germ layers [15]. In adult mammals, high CCN3 expression is observed in endotheliocytes, smooth muscle cells, fibroblasts, and chondrocytes [14, 16,

17]. Besides, Dombrowski et al. first discovered that CCN3 can be produced by regulatory T cells (Treg) [18]. CCN3 exerts biological functions through binding different receptors. It is reported that CCN3 could directly act on endothelial cells by binding integrins $\alpha v\beta 3$ and $\alpha 5\beta 1$ to promote cell adhesion, migration, and cell survival in vitro and induce angiogenesis, while these observations can be blocked by CCN3 inhibitors [14]. Furthermore, the aberrant expression of CCN3 is also involved in fibrosis and cancers [19, 20]. CCN3 may inhibit the activity of NOTCH1 by binding to the extracellular domain of NOTCH1 in CML [21], and the CCN3 protein secreted by prostate cancer (PCa) could recruit macrophages and promote their differentiation into the M2 phenotype [22]. CCN2 is the most well-known fibrosis-related protein in the CCN protein family. At present, the anti-CCN2 antibody (FG-3019) has been used in clinical trials of patients with idiopathic pulmonary fibrosis and has achieved significant therapeutic effects [23]. Barbe et al. found that in animal fibrosis models, FG-3019 treatment can increase the expression level of CCN3 in muscles [24]. CCN3 has been shown as a negative regulator of CCN2 to antagonize the fibrogenesis effect of CCN2 and further to inhibit the progression of fibrosis [25].

Immune-mediated diseases refer to a group of diseases characterized by dysregulated immune responses, eventually leading to the damage of cells, tissues, and even organs. Several articles reported that CCN3 is involved in the regulation of immune cell function, such as the regulation of Treg and hematopoietic stem cell function, while other CCN family molecules are rarely reported in these years [18, 26, 27]. Furthermore, it has been found that the abnormal level of CCN3 is connected with immune regulation and immune-mediated diseases. Although the precise mechanism remains unclear, the insight into CCN3-mediated biological regulation could allow us a better understanding of the emerging role of CCN3 in immune-mediated diseases.

2. CCN3 in Pathophysiological Disorders

2.1. The Role of CCN3 in Endothelial Cell Function. It has been demonstrated that CCN3 maintains cardiovascular homeostasis by regulating VSMC and endothelial cell function [14, 16]. Lin et al. found that purified recombinant human CCN3 could mediate endotheliocyte adhesion through integrins $\alpha v\beta 3$, $\alpha 6\beta 1$, and $\alpha 5\beta 1$. Meanwhile, CCN3 can mediate endothelial cell migration as a ligand of $\alpha 5\beta 1$ and $\alpha v\beta 3$ [14]. In addition, CCN3 exerted an inhibitory effect on VSMC proliferation and migration to resist neointimal hyperplasia. Further study found that CCN3 upregulated the cyclin-dependent kinase inhibitors p15 and p21 partly through the Notch signaling pathway independently of TGF-β signaling [16]. To further explore the function of CCN3 in regulating endothelial inflammation, Lin et al. found that KLF2, a factor that inhibited endothelial proinflammation progress, could increase the expression of CCN3 in HUVECs. Next, by exposing HUVECs infected with adenovirus-CCN3 to TNF-α or IL-1β, the adhesion molecules and VCAM-1 were strongly inhibited. On the contrary, knockdown of CCN3 markedly enhanced VCAM-1 expression induced by TNF-α stimulation. Mechanically, they found that CCN3 exerted a negative effect on NF-κB accumulation induced by inflammatory cytokines [28]. These observations suggest that CCN3 played a vital role in regulating the endothelial cell function.

2.2. The Role of CCN3 in Fibrosis. The process of fibrosis involves a series of sequential and complex steps including the transient activation of fibroblasts, the proliferation of fibroblasts, and the production of excessive extracellular matrix (ECM), which is regulated by numerous cytokines such as transforming growth factor (TGF) and platelet-derived growth factor (PDGF) [29]. Studies have shown that all CCN family members, as matricellular protein, are related to fibrosis [30–33]. CCN2, as the downstream effector of TGF-β, has been implicated in the process of the initial period of fibrosis. Conversely, CCN3 could counter the downstream of the TGF-β signal pathway to inhibit fibrosis. CCN3 and CCN2 act as a yin and yang regulator to adjust the fibrosis development [34].

A previous study has shown that TGF-β regulates CCN2 function through MEK1, YAP1, or TAK1, while inhibiting these molecules will not affect the role of TGF-β on the CCN3 expression in human skin fibroblasts. Therefore, it is believed that the regulation of CCN3 was suppressed by some unknown factors [35]. The previous fibrosis models in vitro and in vivo have proved that CCN3 is a negative regulator of CCN2 and able to block the accumulation of ECM [25, 36]. In the recent TGF-β induced fibrotic models of renal fibroblasts, the increased expression of CCN2 and depressed level of CCN3 were detected in the conditional media [37]. In the chronic fibrosis induced by muscle overuse, researchers observed that the CCN3 expression was improved following the treatment of the CCN2 inhibitor (FG-3019) [24]. Although it was later found that CCN3 did not have a noticeable effect to attenuate the liver fibrosis due to hepatocyte apoptosis which occurred simultaneously [38], the correlations between CCN3 and antifibrosis actions have been identified in a variety of diseases such as chronic overuse muscle fibrosis, glomerular and tubulointerstitial renal fibrosis, and systemic sclerosis [24, 39–41]. Thus, a hypothesis was proposed which also paid attention to the fact that the ratio of CCN2 : CCN3 and balancing the interaction of CCN proteins might be an important measure for the treatment of fibrosis [19].

2.3. The Role of CCN3 in Tumor Proliferation. It is clear that the levels of CCN3 are abnormal in some certain tumors. However, the role of CCN3 in the tumor is exceedingly complex, because the functions of CCN3 have been shown to differ in various types of malignancies [42]. Maillard et al. found that CCN3 was expressed predominantly in prostate cancer cell lines as well as the lymph node metastases compared with normal prostate epithelial cells [43], and similar results were obtained in cervical cancer, bone malignancies, and benign adrenocortical tumors [44–47]. In addition, Chen et al. first reported that prostate cancer- (PCa-) secreted CCN3 had the capacity to recruit macrophages and promote their differentiation to an M2 phenotype, and macrophage migration was induced by conditioned media (CM) from various PCa

cells and was inhibited by an anti-CCN3 neutralizing antibody. These functions of CCN3 might be associated with PCa-derived CCN3-induced focal adhesion kinase (FAK)/AKT/NF-κB signaling, which also lead to increased VEGF expression and increased tube formation in endothelial progenitor cells [22]. These observations suggested that CCN3 was associated with cancer staging and prognosis as well as contributing to tumorigenesis or metastasis formation On the other hand, CCN3 can also negatively regulate some other kinds of tumor growth, such as melanoma, glioblastoma, and chronic myeloid leukemia [42, 48, 49]. The decreased expression of CCN3 was investigated in the invasion melanoma cells, and CCN3 transduction lessened the invasion through restraining the MMP-2 and MMP-9 activities [48]. The cell growth of the K562 CML cell line stably transfected with CCN3 was significantly decreased, especially the number of cells in the subG0 phase increased. Furthermore, the apoptosis of K562 cells treated with CCN3 and imatinib was enhanced, suggesting that CCN3 may affect the process of cell mitosis and enhance imatinib-induced cell apoptosis in CML [49]. Consistently, CCN3 could act as an antiproliferative protein by influencing the cell cycle of glioblastoma cells [50].

3. The Roles of CCN3 Protein in Immune-Related Diseases

3.1. Rheumatoid Arthritis (RA). Previous studies has shown that CCN1, CCN2, CCN4, and CCN5 are highly expressed in OA and RA knee cartilages, while CCN3 and CCN6 can hardly be detected in OA and RA cartilages. However, the CCN3 gene was highly expressed in OA and RA synovial samples compared with normal joint tissues [51]. RA is a chronic systemic autoimmune disease that eventually leads to cartilage and bone destruction and joint dysfunction. However, the role of CCN3 in rheumatoid arthritis (RA) remains elusive. Recently, our data showed that the serum CCN3 level of RA patients was obviously increased in RA, and the immunohistological analysis revealed a considerable increased deposition of CCN3 in the joint tissues from RA patients, but not in the control tissues from OA patients [52]. This may be due to the use of different antibodies in the test. IL-6 and TNF-α are critical inflammatory factors in the RA progression [53]; our data demonstrated that CCN3 positively connected with IL-6 expression but no statistical difference with TNF-α [52]. Our work suggested that CCN3 may serve as a biomarker for inflammation and disease activity in RA, but the mechanism of CCN3 remains to be deeply elucidated. Besides, the expression of CCN1 was also higher in RA but was inversely correlated with RA disease activity [54, 55]. Therefore, further studies on the function of CCN proteins are needed to resolve the specific regulatory mechanism of CCN proteins in the development of RA.

3.2. Osteoarthritis (OA). OA, a major clinical problem among the ageing population, is characterized by articular cartilage degeneration and synovial inflammation. CCN3 was highly expressed in articular chondrocytes in the normal rat articular cartilage [17], but it has an apparent decline in a monoiodoacetic acid- (MIA-) induced osteoarthritic model [56]. Consistently, another research also confirmed that the CCN3 expression was reduced in the cartilage tissue of OA patients and OA rat models [57]. These data suggested that the expression of CCN3 might be a protective role in cartilage degeneration. Of note, exogenous recombinant CCN3 administration increased the accumulation of proteoglycan and the expression of tenascin-C and lubricin, protected the damage of articular cartilage surface, positively modulated chondrogenesis, and attenuated the progress of OA [56]. Furthermore, recombinant CCN3 or CCN3 overexpression could also ameliorate IL-1β-induced osteoarthritis response by reducing extracellular matrix catabolism and inducing cartilage protection in vitro via decreasing the level of HMGB1, reversing the increase of MMP, inhibiting the activation of PI3K/AKT/mTOR pathway, and promoting cell autophagy [57]. However, CCN3 dramatically suppressed the proliferation and activity of osteoblast and has an inhibitory effect on osteoblast differentiation by its involvement of the BMP and Notch signaling pathways, and higher phosphorylation of Smad1/5 was observed in CCN3 knockout mice [58, 59]. CCN3 positively regulates articular chondrocytes but inhibits osteoblast differentiation and acts as an inhibitor of bone regeneration. Therefore, the mechanisms of CCN3 in the bone metabolism need further to be explored.

3.3. Glomerulonephritis (GN). Most of GN are thought to be immune mediated with abnormal regulation of both humoral immunity and cellular immunity, which cause the different sites of glomerular injury such as endothelial cell and mesangial area and result in various histopathological alterations including fibrosis, mesangial cell proliferation, and glomerular sclerosis [60]. Recently, CCN3 was suggested to play a crucial role in the development of some certain types of glomerulonephritis. It was shown that CCN3 could inhibit the fibrotic pathway by reducing the TGF-β-stimulated CCN2 expression and blocking the accumulation of extracellular matrix (ECM) such as collagen type I [25], which was further confirmed an in vitro model of diabetic renal fibrosis [36]. In consistency, exogenous recombinant CCN3 treatment dramatically downregulated the fibrosis-related factor (CCN2, Col1a2, TGF-β1, and PAI-1) mRNA in the kidney cortex of diabetes nephritis [40]. Similar observations were investigated in the culture of human mesangial cells; exogenous rCCN3 effectively controlled ECM formation and improved the TGF-β induced MMP expression [61]. Additionally, Roeyen et al. also found that CCN3 could act as an endogenous inhibitor of mesangial cell growth and a modulator of PDGF-induced mitogenesis in vitro [62]. In the experimental vascular proliferative nephritis model, the expression of glomerular CCN3 was increased in accordance with the decreased proliferation of mesangial cells. Furthermore, the proangiogenic and antiangiogenic effects of CCN3 in experimental glomerulonephritis have been determined [63]. Their observations indicate that CCN3 contributes to repairing glomerular endothelial injury and mesangial proliferation changes. Therefore, the CCN3 protein can be considered a potential therapeutic target for

glomerulonephritis. However, further studies should be explored because the regulation of CCN3 in immune response during the development of nephritis remains unknown.

3.4. Metabolic Diseases. Type 2 diabetes mellitus (T2MD) has not been considered a typical immune-related disease; however, the disorder of the immune system in T2MD have already been found in adipose tissue, the liver, pancreatic islets, the vasculature, and circulating leukocytes which leads to insulin resistance and inflammation eventually. Recent investigation shows that serum CCN3 correlated positively with adiposity-related parameters and insulin resistance indices, which is the first study to focus on the serum concentration of CCN3 in newly diagnosed T2MD (nT2MD) in humans [64], and a strong relationship between plasma CCN3 and obesity was also detected by measuring hundreds of adults suffering from hyperlipidemia and/or receiving lipid-lowering treatment and/or having a high BMI ($>30\,kg/m^2$) [65]. Consistently, it was shown that the $CCN3^{-/-}$ mice gained less body weight and improved the glucose tolerance and insulin sensitivity along with lower inflammation in the adipose tissue compared with wild-type controls when facing high-fat diet, although insulin production remained roughly in equal level. Interestingly, the absence of CCN3 led to a significant decrease expression of several proinflammatory cytokines and chemokines in the adipose tissue, which was associated with a change in the macrophage profile (M1-like to M2-like) [66]. CCN3 can also affect the phagocytosis of macrophages; macrophage from $CCN3^{-/-}$ mice leads to the increase of oxLDL uptake and foam cell formation through upregulated CD36 and SRA1 expressions. At the atherosclerotic lesions, $Apoe^{-/-}$ with CCN3 depletion increased their lipid plaque formation, macrophage infiltration, and the expression of monocyte chemotactic protein 1 compared to $Apoe^{-/-}$mice in vivo with high-fat feed [67]. Additionally, CCN3 has been found to be a new target for the transcription factor, FoxO1, which is a prominent mediator of insulin signaling in pancreatic β-cells. Activation of FoxO1 increased the expression of CCN3 in transgenic mice. On the other hand, CCN3 could inhibit the proliferation of β-cells, leading to the decline of insulin secretion in pancreatic β-cells [68]. Taken together, CCN3 antagonists can be regarded as a potential therapeutic strategy for T2MD. However, most studies have focused on the association between CCN3 and T2MD, while few have reported type 1 diabetes mellitus (T1DM). It is noteworthy that further studies are needed to explore the mechanistic details of CCN3 in T1MD.

3.5. Multiple Sclerosis (MS). It have been demonstrated that CCN3 expression could be detected in the nervous system [15, 69]; the roles of CCN3 in the central nervous system also have gained a lot of attention. Dombrowski et al. firstly reported that CCN3, as a growth-regulating protein, was produced by regulatory T cells (Treg). Anti-CCN3 antibody treatment or depleting CCN3 from Treg-conditioned media could abolish or inhibit the Treg-induced oligodendrocyte-differentiating effect and promyelinating effect. Furthermore, the treatment with recovered CCN3 significantly strengthens brain slice myelination [18]. Subsequent further study has found that increased CCN3 expression was observed in the progression of myelination in vivo. However, there is no significant difference between CCN3 knockout and wild-type control mice in the proliferation and differentiation of oligodendrocyte progenitor cell. Therefore, it is speculated that CNS cells cocultured with glial cells are affected by CCN3, which indirectly affects the differentiation of OPC [70]. A recent data from clinical samples showed that the serum CCN3 level was positively correlated with the CSF CCN3 level. In addition, the CCN3 mRNA expression was higher in peripheral immune cells (PBMC) of MS patients compared with the healthy control group [71]. Therefore, the myelin regeneration function of Tregs and CCN3 can be offered as potential encouraging treatment prospects for multiple sclerosis, while further experiments should be performed to address the mechanism.

3.6. Systemic Sclerosis (SSc). SSc is a chronic connective tissue disease characterized by diffuse or localized skin involvement, which is classified as an autoimmune rheumatic disease. Diffuse microangiopathy, inflammation, autoimmunity, and visceral and vascular fibrosis in multiple organs are mainly pathophysiologic processes of SSc [72]. CCN3 has been proved to be involved as part of the processes above, including antifibrosis and proangiogenesis effects [25, 63]. A study by Lemaire et al. showed that CCN3 plays a counterregulatory role in matrix formation by inhibiting the matrix assembly of fibrillary protein-1, providing a steady-state feedback mechanism for the control of extracellular matrix. These results are directly related to the early diffuse SSc skin [41]. Besides, the dermal capillary damage of the SSc patients' skin was associated with downregulation of CCN3 in dermal vessels and endothelial cells. Blocking CCN3 of human dermal microvascular endothelial cells (HDMECs) can inhibit angiogenesis, and HDMECs can promote angiogenesis of SSc HDMECs [73]. Actually, other CCN family members also participate in the pathological process of SSc [74]. In the bleomycin-induced model of skin scleroderma, the loss of CCN2 resulted in resistance to bleomycin-induced fibrosis, including the decrease of skin thickness, collagen production, and the loss of α-SMA-expressing myofibroblasts [75]. The previous studies have shown that CCN3 is a negative fibrosis of CCN2 and able to block the accumulation of ECM [25, 36]. Thus, CCN3 may be identified as a promising approach for SSc treatment.

4. Conclusion

Numerous basic studies on CCN3 have been explored in immune-related diseases; however, its role and mechanism in the pathogenesis of diseases remain elusive. In the current review, we tried to comprehensively summarize the contribution of CCN3 in the development of immune-mediated disease (Table 1). Of note, the biological effects of CCN3 are best known as the fibrosis inhibitor and proangiogenic factor, and these functions can be observed in many immune-related diseases. However, the regulation of CCN3 on immune cell function is only observed in certain cells, such

Table 1: Immune-related diseases influenced by CCN3.

Diseases	Mechanisms
Osteoarthritis (OA)	Decreases MMP, reduces extracellular matrix catabolism, and decreases the level of proinflammatory cytokines
Glomerulonephritis (GN)	Antifibrosis, inhibits mesangial cell growth, and inhibits the acceleration of ECM
Metabolic diseases	Inhibits the proliferation of pancreatic β-cells, regulates macrophage function, and regulates insulin signaling
Multiple sclerosis (MS)	Regulates Treg function and promotes myelin regeneration
Systemic sclerosis (SSc)	Antifibrosis, proangiogenesis, and controls extracellular matrix deposition
Rheumatoid arthritis (RA)	Decreases inflammatory cytokines

as Treg and macrophages. These findings suggest that CCN3 might indirectly affect the immune cell function, which leads to the development of immune-related diseases. In addition, the receptors of CCN3 should be explored in the other immune cells. Previous studies almost focused on exploring the effects of CCN3 on animal models and in vitro experiment. Recent studies provide more and rich data from clinical samples [52, 64, 71, 73]. Although CCN3 targeted therapy has not been used in clinical immune diseases, we believe that the application of the anti-CCN2 antibody in clinical trials will contribute to further clinical research of CCN3 to a certain extent. As mentioned above, the CCN2 blocking antibody can increase the expression of CCN3 in the muscle and inhibit the progression of fibrosis in the model [24]. This also means that the CCN proteins have the same domain, and their functions show synergistic stimulation or inhibition to a certain extent. Therefore, further studies, particularly a clinical study, are required to fully understand the pathophysiological function of CCN3 in immune-related diseases, which helps to develop immunomodulatory therapeutics against the abnormal immune response.

Data Availability

No data were used to support this study.

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors' Contributions

LP, YL, and YS reviewed the literature and wrote the first draft. YW, NL, and LD reviewed the literature and finalized the manuscript. YW, YL, and LD revised the manuscript. All authors have read and approved the final manuscript. Linan Peng and Yingying Wei contributed equally to this work.

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Research Article

MiR-6869-5p Induces M2 Polarization by Regulating PTPRO in Gestational Diabetes Mellitus

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The role of microRNA (miRNA) in gestational diabetes mellitus has been widely investigated during the last decade. However, the altering effect of miR-6869-5p on immunity and placental microenvironment in gestational diabetes mellitus is largely unknown. In our study, the expression of miR-6869-5p was documented to be significantly decreased in placenta-derived mononuclear macrophages, which was also negatively related to PTPRO. Besides, PTPRO was negatively regulated by miR-6869-5p in placenta-derived mononuclear macrophages. In vitro, miR-6869-5p inhibited macrophage proliferation demonstrated by EdU and CCK-8 experiments. The inflammatory response in macrophages was also significantly inhibited by miR-6869-5p, which could regulate PTPRO as a target documented by luciferase reporter assay. Moreover, miR-6869-5p promoted M2 macrophage polarization and thus restrain inflammation. Accordingly, miR-6869-5p is involved in maintaining placental microenvironment balance by preventing from inflammation and inducing M2 macrophages in gestational diabetes mellitus.

1. Introduction

Gestational diabetes mellitus is a common complication in pregnant females, who usually have normal glucose metabolism or potential abnormal glucose tolerance [1]. The incidence of gestational diabetes mellitus has been increasing during the past few decades. Most patients with gestational diabetes mellitus can return to normal glucose metabolism postpartumly, but they are at an elevated risk of developing type II diabetes in the future. The pathogenesis of gestational diabetes mellitus is unclear yet. Most importantly, abnormal glucose metabolism during gestation can cause pregnancy failure, dystocia, stillbirth, fetal death, and fetal macrosomia increased owing to complicated factors [2]. Therefore, identifying useful way for gestational diabetes mellitus prevention and treatment is urgent.

Mononuclear macrophages are key cells involved in regulating placental immunity and homeostasis. Macrophages can be induced to differentiate into classically activated M1 cells and alternatively activated M2 cells under specific regulators and microenvironment in placenta [3, 4]. Type 2 macrophages (M2) play a critical role in maintaining placental microenvironment balance [5]. Accumulated studies have suggested that microRNAs (miRNAs) participate in the pathogenesis of gestational diabetes mellitus by regulating β cell development, insulin sensitivity, and resistance [6, 7]. It has been well documented that some miRNAs can affect macrophages differentiation and polarization, such as miR-657, miR-145, and miR-221-3p [8–11]. We have previously found miR-657 could induce macrophages to M1 in gestational diabetes mellitus, which is a promising target for disease diagnosis and treatment [8, 12]. MiR-6869-5p has been

firstly found as a cancer suppressor [13]. There is no evidence showing that miR-6869-5p is attributed to gestational diabetes mellitus pathogenesis. We have previously found that the expression of miR-6869-5p was significantly decreased in placenta-derived mononuclear macrophages, which was also negatively related to PTPRO. The bioinformatics analysis has suggested that PTPRO is a potential target of miR-6869-5p. However, little is known about the influence of miR-6869-5p in placental macrophages differentiation and function. In this study, we aim to elucidate the altering effect and potential molecular mechanism of miR-6869-5p on immunity and placental microenvironment in gestational diabetes mellitus, which will provide insight into the disease pathogenesis and gaining promising strategies for disease prevention and therapy.

2. Material and Methods

2.1. Participants and Samples Preparation. Patients with gestational diabetes mellitus (26 cases) and normal pregnancies (23 controls) are enrolled. Inclusion and exclusion criteria are as follows: pregnant women with GDM having full-term cesarean section are enrolled as the case group, while healthy women having full-term pregnancy are regarded as the control group. Those pregnancies with premature or overdue births are all excluded. Table 1 lists the characteristics of participants. The study is approved by the hospital's Institutional Ethics Committee of Affiliated Hospital of Maternal and Child Health, Weifang Medical University. Participants have signed the informed consent. Macrophages are freshly separated from placental tissues, which are divided into small pieces before separation. Placental tissue suspension is used for isolating cells by density gradient centrifugation. Then, we used CD14 positive microbeads (Biolegend, USA) for macrophage purification.

2.2. Cell Line. Human THP-1 cells are cultured in RPMI 1640 with 10-20% fetal bovine serum (Gibco, USA). PMA (100 nM, Sigma, USA) is used to stimulated cells for 48 h to make them differentiate into macrophages. MiR-6869-5p mimics and miR-6869-5p mimic control are used to coculture with macrophages, which are purchased from Genechem Company (Shanghai, China). Lentivirus plasmids with or without overexpression of protein tyrosine phosphatase receptor type O (PTPRO) are used to transfect macrophages.

2.3. CCK-8 and EdU. CCK-8 kit (Vazyme Biotech Nanjing, China) is used to assess cell proliferation. In brief, macrophages (5×10^5/ml per well) with or without overexpression of PTPRO are seeded into 96-well plate for 12 h and, then, are treated by miR-6869-5p mimics and miR-6869-5p mimic control for 24 h and 48 h. 10 μl CCK-8 reagent solution is administrated into cells, which are incubated for 2 h. At last, the optical density (OD) at 450 nm is determined and data are calculated from three independent tests. EdU assay is also performed to estimate macrophage proliferation based on the protocol. Details have been shown in a previous study [8].

2.4. Real-Time PCR. Trizol reagent (Invitrogen, USA) is used to isolate RNAs from placental tissue macrophages and THP-1 cells according to the protocol. RNA (0.5 μg) is used as the template for cDNA synthesis based on the protocol of PrimeScriptTM RT Kit (Takara, Beijing, China). cDNA is applied for PCR amplification by use of Takara SYBR Premix. TaqMan PCR assay kit (ThermoFisher Scientific, USA) is used to determine the expression of miR-6869-5p in cells. Primers are as follows: Human PTPRO, F, TATTGTGAGCCTCCGT GTGT; R, GCCAAGCCTTTTCAGTGACA. Human IL-1β, F, ACGATGCACCTGTACGATCA; R, TCTTTCAACAC GCAGGACAG.

Human TNF-α, F, CCCTGAAAACAACCCTCAGA; R, AAGAGGCTGAGGAACAAGCA. Human GAPDH, F, ACCACAGTCCATGCCATCAC, R, TCCACCACCCTGT TGCTGTA.

Table 1: Characteristics of patients and controls.

n	Patients 26	Controls 23	P value
Age (y)	30.6 ± 4.4	29.2 ± 3.5	0.808
Weight (kg)	70.8 ± 5.5	64.6 ± 4.9	0.409
Infant weight (g)	3708 ± 110.2	3528 ± 124.5	0.283
Gestational weeks	38.1 ± 1.2	39.4 ± 1.2	0.449
Blood pressure			
SBP (mmHg)	119.3 ± 11.1	102.4 ± 10.3	0.274
DBP (mmHg)	71.9 ± 7.2	65.2 ± 8.1	0.538
Glucose metabolism			
Fasting glucose (mmol/L)	5.1 ± 1.0	4.2 ± 0.8	0.043
1 h glucose (mmol/L)	10.8 ± 1.7	6.09 ± 0.72	0.019
2 h glucose (mmol/L)	8.5 ± 1.4	5.3 ± 1.1	0.034
Fasting insulin (mIU/L)	9.6 ± 1.1	7.9 ± 1.3	0.320

2.5. Enzyme-Linked Immune Sorbent Assay (ELISA). As previously described [12], THP-1 macrophages with or without overexpression of PTPRO are incubated in serum-free medium for 12 h. Subsequently, macrophages are transfected by miR-6869-5p mimics or mimics control for 48 hours. IL-1β and TNF-α in cell culture supernatant are determined by ELISA according to the instructions of reagent kits (R & D Systems, USA). The optimal density is finally detected.

2.6. Luciferase Reporter Assay. pGL3 vectors carrying the luciferase reporter gene are used to clone the 3' untranscriptional region (3'UTR) of PTPRO (wild and mutant types). The luciferase activity is estimated using the system of dual luciferase reporter assay. Details have been presented in our previous study [8].

2.7. Flow Cytometry. Macrophages from placenta tissues of gestational diabetes mellitus patients with high or low expression of miR-6869-5p are incubated with FITC-conjuncted CD14 Ab, and PE-HLA-DR-conjuncted Ab, or PE-CD206-conjuncted Ab (Biolegend, San Diego, CA, USA) at room temperature for 30 min. Cells are then centrifugated and harvested for detection by flow cytometry. THP-1 macrophages (5×10^5/ml) with or without overexpression

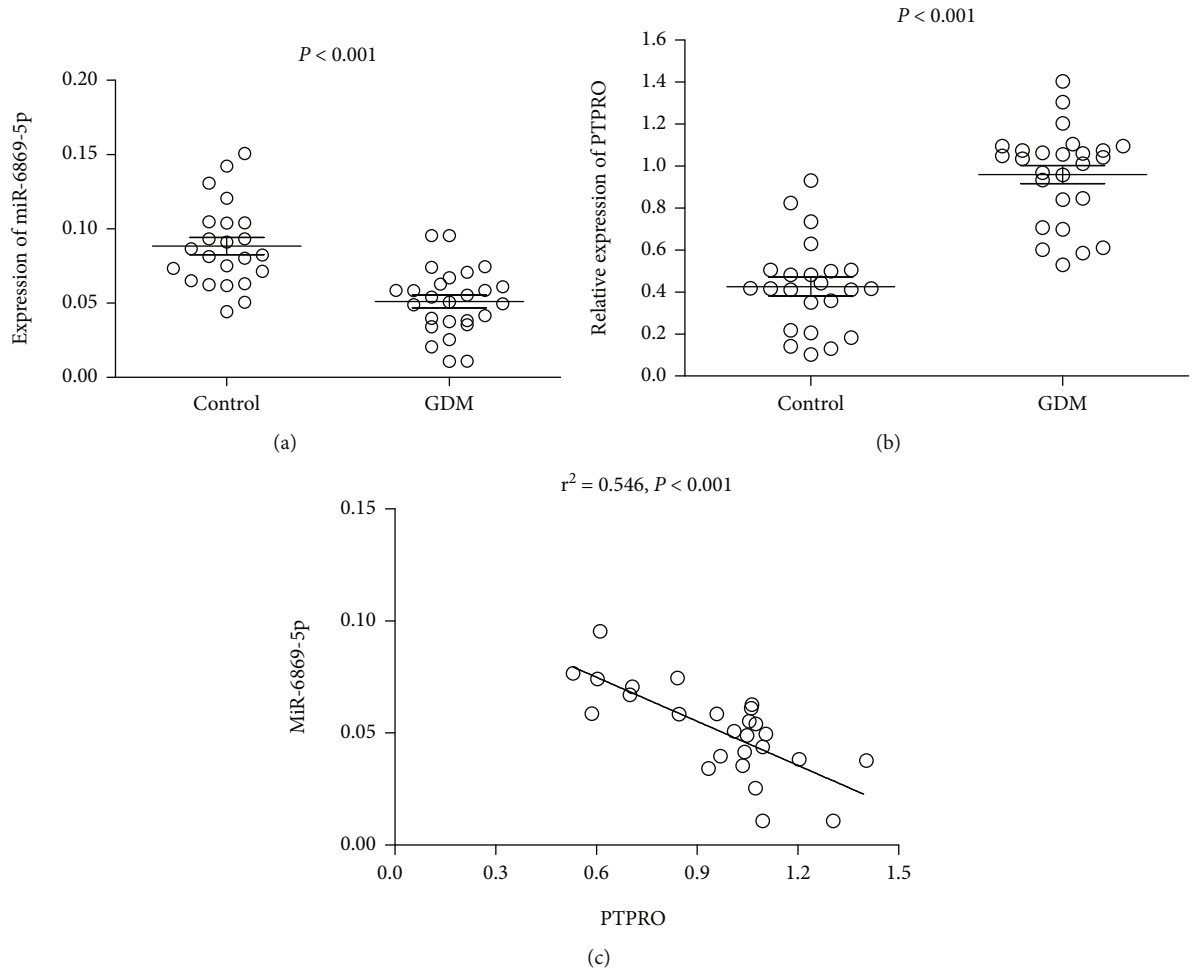

Figure 1: MiR-6869-5p and PTPRO expression in macrophages from placenta. (a) MiR-6869-5p expression in placenta derived macrophages (case/control: 26/23). (b) PTPRO expression in placenta derived macrophages (case/control: 26/23). (c) Association of miR-6869-5p with PTPRO in placenta derived macrophages of gestational diabetes mellitus (GDM) patients ($n = 26$).

of PTPRO are seeded into 24-well plate overnight. Then, cells by miR-6869-5p mimics or mimics control for another 24 h. After incubating with PE-HLA-DR-conjuncted Ab or PE-CD206-conjuncted Ab (Biolegend, San Diego, CA, USA) at room temperature for 30 min, we harvest cells and apply flow cytometry for determination.

2.8. Statistical Analysis. Mean ± SEM is used for data calculation. All results are normally distributed. The GraphPad Software and SPSS Software are used. Differences between two groups are statistically analyzed by use of independent sample Student's T-test for parametric data, while differences among more than three groups are estimated by ANOVA. $P < 0.05$ is considered to be significant.

3. Results

3.1. MiR-6869-5p Was Significantly Decreased in Macrophages from Placenta and Associated with M2 Macrophages Polarization. The expression of miR-6869-5p in placenta-derived macrophages from gestational diabetes mellitus patients was significantly reduced when comparing with that in placenta-derived macrophages from normal pregnancies (Figure 1(a)). Reversely, increased expression of PTPRO was found in placenta-derived macrophages from patients with gestational diabetes mellitus (Figure 1(b)). Negative association between miR-6869-5p and PTPRO was demonstrated regarding their expression in placental tissue-derived macrophages (Figure 1(c)). We also detected the expression of CD206+ macrophages and HLA-DR$^+$ macrophages in placental tissues derived macrophages. Interestingly, CD206$^+$ macrophages but not HLA-DR$^+$ macrophages were significantly increased in those patients with high expression of miR-6869-5p in placental tissue-derived macrophages, while CD206$^+$ macrophages but not HLA-DR$^+$ macrophages were significantly decreased in those patients with low expression of miR-6869-5p in placental tissue-derived macrophages (Figure 2). Accordingly, we hypothesize that miR-6869-5p might regulate macrophage polarization in placental immune microenvironment and induces macrophages towards M2 polarization in gestational diabetes mellitus.

3.2. MiR-6869-5p Inhibited Macrophage Proliferation by Targeting PTPRO. To assure whether miR-6869-5p could

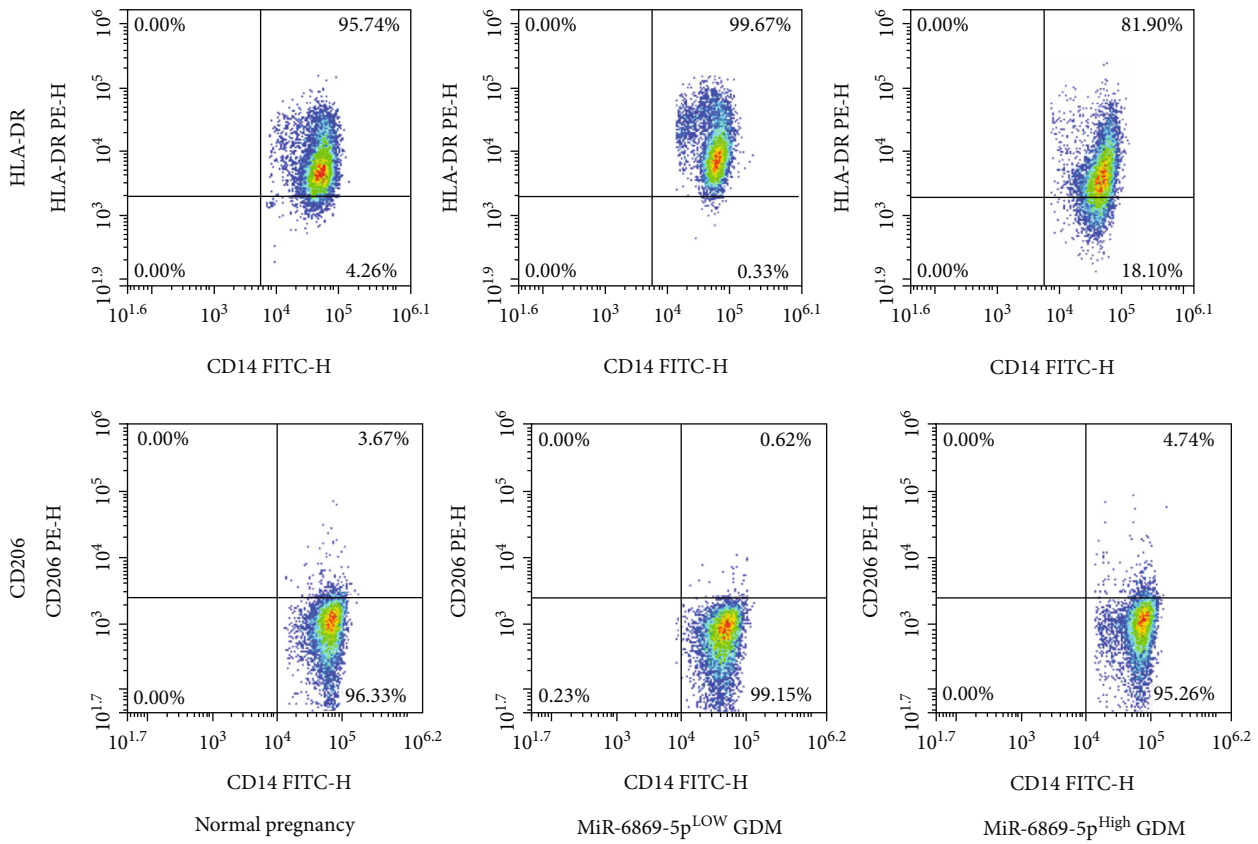

Figure 2: Ratio of CD206$^+$ macrophages and HLA-DR$^+$ macrophages in placenta of GDM patients compared with normal pregnancies.

regulate PTPRO as a target, we screened the Targetscan database and found that miR-6869-5p could recognize the 3'UTR sequence of PTPRO. The predicted consequential pairing of target region (top) and miRNA (bottom) was shown in Figure 3(a). The luciferase reporter assay further demonstrated our hypothesis that PTPRO was a target gene of miR-6869-5p in THP-1 macrophages (Figure 3(b)). In the following experiments, the altering effects of miR-6869-5p on macrophage proliferation were estimated. As evidenced by CCK-8 (Figure 3(c)) and EdU (Figure 3(d)), the proliferation of THP-1 macrophages could be effectively enhanced when PTPRO was overexpressed in macrophages, while miR-6869-5p mimics could successfully rescue its effect because the proliferation of THP-1 macrophages was significantly inhibited in miR-6869-5p mimics treated group compared with miR-6869-5p mimics control group.

3.3. MiR-6869-5p Prevented from Inflammation in Macrophages by Inducing M2 Macrophages. To estimate the effect of miR-6869-5p on macrophage, THP-1 macrophages were treated by miR-6869-5p mimics and simultaneously stimulated by LPS. As shown in Figures 4(a)–4(d), PTPRO overexpression could induce high expression of TNF-α and IL-1β at both levels of mRNA and protein in macrophages. Besides, reduced rate of CD206$^+$ macrophages and elevated rate of HLA-DR$^+$ macrophages was observed when PTPRO was overexpressed in macrophages (Figure 5). However, decreased expression of TNF-α and IL-1β in macrophages was observed when PTPRO-overexpressed cells were treated by miR-6869-5p mimics (Figures 4(a)–4(d)). Moreover, miR-6869-5p mimic-treated macrophages are more likely to differentiate into CD206$^+$ macrophages (Figure 5). Accordingly, miR-6869-5p could rescue the inflammatory response in macrophages induced by PTPRO. Taken together, miR-6869-5p is capable of preventing from inflammation in macrophages by inducing macrophages towards M2.

4. Discussion

Gestational diabetes mellitus is a complication of pregnancy, which poses high risks for both the mother and the fetus. The role of noncoding RNAs in gestational diabetes mellitus has drawing more and more attention in the past few years, such as long noncoding RNA and miRNA [14–16]. Although they do not encode active proteins or polypeptides, many noncoding RNAs are involved in the pathogenesis of gestational diabetes mellitus by RNA-RNA or RNA-protein interactions. Some circulating noncoding RNAs can serve as useful disease biomarkers for the onset and progression of gestational diabetes mellitus [17].

miRNA is a small noncoding RNA, which functions by targeting specific mRNAs. A number of miRNAs have been demonstrated to serve crucial roles in gestational diabetes mellitus by protecting pancreatic β-cell function, affecting insulin resistance, insulin sensitivity as well as liver gluconeogenesis, for instance, miR-143, miR-351, and miR-96 [18–

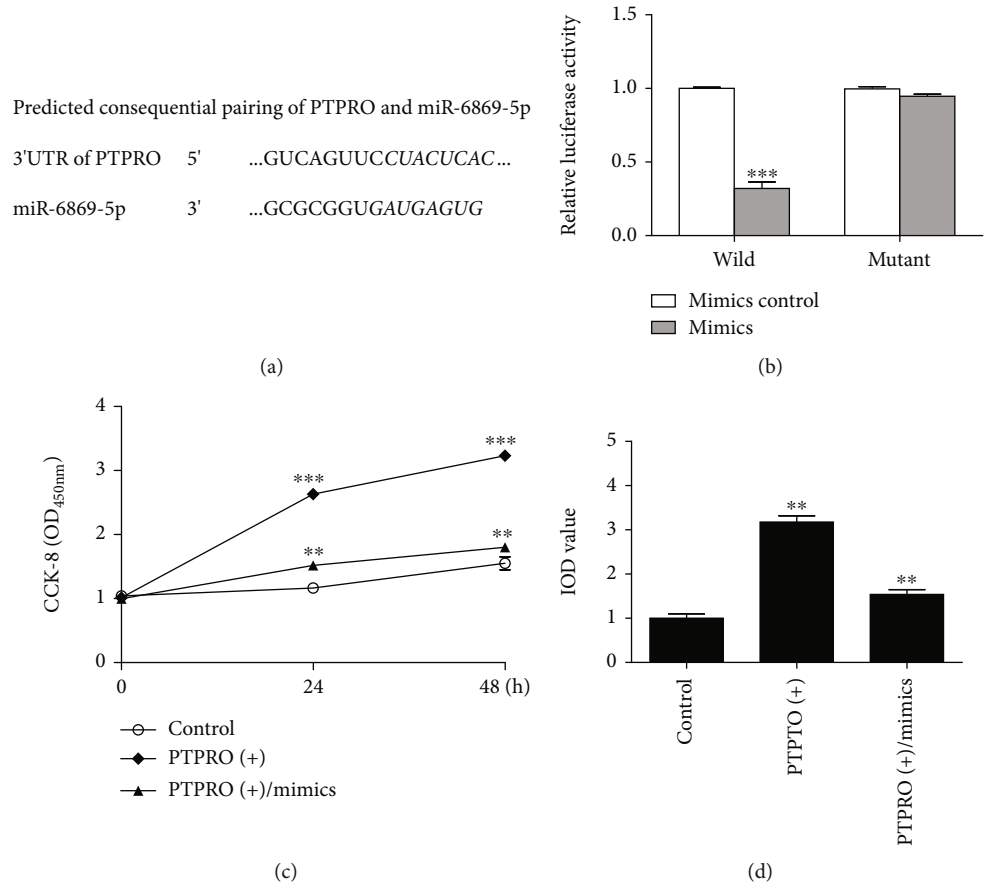

Figure 3: MiR-6869-5p regulated the proliferation of macrophage by targeting PTPRO. (a) The predicted consequential paring of miR-6869-5p and PTPRO 3'UTR. (b) The luciferase reporter assay: the activity of luciferase was significantly decreased in PTPRO wild type group compared with the PTPRO mutant type group in THP-1 macrophages treated by miR-6869-5p mimics ($^{***}P < 0.001$). (c) CCK-8: miR-6869-5p mimics inhibited the proliferation of macrophage although PTPRO was overexpressed (compared with the control group, $^{***}P < 0.001$; compared with the PTPRO (+) group, $^{**}P < 0.01$). (d) EdU: miR-6869-5p mimics inhibited macrophage proliferation (representative pictures of EdU assay and data of three independent experiments; compared with the control group, $^{**}P < 0.01$; compared with the PTPRO (+) group, $^{**}P < 0.01$).

20]. The study by Yan et al. has reported that miR-6869-5p was dysregulated in colorectal cancer and contributed to cancer cell proliferation, invasion, and migration by negatively regulating TLR4/NF-κB signaling pathway [13]. Exosome-encapsulated miR-6869-5p has also been demonstrated to participate in cancer [21]. However, the modifying effect of miR-6869-5p in macrophages mediated gestational disorders has not been evaluated. In this study, we aim to investigate the miR-6869-5p involvement in gestational diabetes mellitus. MiR-6869-5p is significantly downregulated in placenta derived macrophages from gestational diabetes mellitus patients. It is involved in regulating placental immune microenvironment and inducing macrophages towards M2. MiR-6869-5p prevents from macrophage proliferation and inflammation by targeting PTPRO and promoting macrophages polarization to M2 cells. Accordingly, miR-6869-5p can serve as a suppressor in macrophages mediated inflammatory and immune responses in gestational diabetes mellitus. However, the precise molecular mechanism regarding miR-6869-5p regulation in macrophages proliferation and polarization warrants to be elucidated by more future studies.

Mounting data have implicated that macrophages mediated local immunity plays an important role in maintain the balance of immune microenvironment in placenta [22–24]. Also, macrophages play a critical role in adipose tissue inflammation and immunity [3, 25, 26]. To the best of our knowledge, macrophages can be divided into two common cell types, namely, classically activated M1 and alternatively activated M2 [3, 27]. M1 cells usually possess proinflammation activity, while M2 cells exert anti-inflammation effects. The classic markers for M1 macrophages are CD11c, HLA-DR, and TNF-α. The typical markers for M2 macrophages are CD206, CD163, and IL-10. An anti-inflammatory M2 phenotype macrophages is essential for controlling gestational diabetes mellitus [28]. Previously, we have found M1/M2 balance is critical for the maintenance of the maternal-fetal interface immune balance [8]. M1/M2 imbalance would lead to sustained inflammation and immune disorders in the microenvironment of maternal-fetal interface, which may cause premature birth, stillbirth, and so on. Accumulating data have suggested miRNAs participate in the regulating of macrophages proliferation, differentiation, and

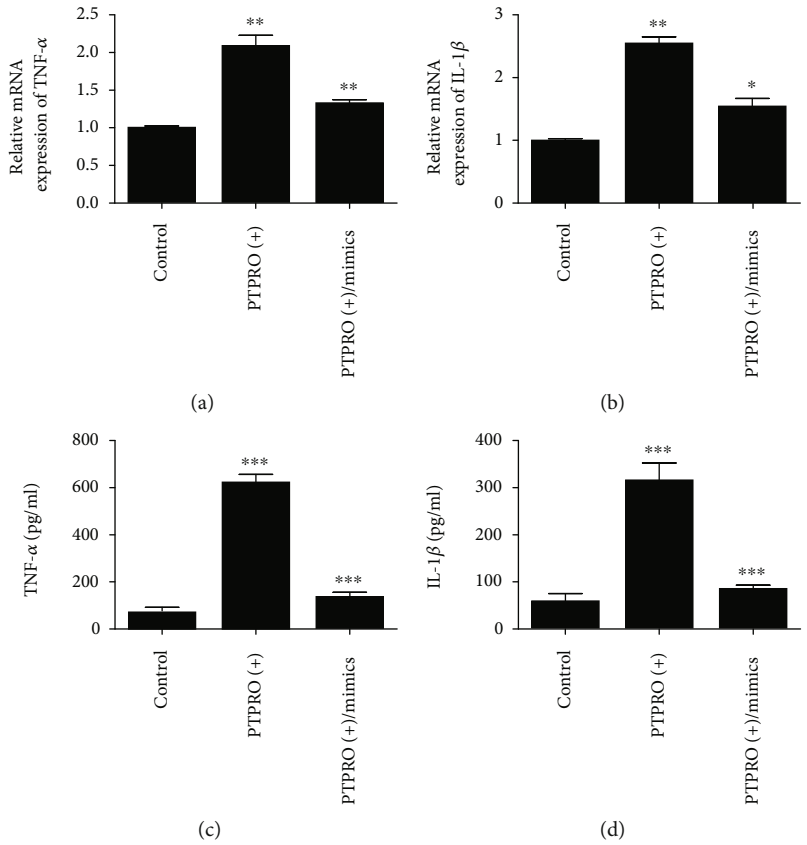

Figure 4: MiR-6869-5p prevented from the production of TNF-α and IL-1β in macrophages (compared with the control group, $^{**}P < 0.01$, $^{***}P < 0.001$; compared with the PTPRO (+) group, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). (a) mRNA expression of TNF-α. (b) mRNA expression of IL-1β. (c) TNF-α in the supernatant of macrophages. (d) IL-1β in the supernatant of macrophages.

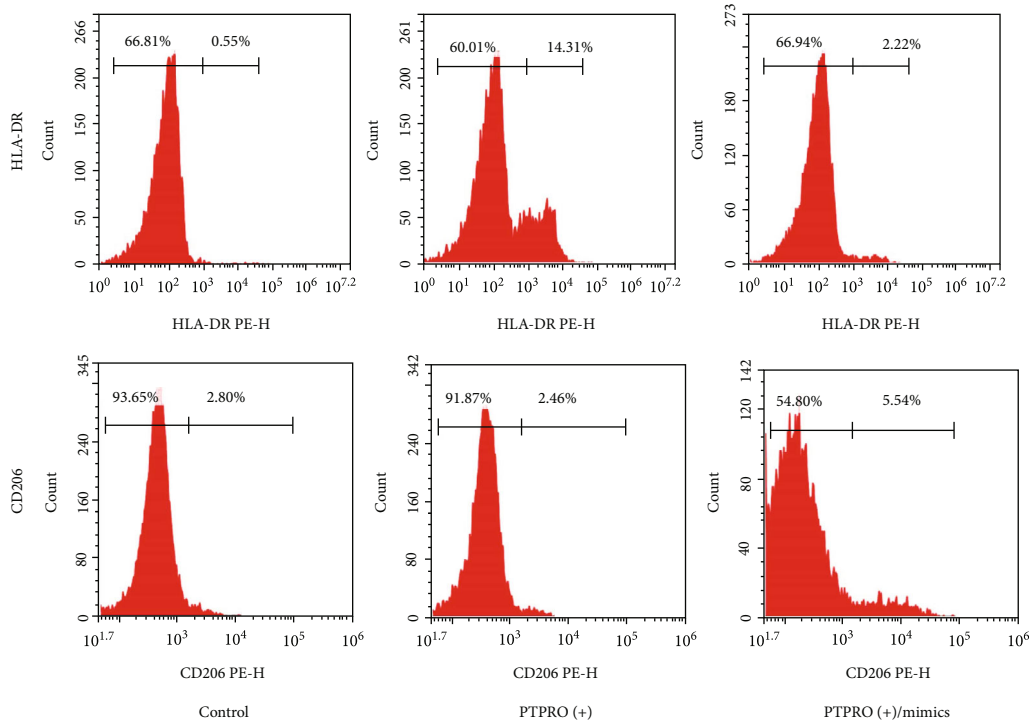

Figure 5: MiR-6869-5p promoted macrophages polarization towards M2.

polarization, which thus contributes to diabetes mellitus [29–31]. Nonetheless, the effect of miRNAs on macrophages polarization in gestational diabetes mellitus is largely unknown. In our study, miR-6869-5p is firstly documented to be positively associated with M2 polarization and protect normal pregnancy in patients with gestational diabetes mellitus. MiR-6869-5p is a promising marker for gestational diabetes mellitus.

PTPRO belongs to protein tyrosine phosphatase family. A number of studies have implicated that PTPRO participates in the regulation of macrophage-mediated inflammatory response, hepatic ischemia reperfusion injury, and tumor immunity [32–35]. In our previous study, PTPRO has been demonstrated to be significantly upregulated in preeclampsia patients, which is also found to be involved in regulating macrophage inflammation in preeclampsia [36]. The current study has firstly suggested that PTPRO is upregulated in placenta-derived macrophages from gestational diabetes mellitus patients. Therefore, we hypothesize that PTPRO may affect the differentiation and function of macrophages and, thus, participate in regulating local immune balance in the placenta. Findings of *in vitro* study have implicated that PTPRO can promote the expression of inflammatory cytokines TNF-α and IL-1β at both levels of mRNA and protein in macrophages. In addition, PTPRO is capable of inducing macrophages polarization towards M1 and enhancing the inflammatory response. As suggested by the bioinformatics analysis, PTPRO is a targeted gene of miR-6869-5p. MiR-6869-5p might negatively regulate PTPRO at the posttranscriptional level in macrophages. Interestingly, miR-6869-5p can prevent from inflammation by inducing higher expression of CD206 and Arg-1 but lower HLA-DR and CD11c in macrophages. As demonstrated by luciferase reporter assay, the well-established target of miR-6869-5p is PTPRO, a key factor in regulating macrophage mediated inflammation and immune disorders. In general, miR-6869-5p possesses anti-inflammation activity by targetedly regulating PTPRO and inducing M2 macrophages ultimately. Nevertheless, whether miR-6869-5p could exert the same effect *in vivo* needs to be investigated in future studies.

In summary, the present study has identified a miR-6869-5p signature involvement in gestational diabetes mellitus, which may contribute to maintain the balance of placental immune microenvironment by targeting PTPRO and inducing macrophages polarization towards M2.

Data Availability

Data can be available from upon requesting for the corresponding author.

Conflicts of Interest

All authors have declared no conflict of interest.

Authors' Contributions

Pingping Wang, Zhenzhi Ma, and Zengyan Wang are cofirst authors.

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Research Article

Cytokines and Water Distribution in Anorexia Nervosa

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In patients with anorexia nervosa (AN), decreased intracellular (ICW), extracellular (ECW), and total body water (TBW) as well as changes in serum cytokine concentrations have been reported. In this exploratory study, we measured body composition and serum cytokine levels in patients with AN ($n = 27$) and healthy controls (HCs; $n = 13$). Eating disorder symptom severity was assessed using the Eating Disorder Examination-Questionnaire (EDE-Q). Body composition was determined by bioimpedance analysis (BIA) which provided information on ICW, ECW, and TBW. Following blood collection, 27 cytokines and chemokines were quantified using multiplex ELISA-based technology: Eotaxin, Eotaxin-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon- (IFN-) γ, interleukin- (IL-) 1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12/IL-23p40, IL-12p70, IL-13, IL-15, IL-16, IL-17A, interferon γ-induced protein- (IP-) 10, macrophage inflammatory protein- (MIP-) 1α, MIP-1β, monocyte chemoattractant protein- (MCP-) 1, MCP-4, thymus and activation-regulated chemokine (TARC), TNF-α, and TNF-β. ICW, ECW, and TBW volumes were significantly lower in patients with AN than in HCs. In the whole sample, GM-CSF, MCP-4, and IL-4 were positively, whereas IFN-γ, IL-6, and IL-10 were negatively associated with all three parameters of body water. In AN participants, we found a statistically significant negative correlation of IL-10 with ICW, ECW, and TBW. Our results suggest an interaction between body water and the cytokine system. Underlying mechanisms are unclear but may involve a loss of water from the gut, kidneys, or skin due to AN-associated inflammatory processes.

1. Introduction

Anorexia nervosa (AN) is characterised by significantly low body weight, an intense fear of weight gain, and disturbed body perception [1]. Compared to healthy individuals, the overall mortality in people with AN is significantly increased with a standardized mortality ratio of ~5 [2]. AN is often accompanied by significant physical health problems, including renal insufficiency, urolithiasis, and disturbances in water and electrolyte balance, e.g., dehydration [3–6]. Bioimpedance analysis (BIA) allows assessment of water distribution and balance in the body, i.e., intracellular (ICW) and extracellular (ECW) water and total body water (TBW). In previous studies, decreased ICW, ECW, and TBW were found in patients with AN [7, 8], and these have been reported to increase during therapy and refeeding [9].

A second important etiopathological factor contributing to AN is inflammation. Meta-analyses indicate that AN is associated with elevated concentrations of the proinflammatory cytokines tumor necrosis factor- (TNF-) α and interleukin- (IL-) 6 [10, 11]. Such inflammatory molecules can be produced by macrophages in the periphery and by astrocytes and microglia in the brain and have been reported to affect systems with a role in the development of AN, i.e., by regulating appetite and food intake, mood, and cognition [12–17].

Although inflammation involving excessive production of proinflammatory cytokines and disturbances in the fluid balance have both been reported [7–11], a potential associa-

tion between cytokine release and water balance has not been explored in patients with AN. The ratio of ECW/TBW, i.e., the oedema index, has been reported to indicate the severity of malnourishment in severely sick patients, independent of their diagnosis, and to be associated with low serum albumin and haemoglobin [18]. Peripheral or pulmonary oedema can be a serious problem in AN, specifically during refeeding [19]. In AN, oedema may reflect heart problems [19] or a deficiency of osmotically potent molecules such as albumin and haemoglobin [6].

Therefore, we analysed body composition data related to water balance (ICW, ECW, TBW, and the oedema index) and potential associations of these parameters with cytokine concentrations in patients with AN.

2. Methods

2.1. Participants. Participants with AN ($n = 55$) and healthy controls ($n = 30$) were recruited as part of a larger study (for full study details, see [20, 21]). Female adults with a primary diagnosis of AN and a BMI < 17.5 kg/m^2 were recruited from Specialist Eating Disorder Services in and around London. HCs, free from a history of or current mental health disorder and physical illness, were recruited via an e-mail circular to students and staff at King's College London. Informed consent was obtained from all participants. The study was conducted in accordance with the Declaration of Helsinki, and the study received ethical approval from the South East London Research Ethics Committee (REC ref: 09/H0807/4).

To be eligible for the present analyses, AN participants were required to be free from autoimmune and/or inflammatory diseases and HCs were required to have a BMI within the healthy range (BMI 18.5-24.9 kg/m^2), leading to the exclusion of five AN participants and one HC. Participants also had to have serum samples available for analysis, resulting in a final sample of 27 participants with AN and 11 HCs.

2.2. Measurements. Height, weight, and body composition were obtained for all participants. Body composition was measured using a portable and noninvasive BIA InBody S10 machine (Biospace Co., Ltd.). This provides data on a range of parameters, including intracellular (ICW) and extracellular (ECW) water, total body water (TBW), and the oedema index (ECW/TBW). Eating disorder symptom severity was assessed using the Eating Disorder Examination-Questionnaire (EDE-Q) [22]. Several additional measures were collected and are reported elsewhere (see [20, 21]).

Following blood sample collection, serum was stored at -80°C prior to use. Serum was thawed at room temperature, and 40 inflammatory markers were quantified simultaneously using multiplex ELISA-based technology provided by the Meso Scale Discovery V-PLEX Human Biomarker 40-Plex Kit (Meso Scale Discovery, Maryland, USA). Plates were scanned on the Mesoscale Scale Discovery MESO Quickplex SQ 120 reader at the Social, Genetic and Developmental Psychiatry (SGDP) Centre, Institute of Psychiatry, Psychology and Neuroscience, King's College London. As the focus of the current study was on cytokines, rather than the broader group of inflammatory markers measured, only data on the following cytokines and chemokines ($n = 27$) were used: Eotaxin, Eotaxin-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon- (IFN-) γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12/IL-23p40, IL-12p70, IL-13, IL-15, IL-16, IL-17A, interferon γ-induced protein- (IP-) 10, macrophage inflammatory protein- (MIP-) 1α, MIP-1β, monocyte chemoattractant protein- (MCP-) 1, MCP-4, thymus and activation-regulated chemokine (TARC), TNF-α, and TNF-β. The cross-sectional comparisons of these data are reported elsewhere [23].

2.3. Statistical Analysis. Statistical analyses were performed in Stata 15 [24]. For demographic, anthropometric, and clinical characteristics, *t*-tests or Mann–Whitney *U* tests (depending on normality) were used to compare AN and HC participants. Due to the presence of outliers and nonnormal distributions, Mann–Whitney *U* tests were performed to compare cytokine data between AN and HC participants, and Spearman's rank correlations were performed to assess the relationship between body water parameters (ICW, ECW, TBW, and ECW/TBW) and cytokines (for the group as a whole and then for the patients and controls separately). The level of significance was set at $p < 0.05$, and as these are exploratory analyses, levels of significance were not adjusted for multiple testing.

3. Results

Demographic, anthropometric, and clinical characteristics are shown in Table 1. As expected, participants with AN reported significantly greater ED symptoms and had lower BMI and body fat percentages, compared to HCs. With regard to body water, ICW, ECW, and TBW were significantly lower in patients with AN than in HCs. There was no significant difference in the ratio between ECW and TBW.

Cytokine levels could not be detected in all serum samples. Therefore, Table 2 depicts the number of participants with undetectable cytokine concentrations for each cytokine in the whole sample. It also informs about the median serum concentrations of cytokines for the HC group and the group of people with AN. IL-6 and IL-15 levels were significantly higher in patients with AN compared to HCs, whereas TNF-β concentrations were lower in people with AN.

Tables 3 and 4 depict all correlations between cytokine concentrations and water distribution variables in the whole sample and in people with AN. In the following paragraphs, we will, however, give more detailed information on the significant correlations.

In the whole sample, ICW was significantly and positively correlated with IL-4 ($r_s(34) = 0.38$, $p = 0.0217$), GM-CSF ($r_s(31) = 0.43$, $p = 0.0121$), IL-1α ($r_s(36) = 0.33$, $p = 0.0409$), TNF-β ($r_s(36) = 0.33$, $p = 0.0414$), and MCP-4 ($r_s(36) = 0.33$, $p = 0.0448$). ICW had a significant negative association with IFN-γ ($r_s(36) = -0.34$, $p = 0.0351$), IL-10 ($r_s(36) = -0.45$, $p = 0.0047$), IL-6 ($r_s(36) = -0.36$, $p = 0.0247$), and IL-15 ($r_s(36) = -0.33$, $p = 0.0466$). In AN participants, ICW was correlated negatively with IL-10

Table 1: Demographic, anthropometric, and clinical characteristics for AN participants and HCs.

	Healthy controls		Anorexia nervosa		Cross-sectional comparison
	N	Mean ± SD	*N*	Mean ± SD	
Age (years)	11	24.82 ± 3.52	27	31.48 ± 11.40	$U = 144$, $z = -1.36$, $p = 0.1735$
EDE-Q global	11	0.73 ± 0.74	27	4.20 ± 1.27	$U = 85.5$, $z = -4.87$, $p < 0.0010$
BMI (kg/m^2)	11	21.13 ± 1.72	27	15.33 ± 2.25	$t(38) = 7.88$, $p < 0.0010$
Body fat (%)	10	17.83 ± 6.29	27	7.76 ± 6.07	$U = -22$, $z = 3.63$, $p = 0.0003$
ICW (l)	11	22.14 ± 2.39	27	17.47 ± 3.39	$t(36) = 4.15$, $p = 0.0002$
ECW (l)	11	13.15 ± 1.45	27	10.64 ± 1.80	$t(36) = 4.11$, $p = 0.0002$
TBW (l)	11	35.28 ± 3.82	27	28.11 ± 5.13	$t(36) = 4.18$, $p = 0.0002$
ECW/TBW ratio	11	0.37 ± 0.01	27	0.38 ± 0.01	$t(36) = -1.66$, $p = 0.1050$

Abbreviations: SD = standard deviation; EDE-Q = Eating Disorder Examination-Questionnaire; BMI = body mass index; ICW = intracellular water; ECW = extracellular water; TBW = total body water.

Table 2: Number of participants with undetectable cytokines for each cytokine in the whole sample ($n = 38$) with median serum concentrations (pg/ml, with interquartile range) of cytokines for HC ($n = 11$) and AN ($n = 27$) participants, with a significance value of group comparison.

	Undetectable[#] (*n* (%))	Healthy controls		Anorexia nervosa		*p*
		N	Median (IQR[†])	*N*	Median (IQR[†])	
Eotaxin	0	11	208.55 (160.77, 252.76)	27	175.47 (155.93, 279.31)	0.7113
Eotaxin-3	0	11	21.36 (17.35, 29.27)	27	15.15 (11.36, 25.58)	0.1147
GM-CSF	5 (13.2%)	11	0.24 (0.06, 0.52)	22	0.19 (0.12, 0.29)	0.6060
IFN-γ	0	11	3.28 (2.68, 4.60)	27	4.64 (2.89, 8.61)	0.1185
IL-1α	0	11	1.13 (0.65, 2.09)	27	1.01 (0.65, 1.29)	0.3761
IL-1β	10 (26.3%)	7	0.21 (0.04, 0.39)	21	0.19 (0.07, 0.25)	0.6907
IL-2	19 (50%)	6	0.12 (0.04, 0.28)	13	0.15 (0.09, 0.25)	0.9301
IL-4	2 (5.3%)	11	0.07 (0.04, 0.13)	25	0.05 (0.03, 0.08)	0.2717
IL-5	4 (10.5%)	10	1.12 (0.92, 1.74)	24	1.29 (0.72, 1.72)	0.7913
IL-6	0	11	0.30 (0.13, 0.42)	27	0.49 (0.35, 1.25)	0.0054
IL-7	0	11	15.45 (10.18, 17.44)	27	12.58 (10.46, 17.27)	0.9359
IL-8	0	11	37.24 (14.00, 118.30)	27	23.09 (10.27, 103.21)	0.3106
IL-10	0	11	0.24 (0.08, 0.26)	27	0.28 (0.11, 0.37)	0.3422
IL-12/IL-23p40	0	11	117.69 (81.15, 138.62)	27	92.00 (68.06, 118.65)	0.1185
IL-12p70	4 (10.5%)	9	0.15 (0.12, 0.19)	25	0.19 (0.11, 0.36)	0.5068
IL-13	14 (36.8%)	7	3.26 (2.13, 8.50)	17	2.44 (1.17, 4.30)	0.1197
IL-15	0	11	2.44 (2.23, 2.57)	27	2.90 (2.70, 3.51)	0.0021
IL-16	0	11	178.76 (138.29, 218.50)	27	183.59 (145.14, 339.36)	0.2274
IL-17A	0	11	1.78 (1.26, 3.20)	27	1.91 (1.08, 2.59)	0.9743
IP-10	0	11	110.93 (81.79, 151.33)	27	115.99 (98.45, 207.33)	0.3937
MIP-1α	0	11	27.16 (21.11, 33.29)	27	23.02 (20.10, 35.20)	0.9230
MIP-1β	0	11	115.67 (62.12, 141.36)	27	81.06 (65.78, 110.10)	0.2809
MCP-1	0	11	208.55 (165.87, 292.65)	27	191.53 (164.41, 241.73)	0.3937
MCP-4	0	11	149.14 (93.31, 195.49)	27	120.65 (86.10, 169.37)	0.3937
TARC	0	11	477.35 (221.30, 607.12)	27	370.33 (263.01, 641.46)	0.9359
TNF-α	0	11	1.59 (1.16, 2.51)	27	1.64 (1.34, 2.42)	0.8343
TNF-β	0	11	0.86 (0.70, 1.21)	27	0.60 (0.49, 0.69)	0.0096

[#]Below fit curve range. [†]25th and 75th percentile reported. Abbreviations: IQR = interquartile range; GMCSF = granulocyte-macrophage colony-stimulating factor; IFN-γ = interferon-γ; IL = interleukin; IP-10 = interferon γ-induced protein-10; MIP = macrophage inflammatory protein; MCP = monocyte chemoattractant protein; TARC = thymus and activation-regulated chemokine; TNF = tumor necrosis factor (TARC).

Table 3: Correlations (r_s) between cytokine concentrations and water distribution variables in the whole sample.

	ICW	ECW	TBW	ECW/TBW ratio
Eotaxin	0.03	0.08	0.06	-0.04
Eotaxin-3	0.17	0.14	0.18	-0.34*
GM-CSF	0.43*	0.45*	0.43*	-0.13
IFN-γ	-0.34*	-0.35*	-0.36*	0.25
IL-1α	0.33*	0.31	0.32*	-0.25
IL-1β	0.06	0.10	0.04	0.34
IL-2	0.09	0.04	0.11	0.29
IL-4	0.38*	0.43*	0.40*	-0.01
IL-5	-0.17	-0.09	-0.11	0.30
IL-6	-0.36*	-0.34*	-0.37*	0.32
IL-7	-0.02	-0.05	-0.01	-0.14
IL-8	-0.05	-0.04	-0.05	0.02
IL-10	-0.45*	-0.45*	-0.46*	0.31
IL-12/IL-23p40	0.18	0.12	0.16	-0.32*
IL-12p70	-0.02	-0.05	-0.02	-0.02
IL-13	0.17	0.07	0.12	-0.22
IL-15	-0.33*	-0.30	-0.32	0.23
IL-16	-0.13	-0.13	-0.14	0.04
IL-17A	0.04	-0.04	0.01	-0.29
IP-10	-0.11	-0.16	-0.13	-0.13
MIP-1α	0.15	0.05	0.10	-0.34*
MIP-1β	0.22	0.17	0.20	-0.22
MCP-1	0.09	0.08	0.11	-0.18
MCP-4	0.33*	0.38*	0.37*	-0.22
TARC	-0.14	-0.12	-0.11	-0.06
TNF-α	0.03	0.02	0.04	-0.09
TNF-β	0.33*	0.26	0.32	-0.30

∗ indicates $p < 0.05$. Abbreviations: GMCSF = granulocyte-macrophage colony-stimulating factor; IFN-γ = interferon-γ; IL = interleukin; IP-10 = interferon γ-induced protein-10; MIP = macrophage inflammatory protein; MCP = monocyte chemoattractant protein; TARC = thymus and activation-regulated chemokine; TNF = tumor necrosis factor (TARC); ICW = intracellular water; ECW = extracellular water; TBW = total body water.

Table 4: Correlations (r_s) between cytokine concentrations and water distribution variables in the AN sample only.

	ICW	ECW	TBW	ECW/TBW ratio
Eotaxin	0.02	0.13	0.09	0.04
Eotaxin-3	-0.11	-0.14	-0.10	-0.28
GM-CSF	0.38	0.39	0.36	-0.13
IFN-γ	-0.20	-0.21	-0.22	0.31
IL-1α	0.26	0.20	0.22	-0.28
IL-1β	-0.05	-0.05	-0.11	0.40
IL-2	-0.01	-0.04	0.02	0.51
IL-4	0.36	0.44*	0.39	0.07
IL-5	-0.38	-0.28	-0.31	0.39
IL-6	-0.23	-0.23	-0.26	0.25
IL-7	0.01	-0.03	0.02	-0.17
IL-8	-0.26	-0.28	-0.28	0.09
IL-10	-0.42*	-0.48*	-0.44*	0.25
IL-12/IL-23p40	0.00	-0.10	-0.04	-0.34
IL-12p70	0.12	0.05	0.10	-0.08
IL-13	0.10	-0.06	0.01	-0.09
IL-15	-0.20	-0.11	-0.15	0.25
IL-16	0.03	-0.00	-0.00	-0.04
IL-17A	0.18	0.04	0.13	-0.37
IP-10	0.06	-0.02	0.03	-0.19
MIP-1α	0.19	0.04	0.14	-0.43*
MIP-1β	-0.01	-0.09	-0.06	-0.26
MCP-1	-0.09	-0.10	-0.06	-0.11
MCP-4	0.30	0.44*	0.36	-0.02
TARC	-0.14	-0.12	-0.13	-0.03
TNF-α	-0.02	-0.05	-0.01	-0.16
TNF-β	0.12	0.04	0.11	-0.19

∗ indicates $p < 0.05$. Abbreviations: GMCSF = granulocyte-macrophage colony-stimulating factor; IFN-γ = interferon-γ; IL = interleukin; IP-10 = interferon γ-induced protein-10; MIP = macrophage inflammatory protein; MCP = monocyte chemoattractant protein; TARC = thymus and activation-regulated chemokine; TNF = tumor necrosis factor (TARC); ICW = intracellular water; ECW = extracellular water; TBW = total body water.

($r_s(25) = -0.42$, $p = 0.0275$), and in HCs, ICW correlated positively with GM-CSF ($r_s(9) = 0.66$, $p = 0.0260$).

In the whole sample, ECW had a significant positive association with IL-4 ($r_s(34) = 0.43$, $p = 0.0083$), GM-CSF ($r_s(31) = 0.45$, $p = 0.0093$), and MCP-4 ($r_s(36) = 0.38$, $p = 0.0181$). ECW significantly and negatively correlated with IFN-γ ($r_s(36) = -0.35$, $p = 0.0312$), IL-10 ($r_s(36) = -0.45$, $p = 0.0047$), and IL-6 ($r_s(36) = -0.34$, $p = 0.0354$). In AN participants, ECW was associated negatively with IL-10 ($r_s(25) = -0.48$, $p = 0.0124$), and positively with IL-4 ($r_s(23) = 0.44$, $p = 0.0288$), and MCP-4 ($r_s(25) = 0.44$, $p = 0.0203$). In HCs, ECW correlated with IL-4 ($r_s(9) = 0.61$, $p = 0.0454$) and GM-CSF ($r_s(9) = 0.68$, $p = 0.0216$).

In the whole sample, TBW was significantly and positively associated with IL-4 ($r_s(34) = 0.40$, $p = 0.0168$), GM-CSF ($r_s(31) = 0.43$, $p = 0.0138$), IL-1α ($r_s(36) = 0.32$, $p = 0.0485$), and MCP-4 ($r_s(36) = 0.37$, $p = 0.0241$). TBW was significantly and negatively correlated with IFN-γ ($r_s(36) = -0.36$, $p = 0.0255$), IL-10 ($r_s(36) = -0.46$, $p = 0.0039$), and IL-6 ($r_s(36) = -0.37$, $p = 0.0215$). In AN participants, TBW was negatively correlated with IL-10 ($r_s(25) = -0.44$, $p = 0.0215$). Figure 1 illustrates this negative correlation. In HCs, ICW correlated positively with GM-CSF ($r_s(9) = 0.66$, $p = 0.0260$).

The ECW/TBW ratio was significantly and negatively associated with IL-12/IL-23p40 ($r_s(36) = -0.32$, $p = 0.0490$), Eotaxin-3 ($r_s(36) = -0.34$, $p = 0.0372$), and MIP-1α ($r_s(36) = -0.34$, $p = 0.0369$). In AN participants, the ECW/TBW ratio was correlated negatively with MIP-1α ($r_s(25) = -0.43$, $p = 0.0241$). In HCs, the ECW/TBW ratio

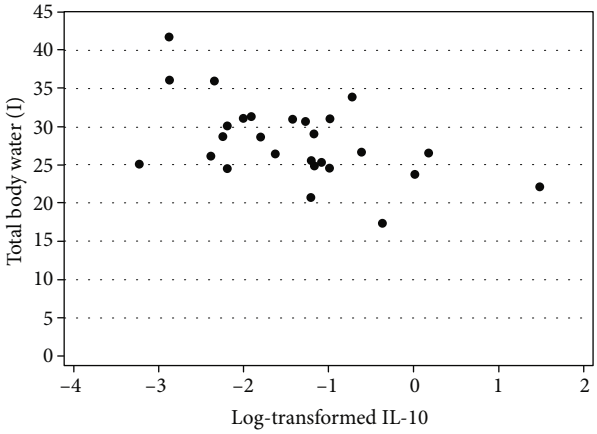

Figure 1: The association between total body water and IL-10 levels (log-transformed for improved presentation) in study participants with anorexia nervosa.

was negatively associated with MCP-4 ($r_s(9) = -0.76$, $p = 0.0062$).

As IL-10 was consistently associated with body water parameters both in the whole sample and the AN sample, we calculated correlations between IL-10 concentrations and body mass. In the whole sample, weight was correlated with IL-10 ($r_s(36) = -0.47$, $p = 0.0027$). In AN participants, weight was significantly associated with IL-10 ($r_s(25) = -0.55$, $p = 0.0028$).

4. Discussion

We replicated reports of decreased ICW, ECW, and TBW in patients with AN [7–9], i.e., the overall amount of water inside and outside of cells was decreased in patients with AN. This was not unexpected, as patients with AN have a lower total body mass and a lower total body volume. However, we found no differences in the oedema index between people with AN and HCs, i.e., there is no apparent shift in water from inside cells to the extracellular matrix.

In the whole study sample, several cytokines were significantly associated with ICW, ECW, and TBW. In particular, GM-CSF, MCP-4, and IL-4 were positively, whereas IFN-γ, IL-6, and IL-10 were negatively associated with all three body water measures. From studies in patients with inflammatory diseases, it appears that cytokines can promote water loss via the gut (by increasing intestinal permeability) [25], via electrolyte and water transport in the kidneys [26], via the lungs (by impeding the reabsorption of salt and water leading to an increased movement of fluid from the lung interstitium to the alveolar lumen [27] or via transdermal water loss [28]). Dehydration and changes in water distribution, in turn, may also influence inflammation and the production of pro-inflammatory cytokines. For example, water restriction is reported to lead to hypovolaemia and reduced kidney perfusion in AN, which can lead to tubular necrosis, repeated urinary tract infection, or nephrocalcinosis [29], and these conditions have been reported to be associated with changes in cytokine signalling [30–32]. However, these mechanisms of the mutual influence of cytokines and the fluid balance may only play a role in people with much higher cytokine concentrations, as can be found in acute inflammatory and infectious diseases. They have not been investigated in HCs or in patients with AN yet. In this context, it is of note that the cited articles refer to experimental and clinical studies from gastrointestinal, renal, lung, and skin research and thus may not be relevant to the physiological regulation of water balance in healthy people and people with eating disorders.

Nevertheless, given that patients with AN had decreased ICW, ECW, and TBW in this and previous studies, and given that some cytokines were statistically associated with parameters of fluid balance in the whole sample, it seems justified to have a more detailed look at the associations between cytokines and ICW, ECW, and TBW in patients with AN. In AN participants, the most notable result is the statistically significant and negative correlation of IL-10 with ICW, ECW, and TBW. IL-10 also correlated negatively with body weight. Thus, IL-10 could be indirectly connected with ICW, ECW, and TBW via the low body weight.

The association of IL-10 with water volumes can be explained in several ways: (a) IL-10 may influence water balance by decreasing fluid intake or by increasing water excretion, (b) hypohydration may lead to an increase of IL-10 production, or (c) weight could influence both IL-10 levels and water balance. However, as body water contributes to weight, these two parameters are not independent. IL-10 regulates the growth and differentiation of B cells, NK cells, cytotoxic and helper T cells, mast cells, granulocytes, dendritic cells, keratinocytes, and endothelial cells [33]. It has also been proposed that it reduces thirst: Acacia catechu, a traditional thirst quencher of South India, has been reported to increase IL-10 production [34], but also a direct effect of IL-10 on thirst was seen in a previous study [35]. Therefore, it is unclear whether IL-10 can reduce thirst in humans. IL-10 is highly secreted in mucosal tissues such as the gut, and studies have confirmed the role of IL-10 in controlling gut inflammation and establishing mutually beneficial commensalism of intestinal microbiota with the mammalian hosts [36]. IL-10 also affects kidney health and function. It can be protective under some conditions but has also been reported to aggravate defects in renal function [37]. Overall, however, it is unclear whether IL-10 can lead to an increased fluid loss via the gut or the kidneys. An additional factor that may influence both IL-10 levels and water content of the body is physical exercise. IL-10 production has been reported to increase after physical exercise [38–40], and physical exercise is also well known as a cause of hypohydration [41]. However, IL-10 levels did not differ between patients with AN and HCs in this study [23]. Therefore, this assumption would be speculation. In fact, we can assume that about 30 percent of people with AN do not exercise regularly [42]. Taken together, there is no literature on the influence of IL-10 on the bodily water content or vice versa.

We found that IL-6 and IL-15 levels were significantly higher in patients with AN compared to HCs, whereas TNF-β concentrations were lower in people with AN. The finding of elevated IL-6 levels is in line with previous meta-analytic research [10]. Interestingly, people suffering from obesity have also been reported to have an increased

production of proinflammatory cytokines such as IL-6. In people with obesity, these proinflammatory cytokines are most likely released by inflammatory cells infiltrating the adipose tissue [43]. This hypothesis that an increase in proinflammatory cytokines is a consequence of inflammatory cells in the adipose tissue cannot be applied to people with AN. In these patients, it has been speculated that increased oxidative stress, chronic physiological and psychological stress, changes in the intestinal microbiota, and an abnormal bone marrow microenvironment contribute to changes in their immune system and thus in cytokine production [44].

Our study has some limitations. Our sample size is relatively small, and we did not control for multiple testing, i.e., our study is exploratory. Our cross-sectional approach limits speculation on the direction of causality. Measurement of body water by BIA uses electrical impedance, i.e., it does not directly determine body water [45], and thus, dehydration and electrolyte imbalances may lead to problems with measurement accuracy [46]. However, it has been suggested that these issues are more related to the measurement of fat mass than to the measurement of body water [46].

5. Conclusion

In conclusion, our results suggest an interaction between body water distribution and the production of certain cytokines, namely, GM-CSF, MCP-4, IL-4, IFN-γ, IL-6, and IL-10. The changes of body water and its distribution may occur via the gut, kidneys, or skin due to AN-associated inflammatory processes. They may be due to the physical health consequences of AN or be in part associated with overexercising in these patients. Further research that employs a larger sample size and a longitudinal approach to address the direction of causality is needed.

Data Availability

The ethics approval of this study does not include sharing the underlying data set. However, Iain Campbell (email: iain.campbell@kcl.ac.uk) may be contacted for specific requests.

Disclosure

The views expressed are those of the authors and not necessarily those of the NHS, the NIHR, or the Department of Health.

Conflicts of Interest

The authors declare no conflict of interest.

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Research Article

Glutamine Administration Attenuates Kidney Inflammation in Obese Mice Complicated with Polymicrobial Sepsis

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Obesity is a well-known public health issue around the world. Sepsis is a lethal clinical syndrome that causes multiorgan failure. Obesity may aggravate inflammation in septic patients. Glutamine (GLN) is a nutrient with immune regulatory and anti-inflammatory properties. Since sepsis is a common contributing factor for acute kidney injury (AKI), this study investigated the effects of GLN administration on sepsis-induced inflammation and AKI in obese mice. A high-fat diet which consists of 60% of calories from fat was provided for 10 weeks to induce obesity in the mice. Then, the obese mice were subdivided into sepsis with saline (SS) or GLN (SG) groups. Cecal ligation and puncture (CLP) was performed to produce sepsis. The SS group was intravenously injected with saline while the SG group was administered GLN one or two doses after CLP. Obese mice with sepsis were sacrificed at 12, 24, or 48 h post-CLP. Results revealed that sepsis resulted in upregulated high-mobility group box protein-1 pathway-associated gene expression in obese mice. Also, expressions of macrophage/neutrophil infiltration markers and inflammatory cytokines in kidneys were elevated. Obese mice treated with GLN after sepsis reversed the depletion of plasma GLN, reduced production of lipid peroxides, and downregulated macrophage/neutrophil infiltration and the inflammatory-associated pathway whereas tight junction gene expression increased in the kidneys. These findings suggest that intravenously administered GLN to obese mice after sepsis alleviated inflammation and attenuated AKI. This model may have clinical application to obese patients with a risk for infection in abdominal surgery.

1. Introduction

Obesity is an important public health issue worldwide because of its high global prevalence. Obesity is mainly caused by high calorie intake and deficiency of physical activity that develop gradually with cellular physiologic changes before the symptoms of diseases become apparent. Obese individuals present systemic markers of chronic low-grade inflammation. Also, obesity results in dysregulation of immune responses [1]. These changes are closely associated with metabolic disturbances that may have adverse impact on host immunity during infection and promote disease progression [1, 2]. Obese subjects are considered frail with increased risk of recurrent nosocomial infections [2]. In the critically ill population, a previous study found that compared to normal weight, obese patients have higher risk of infectious complications that lead to sepsis [3].

Sepsis is a lethal clinical syndrome with multiorgan failure [4]. It is considered that the dysregulated immune reaction in response to infection results in dysregulation between pro- and anti-inflammatory responses that ultimately lead to irreversible multiorgan failure [5]. For critically ill patients, acute kidney injury (AKI) is an independent risk factor for mortality, and the most common contributing factor for AKI is sepsis [6]. The pathogenesis of sepsis-induced AKI is complicated and multifactorial. Although both the pro- and anti-inflammatory mechanisms are involved, inflammation is the key component and proinflammatory cytokine production can be used as predictor of AKI among septic patients [7]. Previous studies found that there were different reactions between obese and lean animals in respect to the septic insult. Obese animals exhibited more severe symptoms than the lean ones and suggest that compared with the lean animals, obesity exaggerates

inflammatory response during sepsis [8–10]. Obesity was considered to be an independent risk factor for mortality in critically ill populations [11] and has become a major concern in septic patients.

Glutamine (GLN) is a nutrient with anti-inflammatory and immunomodulatory properties. Although a multicenter clinical study revealed that GLN administration was associated with an increased mortality among critical patients with multiorgan failure [12], subsequent clinical trials found that parenteral GLN supplementation is safe in surgical intensive care unit (ICU) patients and improved outcomes in stabilized patients with organ function [13, 14]. Previous studies performed by our laboratory revealed that GLN administration attenuates inflammatory reaction and remote organ injury in sepsis [15–17]. GLN was found to elicit a more-balanced T helper cell polarization [16], decreased programmed cell death 1 expression on immune cells [18], and downregulated high-mobility group box protein-1- (HMGB-1-) mediated pathway [19], thus alleviating kidney injury in sepsis. However, the metabolic stress derived from obesity exposed to an acute inflammatory stimulus, such as sepsis, is different from sepsis alone. As we know, there is no study investigating the impact of GLN on inflammation and AKI in obesity complicated with sepsis. This study used a high-fat diet to induce obesity; thereafter, cecal ligation and puncture (CLP) was performed to create a mouse model of obesity with polymicrobial sepsis. We hypothesized that treatment with GLN after sepsis may have beneficial effects on attenuating inflammatory response and subsequent AKI in obese mice concurrent with sepsis.

2. Materials and Methods

2.1. Animals. Male C57BL/6 mice (5 weeks old with ~20 g in body weight) were used in this study. Mice were housed in the Laboratory Animal Center at the Taipei Medical University (TMU; Taipei, Taiwan). The animal center conditions were21 ± 2°Cand relative humidity of 50% ~55% with a 12 h light-dark cycle. Standard rodent chow diet (Purina no. 5001, Fort Worth, TX, USA) and water were provided *ad libitum* during the acclimation period. Care and use of laboratory animals were in compliance with the *Guide for the Care and Use of Laboratory Animals* [20]. The experimental protocols were approved by the Animal Care and Use Committee of TMU.

2.2. Experimental Procedures. In the beginning, mice were randomly assigned to a normal control (NC, $n = 6$) group and a high-fat (HF, $n = 60$) group. Mice in the NC group were provided standard rodent chow diet, while those in the HF group were fed a high-fat diet which consists of 60% kcal as fat [21] for 10 weeks. The composition of the diet is supplied by a commercial company (Research Diets, New Brunswick, NJ, USA) as shown in Table 1. After 10 weeks of feeding, mice in the HF group were subdivided into sepsis with saline (SS, $n = 30$) and sepsis with GLN (SG, $n = 30$) groups, then subjected to CLP as previously described [22]. Mice were injected intraperitoneally with Zoletil (25 mg/kg body weight (BW), Virbac, Carros, France) and Rompun (10 mg/kg BW, Bayer, Leverkusen, Germany) for anesthetization. To open the peritoneum, the abdominal wall was incised with about a 1 cm incision. Then, the cecum was ligated at 50% below the ileocecal valve and was punctured through with a 23-gauge needle. A small drop of feces was squeezed out and smeared onto the abdomen. The abdomen was closed with continuous suture. The CLP operation in all animals was performed by the same technician to ensure consistency. After the operation, sterile saline (4 mL/kg BW) was subcutaneously rehydrated and free access to water and rodent chow. One hundred microliters of 0.25% bupivacaine was administered at the incision site before skin closure to relieve pain after the surgery. Mice in the SS and SG groups were sacrificed at 12, 24, or 48 h post-CLP, respectively, according to their schedule. Mice sacrificed at 12 and 24 h after CLP were injected with a single dose of either saline or GLN (0.75 g GLN/kg BW) intravenously via a tail vein 1 h after CLP. GLN was provided as alanyl-glutamine dipeptide (Dipeptiven; Fresenius Kabi, Homburg, Germany). This dosage was previously shown to have immunomodulatory effects on sepsis [18, 23]. Mice euthanized at 48 h were injected with another dose of saline or GLN 24 h after the first injection to enhance the efficacy of GLN. At the end of the experiment, mice were anesthetized and euthanized by cardiac puncture. Body weight and epididymal tissue weights were recorded. Blood samples were collected and centrifuged to obtain plasma. The peritoneum was opened and irrigated with saline to obtain peritoneal lavage fluid (PLF). Kidneys were excised. All the samples were stored at −80°C for further analysis.

Table 1: Composition of the high-fat diet.

Ingredient	g/kg
Casein	259.13
L-Cysteine	3.89
Maltodextrin	161.96
Sucrose	89.14
Cellulose	64.78
Soybean oil	32.39
Lard	317.44
Mineral mix[1]	12.96
Dicalcium phosphate	16.84
Calcium carbonate, $1H_2O$	7.13
Potassium citrate	21.38
Vitamin mix[2]	12.96
Total	1000

[1]The composition of the mineral mixture is listed as follows (mg/g): calcium phosphate dibasic, 500; sodium chloride, 74; potassium sulfate, 52; magnesium oxide, 24; potassium citrate monohydrate, 20; manganese carbonate, 3.5; ferric citrate, 6; chromium potassium sulfate, 0.55; zinc carbonate, 1.6; cupric carbonate, 0.3; potassium iodate, 0.01; and sodium selenite, 0.01. [2]The composition of the vitamin mixture is listed as follows (mg/g): DL-α-tocopherol acetate, 20; nicotinic acid, 3; retinyl palmitate, 1.6; calcium pantothenate, 1.6; pyridoxine hydrochloride, 0.7; thiamin hydrochloride, 0.6; riboflavin, 0.6; cholecalciferol, 0.25; D-biotin, 0.05; menaquinone, 0.005; and cyanocobalamin, 0.001.

2.3. Measurements of Biochemical Markers and Chemokines in Plasma. Kidney function markers (blood urea nitrogen (BUN) and creatinine (Cre)) were analyzed by the VetTest® Chemistry Analyzer (IDEXX Laboratories Inc., Westbrook, MN, USA). Kidney injury markers (neutrophil gelatinase-associated lipocalin (NGAL)) were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems Inc., Minneapolis, MN, USA). Inflammatory chemokines (monocyte chemoattractant protein-1 (MCP-1) and keratinocyte-derived chemokine (KC)) were also measured by ELISA kits (R&D Systems Inc., Minneapolis, MN, USA). Antibodies (Abs) specific to mouse NGAL, MCP-1, and KC were precoated on the wells of microtiter strips. Plasma samples were incubated and developed with reagents. The absorbance of each well was measured by a spectrophotometer. The analyzing procedures were instructed by the protocols provided by the manufacturer.

2.4. Measurement of Plasma Amino Acid Concentrations. A Waters AccQ-Tag derivatization kit (Milford, MA, USA) was used to prepare the plasma samples. Using the ACQUITY UPLC System (Waters), the samples were applied to ultraperformance liquid chromatography (UPLC) for separation. A Xevo TQ-XS (Waters) mass spectrometer was used for monitoring. The amino acid concentrations were measured by Waters MassLynx 4.2 software and quantified by TargetLynx.

2.5. Inflammatory Mediator Concentrations in PLF. Interleukin-10 (IL-10), tumor necrosis factor-α (TNF-α), MCP-1, and KC were measured by ELISA kits in a microtiter plate (R&D Systems Inc., Minneapolis, MN, USA). Details are mentioned above.

2.6. Messenger (m)RNA Extraction and Analysis of a Real-Time Reverse-Transcription (RT) Quantitative Polymerase Chain Reaction (qPCR). Kidney tissues were homogenized and total RNA was isolated by a TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA pellets were dissolved in RNase-free water and stored at -80°C for further analysis. Using a spectrophotometer, RNA concentrations were quantified at absorbances of 260 and 280 nm. We used a RevertAid™ first-strand complementary (c)DNA synthesis kit (Thermo Fisher Scientific, Vilnius, Lithuania) to synthesize cDNA. cDNA was stored at -80°C until being used. RT was performed by subsequent incubation for 5 min at 65°C, 60 min at 42°C, and 5 min at 70°C. Messenger RNA genes were amplified by a real-time RT-PCR using the 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with SYBR Green I as the detection format. Genes measured in kidney tissues included the high-mobility group box protein (HMGB)-1-associated pathway (HMGB-1, myeloid differentiation factor (MyD)88, toll-like receptor- (TLR-) 4, and nuclear factor- (NF-) κB), inflammatory cytokines and chemokines (IL-6, TNF-α, and MCP-1), macrophage infiltration markers (cluster of differentiation (CD)68 and epidermal growth factor-like module-containing mucin-like hormone receptor-like-1 (EMR-1)), kidney injury molecule-1 (Kim-1), and tight junction protein zonula occluden-1 (ZO-1). Primers used are listed in Table 2. All primers were provided by Mission Biotech (Taipei, Taiwan) based on deposited cDNA sequences (GenBank database, NCBI). A total volume of 25 μL containing Maxima SYBR Green/ROX qPCR Master Mix (2x) (Thermo Fisher Scientific), 100 ng of cDNA, and 40 nM of each primer was used for amplification. The reaction was processed by one cycle of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C, and a final dissociation curve (DC) was analyzed. The gene expression levels were quantified in duplicate by means of a real-time RT-PCR. The relative mRNA expression was calculated by cycle threshold (CT) values and normalized to mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

2.7. Analysis of Myeloperoxidase (MPO) Activity in Kidney Homogenates. Kidney tissue were washed with cold phosphate-buffered saline (PBS) and then homogenized with MPO assay buffer (1 : 10, wt/vol). The homogenates were centrifuged for 10 min at 13,000 g to remove insoluble materials. The supernatants were collected and measured by Myeloperoxidase Activity Assay kit (Abcam, Cambridge, MA, USA). The reagents and samples were added to the 96-well plate and incubated for 1 hr. MPO activities were determined at 412 nm optical density using a spectrophotometer and expressed as a unit/mg protein [23]. The analyzing procedures were instructed by the protocols provided by the manufacturer. A Bradford protein assay reagent kit (Bio-Rad, Richmond, CA) was used to measure protein concentrations.

2.8. Analysis of Thiobarbituric Acid Reactive Substance (TBARS) in Kidney Tissues. Kidney tissues were homogenized at 4°C in a reagent prepared by protease and phosphatase inhibitor (Thermo Fisher Scientific, Vilnius, Lithuania) and Tissue Protein Extraction Reagent (T-PER™, Thermo Fisher Scientific) (1 : 100). The homogenates were centrifuged at 12,000 rpm for 10 min. The supernatants were used for quantifying TBARS. The TBARS consists of malondialdehyde (MDA) and thiobarbituric acid-reacted lipid peroxidation end products. TBARS levels were analyzed by a commercial assay kit (Cayman, MI, USA) and determined at 530-540 nm optical density. The concentrations of TBARS were expressed as μM/mg protein. The Bradford protein assay kit (Bio-Rad) was used to analyze protein concentrations.

2.9. Statistical Analysis. All data are presented as the mean ± standard error of the mean (SEM). Data were analyzed with the GraphPad Prism 5 statistical software program (GraphPad Software, La Jolla, CA, USA). Differences between 2 sepsis groups at the same time point were analyzed by t-test. The comparison among NC and the sepsis groups at three different time points was analyzed by a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. A p value of <0.05 was considered statistically significant.

Table 2: Sequences of oligonucleotide primers used for PCR amplification.

Gene name	Primer sequence (5′→3′)	Accession no.
NF-κB	F: TTAGCCAGCGAATCCAGACC R: AGTTCCGGTTTACTCGGCAG	M61909.1
HMGB-1	F: CCATTGGTGATGTTGCAAAG R: CTTTTTCGCTGCATCAGGTT	NM_010439.4
MyD88	F: CATGGTGGTGGTTGTTTCTGAC R: TGGAGACAGGCTGAGTGCAA	NM_010851.2
TLR4	F: AGAAATTCCTGCAGTGGGTCA R: TCTCTACAGGTGTTGCACATGTCA	NM_021297.2
Kim-1	F: GCATCTCTAAGCGTGGTTGC R: TCAGCTCGGGAATGCACAA	NM_134248.2
MCP-1	F: GATTCACATTTGCGCTGCCT R: TGAGCCTGGGAGATCACCAT	U12470.1
TNF-α	F: ATGGCCTCCCTCTCATCAGT R: TTTGCTACGACGTGGGCTAC	NM_013693.3
IL-6	F: TCCTACCCCAACTTCCAATGCTC R: TTGGATGGTCTTGGTCCTTAGCC	NM_012589.1
CD68	F: TGTTCAGCTCCAAGCCCAAA R: ACTCGGGCTCTGATGTAGGT	NM_001291058.1
EMR-1	F: ACCTTGTGGTCCTAACTCAGTC R: ACAAAGCCTGGTTGACAGGTA	U66889.1
ZO-1	F: GATGTTTATGCGGACGGTGG R: AAATCCAAACCCAGGAGCCC	BC138028.1
GAPDH	F: AACGACCCCTTCATTGAC R: TCCACGACATACTCAGCAC	M32599.1

NF-κB: nuclear factor-κB; HMGB-1: high-mobility group box protein-1; MyD88: myeloid differentiation factor 88; TLR4: toll-like receptor-4; Kim-1: kidney injury molecule-1; MCP-1: monocyte chemoattractant protein-1; TNF-α: tumor necrosis factor-α; IL-6: interleukin-6; CD68: cluster of differentiation 68; EMR-1: epidermal growth factor-like module-containing mucin-like hormone receptor-like-1; ZO-1, zonula occluden-1; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

3. Results

3.1. Changes in Epididymal Adipose Tissue and Body Weights after High-Fat Diet Feeding. The initial BWs did not differ between the NC and HF groups. After 10 weeks of feeding, mice in the HF group had higher epididymal fat weights (NC 0.63 ± 0.02 g vs. HF 2.54 ± 0.09 g, $p < 0.0001$) than those in the NC group. Also, BWs were higher in the HF group than in the NC group (NC 26.4 ± 0.7 g vs. HF 36.5 ± 1.1 g, $p < 0.0001$).

3.2. Plasma Concentrations of Biochemical Markers and Inflammatory Chemokines. Plasma BUN, Cre, NGAL, KC, and MCP-1 concentrations were significantly elevated after CLP. There were no differences in BUN, Cre, and MCP-1 between the SS and SG groups at 12, 24, or 48 h post-CLP. NGAL levels in the SG group were significantly lower at 24 h and KC were lower at 12 h than those in the SS group post-CLP (Table 3).

3.3. Plasma Amino Acid Concentrations. Glutamate, GLN, and branch-chain amino acids (BCAAs) including leucine, valine, and isoleucine levels were measured at 12, 24, and 48 h after CLP. The SS group had lower glutamate and GLN concentrations at 12 and 24 h whereas the BCAAs were higher at 12 h post-CLP when compared to those of the NC group. The SG group exhibited higher glutamate levels at 12 and 24 h and GLN at all the three time points than the SS group after CLP. The isoleucine and valine concentrations were lower at 12 h, while leucine at 12 and 24 h after CLP in the SG group than those in the SS groups (Figure 1).

3.4. PLF Cytokine and Chemokine Levels. Sepsis resulted in elevation of TNF-α, IL-10, KC, and MCP-1 concentrations in obese mice. Compared to the SS group, the SG group exhibited lower TNF-α at 48 h and KC and MCP-1 at 12 h whereas anti-inflammatory IL-10 was elevated at 24 h after CLP (Table 4).

3.5. Inflammatory Gene Expressions in the Kidney. The HMGB-1, MyD88, TLR4, and NF-κB mRNA expressions were upregulated in the sepsis groups than in the NC group. The gene expression of inflammatory mediators including IL-6 and TNF-α was also elevated after sepsis. Compared to the SS group, the SG group had lower gene expressions of MyD88, IL-6, and TNF-α at each time point, TLR4 at 12 and 48 h, and HMGB-1 and NF-κB at 48 h after CLP (Figure 2).

Table 3: Plasma concentrations of kidney function marker and inflammatory chemokine among groups.

	NC	SS12h	SG12h	SS24h	SG24h	SS48h	SG48h
NGAL (μg/dL)	$0.08 \pm 0.01^{*}$	3.52 ± 1.84	2.76 ± 1.08	52.3 ± 5.40	$35.1 \pm 6.50^{\#}$	39.5 ± 32.70	48.0 ± 31.60
BUN (mg/dL)	$18.9 \pm 0.90^{*}$	67.4 ± 8.40	69.1 ± 6.30	97.1 ± 6.10	102.3 ± 6.10	139.9 ± 14.40	130.0 ± 29.30
Cre (mg/dL)	$0.09 \pm 0.01^{*}$	0.14 ± 0.03	0.12 ± 0.01	0.70 ± 0.09	0.71 ± 0.10	1.10 ± 0.28	1.20 ± 0.32
KC (ng/mL)	$0.21 \pm 0.04^{*}$	137.3 ± 36.4	$27.9 \pm 6.6^{\#}$	226.4 ± 143.3	190.3 ± 97.1	4.57 ± 2.15	6.49 ± 1.08
MCP-1 (ng/mL)	$0.03 \pm 0.004^{*}$	2.68 ± 1.96	2.08 ± 1.23	2.70 ± 1.50	3.70 ± 0.18	3.93 ± 3.88	3.14 ± 2.57

Data are presented as the mean ± SEM. NC: normal control group; SS: sepsis group with saline injection sacrificed at 12, 24, and 48 h after cecal ligation and puncture (CLP); SG: sepsis group with glutamine injection sacrificed at 12, 24, and 48 h after CLP; NGAL: neutrophil gelatinase-associated lipocalin-2; BUN: blood urea nitrogen; Cre: creatinine; KC: keratinocyte-derived chemokine; MCP-1: monocyte chemoattractant protein. Differences between 2 sepsis groups at the same time point were analyzed by t-test. The comparison among NC and the sepsis groups at three different time points were analyzed by a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. *Significantly differs from other sepsis groups; $^{\#}$significantly differs from the SS group at the same time point ($p < 0.05$).

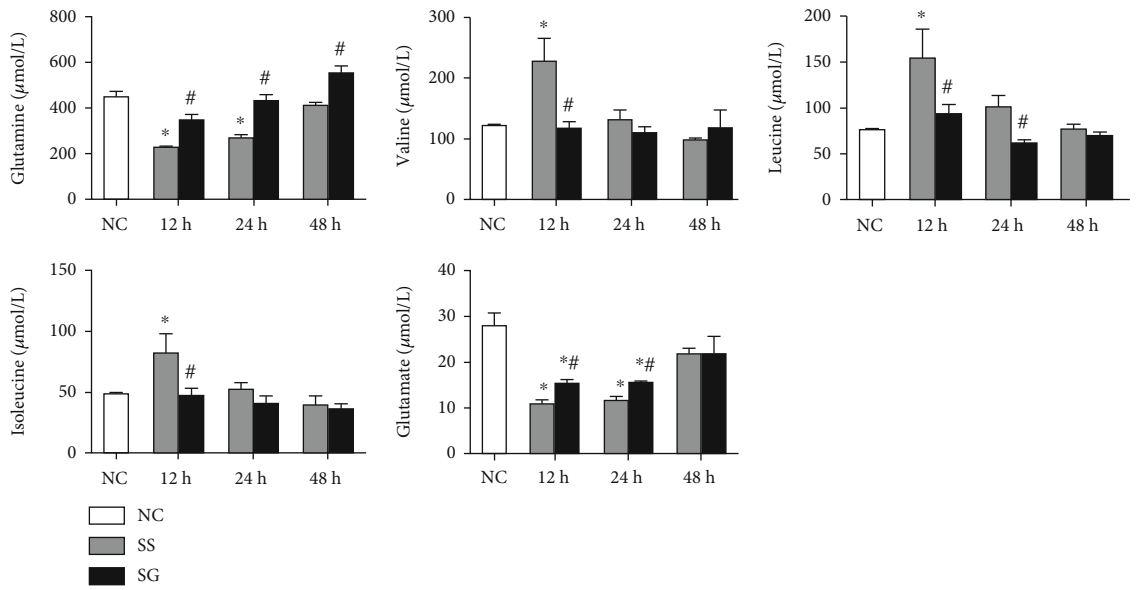

Figure 1: Plasma amino acid concentrations in the normal control (NC) and the sepsis groups at different time points. SS: sepsis group with saline; SG: sepsis group with glutamine. Values are expressed as the mean ± SEM. All data are representative of duplicate measurements at 12, 24, and 48 h after cecal ligation and puncture (CLP) ($n = 8$ for each respective group). Differences between 2 sepsis groups at the same time point were analyzed by t-test. The comparison among NC and the sepsis groups at three different time points were analyzed by a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. *Significantly differs from the NC group; $^{\#}$significantly differs from the SS group at the same time point ($p < 0.05$).

Table 4: The concentrations of inflammatory cytokines and chemokines in peritoneal lavage fluid.

	NC	SS12h	SG12h	SS24h	SG24h	SS48h	SG48h
TNF-α (pg/mg protein)	N.D.	4.88 ± 2.10	4.36 ± 0.84	26.1 ± 2.8	20.8 ± 5.3	46.9 ± 13.2	$18.7 \pm 5.1^{\#}$
IL-10 (pg/mg protein)	N.D.	47.8 ± 36.1	46.0 ± 29.7	309.5 ± 97.7	$693.0 \pm 85.1^{\#}$	89.0 ± 66.9	95.2 ± 69.1
KC (ng/mg protein)	$0.01 \pm 0.003^{*}$	9.67 ± 0.69	$3.39 \pm 1.36^{\#}$	6.91 ± 0.63	5.36 ± 0.44	2.70 ± 1.36	1.19 ± 0.45
MCP-1 (ng/mg protein)	$0.08 \pm 0.001^{*}$	5.17 ± 0.86	$2.45 \pm 0.74^{\#}$	2.73 ± 0.63	2.46 ± 0.82	2.56 ± 1.12	2.03 ± 0.90

Data are presented as the mean ± SEM. The grouping of the experiment is described in the footnote of Table 1. N.D.: nondetectable; IL-10: interleukin-10; TNF-α: tumor necrosis factor-α; KC: keratinocyte-derived chemokine; MCP-1: monocyte chemoattractant protein. Differences between 2 sepsis groups at the same time point were analyzed by t-test. The comparison among NC and the sepsis groups at three different time points was analyzed by a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. *Significantly differs from other sepsis groups; $^{\#}$significantly differs from the SS group at the same time point ($p < 0.05$).

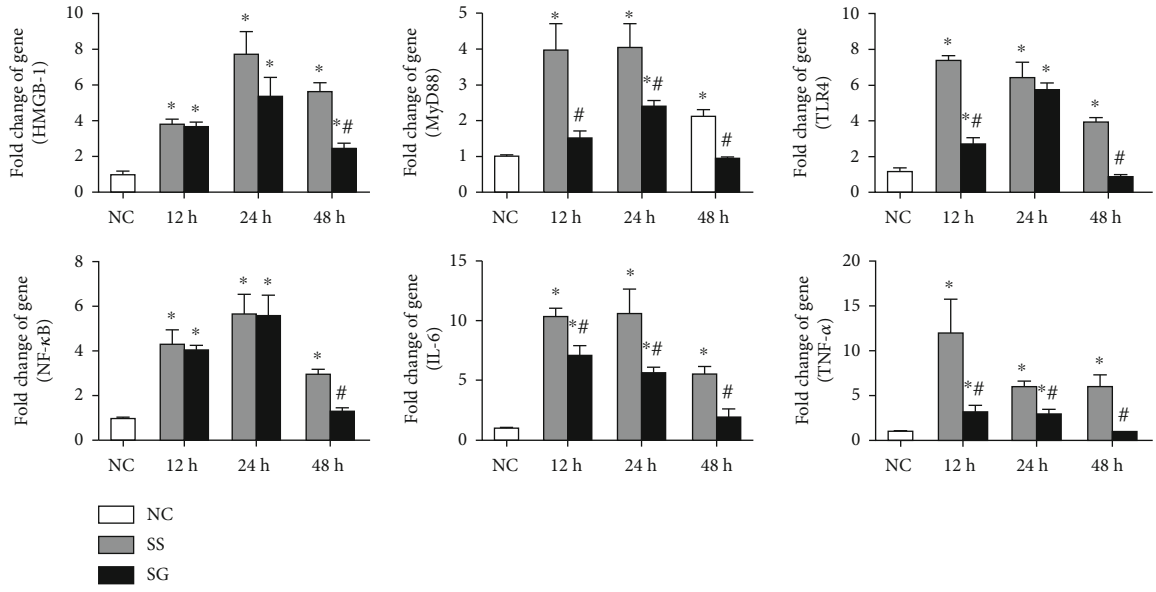

Figure 2: Messenger (m)RNA expressions of high-mobility group box protein-1 pathway-associated genes and subsequent inflammatory cytokines in kidney tissues. NC: normal control; SS: sepsis group with saline; SG: sepsis group with glutamine; HMGB-1: high-mobility group box protein-1; TLR4: toll-like receptor-4; NF-κB: nuclear factor-κB; TNF-α: tumor necrosis factor-α; IL-6: interleukin-6. mRNA changes were quantitated and analyzed by a real-time PCR and were calculated by the comparative CT ($2^{-\Delta\Delta Ct}$) method. mRNA expression levels in the normal control group were used as a calibrator. Values are expressed as the mean ± SEM. $n = 8$ for each group at 12, 24, and 48 h after cecal ligation and puncture (CLP). Differences between 2 sepsis groups at the same time point were analyzed by t -test. The comparison among NC and the sepsis groups at three different time points were analyzed by a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. *Significantly differs from the NC group; #significantly differs from the SS group at the same time point ($p < 0.05$).

3.6. mRNA Expressions of Macrophage Infiltration Markers in the Kidney. Compared to the NC, the macrophage infiltration markers, CD68 and EMR-1, and chemoattractant MCP-1 were all elevated after CLP. The MCP-1 expression was significantly lower in the SG group at 12 and 24 h than in the SS group after CLP. The expressions of CD68 and EMR-1 were lower in the SG group than in the SS group at 48 h. No differences in CD68 and EMR-1 were seen at 12 and 24 h post-CLP between the two sepsis groups (Figure 3).

3.7. mRNA Expressions of Tight Junction and Injury Proteins in the Kidney. The tight junction gene, ZO-1, was significantly elevated in the SG group than in the NC group after sepsis. As compared to the SS group, the SG group exhibited a higher ZO-1 gene expression at 24 and 48 h after CLP. Expression of Kim-1 was increased at 24 and 48 h after CLP. The SG group had a lower Kim-1 expression than the SS group 48 h post-CLP (Figure 4).

3.8. Kidney MPO Activities. MPO activity significantly increased after CLP in both SS and SG groups. Compared to the SS group, the SG group had lower MPO activities at each time point after CLP (Figure 5).

3.9. Kidney Lipid Peroxide Concentrations. The MDA concentrations were higher in the SS groups than in the NC group, while no differences were noted between the SG and NC groups at each time point after CLP. The SG group had lower MDA levels than the SS group at 12 h post-CLP (Figure 6).

4. Discussion

Obesity is a growing challenge around the world. There are increasing numbers of obese patients being admitted to ICUs [24]. There are studies that report that compared to normal weight patients, obesity seems to decrease mortality in the critically ill patients [25, 26]. However, the study also showed that the lower mortality found in the obese patients was abolished when the results were adjusted for comorbidities and sepsis interventions [27]. A clinical study even revealed that mortality of septic patients upon ICU admission was independently associated with obesity [28]. Although discrepancies exist, obesity was found to be correlated with higher infection and exaggerate inflammation [29]. We did not include an obese sham group (without sepsis) in this study, because a former study had shown that compared to the sham group, adipocyte macrophage infiltration and local and systemic inflammation were intensified when obesity coexists with sepsis [30]. Since the kidney is a frequently affected organ in sepsis, the focus of this study was to investigate the impact of GLN on sepsis-induced AKI in obesity. In this study, GLN was administered immediately after CLP. For the group which was sacrificed at 48 h, a second GLN dose was injected 24 h post-CLP. A booster dose was expected to reinforce the efficacy of GLN during the septic state. We found that in the presence of obesity, GLN

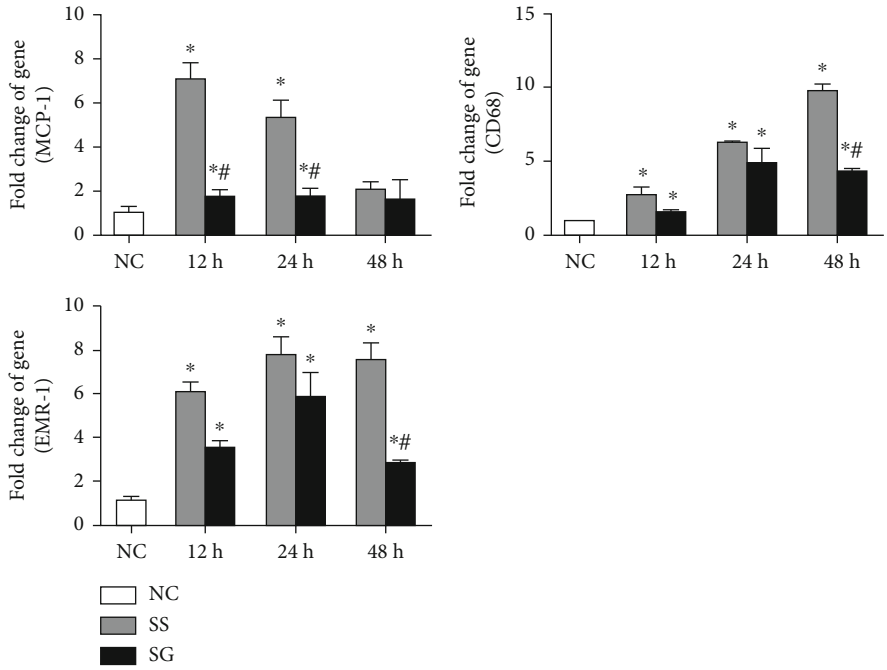

Figure 3: Messenger (m)RNA expressions of macrophage infiltration markers in kidney tissues. NC: normal control; SS: sepsis group with saline; SG: sepsis group with glutamine; CD68: cluster of differentiation 68; EMR-1: epidermal growth factor-like module-containing mucin-like hormone receptor-like-1; MCP-1: monocyte chemoattractant protein-1. mRNA changes were quantitated and analyzed by a real-time PCR and were calculated by the comparative CT ($2^{-\Delta\Delta Ct}$) method. mRNA expression levels in the normal control group were used as a calibrator. Values are expressed as the mean ± SEM. $n = 8$ for each group at 12, 24, and 48 h after cecal ligation and puncture (CLP). Differences between 2 sepsis groups at the same time point were analyzed by t-test. The comparison among NC and the sepsis groups at three different time points were analyzed by a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. *Significantly differs from the NC group; #significantly differs from the SS group at the same time point ($p < 0.05$).

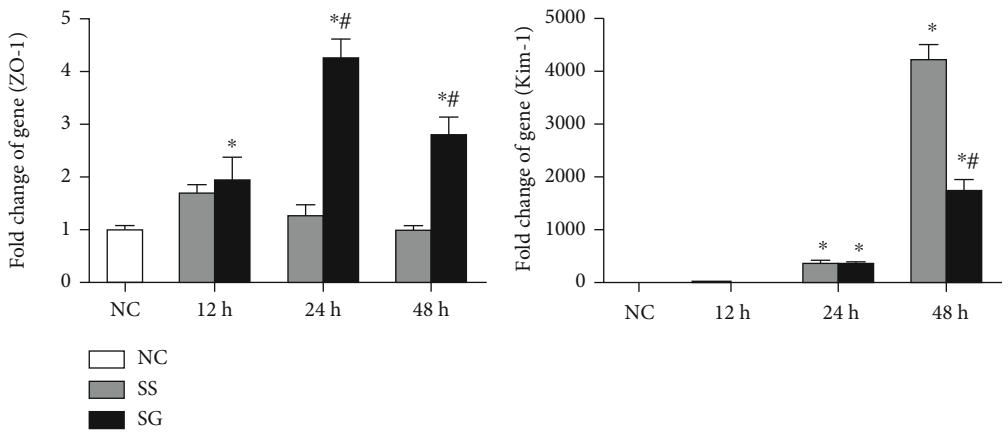

Figure 4: Expressions of genes related to kidney tissue injury. NC: normal control; SS: sepsis group with saline; SG: sepsis group with glutamine; ZO-1: zonula occluden-1; Kim-1: kidney injury molecule. Messenger RNA changes were quantitated and analyzed by a real-time PCR and were calculated by the comparative CT ($2^{-\Delta\Delta Ct}$) method. mRNA expression levels in the normal control group were used as a calibrator. Values are expressed as the mean ± SEM. $n = 8$ for each group at 12, 24, and 48 h after cecal ligation and puncture (CLP). Differences between 2 sepsis groups at the same time point were analyzed by t-test. The comparison among NC and the sepsis groups at three different time points were analyzed by a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. *Significantly differs from the NC group; #significantly differs from the SS group at the same time point ($p < 0.05$).

administration resulted in an elevation in plasma GLN levels and alleviated oxidative stress, and inflammation occurred in the kidney that may protect against sepsis-induced AKI.

We analyzed the gene expressions of HMGB-1-associated mediators. HMGB-1 is a protein released from activated macrophages and damaged tissues which interacts

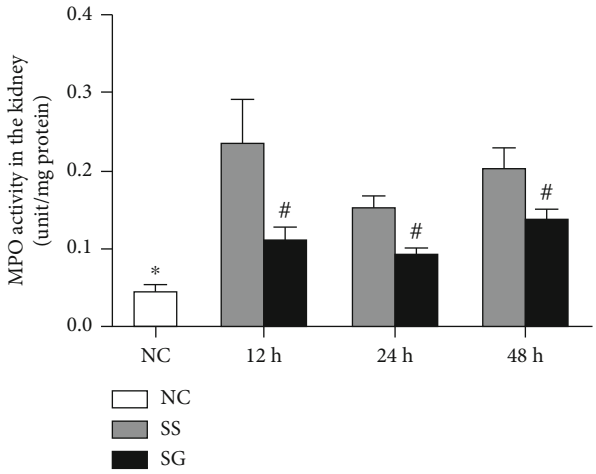

Figure 5: Myeloperoxidase (MPO) activity in kidney tissues. NC: normal control; SS: sepsis group with saline; SG: sepsis group with glutamine. Values are expressed as the mean ± SEM. $n = 8$ for each group at 12, 24, and 48 h after cecal ligation and puncture (CLP). Differences between 2 sepsis groups at the same time point were analyzed by t-test. The comparison among NC and the sepsis groups at three different time points were analyzed by a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. *Significantly differs from other sepsis groups; #significantly differs from the SS group at the same time point ($p < 0.05$).

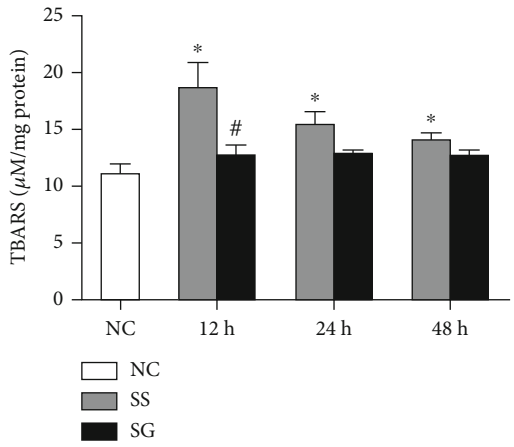

Figure 6: Thiobarbituric acid reactive substance (TBARS) in kidney tissues. NC: normal control; SS: sepsis group with saline; SG: sepsis group with glutamine. Values are expressed as the mean ± SEM. $n = 8$ for each group at 12, 24, and 48 h after cecal ligation and puncture (CLP). Differences between 2 sepsis groups at the same time point were analyzed by t-test. The comparison among NC and the sepsis groups at three different time points were analyzed by a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. *Significantly differs from NC group; #significantly differs from the SS group at the same time point ($p < 0.05$).

with TLRs [31]. HMGB-1 is considered a mediator of systemic inflammation in the relative late phase of sepsis [32]. Previous studies revealed that endogenous HMGB-1 enhances kidney injury during inflammation [33, 34]. MyD88 is an adaptor protein which acts as a connector between TLRs and the downstream kinases [35]. The HMGB-1-mediated pathway activates cellular signaling that ultimately leads to NF-κB activation and subsequent inflammatory mediator production [31]. Kim-1 is a transmembrane tubular protein expressed on epithelial cells in damaged regions. Kim-1 can be considered as an indicator for AKI [36]. In this study, we observed that the HMGB-1-mediated pathway was upregulated; the inflammatory chemokines and Kim-1 expressions increased in the kidney. Consistent with the elevation of plasma Cre and BUN, NGAL levels increased in the sepsis groups. NGAL is expressed by many tissues including the kidney. Under normal conditions, the expression of NGAL is low, but will significantly increase in epithelial damage and inflammation [37]. Thus, NGAL is treated as a useful biomarker of AKI [38]. The upregulated biomarkers mentioned above indicated that kidney inflammation and injury occur in obesity complicated with sepsis. A study by Kolyva et al. also showed that obesity is associated with enhanced proinflammatory cytokine production and exaggerated systemic oxidative stress in septic patients [39].

Bacterial infection may activate both macrophages and neutrophils. The infiltration of these immune cells into kidney tissue results in persistent inflammation and organ injury [40]. In this study, markers of macrophage were analyzed. CD68 is a transmembrane glycoprotein which is well known as a surface marker abundantly expressed by macrophages in inflamed tissues [41]. The F4/80 molecule, also named EMR-1, was established as a unique marker of murine macrophages [42]. In addition, MPO activities in the kidney were analyzed. MPO is an enzyme released by activated neutrophils. A previous study showed that MPO can be used as a marker of neutrophil infiltration and the severity of inflammation in sepsis patients [43]. In this study, kidney expression of CD68 and EMR-1 was elevated and MPO activity was increased in the obese groups with sepsis, suggesting that macrophage and neutrophil infiltration and inflammation occurred during the experimental period.

In this study, GLN was administered 1 or 2 doses after CLP in obese mice. We observed that there were several favorable effects that were not noted in mice with saline injection. *First*, GLN administration maintained plasma GLN and BCAA levels and reversed sepsis-induced depletion of GLN and glutamate. Previous studies showed that the rates of BCAA oxidation and efflux from muscle tissues are enhanced, thus offering energy substrate for the demand under stress and catabolic conditions [44, 45]. The increased BCAA levels observed in the SS group especially at 12 h after CLP may indicate that more severe catabolism exists at this time point. GLN administration provided more fuel source to fulfill the metabolic needed and alleviated catabolism during sepsis. *Second*, GLN attenuated inflammation at the site of injury and the kidney tissues. We observed that TNF-α concentrations reduced while anti-inflammation cytokine IL-10 increased in PLF. Also, the HMGB-1-mediated pathway was downregulated and inflammatory cytokine expressions reduced in the kidneys. Our results are consistent with previous studies that GLN downregulated the HMGB-1 pathway and inhibited NF-κB activation and subsequent downstream target gene expressions [19, 46]. In addition,

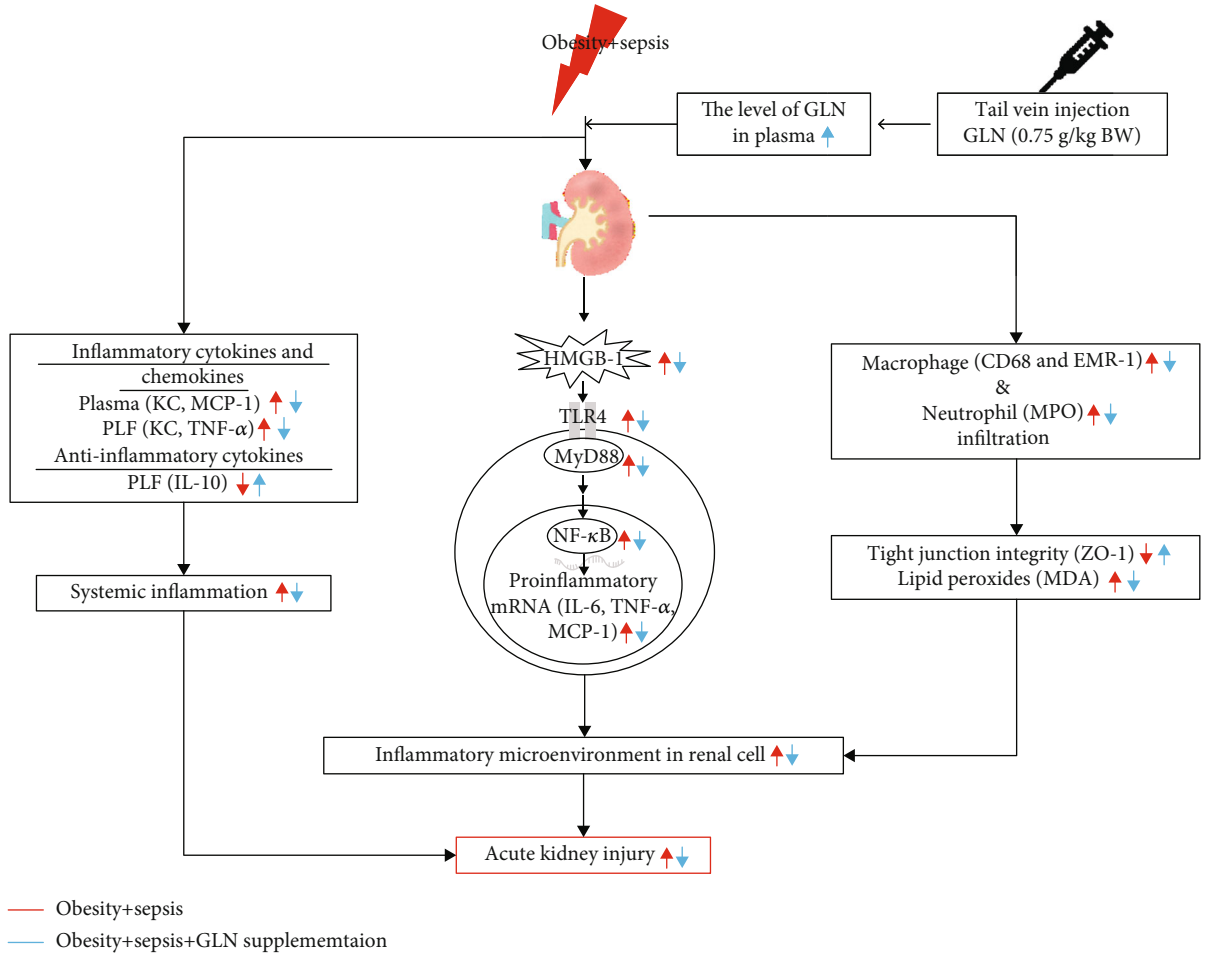

Figure 7: The proposed mechanisms of glutamine (GLN) regulation on attenuating sepsis-induced acute kidney injury (AKI). Obesity exaggerates the severity of sepsis, and AKI is a common complication. Underlying sepsis, immune cells release inflammatory cytokines and chemokines that result in systemic inflammation. In the kidney, upregulation of HMGB-1 activates the NF-κB pathway and subsequent downstream inflammatory mediator production. Activated macrophage and neutrophil infiltration into kidney tissue may worsen the integrity of tight junction and increase the production of lipid peroxides. The inflammatory microenvironment within renal cells ultimately leads to AKI. GLN administration increases the plasma levels of GLN, blocks the inflammatory pathways, and reduces lipid peroxide production, thus ameliorating the occurrence of AKI. Red line means the effects of obesity complicated with sepsis. Blue line means the effects of GLN administration on obesity complicated with sepsis. CD68: cluster of differentiation 68; CLP: cecal ligation and puncture; EMR-1: epidermal growth factor-like module-containing mucin-like hormone receptor-like-1; HMGB-1: high-mobility group box protein-1; KC: keratinocyte-derived chemokine; IL: interleukin; MCP-1: monocyte chemoattractant protein-1; MDA: malondialdehyde; MPO: myeloperoxidase; MyD88: myeloid differentiation factor 88; NF-κB: nuclear factor-κB; PLF: peritoneal lavage fluid; TLR4: toll-like receptor-4; TNF-α: tumor necrosis factor-α; ZO-1, zonula occluden-1.

GLN can activate the expression of the peroxisome proliferator-activated receptor (PPAR). PPAR-γ can be activated by several ligands and has an anti-inflammatory property. GLN is one of the ligands of PPAR-γ. A previous study found that GLN administration to the intestinal lumen induced the expression of PPAR-γ and protected against intestinal inflammation in the postischemic gut [47]. *Third*, GLN administration reduced macrophage and neutrophil infiltration in the kidney. Markers of activated macrophage and neutrophil, including CD68, EMR-1 expression, and MPO activity in kidneys, decreased in the SG group. Consistent with the findings are lower plasma NGAL levels and kidney Kim-1 and MCP-1 whereas higher ZO-1 expressions were noted suggesting that sepsis-associated kidney injury was attenuated when GLN was administered. Previous studies found that GLN sustained the T cell population and modulated a more balanced T helper cell polarization in sepsis that is associated with attenuating inflammation and kidney injury [16, 18].

Despite the anti-inflammatory property of GLN, there may have been other mechanisms that participated in attenuating kidney injury in this study. Oxidative stress and inflammation are underlying disorders occur in both obesity and sepsis [48, 49]. Obesity concurrence with sepsis further intensifies the inflammatory reaction and oxidative stress [29]. GLN is the precursor of an endogenous antioxidant, glutathione [50]. In this study, we found that the production of kidney lipid peroxides was reduced in the SG group. The lower oxidative stress exerted from GLN-associated redox reactions may play a role in alleviating kidney injury. On

the other hand, vascular endothelium damage and dysfunction are correlated with the progression of multiorgan injury in sepsis [51]. Endothelial progenitor cells (EPCs) derived from bone marrow are capable of proliferating and differentiating into mature endothelial cells [52]. Circulating EPCs were shown to be involved in repairing and maintaining vascular endothelium integrity during sepsis [53]. A previous study showed that GLN administration after CLP promotes EPC mobilization, improves vascular function, and protects remote organ injury against sepsis [54]. However, whether GLN administration may initiate endogenous endothelium repair and improve microvascular perfusion, thus alleviating kidney injury, requires further investigation.

As far as we know, this study investigated the effects of GLN on sepsis-induced kidney inflammation and injury in obesity for the first time. Results showed that coexisting with obesity, sepsis resulted in macrophage and neutrophil infiltration in the kidney that may lead to inflammation and injury of the organ. GLN administration after CLP increased plasma GLN levels, attenuated immune cell infiltration in the kidney, and resolved sepsis-induced inflammation and AKI. The proposed mechanisms of GLN regulation on attenuating sepsis-induced AKI is presented as Figure 7. The results presented here showed that GLN may have potential to attenuate inflammation and protect against kidney injury in obesity complicated with sepsis. This model may have clinical application for obese patients with a risk for infection in abdominal surgery.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

Chiu-Li Yeh conceived and designed this study. Li-Han Su performed the study and analyzed the data; Ming-Tsan Lin and Sung-Ling Yeh did part of the analysis and helped to interpret the data. Chiu-Li Yeh, Ming-Tsan Lin, and Sung-Ling Yeh prepared the manuscript. All authors read and approved the final submitted manuscript.

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Research Article

Effect of GLP-1/GLP-1R on the Polarization of Macrophages in the Occurrence and Development of Atherosclerosis

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Aims. To investigate the effect of GLP-1/GLP-1R on the polarization of macrophages in the occurrence and development of atherosclerosis. *Methods*. Totally, 49 patients with coronary heart disease (CHD) and 52 cases of health control (HC) were recruited, all subjects accept coronary angiography gold standard inspection. One or more major coronary arteries (LM, LAD, LCx, and RCA) stenosis degree in 50% of patients as CHD group; the rest of the stenosis less than 50% or not seen obvious stenosis are assigned to the HC group. Flow cytometry were used to detect the percentage of (CD14+) M macrophages, (CD14+CD80+) M1 macrophages, (CD14+CD206+) M2 macrophages, and their surface GLP-1R expression differences in the two groups, using BD cytokine kit to detect the levels of IL-8, IL-1β, IL-6, IL-10, TNF, and IL-12p70. *Results*. GLP-1R expression on the surface of total macrophages and M2 macrophages was different between the CHD group and the HC group ($P < 0.05$). There was no difference in the percentage of total, M1 or M2 macrophages ($P > 0.05$). Concentration of IL-8 in the HC group was higher than that in the CHD group ($P < 0.05$). There is no significant difference in the cytokine IL-1β, IL-6, IL-10, TNF, and IL-12p70 in the two groups ($P > 0.05$). After controlling for potential confounders including age, gender, smoking status (S.S.), drinking status (D.S.), HR, SBP, DBP, PP, TC, TG, HDL-C, LDL-C, GHbA1c, M, M1, M2, GLP-1R_M, GLP-1R_M1, GLP-1R_M2, IL-8, IL-1β, IL-6, IL-10, TNF, and IL-12p70 by multiple linear regression, decreasing Gensini Score was significantly associated with increased percentage of M1 macrophage. *Conclusion*. GLP-1R agonist is independent of the hypoglycemic effect of T2DM and has protective effect on cardiovascular system. GLP-1R may regulate the polarization of macrophages toward M2, thus playing a protective role in the progression of coronary atherosclerosis.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a major risk factor for coronary atherosclerotic heart disease (CHD); CHD is a severe and fatal complication of T2DM [1]. Patients with long course of disease more than 10 years often have retinopathy, which is one of the main causes of blindness and seriously affects the quality of life of the patients [2]. More importantly, atherosclerosis (AS) is the pathological basis of malignant cardiovascular and cerebrovascular events (MACE) such as CHD, acute myocardial infarction (AMI), and stroke [3]. Although AS has always been the core of the research field in cardiovascular and cerebrovascular diseases, this complex disease has still not been conquered by human beings.

Studies have found that a variety of chronic noncommunicable diseases, such as essential hypertension (EH), T2DM, AS, obesity, and nonalcoholic fatty liver disease (NAFLD), are not only related to genetic, environment, and metabolic disorders [4] but also a mild systemic chronic inflammatory reaction; both inflammation and immune response are involved in its occurrence and development to some extent

[5]. Macrophages are innate immune cells, which not merely damage vascular endothelial cells as an initial factor in the process of atherosclerosis, more than run through the whole process of the development of atherosclerosis [6]. Foam cells formed after the phagocytosis of ox-LDL and further release inflammatory cytokines, which also play a vital role in AS [6]. In addition, adaptive immunity represented by T cell subsets such as Th (helper T cell) and Treg (regulatory T cell) is also involved, and other Th2, Th9, Th17, and Th22 subsets remain to be studied [7, 8].

Glucagon-like peptid-1 receptor (GLP-1R) is a G protein-coupled receptor [9]; animal studies shows that activation of GLP-1/GLP-1R pathway can benefit mice with AS, and the mechanism involves the polarization of immune cells [10]. However, most of the current studies have been done at the cellular or animal level. The distribution of GLP-1R on human immune cells and whether it mediates the polarization of human macrophages and then independently make an antiatherosclerosis impact in patients without T2DM is unclear. Therefore, this paper intends to use flow cytometry to analyze the distribution of M1 (proinflammatory) and M2 (anti-inflammatory) macrophages as well as their surface GLP-1R in patients with different degrees of CHD and preliminarily explore the role of cytokine network in GLP-1R-mediated immune cell polarization.

2. Methods

2.1. Subjects. All participants with proposed diagnosis of coronary atherosclerosis (CHD) who were admitted to the Department of Cardiology, Dongfeng General Hospital of affiliated to Hubei University of Medicine, from August to September 2020, people who had clinical symptoms such as chest tightness, were collected after the informed consent. All subjects accept gold standard of cardiac angiography inspection, one or more major coronary arteries, left main (LM), left anterior descending (LAD), left circumflex (LCx), and right coronary artery (RCA) stenosis degree in 50% of patients as CHD group, the rest of the stenosis less than 50%, or no obvious stenosis are assigned to the health control (HC) group. Inclusion criteria are as follows: the Han nationality, chronic myocardial ischemia syndrome, and acute coronary syndrome; all underwent cardiac angiography. Exclusion criteria are as follows: (1) secondary or gestational diabetes mellitus; (2) taking hormonal drugs, such as thyroid hormone or glucocorticoids; (3) rheumatoid arthritis and other autoimmune diseases; (4) severe cardiac, liver, and renal insufficiency; (5) malignant tumor; (6) acute diseases other than myocardial infarction, such as aortic dissecting aneurysm. Finally, 101 people who met the requirements were included in the analysis.

2.2. Data Collection and Experimental Methods. General information was collected for all subjects, including age, sex, smoking, alcohol consumption, heart rate (HR), systolic and diastolic blood pressure (SBP, DBP), and differential pulse pressure (PP). Gensini score was completed according to the guide in 2009 [11]. The peripheral venous blood of fasting patients was numbered in time, and triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and very low-density lipoprotein cholesterol (VLDL-C) were detected by enzymatic method using automatic biochemical analyzer. Determination of glycosylated hemoglobin A1c (GHbAc1) by High Pressure Liquid Chromatography (HPLC).

2.3. Detection of GLP-1R Expression of Macrophages. Blood samples were stored 4°C after sampling and tested within 8 hours to ensure the number of living cells in each sample. Clusters of immune cells are as follows: 1 μL CD3+-labeled antibody, 5 μL CD4+-labeled antibody, 5 μL CD80+-labeled antibody, 10 μL CD14+-labeled antibody, 10 μL CD80+-labeled antibody, and 10-μL CD206+ labeled antibody were added to a 2 mL EP tube and mixed with vortex 3 s. 10 μL antibody was added to the absolute count microsphere test tube (No. Z6410004-10, microsphere number: 51300). Reverse absorption of 30 μL EDTA-K2 anticoagulant whole blood from suspected CHD patients was added to the bottom of the tube. After mixed with vortex 3 s, the blood was incubated at room temperature and dark for 25 min. The hemolytic agent 460 μL for flow cytometer diluted to 1 time was added into the tube, and the tube cap was placed on the tube and gently vortexed for 15 s. The solution was kept away from light for 10 min at room temperature.

Flow cytometer setting is as follows: NovoExpress software was used to conduct automatic sampling cell analysis. The six channel parameters were B530 (CD14 FITC), B572 (CD206 PE), B675 (CD80 PE-CY7), R675 (GLP-1R APC), and R780 (CD4 APC-CY7). Stopping conditions: 5000 M1 macrophages or sample volume up to 450 μL flow rate: high speed, 66 μL/min; sample flow diameter: 16.8 μm well. Plate management: mixing once every 3 wells, flushing once every 3 wells. Blending parameter: speed: 1500 rpm, acceleration time: 2 s, duration: 10 s. Flow cytometry was completed by two experienced personnel and conducted in a blind method with unknown grouping of each sample, so as to avoid systematic errors caused by exposure suspicion bias caused by subjective factors.

2.4. Detection of Cytokines. The collected whole blood anticoagulant tube was put into a centrifuge, centrifuged at 500 g for 5 min, and the upper plasma was absorbed and stored in the cryopreserved tube, which was stored in the -80°C refrigerator for later use. The BD cytokine kit (No. 551811) was used for quantitative analysis of IL-8, IL-1β, IL-6, IL-10, TNF, and IL-12p70.

Preparation of inflammatory cytokine standard: draw 2 mL determination of dilution in a bottle of freeze-dried inflammation factor standard in the bottle, with liquid moving head gently mix recombinant protein, balance for 20 min at room temperature, then transfer the standard to 15 mL polypropylene tube, marked for the highest concentration of standard (5000 pg/mL), other eight 15 mL polypropylene pipe marked as 1 : 2, 1 : 4, 1 : 8, 1 : 16, 1 : 32, 1 : 64, and 1 : 128, respectively, one more negative control was also labeled.

Mixed human inflammatory cytokines capture microspheres: six bottles of capture microspheres were mixed, each

time before complete vortex capture microspheres 3-5 s to make it completely suspended. The above mixed capture microspheres were centrifuged at 200 g for 5 min, carefully absorbed and discarded the supernatant 30 μL, added the equal volume of serum enhanced buffer suspension, in order to reduce the false-positive rate of plasma type samples containing protein, and incubated at room temperature in dark for 30 min.

Plasma sample detection procedures: all eligible plasma samples were stored at -80°C, immediately placed on the ice to thaw. Vortex to mix all plasma samples in the sterile tube, centrifuge 500 g for 5 min. Add 30 μL mixed capture beads to all EP tubes, and vortex the capture beads every 5 EP tubes. Number each EP tube; add 30 μL each sample to the numbered EP tube and incubated in dark for 1.5 hours at room temperature. 1 mL washing buffer was added to each EP tube then centrifuge at 200 g for 5 min. Carefully and continuously absorb and discard the supernatant, leaving about 100 μL of liquid in each EP tube. Add 30 μL human inflammatory cytokine PE secondary antibody to all EP tubes and stir gently. Hatch the EP tube at room temperature for 1 hour without light. Add 1 mL washing buffer to each tube and centrifuge at 200 g for 5 min again. Carefully extract and discard the supernatant from each EP tube. 300 μL washing buffer was added to each EP tube to resuspend the captured microspheres. Samples are transferred to 96-well plates for testing, and random injection sequence was adopted for cytokine detection of all plasma samples.

The cells were automatically collected and analyzed using NovoExpress software. The six-channel parameters were B530 (FITC), B572 (PE), B67 (Per-CP), B780 (PE-CY7), R675 (APC), and R780 (APC-CY7). Stopping conditions: 1800 captured microspheres or samples of 100 μL volume were detected. Flow rate: low speed, 14 μL/min. Flow diameter: 7.7 μm. Threshold: FSC-H greater than 10000, SSC-H greater than 5000 well. Board management: mixing once per well, flushing once per well. Mixing parameters: speed 1000 rpm, acceleration time 0 s, and duration 5 s.

FCAP Software parameters: scattering parameters: FSC-A, scattering peak: 5, clustering parameters: APC-A, reporting parameters: PE-H microsphere A1, A2, A3, A4, A5, and A6 corresponding IL-8, IL-1β, IL-6, IL-10, TNF, and IL-12p70. Quantitative analysis of mixed human cytokine concentrations of standard curve theory in order: 0.00 pg/mL, 19.53 pg/mL, 39.06 pg/mL, 78.13 pg/mL, 156.25 pg/mL, 312.50 pg/mL, 625 pg/mL, 1250 pg/mL, 2500 pg/mL, and 5000 pg/mL of each cytokine standard curve. Fitting standard curve for each cytokine, read the final concentration of each cytokine in each sample for statistical analysis.

2.5. Statistical Analysis. Gensini score exhibits a log-normal distribution after transformed as $\log(X + 2.5)$, and used as a dependent variable in multivariate linear regression model. Measurement data were described by mean ± standard deviation; after one-sample Kolmogorov-Smirnov normality test and variance homogeneity test, variables conforming to normal distribution and homogeneity of variance were tested by T-test, while variables with uneven variance were tested by correction T-test. The rest of the skewed distribution data was described with median, upper, and lower quartile. Counting data is tested by Chi-square. The concentration of cytokines in the two groups were compared with nonparametric Mann-Whitney U test. Spearman correlation analysis was used in two variables. All P values are derived from 2-tailed analyses; $P < 0.05$ have been considered to be of statistical significance. Analyses were performed with IBM SPSS statistics 26.

3. Results

3.1. Clinical Data. In this study, 101 patients were included at last, including 52 patients in the health control (HC) group and 49 patients in the CHD group, whose systolic blood pressure was higher than that in the control group ($P < 0.05$). In addition, compared to the CHD group, the HC group had lower triglyceride levels and higher HDL-c, which is consistent with current perceptions of CHD risk factors. In our study, there were no differences in T2DM distribution or GHbA1c levels between the two groups ($P > 0.05$), which can be used to analyze the difference of GLP-1R expression in macrophages and their subtypes in the two groups. The clinical and biochemical characteristics of the two groups in our research are as follows (Table 1).

3.2. Expression Differences of Total Macrophages, M1 Macrophages, and M2 Macrophages in CHD Group and HC Group: $CD14^+$, $CD14^+CD80^+$, and $CD14^+CD206^+$. Total macrophages were labeled by $CD14^+$ antibody, M1 macrophages by $CD14^+CD80^+$ double-positive antibody, and M2 macrophages by $CD14^+CD206^+$ double-positive antibody. Previous studies have suggested that GLP-1R agonists can improve the risk of cardiovascular disease in diabetic patients, while in this study, there was no difference in the distribution of T2DM between the CHD group and the HC group, thus avoiding the bias caused by the difference in the distribution of diabetic patients between the two groups. In this study, the percentage of total, M1, and M2 macrophages showed no difference between the CHD group and the HC group ($P > 0.05$), indicating that the relative content of macrophages may not be the most critical factor for the development of CHD (Figure 1).

3.3. Expression Differences of GLP-1R on the Surface of Total, M1, and M2 Macrophages in CHD Group and HC Group: $CD14^+GLP\text{-}1R^+$, $CD14^+CD80^+GLP\text{-}1R^+$, and $CD14^+CD206^+GLP\text{-}1R^+$. As mentioned earlier, no difference was found between the two groups in macrophages of different phenotypes, which does not seem to be synergistically demonstrated with the M1 and M2 macrophages effect that we commonly think of. However, we were surprised to find that GLP-1R expression on the surface of macrophages was different in both groups, although the percentage of different phenotypes of macrophages in the CHD group, and the control group was not statistically significant ($P < 0.05$), compared with the control group, the GLP-1R expression on M2 macrophages was higher in the CHD group, and this seems to indicate that macrophages with anti-inflammatory functions can express more

Table 1: Clinical and biochemical characteristics in HC and CHD groups.

	HC group ($n = 52$)	CHD group ($n = 49$)	P value
Age (years)	56.71 ± 10.15	63.59 ± 11.22	0.002
Gender (male, female)	27/25	32/17	0.173
Smoking status (yes, no)	14/38	22/27	0.059
Drinking status (yes, no)	6/46	8/41	0.486
HR (/bmp)	75 (66.25~83.5)	75 (64.5~83.5)	0.534
SBP (mmHg)	127.5 (120~141)	139 (126.5~150.5)	0.015
DBP (mmHg)	78.5 (70.25~87.5)	80 (72~88)	0.809
PP (mmHg)	51.5 (43.5~60)	57 (46~68.5)	0.087
TC (mmol/L)	4.05 (3.54~5.04)	4.21 (3.36~4.98)	0.943
TG (mmol/L)	1.33 (0.83~1.11)	1.81 (1.31~2.84)	0.009
HDL-c (mmol/L)	0.98 (0.86~1.11)	0.90 (0.73~1.14)	0.043
LDL-c (mmol/L)	2.22 (1.82~3.06)	2.01 (1.54~2.94)	0.213
GHbA1c (%)	5.5 (5.3~5.9)	5.8 (5.3~6.75)	0.126
T2DM (yes, no)	5/47	10/39	0.127

HC: health control; CHD: coronary heart disease; HR: heart rate; SBP: systolic blood pressure; DBP: diastolic blood pressure; PP: pulse pressure; TC: cholesterol; TG: triglyceride; HDL-c: high-density lipoprotein cholesterol; LDL-c: low-density lipoprotein cholesterol; GHbA1c: glycosylated hemoglobin A1c; T2DM: type 2 diabetes mellitus.

cardiovascular beneficial GLP-1R. No statistically significant differential expression of anti-inflammatory phenotype was found in M1 macrophages (Figure 2).

3.4. Expression of IL-8, IL-1β, IL-6, IL-10, TNF, and IL-12P70 in the Two Groups. There was a statistically significant difference in IL-8 concentrations between the two groups ($P < 0.05$), while none of the cytokines in IL-1β, IL-6, IL-10, TNF, and IL-12P70 were found to be different between the two groups in our study (Figure 3).

3.5. Correlation between Macrophages and Their Surface GLP-1R and Blood Lipids and Cytokines. We analyzed the correlation between macrophages and their surface GLP-1R and blood lipids and cytokines: (1) in all the studies included, GLP-1R expression on the surface of total macrophages was inversely proportional to TG ($r = -0.221$, $P < 0.05$), the percentage of M1 macrophages was inversely proportional to IL-10 ($r = -0.228$, $P < 0.05$); (2) in the HC group, the percentage of total macrophages was negatively correlated with TG ($r = -0.306$, $P < 0.05$), while the M2 macrophage GLP-1R expression increased with the decrease of IL-8 level ($r = -0.275$, $P < 0.05$); (3) in the CHD group, the percentage of M2 macrophages was inversely proportional to the percentage of total macrophages ($r = -0.328$, $P < 0.05$). However, the expression of GLP-1R in M1 macrophages was directly proportional to TG ($r = 0.325$, $P < 0.05$) (Figure4).

3.6. Effect of M1 Macrophage Percentage on Gensini Score. Log-transformed Gensini score was used as the dependent variable to adjust the potential confounders age, gender, smoking status (S.S.), drinking status (D.S.), HR, SBP, DBP, PP, TC, TG, HDL-C, LDL-C, GHbA1c, M, M1, M2 GLP-1R_M, GLP-1R_M1, GLP-1R_M2, IL-8, IL-1β, IL-6, IL-10, TNF, and IL-12p70. Multiple linear regression analysis was conducted, and the model equation was $Y = 0.024 * \text{Age} + 0.497 * \text{S.S.} - 0.454 * \text{D.S.} - 0.003 * \text{M1}$, and the equation was statistically significant ($P < 0.05$). The coefficient of determination R2 was 0.457, the entry condition was 0.1, and the exclusion condition was 0.15.

4. Discussion

The REWIND experiment [12] is a double-blind randomized placebo-controlled trial, which found that both middle-aged and elderly patients with T2DM who had previous cardiovascular diseases or had cardiovascular disease risk factors could benefit from the use of liraglutide. This suggests that the beneficial inhibitory effects of GLP-1R agonists on the occurrence and progression of cardiovascular diseases and the mechanism may not entirely dependent on the treatment of T2DM; therefore, our study included patients with CHD or not confirmed by coronary angiography and compared the distribution of macrophages and their surface GLP-1R without bias caused by the difference in T2DM distribution or GHbA1c level between the two groups. Data shows that the expression of GLP-1R on total and M2 macrophages was different between the CHD group and HC group ($P < 0.05$), and the expression of GLP-1R was higher in the HC group. The expression level of GLP-1R on the surface of M1 macrophages or the percentage of total, M1, and M2 macrophages showed no significant difference between the two groups. This suggests that: (1) in the chronic inflammatory process of CHD, changes in GLP-1R expression level are more sensitive than relative macrophage content; (2) the reduction of GLP-1R on beneficial anti-inflammatory type (M2) macrophage in CHD patients may explain that GLP-1R receptor agonists are independent of glycemic control effects, thus benefits for CHD.

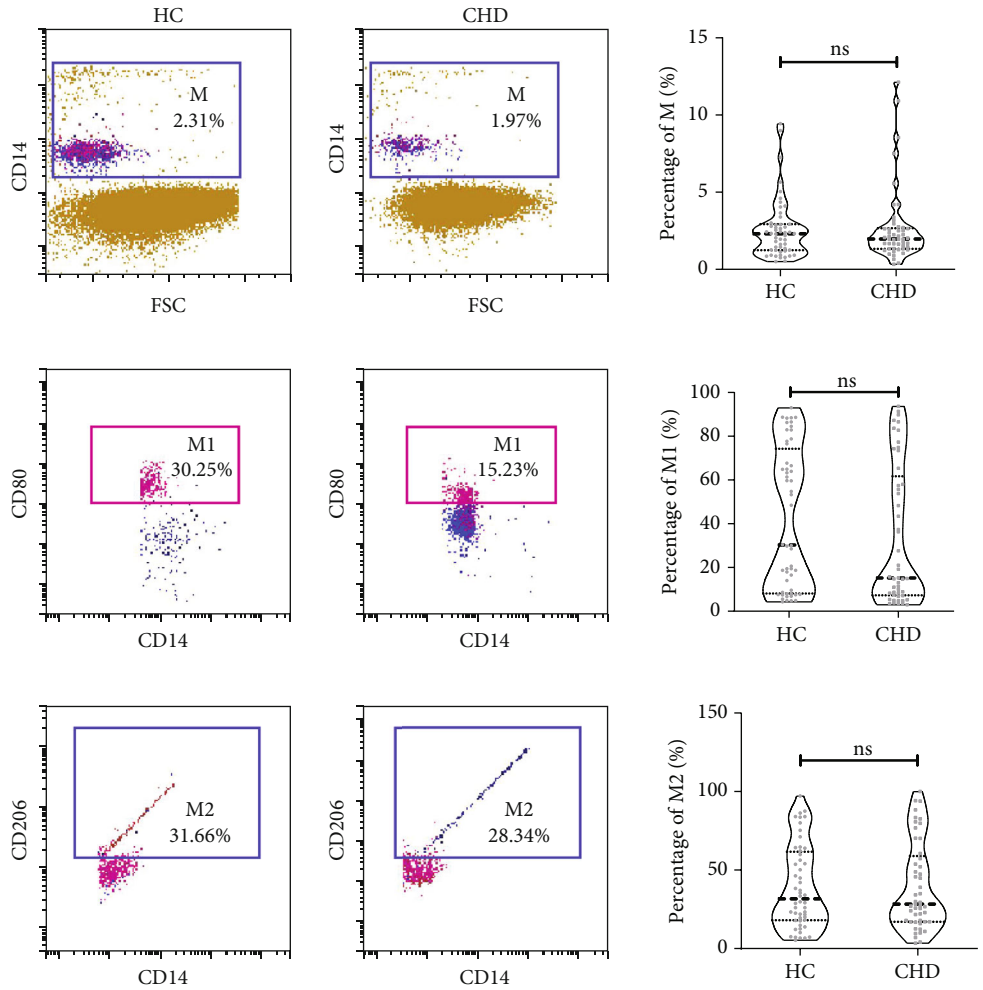

Figure 1: Expression difference of total macrophage, M1, and M2 macrophage between CHD group and HC group. Data are represented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$. M: macrophage, labeled by CD14+ molecule; M1: M1 macrophage, labeled by CD14+ and CD80+ molecule; M2: M2 macrophage, labeled by CD14+ and CD206+ molecule.

DPP-4 inhibitors can inhibit the degradation of human native GLP-1 by DPP-4 enzyme and indirectly stimulate GLP-1R. Jawahar L. Mehta et al. [13] used PMA to block THP-1 macrophages and coincubate them with ox-LDL; inhibition of NLRP3 Toll-like receptor (TLR4) and IL-1 expression by DPP-4 inhibitors was observed, as well as upregulation of GLP-1R expression; while the blocking effect of PMA was relieved by GLP-1R agonists such as liraglutide, this study further demonstrates the inflammatory state and immune response of atherosclerosis. Our data shows that GLP-1R expression levels were indeed higher in total and M2 macrophages in the control group and not the percentage of the inflammatory cells itself, which suggests that GLP-1R may play a more central role than inflammatory cells themselves in the regulation of inflammatory nodes in atherosclerosis; in turn, it also supports the anti-inflammatory effect of GLP-1R in CHD.

Existing studies have shown that inflammatory response plays a vital role in the occurrence and development of CHD, T2DM, chronic obstructive pulmonary disease (COPD), chronic hepatitis, periodontitis [14], Alzheimer's disease (AD), etc. IL-6 [15, 16] has been shown to be negatively correlated with heart rate variability (HRV) in CHD women, elevated HRV is associated with parasympathetic excitation, the increased threshold of ventricular fibrillation is a protective factor, and this study reconfirms that IL-6 is a risk factor for CHD. Besides, IL-8, IL-10, and MCP-1, as a classic inflammatory cytokine, have been extensively studied in CHD [17–19]. Among the 6 cytokines involved in our study, IL-10 was the only anti-inflammatory cytokine, while the rest were proinflammatory cytokines. Nevertheless, our data showed that there was a statistically significant difference in IL-8 concentrations between the two groups ($P < 0.05$), while IL-1β, IL-6, TNF, and IL-12P70 revealed no significant difference.

Similar to the results of Karina Vargas-Sanchez's result, GLP-1R was not associated with any inflammatory markers [20]. Since the surface of natural killer T cells (NKT) also expresses GLP-1R, and this pathway can enhance the expression of anti-inflammatory cytokines such as IL-10 [21]; this may partly explain the higher IL-10 levels in Karina Vargas-Sanchez and our study in the disease group, although

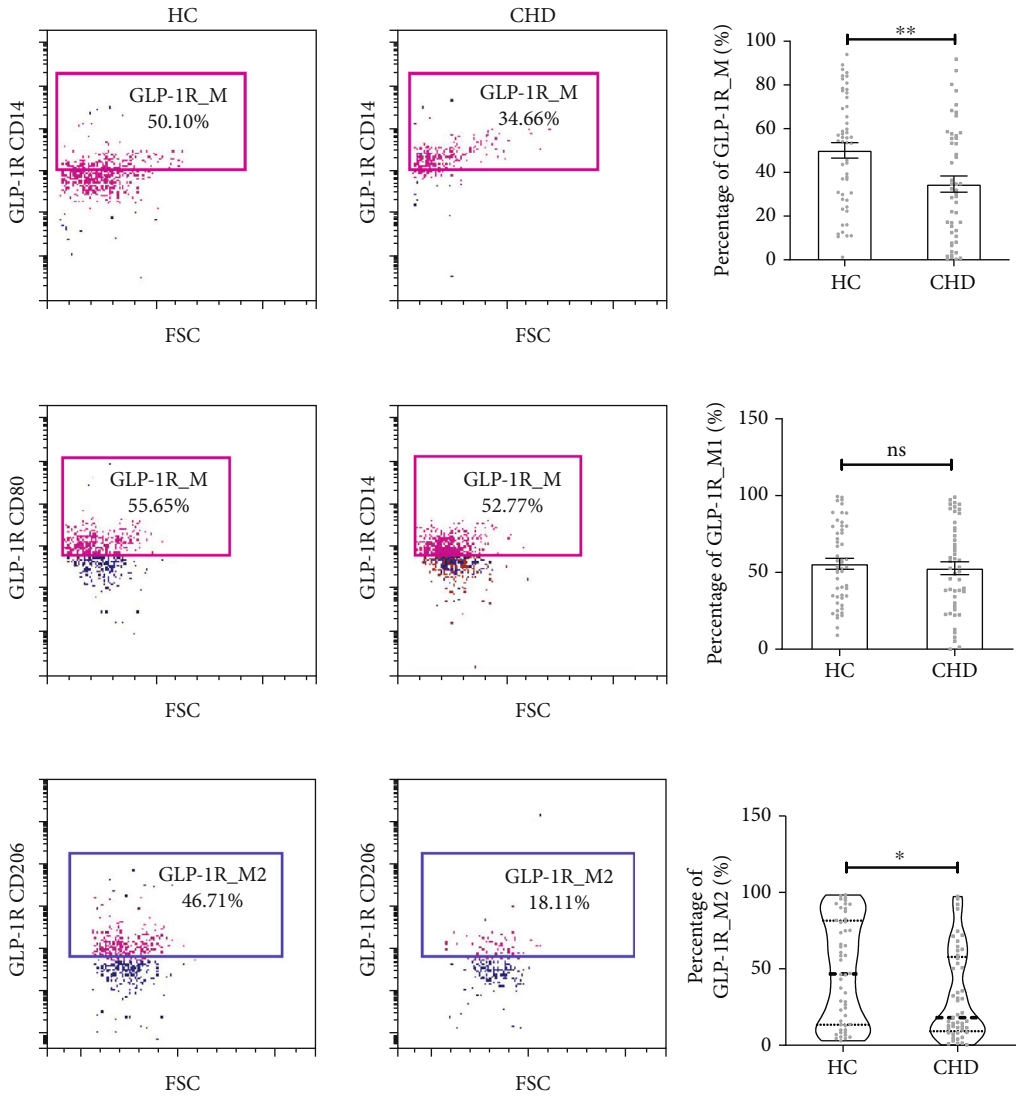

Figure 2: Expression difference of GLP-1R on the surface of total macrophage, M1, and M2 macrophage between two groups. Data are represented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$. GLP-1R_M: GLP-1R on the surface of macrophage, labeled by CD14+ and GLP-1R+ molecule; GLP-1R_M1: GLP-1R on the surface of M1 macrophage, labeled by CD14+, CD80+, and GLP-1R+ molecule; GLP-1R_M2: GLP-1R on the surface of M2 macrophage, labeled by CD14+, CD206+, and GLP-1R+molecule.

there was no statistically significant difference in IL-10 between the two groups in our study. Moreover, our sample is peripheral blood-derived cytokines, which can well reflect the downstream results of the aforementioned systematic regulation of human effects.

Other possibilities we speculate about for the above phenomenon are as follows: (1) since the diagnostic standard of CHD is 50% of the stenosis degree of one or more major coronary arteries as the limit, the subjects who did not reach the stenosis degree but still had slight plaque formation were included in the HC group, leading to the fact that the HC group was not completely free of plaque formation, but more than that, if a statistically significant difference was found between the CHD group and the population with stenosis between 0 and 50 percent, it would be easier to identify patients without stenosis at all; (2) compared with acute inflammation caused by severe trauma or pathogen infection, CHD is a mild chronic inflammation of the circulatory system [19], with low baseline concentration of inflammatory or anti-inflammatory cytokine and no significant change in a short term; it is possible that a large sample size of a multicenter, large-scale population would reveal statistical differences [17]; (3) mild inflammation is common in a variety of chronic diseases, and the exclusion criteria of this study is limited to acute inflammation, infective inflammation, and patients with autoimmune diseases or take immunosuppressant, etc., other than cardiovascular system of chronic inflammatory diseases [22] such as mild liver dysfunction or mild chronic gastritis patients has not been ruled out strictly; what is more, too strict rule out conditions limit the extrapolation of the results of the study and against the scenario of the real world; (4) IL-1, IL-12, and TNF-α can be used as the characteristic molecule of M1 macrophages, while IL-10 and IL-4 can be used as the characteristic

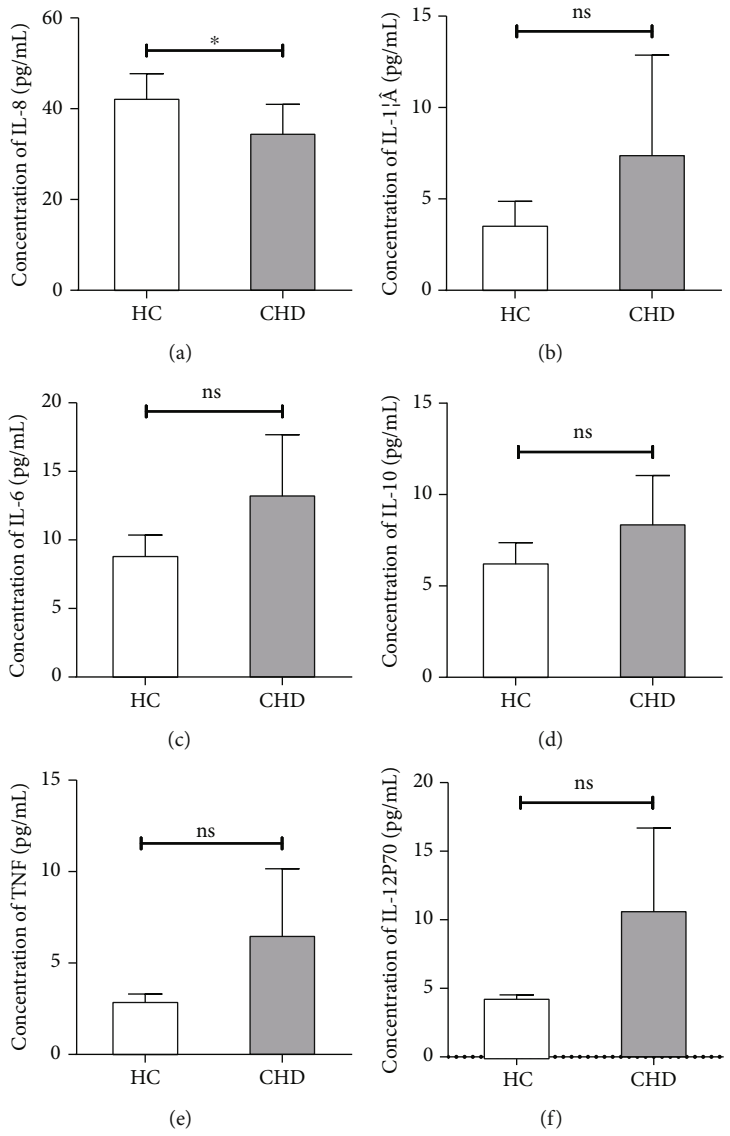

Figure 3: Plasma cytokine concentrations of IL-8, IL-1, IL-6, IL-10, TNF, and IL-12P70 in patients with coronary heart disease and health control. Data are represented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$.

molecule of M2 macrophages [23]. As mentioned in this study, there was no difference in the percentage of M1 macrophages or M2 macrophages between the HC group and the CHD group; this may explain why IL-12, IL-4, IL-1, and IL-10 were not different between the two groups.

Atherosclerosis is closely related to lipid metabolism and inflammatory reaction [24, 25]. In the 101 people included in this study, correlation analysis shows that the GLP-1R expression level of total macrophages was inversely proportional to TG; TG is a known and recognized cardiovascular risk factor [26], indicating that GLP-1R negatively regulates TG in both patients with CHD and CS population. In the HC group, the expression level of GLP-1R M2 was negatively correlated with IL-8, as a chemokine of neutrophils; IL-8 promotes the migration of neutrophils to the inflammatory site and blocks cholesterol efflux by inhibiting the expression of ABCA1 [27], thus promoting the inflammatory response. Laura J. den Hartigh [28] stimulated human monocytes with TG and lipoprotein lipase, the degradation product of VLDL, then observed that monocytes initiate adhesion to endothelial cells, and detected the up-regulation of IL-8 expression, thus supporting our results. Combined with flow cytometry detection in this study, it was found that the expression level of M2 macrophages in the HC group was higher than that in the CHD group; we speculated that, compared with the CHD group, the enhanced protective factors represented by the up-regulated GLP-1R expression of M2 macrophages in the HC group were the main reasons.

Though in the CHD group, the expression level of GLP-1R_M1 was positively correlated with TG, and we have observed that GLP-1R on the cell membrane of total, M1, and M2 macrophages was lower than that of the HC group. We thus speculated that the high expression of GLP-1R was related to the increase in the number of M1 macrophages, or, as a negative feedback regulatory compensation mechanism of the body, M1 macrophages could be highly expressed

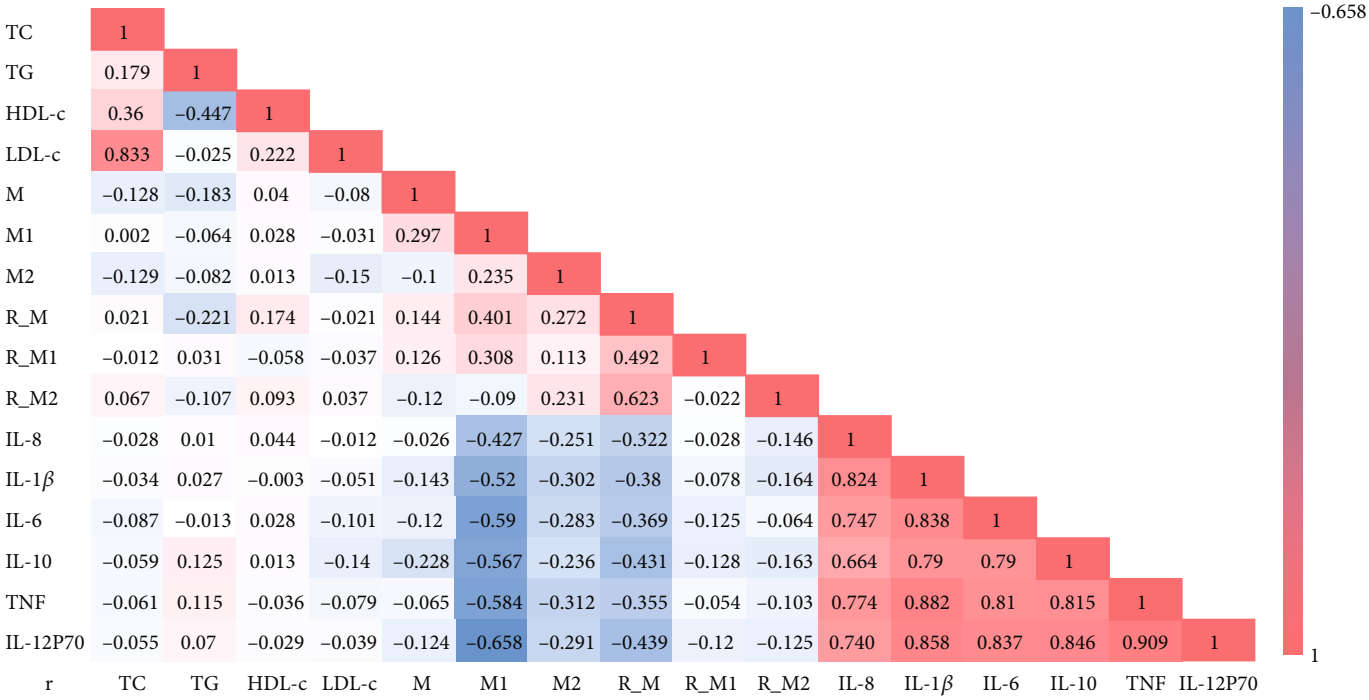

Figure 4: Correlation analysis between lipid markers, macrophage, GLP-1R, and cytokines in total 101 people. Spearman rank correlation was used. Value is correlation coefficient, bold black font represents $P < 0.05$, bold red font means $P < 0.01$. Colors represent different correlation coefficient as indicated by the color bar. R_M: GLP-1R_M; R_M1: GLP-1R_M1; R_M2: GLP-1R_M2.

in GLP-1R. Although this study did not show a difference in the percentage of M1 macrophages between the two groups, clinical studies with a larger sample size may show a difference to explain this phenomenon, and the specific mechanism and causal relationship need to be further studied. Luo et al. [29] inhibited the ROS, NLRP3, and Caspase-1 of HUVECs by using GLP-1R agonist Dulaglutide and also inhibited the maturation of IL-1 and IL-18. Therefore, GLP-1R may mediate the polarization of macrophages and release different inflammatory small molecules through NLRP3/Caspase1 mechanism, so as to regulate the development of AS.

The massive immune system composed of immune cells, the immune organs, and the inflammatory response participates in physiological and pathological conditions of the body all the time; the role and mechanism of the newly discovered macrophage-subtype T cell subtype in atherosclerosis need to be further studied. Like most clinical studies, CHD group and the HC group are according to the diagnostic gold standard of coronary angiography and intravascular ultrasound to distinguish, but the technology itself, provides only stenosis site and degree of structural information, such as functional index score blood flow reserve (FFR) has not been collected and assist for the illness subgroup analysis. Last but not least, it is hoped to find disease biomarkers of CHD [30], such as C-reactive protein (CRP), IL-6, serum amyloid A (SAA), CD40/CD40 ligand (CD40L), and heat shock protein 60 (Hsp60) [31] and 70 (Hsp70), but their specificity needs to be improved; therefore, more sensitive and specific inflammatory disease biomarker still need to be studied.

5. Conclusion

GLP-1R agonist is independent of the hypoglycemic effect of T2DM and has protective effect on cardiovascular system. GLP-1R may regulate the polarization of macrophages toward M2, thus playing a protective role in the progression of coronary atherosclerosis.

Data Availability

Supporting data of the findings in our study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest.

Authors' Contributions

Handong Yang and Jun Chen designed the study. Li Yang and Long Chen collected the clinical data and completed the experiment. Yang Li, Dongfeng Li, Hao Xu, Jishun Chen, Xinwen Min, Meian He, Tangchun Wu, Jixin Zhong, and Jun Zhao (Hubei University of Medicine) completed the statistical analysis and interpreted the results. The manuscript was drafted by Li Yang and Chen Jun and corrected by Li Yang and Handong Yang.

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Research Article

Laboratory Predictors of COVID-19 Mortality: A Retrospective Analysis from Tongji Hospital in Wuhan

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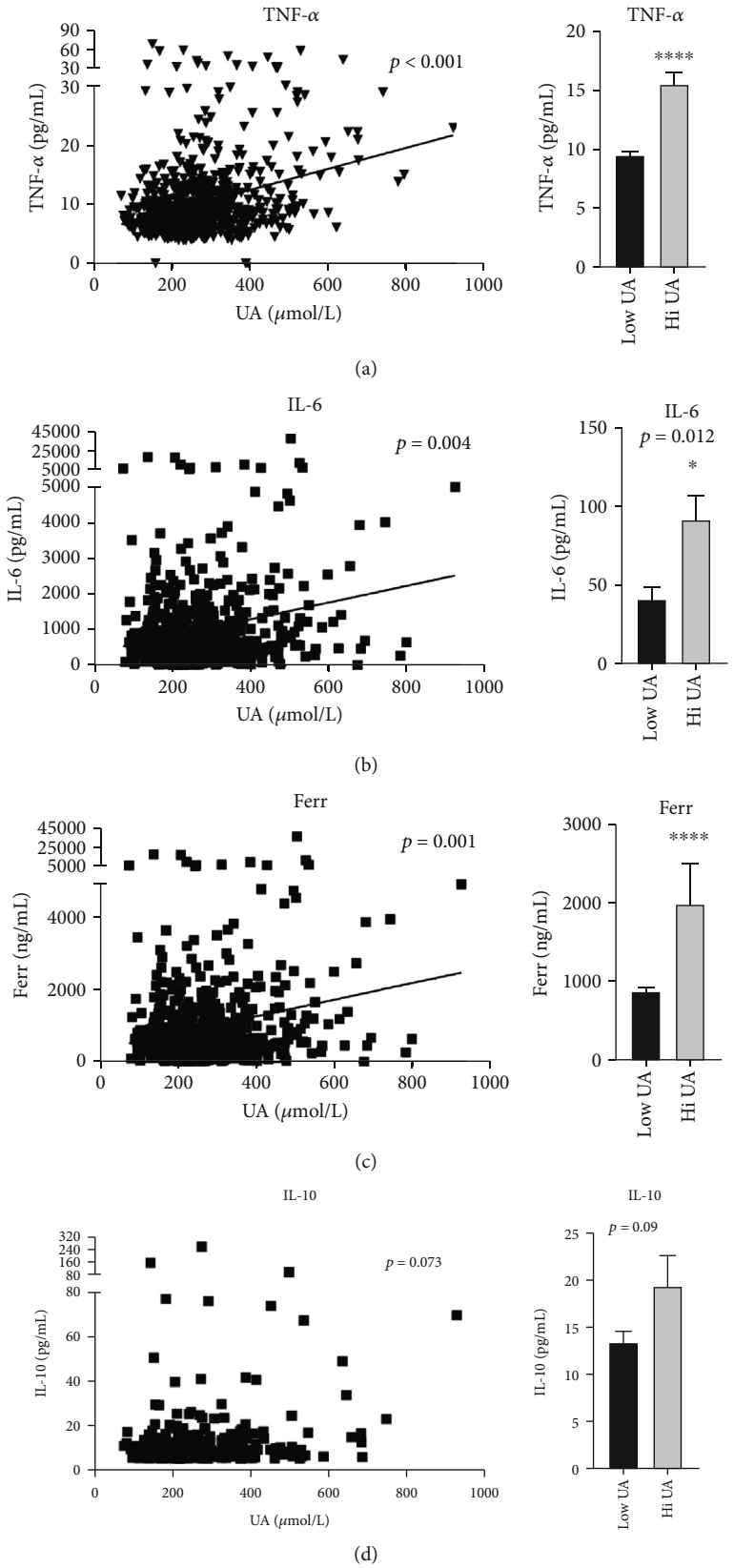
Background. Novel coronavirus disease 2019 (COVID-19), an acute respiratory disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), rapidly progressed to a global pandemic. Currently, there are limited effective medications approved for this deadly disease. *Objective*. To investigate the potential predictors of COVID-19 mortality and risk factors for hyperinflammation in COVID-19. *Methods*. Retrospective analysis was carried out in 1,149 patients diagnosed with COVID-19 in Tongji Hospital, Wuhan, China, from 1/13/2020 to 3/15/2020. *Results*. We found significant differences in the rates of hyperuricemia (OR: 3.17, 95% CI: 2.13-4.70; $p < 0.001$) and hypoalbuminemia (OR: 5.68, 95% CI: 3.97-8.32; $p < 0.001$) between deceased and recovered patients. The percentages of hyperuricemia in deceased patients and recovered patients were 23.6% and 8.9%, respectively, which were higher than the reported age-standardized prevalence of 6.2% in Chinese population. Of note, the percentages of both IL-6 and uric acid levels in survived COVID-19 patients were above 90%, suggesting that they might be good specificity for indicators of mortality in COVID-19 patients. The serum level of uric acid (UA) was positively associated with ferritin, TNF-α, and IL-6 but not with anti-inflammatory cytokine IL-10. In addition, the levels of these proinflammatory cytokines in COVID-19 patients showed a trend of reduction after uric acid lowering therapy. *Conclusions*. Our results suggest that uric acid, the end product of purine metabolism, was increased in deceased patients with COVID-19. In addition, the serum level of uric acid was positively associated with inflammatory markers. Uric acid lowering therapy in COVID-19 patients with hyperuricemia may be beneficial.

1. Introduction

Coronavirus disease 19 (COVID-19) caused by the ribonucleic acid (RNA) virus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), emerged in Wuhan City, Hubei Province, China, has spread rapidly across the world. At the time of drafting this manuscript (Oct. 19, 2020), the worldwide death toll from the COVID-19 pneumonia eclipsed 1,000,000 [1], and the number of people infected continues to slowly climbed upward. Although predictors such as high-sensitivity C-reactive protein (hsCRP), aspartate aminotransferase (AST), and D-dimer for mortality of COVID-19 patients had been determined [2], more risk predictors and prognostic factors still desperately needed to been found to improve the treatment programs for infected patients, especially for patients with other underlying diseases (such as identified risk factors indicator cardiac troponin I to preexisting concurrent cardiovascular or cerebrovascular diseases [3]; BMI for COVID-19 severity in the population living with diabetes in hospital admission [4]).



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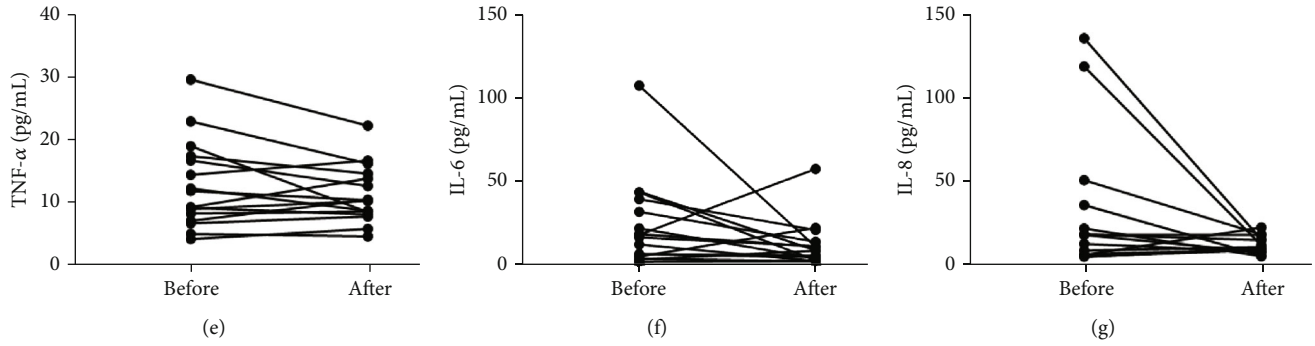

Figure 1: Uric acid is associated with serum levels of TNF-α, IL-6, and ferritin in COVID-19 patients. (a) Left, serum uric acid (UA) is associated with serum TNF-α level; right, serum TNF-α level in patients with a UA level of lower or higher than 400 μmol/L. (b) Left, association between serum UA and IL-6 levels; right, serum IL-6 level in patients with a UA level of lower or higher than 400 μmol/L. (c) Left, association between serum UA and ferritin levels; right, serum ferritin level in patients with a UA level of lower or higher than 400 μmol/L. (d) Left, association between serum UA and IL-10 levels; right, serum IL-10 level in patients with a UA level of lower or higher than 400 μmol/L. (e) Serum TNF-α level before and after uric acid lowering therapy. (f) Serum IL-6 level before and after uric acid lowering therapy. (g) Serum IL-8 level before and after uric acid lowering therapy.

2. Materials and Methods

2.1. Participants and Clinical Variables. We analyzed and observed serological tests result a number of laboratory parameters may serve as predictors of disease progression in 1,149 in patients diagnosed with COVID-19 in Tongji Hospital, Wuhan, China, from 1/13/2020 to 3/15/2020 and performed statistical analysis to estimate ORs and 95% CIs of mortality. The correlation between serum uric acid and other inflammatory factors and the content of these inflammatory factors before and after treatment were compared.

2.2. Statistical Analysis. Categorical variables were reported as percentages. Combined odds ratios (ORs) and 95% CIs were evaluated as effect size using uni- and multivariate analyses. We used linear regression to evaluate any association between two variables. p values less than 0.05 were considered statistically significant.

2.3. Ethics. The study was performed in accordance with the ethical standards laid down in the Declaration of Helsinki. Our work has been reviewed and approved by Tongji Hospital Ethics Committee.

3. Results

In univariate analyses, patients who died from COVID-19 had higher hyperinflammation markers than patients who survived: lactate dehydrogenase (LDH, OR: 25.14, 95% CI: 17.06-37.53; $p < 0.0001$), AST (OR: 5.08, 95% CI: 3.67-7.05; <0.0001), alanine aminotransferase (ALT, OR: 1.52, 95% CI: 1.07-2.14; $p = 0.018$), ferritin (OR: 12.92, 95% CI: 5.74-37.0; $p < 0.0001$), and inflammatory cytokines TNF-α (OR: 4.34, 95% CI: 2.90-6.59; $p < 0.0001$), Interleukin-6 (IL-6, OR: 68.63, 95% CI: 31.02-182.30; $p < 0.0001$), Interleukin-8 (IL-8, OR: 7.60, 95% CI: 4.26-13.80; $p < 0.0001$), and anti-inflammatory cytokines Interleukin-10 (IL-10, OR: 8.06, 95% CI: 3.56-20.75; $p < 0.0001$). In addition to these previously confirmed indicators of COVID-19 disease severity [5, 6], we found significant differences in the rates of hyperuricemia (OR: 3.17, 95% CI: 2.13-4.70; $p < 0.001$) and hypoalbuminemia (OR: 5.68, 95% CI: 3.97-8.32; $p < 0.001$) between deceased and recovered patients. The percentages of hyperuricemia in deceased patients and recovered patients were 23.6% and 8.9%, respectively, which are higher than the reported age-standardized prevalence of 6.2% in Chinese population [7]. Of note, the percentages of both IL-6 and uric acid (UA) levels in survived COVID-19 patients were above 90%, suggesting that these two factors have a good specificity when used as indicators for COVID-19 mortality.

Interestingly, the serum level of uric acid is positively associated with ferritin, TNF-α, and IL-6 but not with anti-inflammatory cytokine IL-10 (Figures 1(a)–1(d)). Patients with a serum uric acid level over 400 μmol/L had higher serum levels of TNF-α, IL-6, and ferritin. To investigate if the increase of uric acid contributes to the hyperinflammatory status in COVID-19, we analyzed cytokine levels before and after administration of uric acid lowering agents (febuxostat or benzbromarone). A total of 16 COVID-19 patients with an acute gout attack during the hospital stay were examined for cytokine profile before and after the treatment with uric acid lowering therapy. After an average of 7 days of therapy, there was a trend toward reduced serum levels of IL-6, IL-8, and TNF-α (Figures 1(e)–1(g)). There were 9 out of the 16 patients taking uric acid lowering agents that had a serum uric acid level over 400 μmol/L. While 8 out of these 9 patients recovered from COVID-19, only 74 out of the 121 hyperuricemia patients without uric acid lowering agents survived (88.9% vs. 61.2%; $p = 0.096$).

4. Discussion

We observed in this study that deceased COVID-19 patients had a higher rate of hyperuricemia. Given the fact that biomarkers of tissue damage such as LDH, AST, ALT, and ferritin were increased in severely ill or deceased COVID-19 patients (Table 1 and references [5, 6]), increased percentages

Table 1: Laboratory parameters in 1,149 COVID-19 patients hospitalized in Tongji hospital between 1/13/2020 and 3/15/2020.

	Deceased, *n* (%)	Survived, *n* (%)	OR (95% CI)	*p*
Age (years)				
<60, *n* (%)	41 (19.0)	522 (55.9)	1.00	—
≥60, *n* (%)	175 (81.0)	411 (44.1)	3.36 (2.36-4.89)	<0.0001
Gender				
Male, *n* (%)	145 (67.1)	477 (47.9)	2.23 (1.63-3.09)	<0.0001
Female, *n* (%)	71 (32.9)	486 (52.1)	1.00	—
IL-6 (pg/mL)				
>150, *n* (%)	61 (43.3)	6 (1.1)	68.63 (31.02-182.30)	<0.0001
≤150, *n* (%)	80 (56.7)	540 (98.9)	1.00	—
IL-10 (pg/mL)				
> 15, *n* (%)	72 (68.6)	44 (29.7)	8.06 (3.56-20.75)	<0.0001
≤ 15, *n* (%)	33 (31.4)	104 (70.3)	1.00	—
IL-8 (pg/mL)				
> 100, *n* (%)	32 (24.8)	22 (4.2)	7.60 (4.26-13.80)	<0.0001
≤ 100, *n* (%)	97 (75.2)	507 (95.8)	1.00	—
TNF-α (pg/mL)				
> 100, *n* (%)	83 (65.4)	159 (30.3)	4.34 (2.90-6.59)	<0.0001
≤ 100, *n* (%)	44 (34.6)	366 (69.7)	1.00	—
Uric acid (μmol/L)				
> 400, *n* (%)	48 (23.6)	82 (8.9)	3.17 (2.13-4.70)	<0.0001
≤ 400, *n* (%)	155 (76.4)	840 (91.1)	1.00	—
Ferritin (ng/mL)				
> 400 (M)/300 (F), *n* (%)	123 (96.1)	337 (65.6)	12.92 (5.74-37.0)	<0.0001
≤ 400 (M)/300 (F), *n* (%)	5 (3.9)	177 (34.4)	1.00	—
Albumin (g/L)				
≥ 35, *n* (%)	40 (19.9)	537 (58.4)	1.00	—
< 35, *n* (%)	161 (80.1)	382 (41.6)	5.68 (3.97-8.32)	<0.0001
LDH (U/L)				
> 450, *n* (%)	123 (62.4)	57 (6.2)	25.14 (17.06-37.53)	<0.0001
≤ 450, *n* (%)	74 (34.6)	862 (93.8)	1.00	—
ALT (U/L)				
> 40, *n* (%)	57 (28.4)	189 (20.6)	1.52 (1.07-2.14)	0.018
≤ 40, *n* (%)	144 (71.6)	728 (79.4)	1.00	—
AST (U/L)				
> 40, *n* (%)	102 (50.7)	153 (16.7)	5.08 (3.67-7.05)	<0.0001
≤ 40, *n* (%)	99 (49.3)	764 (83.3)	1.00	—
AST/ALT				
> 1, *n* (%)	159 (79.1)	533 (60.3)	2.48 (1.74-3.61)	<0.0001
≤ 1, *n* (%)	42 (20.9)	364 (39.7)	1.00	—

ALT: alanine aminotransferase; AST: aspartate aminotransferase; IL-6: interleukin 6; IL-8: interleukin 8; IL-10: interleukin 10; LDH: lactate dehydrogenase; TNF-α: tumor necrosis factor α.

of hyperuricemia in COVID-19 patients, especially in deceased patients, may be a result of tissue damage/cell death. Uric acid has been characterized as a danger signal to alarm immune system to cell injury and initiate immune responses to clear damaged cells/tissues [8]. Uric acid is a degradation product of purines and presents in the blood at a high concentration approaching to the saturation threshold. In the event of tissue damage, intracellular stores of uric acid are released out of the cells, which may cause hyperuricemia and form crystals. Crystallized form of uric acid is a strong inducer of NLRP3 inflammasome, inciting robust inflammation. Nucleated uric acid is able to stimulate the production of inflammatory cytokines such as IL-1, TNF-α, and IL-6 in gout, a classic disease caused by hyperuricemia. In this case series, we observed positive correlations between uric acid and inflammatory markers in patients with COVID-19. In

addition, uric acid lowering treatment with febuxostat and/or benzbromarone reduced levels of proinflammatory cytokines in COVID-19 patients. There was also a trend toward reduced mortality rate in hyperuricemia patients with uric acid lowering therapy. These results suggest that uric acid lowering therapy may be beneficial in COVID-19 patients with hyperuricemia. However, a further investigation with larger sample size and randomized controls is required to confirm the beneficial effects of uric acid lowering therapy in reducing proinflammatory cytokines.

In summary, uric acid released from the injured tissues/cells may serve as a danger signal to amplify the hyperinflammatory response in severe COVID-19 cases. Uric acid lowering therapy in COVID-19 patients with hyperuricemia patients may be able beneficial.

Data Availability

Derived data supporting the findings of this study are available from the corresponding author (JZ, LD, QG) on request.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

All the authors contributed substantially to the work. Drs. Zhong, Dong, and Gong contributed equally to this article.

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Research Article

Cytomegalovirus Infection and Its Relationship with Leukocyte Telomere Length: A Cross-Sectional Study

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Background. Telomeres undergo shortening with each cell division, which could be accelerated by infection. The association between virus infection and telomere length is poorly understood. In the present study, we investigated the putative associations between leukocyte telomere length (TL), cytomegalovirus (CMV) infection, and C-reactive protein (CRP) in a national representative sample of noninstitutionalized population. *Methods*. We analyzed data that was collected in a cross-sectional setting, where 3,987 participants were enrolled with available data on telomere length. The association between telomere length with previous CMV infection and CRP was analyzed using multivariable linear regression models. We further tested if obesity, measured by body mass index (BMI), and smoking could modify this relationship. *Results*. In total, around 46% percent of the study population were men and 54% were women. Average ages were 35.1 years for men and 35.0 years for women. One unit increase of CMV antibody IgG titer was associated with -0.07 (95% confidence interval: -0.12, -0.01) unit decrease of leukocyte TL when sex was adjusted for. After additionally adjusting for BMI and smoking status, the magnitude of the association was only slightly decreased to -0.06 (95% confidence interval: -0.11, -0.01). The effect sizes were comparable after additionally adjusting for CRP. These analyses imply that previous CMV infection affects leukocyte TL through pathways other than CRP. *Conclusions*. Previous CMV infection was associated with shorter leukocyte TL. This association was independent of CRP.

1. Background

Telomeres are repeated sequences of nucleotides with protecting proteins at the end of chromosomes. The length of telomeres (TL) shortens as cell divides. TL has therefore been acknowledged as a putative predictive biomarker for biological aging [1]. TL shortens as people become older and has been reported to be a risk factor for metabolic disorders like diabetes [2, 3], cardiocerebrovascular disease, and metabolic syndrome [4–7], among others like dementia and cancer [8–14]. Its association with immunological functions and virus infection has, however, rarely been investigated [15].

Chronic cytomegalovirus (CMV) infection significantly influences the immune system and has been found to be one of the main determinants of immune senescence in the elderly [16, 17]. The serum titer of CMV antibody is widely used and measured in both research and clinics as one of the biomarkers for CMV infection. However, the role of CMV infection/antibody titer in cellular senescence (e.g., biological aging as measured by leukocyte TL) has rarely been extensively examined. A landmark report on this topic was a study of 159 healthy individuals from the Netherlands, which found that telomere length shortening was even more pronounced in CMV-seropositive individuals [18]. A more recent study using longitudinal data also found that CMV infection and inflammatory biomarkers were associated with mean levels of TL [19]. Another study, however, did not find significant differences of TL in participants with CMV seropositive and negative from a cross-sectional survey [20].

Additionally, studying the putative association between TL and CMV could supply further knowledge regarding the functions of infection and immune system in biological aging. Because C-reactive protein (CRP) was in a close relationship with both TL and CMV infection, this relationship between TL and CMV infection could therefore be confounded or mediated by CRP. We hypothesized, throughout

the manuscript, that higher levels of CMV antibody could be a risk factor for shorter leukocyte TL. This relationship may be also influenced by CRP. The analysis will be based on data from the National Health and Nutrition Examination Survey (NHANES).

2. Methods

2.1. Study Materials. The NHANES has been a continuous population-based survey led and conducted by the Centers for Disease Control and Prevention (CDC) with the primary aim to estimate the prevalence of various chronic disease, health status, and nutritional conditions among noninstitutionalized population across the US on a regular basis since long. The present study extracted data from the 1999-2000 and 2001-2002 cycles. During these periods, the study participants donated blood samples and other biomaterials. The leukocyte TL was also measured thereafter [21].

2.2. Telomere Length Measurement. The measurement procedures and standard operation process for telomere length assessment were reported elsewhere previously [21, 22]. The actual DNA processing procedure was conducted by the laboratory of the NHANES Division in the Centers for Disease Control and Prevention, USA. DNA samples were extracted from peripheral blood samples and then stored at -80°C. The TL measurement was performed later in another laboratory in UCSF. A standard quantitative polymerase chain reaction (PCR) method was employed for the assessment. This method was able to calculate the relative TL as commonly presented as the *T*/*S* ratio in previous publications. On average, 98.7% of the samples passed the quality control process.

2.3. Cytomegalovirus Antibody Ascertainment. The detailed procedures for the CMV antibody assessment were described on the official website (https://wwwn.cdc.gov/Nchs/Nhanes/2001-2002/SSCMV_B.htm). In short, CMV-specific IgG was measured with an ELISA method. Optical density shows whether the antibody titer was low or high.

2.4. Statistical Analysis. We log-transformed leukocyte TL and CRP and employed multivariable linear regression models to estimate and test the association between leukocyte TL and CMV antibody. We presented continuous variables by mean and standard deviations and category variables as the number and proportions. Different regression models were used to calculate these associations. Firstly, we estimated the crude association in which the leukocyte TL is the dependent variable and independent variables are CMV antibody, ethnicity, age, and sex. We further adjusted for body mass index (BMI) in the second model. The third model was moreover adjusted for smoking status. The fourth model was controlled for CRP. We also recorded CMV antibody and CRP to be a categorical variable by quartiles and presented the analyses as well. Because NHANES used complex survey designs, weights for the sampling were considered in the multivariable analyses using the survey package in R. We used R 3.6 for all statistical analysis and $P < 0.05$ as a statistical significance level.

Table 1: Basic characteristics of study participants in NHANES.

Variables	Men ($n = 1816$)	Women ($n = 2171$)
Age	35.1 (0.3)	35.0 (0.3)
Telomere length (*T*/*S* ratio)	1.12 (0.02)	1.13 (0.02)
Cytomegalovirus antibody (AU/mL)	1.09 (0.04)	1.48 (0.03)
Body mass index (kg/m^2)	27.6 (0.2)	27.9 (0.2)
C-reactive protein (mg/dL)	0.29 (0.01)	0.45 (0.02)
Smoking	51.0%	42.1%
Ethnicity		
Non-Hispanic White	10.1%	8.4%
Non-Hispanic Black	7.1%	8.1%
Mexican American	68.7%	67.8%
Others	14.1%	15.7%

3. Results

Table 1 presents the demographic characteristics for men and women. 3,987 participants had both leukocyte TL and CMV antibody data. 1,816 (46.0%) were men and 2,171 (54.0%) were women. Men and women had similar average ages (35.1 and 35.0 years for men and women, respectively). While women had slightly longer TL compared with men (1.13 and 1.12 T/S ratio), it is not statistically significant. CMV antibody was higher in women (1.48 and 1.09 AU/mL).

Table 2 describes the associations between CMV antibody and leukocyte TL in this study population. We first categorized CMV antibody to a categorical variable with four equal groups by quartiles and then used the categorical CMV antibody as an independent variable to investigate its association with leukocyte TL. Compared with people in the lowest CMV antibody group, people in the highest CMV antibody group had -0.18 (95% confidence interval: -0.32, -0.05) lower leukocyte TL in the first model, where age and sex were adjusted for. The magnitude of this association was comparable when taking into account BMI in the second model ($\beta = -0.18$, 95% CI: -0.31, -0.04). When adjusting for smoking status, the association was lowered to -0.17 (95% CI: -0.30, -0.03). We also modeled CMV antibody as a continuous variable and found that one unit increase of CMV antibody was associated with -0.06 (95% CI: -0.11, -0.01) decrease of leukocyte TL when adjusting for age, sex, BMI, and smoking status in the third model. We tested the interaction term between sex and CMV antibody and did not find statistical significance.

Likewise, we studied the association of CRP with leukocyte TL and presented the results in Table 3. The same modeling strategies were used as CMV antibody described above. In Model 3, after controlling for age, sex, BMI, and smoking status, people in the highest group of CRP levels had -0.22 (95% CI: -0.33, -0.11) lower levels of leukocyte TL compared with those in the lowest group of CRP levels. One unit increase of CRP antibody was associated with -0.06 (95% CI: -0.11,-0.01) decrease of leukocyte TL when

Table 2: Association between cytomegalovirus antibody and telomere length in NHANES.

Cytomegalovirus antibody	Model 1 (crude model)	Model 2 (age, sex adjusted model)	Model 3 (multivariable adjusted model)
Q1	0 (reference)	0 (reference)	0 (reference)
Q2	0.03 (-0.07, 0.13)	0.04 (-0.05, 0.14)	0.04 (-0.05, 0.14)
Q3	-0.04 (-0.18, 0.10)	-0.04 (-0.17, 0.10)	-0.03 (-0.17, 0.10)
Q4	-0.18 (-0.32, -0.05)	-0.18 (-0.31, -0.04)	-0.17 (-0.30, -0.03)
Continuous	-0.07 (-0.12, -0.01)	-0.07 (-0.12, -0.01)	-0.06 (-0.11, -0.01)

Q: quartile.

Table 3: Association between C-reactive protein and telomere length in NHANES.

C-reactive protein	Model 1 (crude model)	Model 2 (age, sex adjusted model)	Model 3 (multivariable adjusted model)
Q1	0 (reference)	0 (reference)	0 (reference)
Q2	-0.10 (-0.19, 0.001)	-0.08 (-0.17, 0.01)	-0.08 (-0.17, 0.01)
Q3	-0.17 (-0.35, 0.01)	-0.13 (-0.29, 0.02)	-0.13 (-0.29, 0.03)
Q4	-0.28 (-0.42, -0.15)	-0.23 (-0.34, -0.11)	-0.22 (-0.33, -0.11)
Continuous	-0.08 (-0.12, -0.03)	-0.06 (-0.10, -0.02)	-0.06 (-0.09, -0.02)

Q: quartile.

Table 4: Joint association of cytomegalovirus antibody and CRP with telomere length in NHANES.

Variables	Model 4 (additionally CRP-adjusted model)
Cytomegalovirus antibody	
Q1	0 (reference)
Q2	0.04 (-0.05, 0.14)
Q3	-0.03 (-0.17, 0.11)
Q4	-0.16 (-0.29, -0.02)
Continuous	-0.06 (-0.11, -0.01)
C-reactive protein	
Q1	0 (reference)
Q2	-0.07 (-0.17, 0.02)
Q3	-0.13 (-0.29, 0.04)
Q4	-0.25 (-0.32, -0.11)
Continuous	-0.06 (-0.09, -0.02)

Q: quartile.

controlling for age, sex, BMI, and smoking status in the third model.

In Table 4, we showed the results of the joint analyses of CMV antibody and CRP with leukocyte TL. We found that the associations of CMV antibody and CRP with leukocyte TL were independent of each other. The effect magnitudes were similar to those in Tables 2 and 3. We did not find a significant interaction effect of CMV antibody and CRP.

4. Discussion

In the present study, we tested the hypothesis that previous CMV infection (measured by antibody) was associated with shorter leukocyte TL and this relationship might be modulated by CRP. We investigated this association in a large cohort, which is a population-based survey using the NHANES data. We found that higher levels of previous CMV infection were associated with shorter leukocyte TL. This observed association only slightly changed after controlling for multiple covariates including age, sex, BMI, and smoking status. Additionally controlling for CRP in the multivariable regression models did not change the effect size too much, which might suggest that previous CMV infection influences leukocyte TL through biological pathways beyond CRP.

Leukocyte TL has been reported to be a predictor for various aging-related diseases and to be influenced by stress and inflammation. However, its association with infection, particularly CMV infection, has seldom been investigated. Addressing these associations is of significance in enhancing our knowledge of how the underlying mechanisms could affect different aspects of immune and biological aging. A previous clinical study reported that previous CMV infection could induce a strong decrease in T cell TL [18]. However, the Berlin BASE-II study performed a similar comparative analysis of leukocyte TL in CMV-positive and CMV-negative individuals and only found leukocyte TL to be slightly longer in CMV-negative participants ($P = 0.056$) [20]. This study did not perform multivariable analyses to control the potential confounding factors. A recent study using data from the Baltimore Longitudinal Study on Aging employed sophisticated models with adjustment for more confounders and reported that CMV infection was associated with shorter leukocyte TL [19]. Studies examining the association of CRP with leukocyte TL were more than that of CMV infection. Most of these studies observed shorter TL to be related to higher CRP levels [23–25]. A recent Mendelian randomization study using CRP genetic variants suggested that CRP could a causal risk factor for TL [26]. Our analyses were largely comparable to these results, but with larger sample sizes.

Previous studies also reported inflammation was associated with CMV infection [27, 28]. It is thus natural to hypothesize that inflammation (measured by CRP) could moderate the associations between previous CMV infection and leukocyte TL. In our study, we examined this hypothesized relationship. However, the findings did not support this hypothesis. When adjusting for CRP in the regression models, the magnitude of the observed association was attenuated slightly. This implies that CRP might not be an important mediator for the association of previous CMV infection and TL. The influence of previous CMV infection on shorter TL might be through other pathways.

Several strengths and limitations for the present study should be acknowledged. First, leukocyte TL was processed and assessed in a lab using well-established PCR methods. Second, the population recruited in this study were chosen as a random sample by NHANES. The sample size was larger compared with previous studies and could be representative of the age span of the participants studied, which allowed the generalization. Lastly, we adjusted for a number of potential confounders in the multivariable regression analyses including age, BMI, smoking status, and sex. These results in general suggest that our observed association for previous CMV infection and leukocyte TL was not dependent of the studied potential confounding variables. However, a few limitations should also be noted. The design of our cross-sectional study makes it hard to infer causality because of the limitations of the data collection. In this study, all these biomarkers were measured from the blood that was collected at the same time. Therefore, it is difficult to make difference between the causes and consequences. Additionally, there might be other variables that could potentially confound the observed association that we did not control. Residual confounding should be taken into account of or adjusted for in either the study design or analysis stage in future studies.

In summary, our present study demonstrates that previous CMV infection was associated with shorter leukocyte TL in the study participants of NHANES and this association was independent of CRP, suggesting that previous CMV infection may affect TL through other pathways. Our analyses could shed light on the underlying biology of immune function and its roles in biological aging.

Data Availability

The data were provided in the supplementary file.

Ethical Approval

The investigation has been conducted in accordance with ethical standards and according to the Declaration of Helsinki and according to national and international guidelines. National Center for Health Statistics Ethics Review Board approved the NHANES survey.

Consent

Written informed consent was obtained in NHANES.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

All authors contributed to the design, analyses, and writing of the manuscript.

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Supplementary Materials

This supplementary file contains the full original data analyzed in this article. Each row refers to one study participant and each column refers to one variable. *(Supplementary Materials)*

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Research Article

Factors Related to Bone Metabolism in Kidney Transplant Recipients

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This study is aimed at establishing the prevalence of osteoporosis and osteopenia in kidney transplant recipients (KTRs) and determining the risk factors for bone mass loss. We invited KTRs who were under regular follow-up at Jiangxi Provincial People's Hospital Affiliated with Nanchang University to attend an assessment of osteoporotic risk assessed by questionnaire, biochemical profile, and dual-energy X-ray absorptiometry (DXA) scanning of the lumbar spine, total hip, and femoral neck. Binary logistic regression models were used to investigate the relationship between the different variables and bone mass density (BMD). A total of 216 patients satisfied the inclusion criteria. The group consisted of 156 men (72.22%) and 60 women (27.78%), and the mean age was 41.50 ± 9.98 years. There were 81 patients with normal bone mass (37.50%) and 135 patients with bone mass loss (62.50%). Logistic regression analysis showed that a higher phosphorus value and higher alkaline phosphatase concentration and a longer use of glucocorticoids were risk factors for bone mass loss in KTRs, and maintaining an appropriate weight and exercising an appropriate number of times per week helped to maintain bone mass.

1. Introduction

Kidney transplantation is a common and effective treatment modality for end-stage renal failure. Successful transplantation is capable of reversing many complications of renal failure; however, disturbances in bone and mineral metabolism may persist and be associated with a high risk of fracture, morbidity, and mortality. Kidney transplant recipients (KTRs) are known to have an increased risk of bone loss, and fracture risk is also higher than those for the general population and patients on dialysis [1–4]. At present, the focus of kidney transplantation research is mainly on the maintenance of renal function after transplantation and the side effects of immunosuppressive agents. The prevalence of osteoporosis of KTRs is estimated to be close to 30% [3], and an estimated 22.5% of patients will experience a fracture within the first 5 years following transplantation [4]. Intuitively, any treatment intervention to preserve bone mass density (BMD) in KTRs should be directed at the underlying cause; thus, identifying the risk factors for this complex pathophysiological situation is an attractive proposition. Bone loss after renal transplantation has not been well quantified in KTRs nor have the factors that may contribute to bone loss in this population been well elucidated. Therefore, this cross-sectional study was designed to establish the prevalence of bone loss and osteoporotic fractures and evaluate the risk factors for bone health in KTRs.

2. Materials and Methods

2.1. Patients. We invited 234 KTRs who were under regular follow-up by Jiangxi Provincial People's Hospital Affiliated with Nanchang University to attend an assessment of osteoporotic risk from August 16, 2018, to September 16, 2019. Exclusion criteria included systemic illness, prolonged immobilization, liver disease, Cushing syndrome, and

chronic gastrointestinal disease (chronic diarrhea or malabsorption). Patients with a history of thyroid disease before or after transplantation (hyperthyroidism or hypothyroidism) were excluded. The inclusion criteria included age 18 years or older, completed kidney transplant, and sign an informed consent. A total of 216 patients did not meet the exclusion criteria and met the inclusion criteria.

2.2. Methods. Bone health risk was assessed by questionnaire, biochemical profile, and dual-energy X-ray absorptiometry (DXA) scanning at the lumbar spine, total hip, and femoral neck. The contents of the questionnaire include age, sex, education, marriage, hemodialysis duration, age at the start of dialysis, age at transplantation, age of menopause, milk intake, exercise sessions per week, smoking habit, time outdoors, alcohol abuse, fracture, reason for the renal failure, renal source and duration of glucocorticoid use (glucocorticoids are converted to prednisone), average daily glucocorticoid dose, duration of cyclosporine, duration of tacrolimus, duration of mycophenolate mofetil (MMF), and exogenous intake of vitamin D and calcium (pre- and posttransplantation). Height and weight were measured, and the BMI was calculated.

Cumulative doses of glucocorticoids were calculated from outpatient and inpatient case data records, which included pulsed doses of intravenous methylprednisolone given during transplant rejection episodes. However, other immunosuppressants were given in varied doses according to the concentration of the drug. Thus, it is difficult to calculate the cumulative exposure, so the variable we use is the duration of use. Smoking history was defined as continuous or cumulative smoking for 6 months or more, and alcohol abuse was defined as an average daily alcohol intake ≥3 U (1 U≈285 ml standard beer/30 ml liquor/120 ml wine). Fracture information was obtained from the medical history and thoracolumbar anterolateral radiographs.

Routine laboratory tests including creatinine (CR), albumin (ALB), serum calcium, phosphorus, alkaline phosphatase (ALP), blood urea nitrogen (BUN), carbon dioxide combining power (CO2CP), total cholesterol (TC), triacylglycerol (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and fasting blood glucose (FBG) were measured using an automated multichannel analyzer (Olympus AU 800 automated multichannel analyzer, UK). Serum intact parathyroid hormone (iPTH), 25(OH)D (25-hydroxyvitamin D3), N-terminal propeptide of type 1 collagen (P1NP), and β-isomerized C-terminal telopeptide of type 1 collagen (β-CTX) were assessed by an automatic electrochemical luminescent immunoassay (Roche Cobas e601 automatic electrochemical luminescent immunoassay system, Switzerland).

BMD measured in grams per square centimeter was determined using DXA (Hologic Discovery 89098 densitometer, Waltham, MA, USA) for the lumbar spine (L1-L4 in the anteroposterior direction), total hip, and femoral neck. BMD was expressed in standard deviation units as t scores (comparison with the young adult mean) or as z scores (comparison with the age-matched mean). The following are the scores according to the diagnostic criteria of osteoporosis published by the World Health Organization (WHO) in 1994 [5]: postmenopausal women and men over 50 years old: t value ≥-1.0 SD indicates normal bone mass, a t value between -1.0 and -2.5 indicates osteopenia, and a t value ≤-2.5 indicates osteoporosis; premenopausal women and men younger than 50 years of age: z value ≤-2.0 is "below the expected range for age", and z value >-2.0 is "within the expected range for age". In addition, osteoporosis is also diagnosed in patients with brittle fractures. To facilitate the study, osteopenia, osteoporosis, and "below the expected range for age" are collectively associated with "bone mass loss", and the rest of the population has "normal bone mass."

2.3. Statistical Analysis. Demographics and other characteristics were summarized using descriptive statistics with continuous variables that were normally distributed and reported as the mean ± standard deviation (SD), and categorical variables are presented as numbers (percentage) or for nonparametric data as the median (interquartile range). Continuous variables were compared using Student's t-tests, the statistical verification value was expressed as t, and a p value of less than 0.05 was considered statistically significant. Pearson's χ^2 test or Fisher's exact test was used to compare categorical variables, the statistical verification value was expressed as χ^2, and a p value of less than 0.05 was considered statistically significant. Ordinal data were compared using the Mann-Whitney U test, the statistical verification value was expressed as z, and a p value of less than 0.05 was considered statistically significant. Univariate variables were included in the binary logistic regression analysis model, and stepwise regression analysis was used. A p value of less than 0.05 was considered statistically significant. All statistical analyses were performed using SPSS version 23.0 (Version 22; SPSS Inc., Chicago).

2.4. Informed Consent Was Obtained from all Patients, and the Local Ethics Committee Granted the Approval

3. Results

3.1. Descriptive Characteristic. A total of 216 patients satisfied the inclusion criteria. The group consisted of 156 men (72.22%) and 60 women (27.78%), among whom 20 (9.26%) were postmenopausal and 31 (14.35%) men were aged 50 or older. The mean age was 41.50 ± 9.98 years. We divided the patients into two groups: 81 patients with normal bone mass (37.50%) and 135 patients with bone mass loss (62.40%). Of the 20 postmenopausal women, 9 had osteoporosis, and 8 had osteopenia, which combined accounted for 85% of these postmenopausal participants. Among the 40 premenopausal women, 19 patients had bone mass loss, accounting for 47.50%. There were 7 cases (3.24%) with a history of brittle fracture. The anatomic fracture sites were as follows: including the spine ($n = 2$), forearm ($n = 2$), leg ($n = 2$), rib ($n = 1$), and hip ($n = 1$), ages ranged from 36-58 years, and the mean age was 47.43 ± 8.56 years. Seventy patients (32.41%) had smoking habits, and 41 patients (18.98%) abused alcohol. There were 15 KTRs (6.94%) who had diabetes listed as one of the diagnoses (among them, 4

patients had a history of diabetes, and 11 patients had fasting blood glucose greater than 7 mmol/L by monitoring, where postprandial blood glucose was not detected for 2 hours), and 8 of those were diagnosed with bone mass loss.

The cause of end-stage renal failure was glomerulonephritis in 68 patients, polycystic kidney in 4, hypertensive nephropathy in 25, diabetic nephropathy in 2, IgA nephropathy in 22, systemic lupus erythematosus in 1, and gouty nephropathy in 3, and in 87 cases, the cause was unknown or missing. Almost all kidney transplant recipients had been on long-term dialysis pretransplantation (214/216 99.07%). Patients underwent dialysis for a median of 19.04 months before transplantation. Most of the kidney supply is from unrelated donors (180/216, 83.33%).

None of the recipients had ever been treated with bisphosphonates, denosumab, calcitonin, or teriparatide. Seventy-one patients were taking vitamin D agents or calcium agents (32.87%) before transplantation and 9 patients after transplantation (4.17%). All subjects had received immunosuppression treatment with glucocorticoids, tacrolimus, MMF, or cyclosporine. Among the 216 patients, a serum calcium level higher than 3 mmol/L was rarely observed; hypercalcemic episodes (defined as total serum calcium > 2.62 mmol/L) were reported in 19 and 8.79% of KTRs, whereas hypophosphatemia (phosphorus < 2.5 mg/dl) was reported in 8 and 3.70% of KTRs. We defined insufficiency as 25 − OHD < 30 ng/ml, and it was reported in 100 and 46.30%; deficiency as 25 − OHD < 20 ng/ml, which was reported in 61 and 28.24%; and severe deficiency as 25 − OHD < 10 ng/ml, which was reported in 9 and 4.17%. More KTRs were in CKD stages 1 and 2, and fewer were in stages 3-5.

The baseline demographic, anthropometric, and lifestyle variables of the patients are presented in Table 1. There were statistically significant differences in weight, age, BMI, and exercise sessions per week between the low bone density group and the normal bone density group ($p < 0.05$).

The biochemical characteristics of the patients are presented in Table 2. There were statistically significant differences in phosphorus levels between the low bone density group and the normal bone density group ($p < 0.05$).

All patients were treated with glucocorticoid, tacrolimus, and cyclosporine or glucocorticoid, tacrolimus, and MMF immunosuppressive therapy after transplantation.

The use of immunosuppressive drugs in the two groups of patients is shown in Table 3. There were statistically significant differences in the duration of glucocorticoids, cumulative glucocorticoids, duration of cyclosporine, and duration of MMF between the low bone density group and the normal bone density group ($p < 0.05$).

3.2. Binary Logistic Regression. The two groups of statistically significant variables were included in the binary logarithmic regression model in the above three tables, and logistic regression analysis showed that phosphorus, alkaline phosphatase, BMI, exercise sessions per week, and the duration of glucocorticoids were the factors that truly affected the BMD of KTRs. Among them, phosphorus, alkaline phosphatase, and the duration of glucocorticoid use were the risk factors affecting bone mineral density. BMI and weekly exercise sessions were protective factors that affected bone mineral density (Table 4).

4. Discussion

The decrease in BMD measured by DXA occurs in the first 12 months after transplantation and seems to slow down thereafter but at significantly lower levels than in healthy controls [6]. The incidence of bone mass loss was found to be 62.4% based on our previously defined criteria, which is consistent with most studies so far. The prevalence of bone mass loss in KTRs is higher, which indicates that while paying attention to cardiovascular disease and transplant function in kidney transplant patients, bone metabolism should also be considered. A systematic literature review by Naylor et al. found that fracture rates ranged from 3.3 to 99.6 fractures per 1,000 person-years [7]. The overall fracture risk after renal transplantation is 3.6–3.8-fold higher than that in healthy individuals and is 30% higher during the first 3 years after transplantation than that in patients before transplantation [8, 9]. However, to our surprise, the prevalence of fractures was not as high as we thought; the prevalence of fracture was only 3.24% in our population. The differences may be due to the different definitions of fractures used and the different characteristics of the population. However, overall, this group will have a higher fracture risk than the general population, leading to an associated increase in morbidity and mortality.

Our studies showed an independent association between exercise sessions per week, BMI, and bone mass in KTRs. This suggests that maintaining an appropriate weight and weekly exercise routine may have positive implications for maintaining bone mass in KTRs. We found that smoking and milk intake do not affect bone health as much, and it may take a longer time to observe and require a larger sample size to discover their effects on bone mass and fractures. The duration after transplantation and duration of dialysis in KTRs also did not affect bone mass. A prior study demonstrated that BMD increased or remained stable several years after transplantation [10]. In our study, we did not observe a relationship between age, sex, and bone mass of KTRs, probably because the average age of the study population is young, and most of them were premenopausal women and mature men.

Abnormal phosphorus and calcium concentrations are common and fluctuate widely in the first year after kidney transplantation. Therefore, the KDIGO 2017 guideline update recommends that serum calcium and phosphorus levels be measured at least weekly in the immediate post-kidney transplant period until stable [11]. In our study, hyperphosphatemia was a significant factor for the negative effect on bone density. Hyperphosphatemia is usually seen in patients with delayed graft function or in patients with advanced CKD. For patients with CKD G3a–G5D, the 2019 Chinese Guidelines suggest lowering elevated phosphate levels toward the normal range. For patients whose serum phosphorus exceeds the target value, the guidelines suggest reducing dietary phosphorus intake (800-1,000 mg/day) alone or in combination with other phosphorus reduction

Table 1: Baseline demographic, anthropometric, and lifestyle variables of the patients.

	Normal bone density ($n = 81$)	Low bone density ($n = 135$)	$t/\chi^2/z$	p
Height*	162.57 ± 6.54	162.12 ± 7.08	$t = 0.465$	0.643
Weight*	59.65 ± 9.59	56.52 ± 9.93	$t = 2.276$	0.024
Age at transplantation*	37.84 ± 7.72	36.84 ± 10.49	$t = 0.805$	0.422
BMI*	22.52 ± 3.04	21.44 ± 3.19	$t = 2.444$	0.015
Sex†			$\chi^2 = 0.190$	0.663
Male	57 (36)	98 (63.2)		
Female	24 (40)	36 (60.0)		
Age†			$\chi^2 = 27.882$	≤0.001
18-29	8 (30.8)	18 (69.2)		
30-39	20 (32.3)	42 (67.7)		
40-49	48 (57.1)	36 (42.9)		
≥50	5 (11.4)	39 (88.6)		
Education†			$\chi^2 = 1.691$	0.429
Illiteracy-primary	10 (45.5)	12 (54.5)		
Middle school, high school, or technical secondary school	55 (38.7)	87 (61.3)		
College and above	16 (30.0)	36 (69.2)		
Marriage†			$\chi^2 = 3.437$	0.064
Married	74 (40.0)	111 (60.0)		
Unmarried	7 (22.6)	24 (77.4)		
Milk intake†			$\chi^2 = 2.670$	0.104
Yes	20 (48.8)	21 (51.2)		
No	61 (35.1)	113 (64.9)		
Outdoor time†			$\chi^2 = 0.772$	0.441
<30 min	14 (32.6)	29 (67.4)		
≥30 min	67 (39.0)	105 (61.0)		
Exercises sessions per week†			$\chi^2 = 2.124$	0.035
Seldom	29 (29.9)	68 (70.1)		
1 to 4 times a week	15 (41.7)	21 (58.3)		
More than five times a week	37 (45.1)	45 (54.9)		
Smoking habit†			$\chi^2 = 0.035$	0.852
Yes	27 (38.6)	43 (61.4)		
No	54 (37.0)	92 (63.0)		
Alcohol abuse†			$\chi^2 = 1.119$	0.291
Yes	18 (43.9)	23 (56.1)		
No	63 (36.0)	112 (64.0)		
Duration after transplantation†			$\chi^2 = 4.614$	0.033
1-12 mo	20 (41.7)	28 (58.3)		
12-36 mo	28 (45.9)	33 (54.1)		
36-60 mo	13 (40.6)	19 (59.4)		
60-120 mo	14 (26.9)	38 (73.1)		
≥120 mo	6 (26.1)	17 (73.9)		
Duration of dialysis§ mo	18 (6, 24)	12 (6, 24)	$z = -0.689$	0.491

Abbreviations: BMI: body mass index; mo: month; min: minute. *Continuous variables conforming to the assumption of normal distribution and the assumption of homogeneity were compared using Student's t-tests. †Pearson χ^2 test or Fisher exact test was used to compare categorical variables. §Ordinal data were compared using the Mann-Whitney U test. A p value of less than 0.05 was considered statistically significant.

Table 2: The baseline biochemical characteristics of the patients.

	Normal bone density ($n = 81$)	Low bone density ($n = 135$)	t/z	p
Creatinine[§]	109 (88.5, 135)	111 (90, 139)	$z = -0.477$	0.634
BUN[§]	6.38 (5.28, 7.215)	6.5 (5.02, 8.62)	$z = -1.153$	0.249
FBG[§]	5.1 (4.9, 5.5)	5.2 (4.9, 5.5)	$z = -0.135$	0.892
Calcium[§]	2.44 (2.37, 2.52)	2.42 (2.35, 2.52)	$z = -0.352$	0.725
Albumin[§]	47.4 (44.6, 48.7)	47 (44.9, 48.4)	$z = -0.660$	0.509
Phosphorus[§]	0.96 (0.83, 1.05)	1.03 (0.87, 1.11)	$z = -2.190$	0.029
CO2CP[§]	23.9 (22.3, 26.45)	23.8 (22, 25.7)	$z = -0.538$	0.591
iPTH[§]	77.83 (78.05, 111.15)	75.36 (52.95, 110.48)	$z = 1.652$	0.200
ALP[§]	68.5 (57, 91.75)	77 (63.5, 100)	$z = -2.038$	0.042
TC[§]	5.01 (4.495, 5.805)	5.1 (4.56, 5.77)	$z = -0.569$	0.569
TG[§]	1.44 (1.025, 1.7)	1.28 (1, 1.65)	$z = -0.776$	0.438
LDL-C[§]	2.53 (2.06, 3.01)	2.54 (2.08, 2.98)	$z = -0.355$	0.722
HDL-C*	1.49 ± 0.37	1.53 ± 0.4	$t = -0.763$	0.446
P1NP[§]	55.59 ± 5 (40.25, 71.92)	61.81 (43.44, 88.89)	$z = -1.843$	0.065
βCTX[§]	0.68 (0.4, 0.92)	0.69 (0.47, 0.96)	$z = -0.838$	0.402
25(OH)D[§]	23.71 (17.9, 29.44)	22.19 (16.22, 28.85)	$z = -0.716$	0.474

Abbreviations: BUN: blood urea nitrogen; FBG: fasting blood glucose; CO2CP: carbon dioxide combining power; iPTH: serum intact parathyroid hormone; ALP: alkaline phosphatase; TC: total cholesterol; TG: triacylglycerol; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; P1NP: N-terminal propeptide of type 1 collagen; β-CTX: β-isomerized C-terminal telopeptide of type 1 collagen; 25(OH)D: 25-hydroxyvitamin D3. *Continuous variables conforming to the assumption of normal distribution and the assumption of homogeneity were compared using Student's t-tests. [§]Ordinal data were compared using the Mann-Whitney U test. A p value of less than 0.05 was considered statistically significant.

Table 3: The use of immunosuppressive drugs in the patients.

	Normal bone density ($n = 81$)	Low bone density ($n = 135$)	$t/\chi^2/z$	p
Duration of glucocorticoid[§]	26 (9, 52)	41 (15, 88)	$z = -2.492$	0.013
Current dose of glucocorticoid*	8.76 ± 2.31	8.36 ± 2.56	$t = 1.382$	0.241
Cumulative of glucocorticoid[§]	10435 (5580, 17510)	14060 (7335, 26190)	$z = -2.265$	0.025
Average daily glucocorticoid dose[§]	12.905 (10.79, 18.02)	11.509 (9.78, 15.28)	$z = 0.643$	0.423
Duration of tacrolimus[§]	22 (6, 42.5)	23 (3, 60)	$z = -0.14$	0.886
Duration of cyclosporine[§]	0 (0, 0)	0 (0, 0)	$z = -2.150$	0.032
Duration of MMF[§]	26 (8.5, 50)	38 (12, 88)	$z = -2.168$	0.030

Abbreviations: MMF: mycophenolate mofetil. *Continuous variables conforming to the assumption of normal distribution and the assumption of homogeneity were compared using Student's t-tests. [§]Ordinal data were compared using the Mann-Whitney U test. A p value of less than 0.05 was considered statistically significant.

Table 4: Binary logistic regression analysis of bone mineral density.

	B	S.E.	P	OR	OR 95% CI	
					Lower	Upper
BMI	-0.149	0.055	0.007	.0862	0.773	0.960
Weekly exercise sessions	-0.335	0.170	0.049	0.715	0.512	0.998
Phosphorus	2.447	0.970	0.012	11.552	1.725	77.354
ALP	0.016	0.006	0.006	1.016	1.005	1.028
Duration of glucocorticoid use	0.011	0.004	0.007	1.011	1.003	1.018
Constant	-.236	1.717	.891	1.266		

Abbreviations: BMI: body mass index; ALP: alkaline phosphatase; B: regression coefficient; S.E.: standard error; OR: odds ratio; OR 95% CI (lower and upper): OR 95% confidence interval (lower and upper).

treatments [12]. No correlation was found between calcium and low bone density.

Vitamin D insufficiency and deficiency are extremely widespread among KTRs. The reported prevalence of vitamin D insufficiency after transplantation is 51-97%, and deficiency is 26%-33% [13, 14]. Our data were similar; vitamin D insufficiency accounted for 74.5%, and deficiency accounted for 28.2%, much higher than those who did not undergo kidney transplantation. Most patients in this study were not supplemented with vitamin D and its derivatives. Different regions, different populations, and different seasons are also important factors that determine the vitamin D status of KTRs.

The study found that increased ALP is an independent risk factor for low bone mass. Elevated ALP indicates a high conversion state of bone, and the rate of bone loss is accelerated. Our study did not find a relationship between iPTH and bone mass loss. The association between high iPTH levels and low bone mass might be mainly prevalent in short-term transplant patients. Over the very long term after transplantation, iPTH levels decrease and lose an association with BMD. Bone biochemical indicators such as PINP and β-CTX were found to have nothing to do with bone density.

Glucocorticoids are commonly prescribed for KTRs and have a profound inhibitory effect on bone formation by targeting osteoblast proliferation and differentiation while stimulating apoptosis of osteoblasts and osteocytes, thereby reducing bone turnover and synthesis [15]. In addition, glucocorticoids influence the synthesis of IGF-1, an osteoblast activator, by inhibiting IGF-1 gene transcription [16]. In our population, all KTRs were prescribed with glucocorticoids, and focusing on the duration of glucocorticoid use may show the true relationship between hormones and bone density. Early glucocorticoid withdrawal has been associated with a significant reduction (31%) in fracture risk and fracture-induced hospitalization among patients [17]. We also found that the longer the use of glucocorticoids, the lower was the bone density. However, the cumulative, current, and average daily dosage of glucocorticoids in our patients had no association with bone mass loss. In addition, BMD [18, 19] and fractures [20] decrease with prednisolone-sparing, prednisolone-withdrawal, and prednisolone-limiting protocols. The skeletal effects of other immunosuppressive agents remain uncertain.

Supplementation with vitamin D has been shown to improve components of mineral and bone disease, such as reduced PTH levels and possibly improved bone mineral density. Josephson et al. found that treatment with calcitriol (0.25 μg/d) and calcium (1 g/d) led to a significant 4.8% gain in FN-BMD after 12 months and unchanged LS-BMD [21]. In our study, the number of cases of vitamin D supplementation before and after transplantation was too small to determine the relationship between bone mass losses. Most recommend the use of an adequate dose of vitamin D to correct vitamin deficiency and maintain a serum 25(OH)D level of >30 ng/ml.

Management of posttransplant bone disease is challenging. This study found that the special population of KTRs has a very high incidence of low bone mass, which is higher than the general population of the same age. With increasing age, the incidence of osteoporosis and fracture in this population will further increase, bringing a large economic burden to families and society. Reducing the time of steroid exposure, maintaining an appropriate body weight and the number of weekly exercise sessions, and correcting abnormal phosphorus metabolism may help maintain the bone mass of KTRs. The contribution of this study is that these people have hardly received any medical intervention for the prevention and treatment of osteoporosis, which shows the natural changes in the bone health status of KTRs. Our study has some limitations. First, it was conducted in a single center and is a cross-sectional study that does not truly reveal the causal relationship between variables and bone mass loss. Due to the small number of fracture events, we cannot determine whether there is a correlation between the changes in bone density and fractures.

Data Availability

Due to respect for and protection of patient privacy, the data generated and/or analyzed in this study are not publicly available. However, they are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare no conflicts of interest.

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