Detrimental Effect of C-Reactive Protein on the Cardiometabolic Cells and Its Rectifying by Metabolic Surgery in Obese Diabetic Patients

Background: High-sensitivity C-reactive protein (hs-CRP) has been regarded as a biomarker of low-degree inflammation in illness; however, whether CRP exerts its pathogenic effect on the cardiometabolic system remains unknown. Aside from the beneficial effects of metabolic surgery on cardiometabolic system, its impact on inflammation still worth examining. Thus, this study aims to investigate the effect of CRP on adipose and vascular cells, and their responses to metabolic surgery in obese diabetic patients.

Patients and Methods: The expression of CRP and RAS- and ERK-related factors in the adipocytes and VSMCs were measured. Obese patients with type 2 diabetes who underwent metabolic surgery were followed up for 2 years thereafter. Laboratory tests, which included serum hs-CRP levels and visceral fat thickness (VFT), were obtained before and after surgery.

Results: CRP administration significantly and dose-dependently increased the intracellular-free calcium concentration ([Ca2+]i) in cultured adipocytes and in the VSMCs. CRP administration significantly increased ACE, Ang II, AT1R and p-ERK expressions, but reduced ACE2 expression in both the adipocytes and VSMCs. Clinical study showed that VFT was closely associated with serum hs-CRP. Furthermore, VFT and serum hs-CRP were found to be highly associated with blood pressure. Finally, metabolic surgery remarkably decreased blood pressure, visceral fat and serum hs-CRP levels.

Conclusion: CRP has a detrimental effect on cardiometabolic cells, aside from functioning merely as a biomarker. Serum hs-CRP levels are highly associated with hypertension and visceral obesity, which can be antagonized by metabolic surgery in obese diabetic patients.

Keywords: CRP, metabolic surgery, type 2 diabetes

Introduction
Diabetes and obesity often coexist, and hypertension is twice as frequent in obese patients with type 2 diabetes compared with non-diabetic patients.1,2 Additionally, obese patients with type 2 diabetes have a greater risk of cardiovascular than non-obese diabetic individuals.3 Accordingly, obesity, diabetes, and hypertension are commonly clustered as a metabolic syndrome because of their common pathogenesis, such as insulin resistance, activation of the renin-angiotensin-aldosterone system, oxidative stress, and abnormalities of the immune system.4,5 Extensive experimental studies have shown that inflammation is closely linked with hypertension, obesity and diabetes,6,7 while clinical studies have demonstrated that obese diabetic patients have increased total leukocyte counts, which are correlated with
insulin sensitivity and partially mediated by inflammatory changes in adipose tissue.\textsuperscript{8,9} Thus, chronic, low-grade inflammation contributes to the development of cardiometabolic diseases.

In the past decade, hs-CRP has been regarded as a biomarker of low-degree inflammation in illness.\textsuperscript{10,11} As a reaction protein in acute inflammation, CRP expresses in the liver and pancreas.\textsuperscript{12,13} Its synthesis and expression are regulated by activated monocytes, fibroblasts and some cytokines, with interleukin (IL)-6 and tumor necrosis factor (TNF)-alpha acting as the main stimulants of CRP synthesis and secretion.\textsuperscript{14,15} Several cross-sectional clinical trials have suggested that obesity is intimately related to the level of CRP.\textsuperscript{16,17} Moreover, serum CRP levels were related to blood pressure, blood lipids, and glucose.\textsuperscript{18,19} Baseline serum hs-CRP levels in healthy subjects are a good predictor for the future incidence of cardiovascular disease, stroke, sudden death and peripheral vascular diseases.\textsuperscript{20,21} Aside from the liver, adipose tissue has also been implicated in the regulation of CRP production, mediated by inflammatory factors.\textsuperscript{22,23}

Although CRP is extensively applied as a biomarker of low-degree inflammation in clinical settings, whether CRP itself participating in the pathogenesis of the cardiometabolic diseases remains unknown. The renin-angiotensin system (RAS) and extracellular signal-regulated kinases (ERK)1/2 play a crucial role in cardiovascular remodelling and dysfunction, but how CRP affects RAS and ERK 1/2 is still unclear.\textsuperscript{24} Weight reduction and the administration of hypoglycaemic and antihypertensive drugs have been shown to have anti-inflammatory effects.\textsuperscript{25} In addition, metabolic surgery has been shown to significantly improve hyperglycaemia and obesity, as well as the control of hypertension.\textsuperscript{26,27} However, it is unclear whether metabolic surgery improves cardiometabolic dysfunction through directly antagonizing inflammation. Thus, this study aims to investigate the effect of CRP on RAS and ERK in both adipose and vascular tissues, as well as the impact of metabolic surgery on blood pressure and inflammation in obese diabetic patients.

### Materials and Methods

#### Cell Culture and Treatment

**Induced Differentiation of 3T3-L1 Preadipocytes**

The method used for differentiation of murine 3T3-L1 adipocytes was described before. Briefly, murine 3T3-L1 cell line purchased from the Institute of Cell Biology, Shanghai Academy of Chinese Sciences. Cells were cultured in Dulbecco’s modification of eagle medium (DMEM) high glucose (25 mM), 15% fetal bovine serum (FBS), and 1% penicillin/streptomycin. Cells incubation condition was humidified with air containing 5% CO\textsubscript{2}. All evaluation performed on cells between 15 and 35 passages. 3T3-L1 cells, $3 \times 10^5$/well, cultured in the 6-well plate to reach 90% confluency. Then, cells differentiated into adipocytes using 3T3-L1 differentiation kit and according to the manufactures instructions. Briefly, 3T3-L1 cells culture medium was replaced with differentiation medium (DMEM/F12, 1:1) with 10% FBS, insulin 1.5 µg/mL, dexamethasone 1 µM, IBMX 500 µM, and rosiglitazone 1 µM and incubated for 3 days. Next, differentiation medium replaced with maintenance medium (1 µM of insulin to 1 mL of DMEM/F12 (1:1) with 10% FBS). According to the manufacturer’s instructions, maintenance medium changed every 2–3 days.

**Primary Culture and Passage of VSMC**

Thoracic aorta was removed from anaesthetized healthy male Wistar rats (provided by the experimental animal center of Daping Hospital of Third Military Medical University), and transferred to serum-free DMEM culture medium. Medium membrane of blood vessel was separated and transferred into penicillin vial containing adequate amount of serum-free DMEM medium. The blood vessel strips were cut into 1 mm * 1 mm sized tissue blocks by ophthalmic scissors, and placed in 37°C and 5% CO\textsubscript{2} incubator for 3 h to attach to the bottom of the bottle. The cells were cultured for 4 days and then changed into fluids. The cells were fused and passed. Cellular immunohistochemical staining with anti-alpha sm-actin antibody showed that the brown positive staining of intracytoplasmic myofibrils was VSMC.

**Cell Treatment**

The differentiated 3T3-L1 adipocytes and VSMC were divided into control group, 0.1µg/mL CRP intervention group and 1µg/mL CRP intervention group. After 24 h of intervention, the total cell protein was extracted, and the related protein expression including ACE, ACE 2, Ang II, AT1R, P-ERK, ERK, was detected by Western blotting according to the manufacturer’s instructions, β-actin used for normalization. CRP mRNA in 3T3-L1 adipocytes were detected by RT-PCR according to the manufacturer’s instructions, GAPDH was used for normalization. The oligonucleotides, used as PCR primers, were as follows: 5'-CTT ACG CTA CCA AGA CGA-3' and 5'-TCC CAC CAA AGA CTG ATT-3' for murine CRP, GAPDH 5'-ACG GCA AATTCA ACG GCA CAG TCA-3' and 5'-TGG GGG CAT CGG CAG AAG G-3'.
Measurement of Ca$^{2+}$ Fluorescence

The method used for measurement of Ca$^{2+}$ fluorescence was described before. 3T3-L1 adipocytes and VSMC seeded on coverslips or freshly isolated cardiac mitochondria were stained with Fura-2AM (2 mM; Sigma-Aldrich) or Rhod-2AM (5 mM; Thermo Fisher Scientific) calcium fluorescent indicator for the cytosolic or cardiac mitochondrial Ca$^{2+}$, respectively. The cells or mitochondria were incubated for 60 min at room temperature and then washed to remove the extraneous dye using a confocal microscope (LCS SP8 STED; Leica). Fura-2 fluorescence was measured using a 510 nm emission wavelength and 340 and 380 nm excitation; emission at 580 nm and excitation at 550 nm were used for Rhod-2.

Immunofluorescence

3T3-L1 preadipocytes were fixed with 4% paraformaldehyde at room temperature for 10 min and then bathed in 0.2% Triton X-100 for 10 min. The cells were washed with PBS, then blocked with 10% fetal bovine serum for 30 min and incubated with antibodies against PKD2LI (1:100, AB9084, rabbit; Merck), NCX1 (1:100, ab2869, mouse; Abcam), or ATP5A (1:100, ab14748, mouse; Abcam) overnight at 4°C. The cells were washed three times and then incubated with fluorescent dye-labeled secondary antibodies (Santa Cruz) at room temperature for 30 min. DAPI was used to stain the nuclei for 5 min. Images were acquired using a confocal microscope (LCS SP8 STED; Leica).

Clinical Study

Patients

In order to conduct an analysis of the relationship between CRP and multiple metabolic factors, we performed a prospective single-centre cohort trial that was in accordance with the STROBE guidelines for the presentation of clinical trials and the principles of the Declaration of Helsinki. The protocol is registered on the office site (ClinicalTrials.gov Identifier: NCT02050984). The institute’s ethics committee approved this study.

A total of 127 patients who were suffering from metabolic diseases in this hospital between January 2010 and December 2018 were recruited in this study. The diagnosis of metabolic syndrome, diabetes, obesity and hypertension was based on AHA, ADA and IDF criteria. RYGB and sleeve gastrectomy were both included in the operation. The main exclusion criteria included the following: patients with more than 15 years of diabetic duration or islet β cell function failure, patients with any other conditions that prevent them from undergoing surgery (including recent infections, pregnancy, coronary artery disease, cerebrovascular disease, liver or renal dysfunction, tumors, or mental disease), as well as being enrolled in another trial within the last 3 months.

Data Collection and Assessment

Patients who underwent metabolic surgery completed their evaluations both before and after the operation, and were subsequently followed up for 2 years at 3,6,12, and 24 months, respectively. Baseline clinical data were collected, including age, sex, abdominal circumference, and blood pressure. At each follow-up, some general clinical data needed to be collected repeatedly; laboratory tests were performed after an overnight fast, including measurements of fasting plasma glucose, glycosylated haemoglobin A1c (HbA1c), serum insulin, triglyceride, cholesterol, uric acid, and high-sensitivity C-reactive protein (hs-CRP) levels. Visceral fat thickness (VFT) was also examined by an abdominal fat ultrasound. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the following equation: [(Fasting insulin (FINS, mIU/L) × Fasting plasma glucose (FPG, mmol/L))/22.5].

Statistical Analysis

The data were expressed as mean ± SEM or SD for normally distributed variables and median (25th and 75th percentiles) for non-normally distributed variables. Comparisons between the groups in cell study were made using a two-tailed unpaired Student’s t-test or one-way ANOVA with Bonferroni’s post-hoc test. Comparisons of variables before and after treatments were analysed using the Wilcoxon signed-rank matched pair test. The $\chi^2$ test was used for categorical variables. Spearman’s nonparametric correlation analysis was performed to determine the relationships between hs-CRP changes and other factors. A two-tailed p<0.05 was considered statistically significant, and all the results were analysed using SPSS 18.0.

Results

Baseline Characteristics of the Participants

All patients who underwent metabolic surgery were divided into non-obese (BMI<28) and obese (BMI≥28) groups. The baseline characteristics of the participants are presented in Table 1. Aside from BMI, obese patients had larger waist
Table 1 Baseline Characteristics of the Patients Who Underwent Metabolic Surgery

|              | BMI<28 (n=42) | BMI≥28 (n=85) |
|--------------|---------------|---------------|
| Age (year)   | 48 (44–54)    | 35 (28–57)    |
| Gender (M/F) | 20/22         | 34/51         |
| BMI (kg/m²)  | 26.0 (24.6–27.2) | 31.8 (29.9–38.2) ** |
| WC (cm)      | 90 (85–94)    | 105 (98–116)** |
| VFT (cm)     | 8.6 (7.5–24)  | 12.3 (10.5–29)** |
| SBP (mmHg)   | 125 (120–135) | 130 (120–142)** |
| DBP (mmHg)   | 76 (70–83)    | 81 (76–90)** |
| hs-CRP (mmol/L) | 2.0 (1.38–3.41) | 4.27 (2.09–8.53)** |
| FPG (mmol/L) | 7.5 (5.7–9.6) | 7.0 (5.5–9.5) |
| HOMA-IR      | 3.3 (1.7–5.4) | 6.5 (3.9–9.1)** |
| HbA1c (%)    | 7.8 (6.8–9.5) | 7.6 (6.0–8.7) |
| TC (mmol/L)  | 4.8 (4.0–5.4) | 4.7 (4.1–5.3) |
| TG (mmol/L)  | 1.8 (1.2–2.7) | 2.2 (1.5–3.1) |
| HDL-c (mmol/L) | 1.1 (0.9–1.3) | 1.0 (0.8–1.1) |
| LDL-c (mmol/L) | 2.7 (2.2–3.1) | 2.8 (2.3–3.3) |
| UA (umol/L)  | 324.8 (260.3–413.7) | 396.8 (340.2–475.2)** |

Notes: *p<0.05, **p<0.01, compared with BMI<28.

Abbreviations: BMI, body mass index (calculated as weight in kilograms divided by height in meters squared); WC, waist circumference; HbA1c, glycosylated haemoglobin; VFT, visceral fat thickness; SBP, systolic blood pressure; DBP, diastolic blood pressure; FPG, fasting plasma glucose; FINs, fasting insulins; HOMA-IR, homeostasis model assessment-insulin resistant; UA, uric acid; TC, total cholesterol; TG, triglycerides; LDL-c, low-density lipoprotein cholesterol; HDL-c, high density lipoprotein cholesterol; hs-CRP, high-sensitivity C-reactive protein.

Effects of hs-CRP on Preadipocytes and Vascular Cells
hs-CRP is well known to contribute the metabolic syndrome and atherosclerosis, but it remains elusive whether adipose tissue produces this inflammatory factor and how it affects the adipocyte and vascular cells. The adipose tissue of human mesentery was derived from the greater omentum which was removed by metabolic surgery. The use of human tissue specimen had been informed consent before operation. Immunofluorescence staining showed that CRP expressed in both in 3T3-L1 preadipocytes (Figure 1A) and in human adipose tissue, and mainly distributed in the cell membrane (Figure 1B). CRP mRNA and protein expression were also detected in human mesenteric adipose tissue (Figure 1C and D). We also showed that the administration of CRP significantly increased intracellular-free calcium concentration [Ca²⁺], in 3T3-L1 preadipocytes (F340/F380 1.06±0.20, n=6) (Figure 1E), and in cultured VSMC (F340/F380 1.16±0.09, n=6), compared with control cells (Figure 1F). In addition, the administration of CRP dose-dependently increased [Ca²⁺], in 3T3-L1 cells and cultured VSMC. These results suggest that CRP not only exists in adipocyte tissue but also has an impact on both adipocyte and the vascular cells.

Effects of CRP on RAS/ERK Pathways in Adipocytes and Vascular Smooth Muscle Cells
Then, we examined the effect of CRP on calcium signalling and the RAS/ERK pathways, which play a crucial role in the pathogenesis of hypertension and obesity.5,31 We showed that the administration CRP significantly increased the expression of ACE, Ang II, AT1R and p-ERK in mature 3T3-L1 adipocytes in a concentration-dependent manner, while significantly reduced the expression of ACE2 (Figure 2A). Similarly, CRP administration for 24 h significantly increased the expressions of ACE, Ang II and AT1R in a dose-dependent manner, and increased p-ERK expression but reduced the ACE2 expression in cultured VSMCs (Figure 2B). These results indicate that CRP has direct effects on the RAS/ERK pathways in both adipocyte tissue and vascular cells.

Plasma hs-CRP Level Was Associated with Blood Pressure and Obesity
Having found that CRP directly stimulates RAS and ERK in adipocytes and vascular cells,12,32 we then investigated whether hs-CRP was associated with obesity and blood pressure in patients. We showed that plasma hs-CRP levels were positively correlated with fat thickness (VFT), WC and SBP in patients (p<0.01) (Figures 3A–C). A similar positive correlation among SBP, WC and VFT was demonstrated in patients (p<0.01) (Figures 3D and E). These results indicate that abdominal fat and CRP are highly associated with blood pressure.

Effects of Metabolic Surgery on CRP and Blood Pressure
Surgical therapy can antagonize hypertension and obesity, but its mechanism for doing so remains elusive. We aimed...
to understand whether the antihypertensive effects of metabolic surgery were associated with the reduction of hs-CRP. During our long-term follow-up, we found that metabolic surgery time-dependently decreased both systolic and diastolic blood pressure (Figures 4A and B). Additionally, a significant decrease of BP occurred at 12 months after the operation. CRP also showed a time-dependent decline, with a rapid reduction at 3 months following the operation, which was consistent with the declining trend in patients’ blood pressure (Figure 4C). These results suggest that the antihypertensive effects of metabolic surgery could be associated with the reduction

Figure 1 CRP distribution and effects on the cytosolic calcium levels in cultured cells. Immunofluorescence staining of CRP in 3T3-L1 preadipocytes (A) and adipose tissue (B), green fluorescence represents CRP distribution, while blue fluorescence represents the nucleus (A and B). Representative RT-PCR and Western blot of CRP from human mesenteric adipose tissue (C and D). The quantitative results are shown below. Data are expressed as mean ± SD. Representative traces and ratio fluorescence imaging showed the effect of CRP (1 µg/mL) on calcium ([Ca^{2+}]) in cultured 3T3-L1 preadipocytes (E) and vascular smooth muscle cells (VSMC) (F). The quantitative results and dose-dependent CRP effects on [Ca^{2+}] are shown (n=6 in independent experiments). Data are expressed as mean ± SD, **p<0.01 versus control.
of the inflammatory factor related to the decrease of CRP, which thereby affected blood pressure in patients who underwent metabolic surgery.

**Discussion**

The major findings in this study are as follows: diabetic patients with obesity had higher blood pressure, more visceral fat accumulation and higher serum hs-CRP levels. Moreover, serum hs-CRP levels were highly correlated with blood pressure and visceral fat accumulation in patients. Adipose tissue also produced CRP, and CRP directly activated RAS and ERK in both adipose and vascular tissues. Metabolic surgery not only improved abdominal obesity and hypertension, but also lowered serum hs-CRP. This study highlights the fact that CRP itself contributes to the development of hypertension and fat illness in diabetic patients, which can be antagonized by surgical treatment.

It is documented that visceral adiposity is closely associated with hypertension and diabetes. Excess visceral fat accumulation causes chronic low-grade inflammation and initiates the progression of cardiovascular diseases. One study showed a significant correlation between visceral adiposity and hs-CRP in Japanese subjects. Our previous observations demonstrated that serum hs-CRP was associated with the component of metabolic syndrome and correlated with target organ damage in patients with metabolic syndrome. The present study also found that abdominal obesity was highly correlated with blood pressure and serum hs-CRP in obese diabetic patients. Although the liver is the main site of CRP production, CRP is also produced in adipose tissue. Additionally, adipose tissue is an active endocrine organ and secretes a variety of bioactive factors, such as adipokines, including tumor necrosis factor (TNF)-α, monocyte chemoattractant protein (MCP)-1, and interleukin (IL)-6. All of these factors are stimulants of CRP synthesis and secretion. In addition to showing that CRP produced in adipose tissue, our study also demonstrated that the administration of CRP remarkably raised [Ca^{2+}], a universal regulator of various cellular functions, in both adipocytes and VSMCs. In blood vessels, Ca^{2+} is the central element...
of excitation-contraction coupling, but it also impacts diverse signalling cascades and influences the regulation of gene expression. Disturbances in cellular Ca\(^{2+}\)-handling and alterations in Ca\(^{2+}\)-dependent kinase pathways are pivotal characteristics related to cardiovascular remodeling and dysfunction. This study’s indication that CRP initiates a Ca\(^{2+}\)-dependent regulatory pathway might offer broad therapeutic potential.

Although hs-CRP is clinically useful as a biomarker for CV risk prediction, some mechanistic studies suggest that CRP itself is likely involved in the pathogenesis of cardiovascular diseases.\(^{38}\) Procter et al identified the impact of inflammation (myeloperoxidase and CRP) and impaired nitric oxide (NO) signaling in acute and chronic atrial fibrillation (AF).\(^ {39}\) The activation of RAS is associated with the pathogenesis of obesity and hypertension.\(^ {40}\) Angiotensin I (Ang I) is cleaved by angiotensin-converting enzyme (ACE) to Ang II, which is metabolized by ACE2 to Ang-(1-7). Ang II binds to Ang II type 1 receptors (AT1Rs), which causes vasoconstriction, inflammation, fibrosis, and apoptosis. Reduced ACE2 shifts the balance in the RAS toward the Ang II/AT1 Raxis, resulting in the
We showed that CRP dose-dependently increases the expressions of ACE, AngII and AT1R proteins, but inhibits ACE2 expression in both adipocytes and VSMCs, suggesting the detrimental effect of CRP on vasculatures is connected with an imbalance of RAS. Ang II/ATR1 signalling also leads to the activation of several downstream kinase pathways, such as extracellular signal-regulated kinases (ERK)1/2. Additionally, Pardina et al., found that morbidly obese patients had a greater amount of CRP in their plasma and adipose tissue compared with control subjects, and that plasma CRP can reflect decreases in inflammation and improvements with bariatric surgery-mediated changes in gut hormones, nutrient sensing, intestinal glucose utilization, microbiota, and bile acid metabolism in diabetes and obesity have been proposed to function as such mechanisms. Our previous study demonstrated that metabolic surgery ameliorates hypertension through the inhibition of the activation of the sympathetic nervous system in both hypertensive patients and genetic hypertensive rats. Additionally, Pardina et al, found that morbidly obese patients had a greater amount of CRP in their plasma and adipose tissue compared with control subjects, and that plasma CRP can reflect decreases in inflammation and improvements with bariatric surgery-related weight loss. Our previous study showed that metformin reduced abdominal obesity and serum hs-CRP levels in obese hypertensive patients without diabetes. In this study, we further demonstrated that metabolic surgery not only lowered the blood pressure and body weight, but also reduced serum hs-CRP levels. This suggests that the inhibition of inflammation and its detrimental effects on cardio-metabolic organs could be one of the key mechanisms underlying the success of metabolic surgery.

**Conclusions**

In conclusion, our study demonstrates that CRP is highly correlated with hypertension and visceral adiposity. CRP is not only a biomarker, but is also a participating factor in the pathogenesis of obesity and hypertension, where it functions through impacting the RAS and ERK pathways. Our findings can, therefore, be understood as providing key insights into the beneficial effects of metabolic surgery, which, as we show, could be partly associated with antagonizing inflammation. Thus, metabolic surgery may represent a promising intervention in obese hypertensive patients with diabetes.

**Abbreviations**

BMI, body mass index; WC, waist circumference; HbA1c, glycosylated haemoglobin; VFT, visceral fat thickness; SBP, systolic blood pressure; DBP, diastolic blood pressure; FPG, fasting plasma glucose; FINS, fasting insulins; HOMA-IR, homeostasis model assessment-insulin resistant; UA, uric acid; TC, total cholesterol; TG, triglyceride; LDL-c, low-density lipoprotein cholesterol; HDL-c, high-density lipoprotein cholesterol; hs-CRP, high-sensitivity C-reactive protein.

**Data Sharing Statement**

All data generated or analyzed during this study are included in this published article and information about experimental sessions and results are available from the corresponding author on reasonable request.

**Ethics and Consent Statement**

The animal experiments were performed in compliance with and were approved by the ethics committee of the Daping Hospital, the Third Military Medical University. All experiments were performed following institutional guidelines and regulations of ethics committee of the Daping Hospital, the Third Military Medical University. All patients provided written informed consent.

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**Author Contributions**

All authors made substantial contributions to conception and design, acquisition of data, or analysis and
interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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**Disclosure**
The authors declared that they have no conflicts of Financial interests/non-financial interests in this work.

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