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# Mucosal Biomarkers of Colorectal Cancer Risk Do Not Increase at 6 Months Following Sleeve Gastrectomy, Unlike Gastric Bypass

*Prashant Kant[1], Sarah L. Perry[1], Simon P. Dexter[2], Amanda D. Race[3], Paul M. Loadman[3] and Mark A. Hull[1]*

**Objective:** The hypothesis that sleeve gastrectomy (SG) is not associated with an increase in mucosal colorectal cancer (CRC) biomarkers, unlike Roux-en-Y gastric bypass (RYGB), was tested.

**Design and Methods:** Rectal mucosa, blood, and urine were obtained from morbidly obese patients ($n = 23$) before and after (median 28 months) SG, as well as from nonobese controls ($n = 20$). Rectal epithelial cell mitosis and apoptosis, crypt size/fission, and pro-inflammatory gene expression were measured, as well as systemic inflammatory biomarkers, including C-reactive protein (CRP).

**Results:** The mean pre-operative body mass index in SG patients was 65.7 kg/m$^2$ (24.7 kg/m$^2$ in controls). Mean excess weight loss post-SG was 38.2%. There was a significant increase in mitosis frequency, crypt size, and crypt fission (all $P < 0.01$) in SG patients *versus* controls, as well as evidence of a chronic inflammatory state (raised CRP and mononuclear cell p65 NF$\kappa$B binding), but there was no significant change in these biomarkers after SG, except CRP reduction. Macrophage migration inhibitory factor mRNA levels were increased by 39% post-SG ($P = 0.038$).

**Conclusions:** Mucosal biomarkers of CRC risk do not increase at 6 months following SG, unlike RYGB. Biomarkers of rectal crypt proliferation and systemic inflammation are increased in morbidly obese patients compared with controls.

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## Introduction

The number of bariatric surgical procedures that are performed continues to increase in line with the increasing global prevalence of obesity (1,2). Several bariatric surgical techniques are performed routinely including purely "restrictive" procedures such as gastric banding, as well as procedures that are believed to induce weight loss via a combination of restrictive and malabsorbtive mechanisms such as Roux-en-Y gastric bypass (RYGB) (1). Sleeve gastrectomy (SG) is now performed more widely, either alone or as part of a two-stage procedure followed by a duodenal switch (3).

A link between obesity and increased colorectal cancer (CRC) risk is firmly established (4). However, the effect of bariatric surgery on future CRC risk remains unclear. Initial reports suggested that bariatric surgery was associated with a reduction in overall cancer risk and risk associated with some obesity-related cancers including postmenopausal breast cancer (5-7). However, these studies were too small to determine specific effects on CRC risk. In a large population-based study in Sweden, we recently reported that the standardized incidence ratio (SIR) for CRC risk actually increased with time after bariatric surgery such that the SIR was 2 (95%CI 1.5-2.6) 10 years after surgery compared with a SIR of 1.25 for a group of obese patients who did not undergo bariatric surgery, which remained stable over time (8). The increased CRC SIR was shared by those who underwent restrictive procedures (limited to gastric banding and vertical-band gastroplasty), as well as the more common RYGB (8). These observational findings concurred with our previous colorectal mucosal biomarker studies, in which a mucosal hyper-proliferative state (increased epithelial cell mitosis frequency and crypt size) persisted at least three years after RYGB compared with pre-operative morbidly obese patients and normal weight controls (9,10).

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Possible mechanistic explanations for increased CRC risk after bariatric surgery include increased colorectal mucosal exposure to bile acids (11,12)and/or a carcinogenic metabolomic profile of intestinal luminal content secondary to postoperative changes in diet and/or gut microflora (13,14).

As the "restrictive" procedure SG is increasingly performed at the expense of RYGB and results in similar degrees of weight loss (3), we tested the hypothesis that SG would not be associated with an increase in the colorectal mucosal biomarkers that we had observed in our previous studies of colorectal mucosal changes after "malabsorbtive" RYGB.

Obesity is recognized as a state of chronic, low-grade, systemic inflammation (4). One hypothesis is that there is subclinical colorectal mucosal inflammation in obesity that could drive colorectal carcinogenesis. Therefore, we measured levels of mucosal pro-inflammatory gene expression, and changes following SG, in obese patients and nonobese controls, in comparison with levels of systemic inflammatory biomarkers such as C-reactive protein (CRP).

# Methods

All aspects of the study were approved by the Leeds (East) Research Ethics Committee (07/Q1206/29 and 08/H1311/42). Written, informed consent was obtained from all participants.

## Study subjects and study pathway

Consecutive patients scheduled for SG at St James's University Hospital and The Nuffield Hospital, Leeds were contacted 2 weeks prior to surgery. Patients were eligible if aged between 18 and 65 years with a body mass index (BMI) $\geq$35 kg/m$^2$ and if able to provide informed consent. The control group consisted of patients aged 18-65 years with a BMI 18.5-29.9 kg/m$^2$, who were undergoing anal canal surgery, for which bowel preparation was not required. Patients were excluded if there was a history of significant gastrointestinal disease (including CRC, colorectal adenoma, inflammatory bowel disease, or coeliac disease), previous colorectal surgery, bleeding diathesis, current anticoagulation therapy or regular use of aspirin or other nonsteroidal anti-inflammatory drugs (NSAIDs; defined as $\geq$4 doses per week for more than three months).

A full medical and drug history was taken prior to SG as described (9). Height, weight, and BMI were measured. Venous blood was obtained and urine was collected the day before surgery. Six rectal mucosal biopsies were obtained by rigid sigmoidoscopy under general anaesthetic immediately prior to surgery. Biopsies were taken from 8-10 cm above the dentate line on the posterior wall of the unprepared rectum using 2.2-mm jaw, single-use biopsy forceps (Boston Scientific, MA). Any patient with macroscopic evidence of mucosal inflammation or polyps was excluded. SG patients underwent repeat assessment and sampling in clinic approximately 6 months after SG.

## Blood, urine and mucosal biopsy handling

Serum and plasma were obtained using SST II advance™ and EDTA tubes (both BD Vacutainer®), respectively, and stored at −80°C. Peripheral blood mononuclear cells (PBMCs) were isolated in sodium citrate tubes (BD Vacutainer® CPT™), which were centrifuged at 1500 *g* for 20 mins at 20°C within 2 h of venepuncture.

Twenty milliliter of urine was collected in a sterile container and stored at −80°C. All urine samples were frozen within 2 h of collection.

Two rectal biopsies were placed into Carnoy's fixative for 2 h at 20°C and then transferred to 70% (v/v) ethanol for storage at 4°C (9). Two biopsies were fixed in 10% formalin for 24 h and then embedded in paraffin. One biopsy was placed in 500 $\mu$L Tris-buffered saline (TBS), pH 7.4 for measurement of *ex vivo* $PGE_2$ synthesis as described (9). One biopsy was placed in 1 ml of TRIzol® (Invitrogen, Paisley) and stored at −80°C.

## Laboratory analyses

*Cytokine and CRP measurement*. Levels of serum TNFα and IL-6 were measured using Quantkine® Immunoassays (R&D Systems, Minneapolis). Serum CRP levels were analyzed using a high-sensitivity immunoassay (Advia 1650/2400, Siemens HealthCare Diagnostics, Newbury, UK). The lower limit of detection was 0.2 mg/L.

*Whole crypt microdissection and mitosis counting*. The number and position of mitotic epithelial cells in microdissected rectal crypts was determined by one observer (PK) as described in Ref. (9). Inter-observer agreement between this study and the previous RYGB study (9) has previously been confirmed (10).

*Immunohistochemistry*. Immunohistochemistry for neo-cytokeratin (CK) 18 was performed as described in Ref. (9).

*PBMC culture and NFκB DNA binding assay*. PBMCs were washed twice in Dulbecco's phosphate-buffered saline (PBS; Invitrogen). Cells were then counted with a haemocytometer. PBMCs were re-suspended in RPMI 1640 with GlutaMAX™ supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% (v/v) fetal calf serum (Harlan Seralabs, UK) and cultured at 37°C in the presence of 5% $CO_2$ for 2 h in the presence or absence of 1 μg/ml *Escherichia coli* serotype 026:B6 lipopolysaccharide (LPS; Sigma).

Nuclear protein was isolated using a nuclear protein extraction kit (Active Motif, CA, USA). The protein concentration was measured using a colorimetric DC™ Protein Assay kit (Bio-Rad, CA, USA). NFκB binding activity was measured using the TransAM NFκB Family Transcription Factor Assay Kit™ for p50 (NFκB1), P52 (NFκB2), p65 (Rel A), Rel B and c-Rel subunits (Active Motif) on 5 $\mu$g total protein samples. Data are presented as the binding activity, measured as the absorbance at 450 nm using an Opsys MR spectrophotometer (Dynex Technologies, Worthing, UK) relative to the same amount of control human B cell lymphoblast nuclear extract.

Preliminary experiments demonstrated that there was significant degradation of NFκB binding in nuclear extracts stored at −80°C when measured 9 months apart (data not shown). Therefore, all NFκB DNA binding assays were performed strictly 9 months after nuclear protein extraction.

*Real-time -PCR*. Total RNA was isolated and cDNA synthesized as described in Ref. 9. SYBR Green™ real-time PCR (Applied Biosystems, Warrington, UK) was carried out using an ABI PRISM®

7900HT Sequence Detection System (Applied Biosystems) as described in Ref. 9. Primers used are detailed in Supporting Information Table 1. Each reaction cycle consisted of heating to 90°C for 2 min, followed by 10 min at 50°C. This was followed by 40 amplification cycles of heating to 95°C for 15 seconds followed by cooling at 60°C for 1 min. Each sample was performed in duplicate and the absence of cDNA served as a "no template" negative control. Individual pro-inflammatory gene transcript levels were quantified by the $\Delta C_t$ method using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the reference gene transcript (9).

*Urinary PGE-M assay by liquid chromatography-tandem mass spectrometry.* Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed on an Acquity Ultra Performance LC™ (UPLC™) System linked with a Quattro Premier™ XE (Waters Corp, Milford, USA) bench-top tandem quadrupole mass spectrometer operated in multiple reaction monitoring (MRM) mode. Samples were prepared and extracted using solid phase extraction as described in Ref. 15. Separation was achieved using an Acquity UPLC BEH C8 Column, 2.1 × 100 mm, 1.7 μm (Waters, Milford, USA) and a mobile phase A of 95:5, 0.1% (v/v) acetic acid:acetonitrile, and mobile phase B of 50:50, 0.1% (v/v) acetic acid:acetonitrile. Samples were separated by a gradient of 98-40% mobile phase A over 15 min at 300 μl/min. The mass spectrometer was operated in negative ion mode as described in Ref. 15, with the exception that the cone voltage was set at 19 V. MRM channels were established for PGE-M ($m/z$ 385.2 > 336.2) and the internal standard PGE-Md6 ($m/z$ 391.2 > 339.2) (both Cayman Chemical, Ann Arbor, MI). PGE-M was quantified against the internal standard with calibration using 10-100 ng/ml authentic PGE-M. The limit of detection was 1 ng/ml.

The urinary creatinine (Cr) concentration in mg/ml was measured using an ADVIA 1800 analyzer (Siemens). The urinary PGE-M concentration is presented as ng PGE-M/mg Cr.

The $PGE_2$ level in rectal biopsy-conditioned TBS was measured by immunoassay (Cayman Chemical) as described in Ref. 9.

*Statistical analysis.* Data were analyzed for normality using the Shapiro-Wilk test. Normally distributed and log transformed data were compared using Student's $t$-test and presented as the mean and 95% confidence interval (CI). Unpaired categorical data were compared using the $\chi^2$ or Fisher's exact test. Categorical data were compared using the Mann-Whitney U test and presented as the median value and inter-quartile range (IQR). For paired data, the Student's paired $t$-test or Wilcoxon signed rank test was used. Paired categorical data were compared using the McNemar test. Pearson's coefficient was used to test for correlations between normally distributed variables, whilst Spearman's coefficient was used for non-normally distributed data.

# Results

## Clinical characteristics of the non-obese/morbidly obese cohorts and outcomes after SG

Between October 2008 and July 2010, 23 out of 35 potential SG patients consented to participate in the study and provide blood, urine, and rectal mucosal samples. Seven patients declined to participate and five eventually underwent RYGB.

**TABLE 1** Clinical characteristics of nonobese control and morbidly obese (presleeve gastrectomy) patients

| | Control | Morbidly obese | P value[a] |
|---|---|---|---|
| n | 20 | 23 | |
| Mean BMI (SEM) | 24.7 (0.6) | 65.7 (2.4) | < 0.001 |
| Mean age (range) | 45 (22-63) | 46 (30-63) | 0.75 |
| Sex (male:female) | 8:12 | 8:15 | 0.76 |
| Smoking status (%)[b] | 12 (60) | 7 (30) | 0.052 |
| Never smoked | 3 (15) | 10 (44) | 0.054 |
| Former smoker | 5 (25) | 6 (26) | 0.99 |
| Current smoker | | | |
| Diabetes mellitus (%) | 2 (10) | 7 (33) | 0.13 |
| NSAID use[c] (%) | 2 (10) | 9 (40) | 0.03 |

[a]Normally distributed data (BMI and age) were compared using the unpaired $t$-test. Differences in sex were compared by the $\chi^2$ test. Differences in all other categorical data between the two groups were compared by Fisher's exact test.
[b]Never smoked defined as a lifetime smoking consumption <100 cigarettes; former-smoker defined as a lifetime smoking consumption >100 cigarettes ceasing >1 year previously; current smoker defined as either presently smoking or ceasing <1 year ago.
[c]The use of any dose of NSAID (aspirin or nonaspirin NSAID) during the preceding 28 days.

Postoperative samples were obtained a median 28 weeks (IQR 27-31 weeks) after SG from 21 patients. Two patients withdrew consent. In one case, postoperative rectal mucosal samples were not obtained as anticoagulant therapy had been started after surgery. Urine samples were not obtained in two patients before SG and in another patient after SG, giving 18 paired urine samples.

Pre- and postoperative BMI values are displayed in Supporting Information Figure 1. The mean individual weight loss was 44.9 (standard error of the mean [SEM] 3.6) kg, equating to a mean individual excess weight loss (EWL) of 38.3 (SEM 3.8) %.

Twenty nonobese patients scheduled for either haemorrhoidectomy ($n$ = 19) or anal sphincter collagen injection ($n$ = 1) consented to provide blood, urine, and rectal mucosal samples at the time of surgery. Urine samples were not obtained in eight cases.

The two groups were well matched for age and sex (Table 1. However, there were significantly more subjects who used a NSAID and former smokers in the morbidly obese group, as well as an expected nonsignificant excess of individuals with diabetes.

## Morbid obesity is associated with a rectal mucosal hyper-proliferative state that does not alter 6 months after SG

Consistent with our previous studies of RYGB patients (9,10), morbidly obese patients had a significantly higher crypt mitosis frequency ($P$ < 0.01) and significantly larger crypts compared with nonobese controls ( $P$ < 0.01; Figure 1A,B and Table 2. Morbidly obese subjects had a significantly greater percentage of mitotic epithelial cells in crypt zones 4 and 5, indicating an expanded proliferative compartment (Figure 1C and Table 2. Morbidly obese patients also exhibited more crypts in fission than nonobese controls ( $P$ < 0.01; Figure 1D and Table 2.

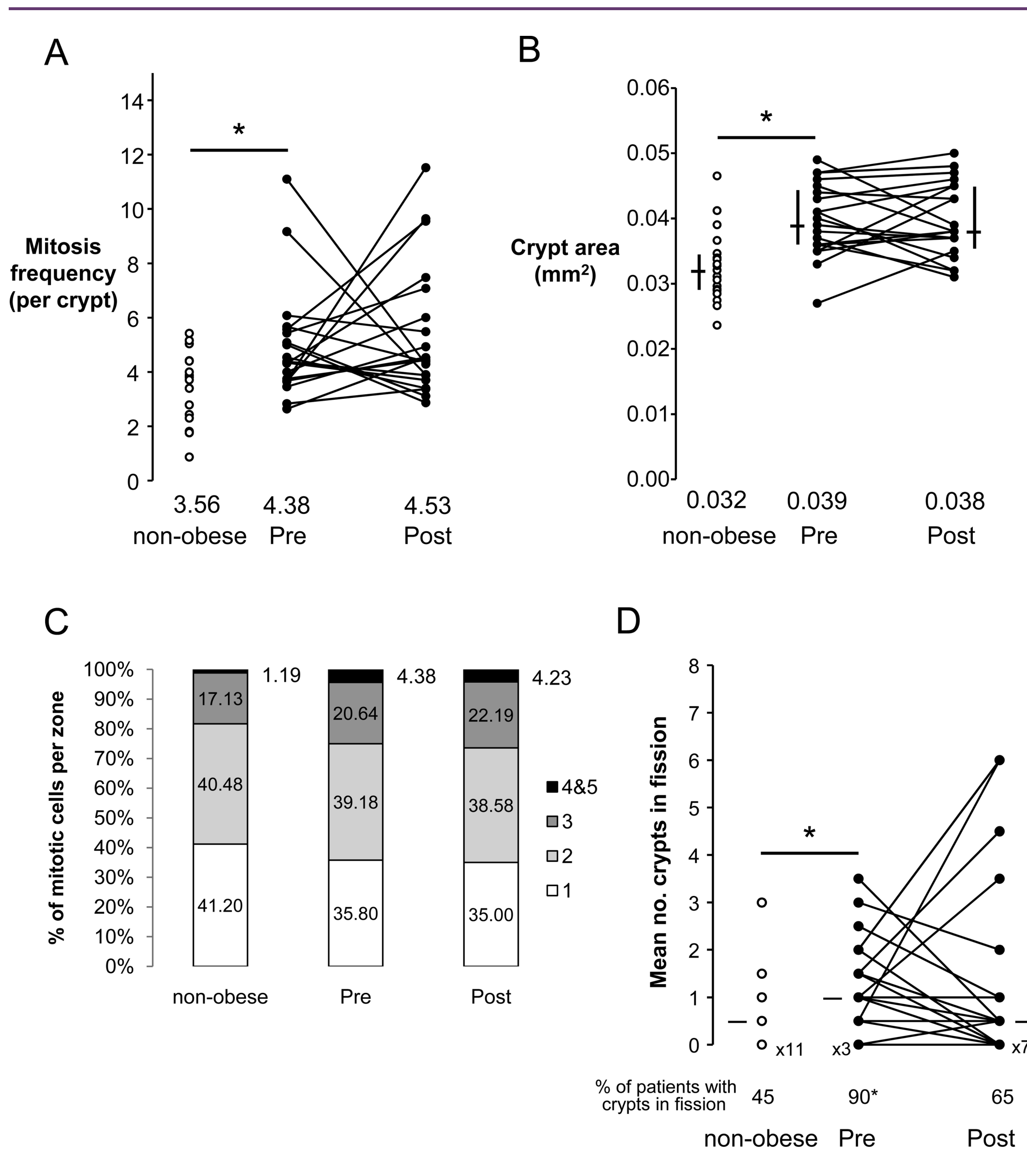


**FIGURE 1** Rectal mucosal crypt biomarker values in patients before and after SG compared with nonobese controls. (**A**) Mitosis frequency (mean number of mitotic cells per crypt). Open circles represent data from nonobese patients ($n$ = 20). Filled circles joined by a line represent data from morbidly obese patients before (Pre) and after (Post) SG ($n$ = 20 pairs). Values below the graph represent the median value for each group. * $P$ = 0.006; Mann-Whitney test. (**B**) Mean rectal crypt area. Open circles represent data from nonobese patients ($n$ = 20). Filled circles joined by a line represent data from morbidly obese patients before (Pre) and after (Post) SG ($n$ = 20 pairs). Horizontal and vertical bars represent the median and IQR values for each dataset respectively. Values below the graph represent the median value for each group. *$P$ = 0.002; Mann-Whitney U test. (**C**) Mitosis crypt distribution in crypt zones 1-5 in nonobese controls and patients before (Pre) and after (Post) SG. The numbers in each bar are mean percentage values for each zone. Because of the small numbers in zones 4 and 5, these values were combined. *$P$ = 0.003; Mann-Whitney U test. (**D**) The number of crypts in fission. Open circles represent data from nonobese patients ($n$ = 20). Filled circles joined by a line represent data from morbidly obese patients before (Pre) and after (Post) SG ($n$ = 20 pairs). Adjacent bars demonstrate the median value for each dataset. The number of patients exhibiting no crypt fission (xN) is displayed next to the zero values for each dataset. Numbers below the graph represent the percentage of individuals exhibiting equal to or more than one crypt in fission. The percentage of patients exhibiting crypt fission was significantly higher in morbidly obese patients prior to SG compared with nonobese patients. *$P$ = 0.002; $\chi^2$ test.

Neo-CK18-positive apoptotic epithelial cells in rectal biopsies were sparse as described previously (9). There were no significant differences between the apoptosis index or the percentage of patients numbers with at least one neo-CK18-positive cell-containing crypt between morbidly obese subjects and nonobese controls ($P$ = 0.85; Table 2.

By contrast with the effect of RYGB on rectal epithelial cell kinetics (9), there was no significant change in the crypt mitosis frequency ($P$ = 0.46), crypt size ($P$ = 0.98), or the percentage of mitotic cells in zones 4 and 5 at 6 months after SG compared to pre-operative values ($P$ = 0.92; all Wilcoxon signed rank test; Figure 1A-C and Table 2. There was also no significant difference in the number of

**TABLE 2** Summary of biomarker comparisons between nonobese controls and patients before (pre) and after (post) sleeve gastrectomy

| | | SG | |
|---|---|---|---|
| | Nonobese | Pre | Post |
| Weight (kg)[a] | 72.1 ± 2.3 | 188.7 ± 9.0 | 143.8 ± 7.7 |
| BMI (kg/m$^2$ )[a] | 24.7 ± 0.6 | 65.7 ± 2.4 | 50.1 ± 2.0 |
| % Excess weight loss[a] | - | - | 38.3 ± 3.8 |
| Rectal crypt measurements | | | |
| Mitosis frequency (per crypt) | 3.6 (2.3-4.3) | 4.4[b] (3.7-5.5) | 4.5 (3.7-6.8) |
| Crypt area (mm$^2$) | 0.032 (0.029-0.035) | 0.039[b] (0.036-0.045) | 0.038 (0.036-0.045) |
| Crypt branching | | | |
| Number of branching crypts/patient | 0.5 (0-3) | 1.0[b] (0-3.5) | 0.5 (0-6) |
| % patients with ≥ 1 branching crypt | 45 | 90[b] | 65 |
| Apoptosis | | | |
| M30 positive crypts/patient | 0.68 (0.24-1.0) | 0.54 (0.31-0.81) | 0.17 (0.17-0.17) |
| % patients with M30-positive crypts | 33.3 | 25 | 5 |
| Mucosal mRNA | | *Fold difference from nonobese*[c] | *Fold increase from pre-SG level*[d] |
| MIF | - | 1.24 (0.98-1.56) | 1.39 (1.04-1.87)[b] |
| COX-1 | - | 1.10 (0.96-1.26) | 0.57 (0.26-1.13) |
| COX-2 | - | 0.31 (0.26-0.38) | 1.79 (0.93-3.49) |
| TNFα | - | 0.83 (0.77-0.89) | 1.03 (0.41-2.62) |
| IL-6 | - | 0.90 (0.72-1.12) | 1.40 (0.62-3.20) |
| IL-1β | - | 0.68 (0.58-0.79) | 0.98 (0.51-1.89) |
| Urinary PGE-M | 11.8 (9.6-29.2) | 16.3 (12.1-24.2) | 10.9 (8.4-23.4) |
| Systemic inflammatory markers | | | |
| CRP (mg/l) | 0.5 (0.2-2.0) | 17.5 (10.5-25.3)[b] | 13.5 (3.4-16.3)[e] |
| TNFα (pg/ml) | 1.07 (0.82-1.21) | 1.99 (1.42-2.44) | 1.55 (1.35-2.21) |
| IL-6 (pg/ml) | 0.72 (0.24-1.78) | 5.01 (2.18-8.31) | 5.35 (3.27-6.95) |
| PBMC NFκB binding activity | | | |
| p50[a] | | | |
| Unstimulated | 0.33 ± 0.03 | 0.37 ± 0.02 | 0.41 ± 0.03 |
| LPS stimulated | 0.50 ± 0.03 | 0.55 ± 0.02 | 0.57 ± 0.03 |
| p65[a] | | | |
| Unstimulated | 0.42 ± 0.04 | 0.62 ± 0.04[b] | 0.64 ± 0.04 |
| LPS stimulated | 0.62 ± 0.08 | 0.99 ± 0.08[b] | 1.06 ± 0.05 |

[a]Values indicate the mean ± SEM. All other values are stated as the median (IQR).
[b]Indicates statistically significant difference compared with nonobese group; $P \leq 0.05$.
[c]Fold difference between the nonobese and pre-SG group is relative to GAPDH expression.
[d]Fold increase and 95% confidence interval are converted from the mean $\Delta\Delta C_t$ values
[e]Indicates a statistically significant difference compared with pre-SG values; $P \leq 0.05$

crypts in fission or the proportion of patients exhibiting crypts in fission 6 months after SG compared with baseline values ($P = 0.18$; McNemar test; Figure 1D and Table 2.

## Changes in systemic inflammation biomarkers following SG

As predicted by existing data (9,10), morbidly obese patients prior to SG had significantly higher levels of serum CRP, IL-6, and TNFα than nonobese patients ($P < 0.001$; Mann-Whitney U test; Figure 2 and Table 2.

Weight loss following SG was associated with a significant reduction in serum CRP ($P < 0.01$; Wilcoxon signed rank test; Figure 2 and Table 2, the magnitude of which correlated with the degree of postoperative weight loss at 6 months (Supporting Information Figure 2). However, there were no significant changes in serum IL-6

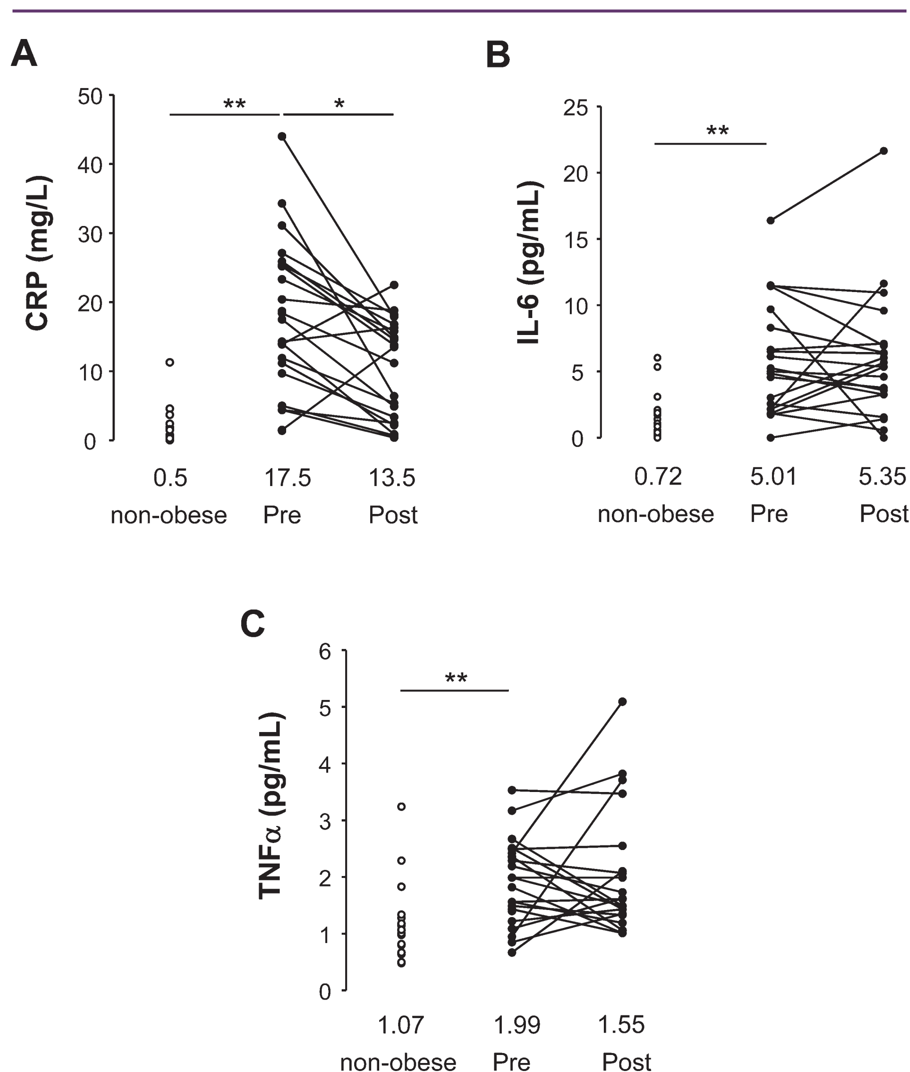


**FIGURE 2** Serum levels of CRP, IL-6 and TNFα in patients before and after SG compared with nonobese controls. Open circles represent (**A**) CRP, (**B**) IL-6, and (**C**) TNFα data from nonobese patients ($n = 20$; multiple data points overlap). Filled circles joined by a line represent data from morbidly obese patients before (Pre) and after (Post) SG ($n = 21$ pairs). Values below the graph represent median serum concentration values. **$P < 0.001$; Mann-Whitney U test. *$P < 0.01$; Wilcoxon signed rank test.

or TNFα levels ($P = 0.83$ and 0.89, respectively; Wilcoxon signed rank test) 6 months after SG (Figure 2 and Table 2).

We also examined NFκB DNA binding in PBMCs as a biomarker of innate immune activation in obesity (16). We first confirmed that p65 (Rel-A) and p50 are the NFκB subunits that exhibit the highest level DNA binding in obese human PBMCs (Supporting Information Figure 3). We then restricted our analysis to p65 and p50 DNA binding in nuclear extracts from PBMCs isolated from nonobese controls and morbidly obese patients, before and after SG. Activation with LPS acted as a positive control for NFκB binding with a consistent increase in NFκB binding observed upon LPS stimulation in all three groups (Figure 3A). Binding of p65 was increased in both un-stimulated and LPS-stimulated PBMCs from morbidly obese patients compared with nonobese individuals (Figure 3B,C and Table 2). However, in keeping with the absence of change in serum TNFα levels after SG, there was no significant difference between NFκB binding in PBMCs before and after SG, cultured in either the presence or absence of LPS (Figure 3B,C and Table 2). Interestingly, there was no difference in DNA binding of the p50 subunit in PBMCs between morbidly obese patients and nonobese controls despite the fact that LPS stimulation induced significant p50 binding in all three groups (Table 2).

## Mucosal and systemic $PGE_2$ synthesis in morbidly obese patients

There was no significant difference in *ex vivo* mucosal $PGE_2$ synthesis between morbidly obese patients and nonobese controls (data not shown).

We also measured urinary 11α-hydroxy-9, 15-dioxo-2, 3, 4, 5-tetranor-prostane-1, 20-dioic acid (PGE-M) levels as a marker of

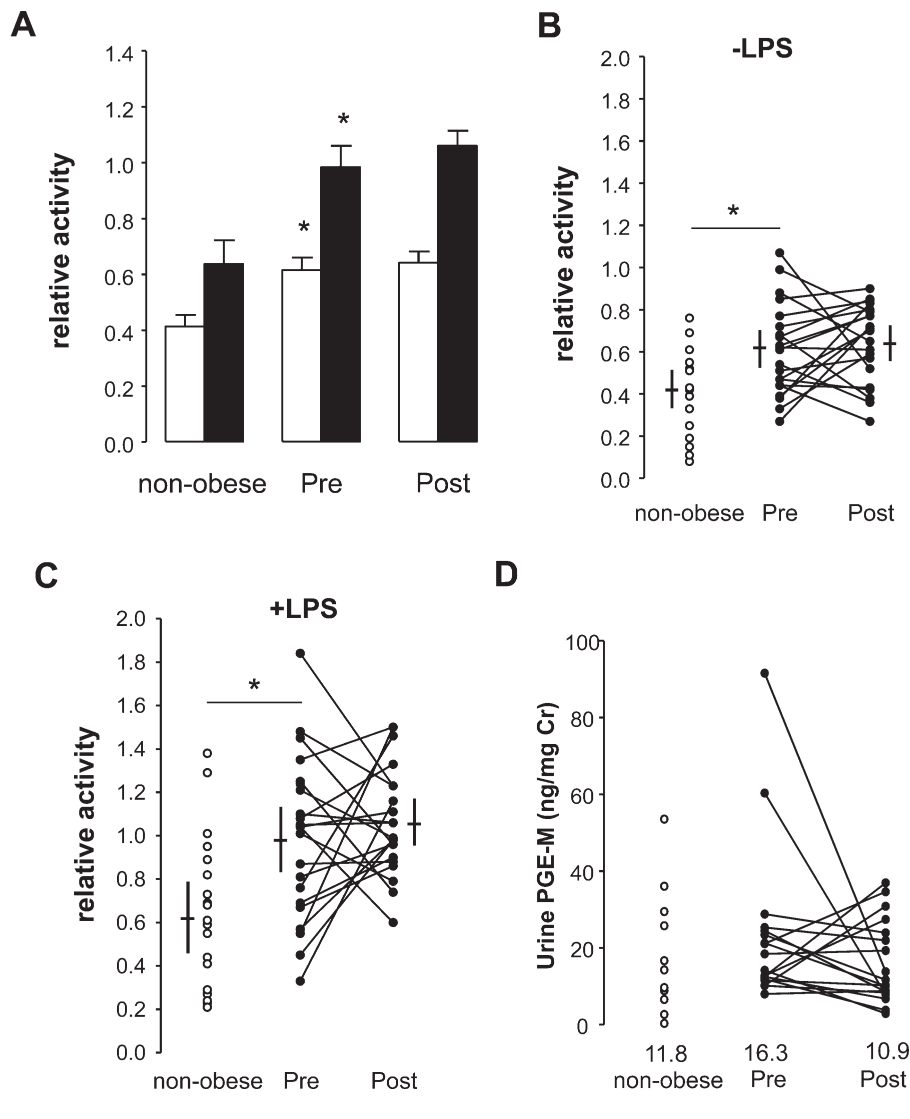


**FIGURE 3** NFκB p65 DNA binding in PBMCs from patients before and after SG compared with nonobese controls. (**A**) p65 DNA binding in PBMCs from nonobese controls and patients before (Pre) and after (Post) SG. Open bars represent the mean relative DNA binding in un-stimulated PBMCs, black bars represent mean relative DNA binding in LPS-stimulated PBMCs. Error bars represent the SEM. *$P < 0.01$ for the difference between the nonobese values and morbidly obese group data before SG. (**B**) Individual patient PBMC p65 DNA binding data. Open circles represent DNA binding in un-stimulated PBMCs from nonobese patients. Filled circles joined by a line represent data from morbidly obese patients before (pre) and after (post) SG. Adjacent bars represent the mean and 95% CI. *$P < 0.01$ for the difference between the nonobese values and morbidly obese group data before SG. (**C**) Corresponding p65 DNA binding values from LPS-stimulated PBMCs. *$P < 0.01$ for the difference between the nonobese values and morbidly obese group data before SG. *$P < 0.01$ for the difference between the nonobese values and morbidly obese group data before SG. (**D**) Urinary PGE-M levels in nonobese controls and morbidly obese patients before and after SG. Open circles represent data from nonobese patients ($n = 12$). Filled circles joined by a line represent paired data from morbidly obese patients before (Pre) and after (Post) SG ($n = 18$). Values below the graph represent the median urinary PGE-M level for each group.

systemic $PGE_2$ production (15). The coefficient of variation for 10 and 100 ng/ml authentic PGE-M was 11.3% and 9.6%, respectively, ($n = 9$ for both). Although median levels of urinary PGE-M were lower in the nonobese group compared with the morbidly obese group prior to SG, this difference did not reach statistical significance (Figure 3D; $P = 0.32$; Mann-Whitney U test). There was also no difference in urinary PGE-M levels or in morbidly obese patients before and after SG ($P = 0.25$; Wilcoxon signed rank test).

## Changes in rectal mucosal pro-inflammatory gene expression after SG

There was no significant difference in the mean GAPDH $C_t$ values between nonobese individuals and morbidly obese patients prior to SG (Supporting Information Figure 4).

$\Delta C_t$ values for individual pro-inflammatory gene transcripts varied widely (Figure 4A), but were normally distributed and so were

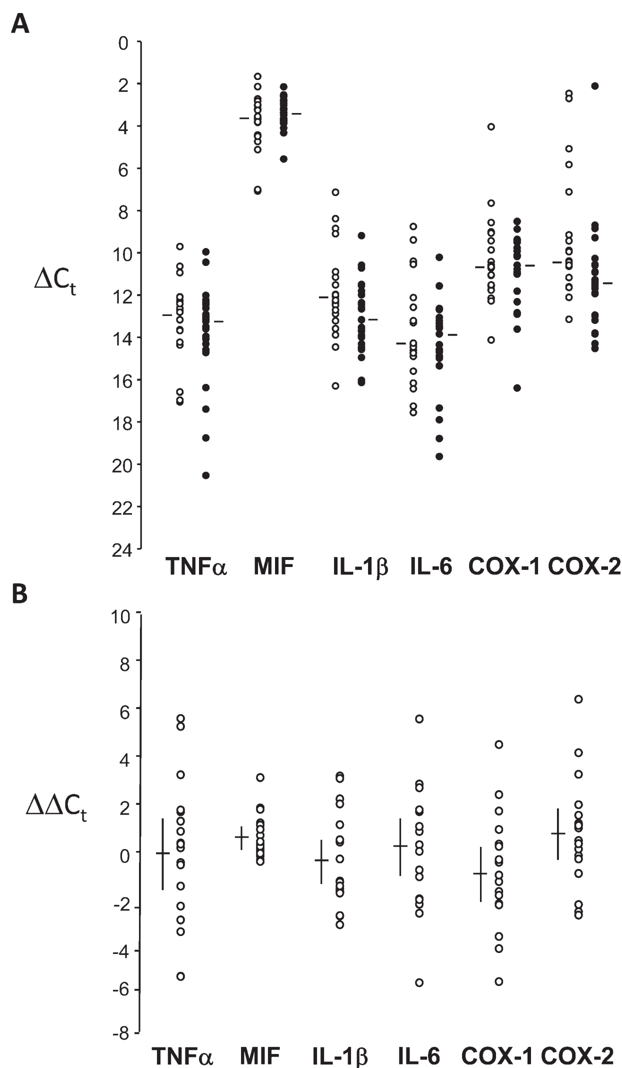


**FIGURE 4** Relative expression of rectal mucosal pro-inflammatory genes in non-obese individuals and morbidly obese patients before and after SG as measured by RT-PCR. (**A**) The distribution of $\Delta C_t$ values (normalised to GAPDH) for nonobese controls (open circles) and morbidly obese individuals before SG (closed circles). Note the inverted y-axis with lower $\Delta C_t$ values indicating a *higher* level of gene expression (relative to GAPDH) than higher $\Delta C_t$ values. Adjacent bars represent the median $\Delta C_t$ value for each group of $\Delta C_t$ values. (**B**) Changes in rectal mucosal transcript expression following SG. Each data point represents the $\Delta\Delta C_t$ value of an individual patient for a particular gene ($\Delta C_{tPre\text{-}SG}$ minus $\Delta C_{tPost\text{-}SG}$) normalised to GAPDH expression. Positive $\Delta\Delta C_t$ values represent an increase in transcript expression following SG and negative values represent a reduction. Adjacent bars represent the mean $\Delta\Delta C_t$ value; error bars denote the 95% confidence interval. *There was a statistically significant increase observed in MIF mRNA levels following SG ($P = 0.038$; paired *t*-test).

compared using parametric tests. There were consistently high levels of migration inhibitory factor (MIF) mRNA in rectal mucosal biopsies compared with transcript levels for the other genes examined, expression of which was more variable than MIF (Figure 4A). There was no significant difference in mRNA levels of any pro-inflammatory gene between nonobese patients and morbidly obese patients prior to SG surgery (Figure 4A and Table 2).

Paired analysis of GAPDH $C_t$ values before and after SG demonstrated that surgery also had no significant effect on reference GAPDH mRNA levels ($P = 0.13$; Mann-Whitney U test; Supporting Information Figure 4). Moreover, $\Delta C_t$ values before and after SG were normally distributed. There was a small, but significant, 39% increase in *MIF* mRNA level following SG (mean fold change [calculated as the $2^{\Delta\Delta Ct}$ value] 1.39; $P = 0.038$, paired *t*-test). However, there were no significant changes in the expression of TNFα, IL-1β, IL-6, COX-1, or COX-2 over the same time period (Figure 4B and Table 2).

## Discussion

Rectal mucosal crypt biomarkers of CRC risk did not increase 6 months after SG, unlike RYGB (9,10), in our small study. In the light of our recent observation that CRC risk may increase with time after RYGB, gastric banding, and vertical-band gastroplasty (8), it will be important to determine long-term colorectal neoplastic risk in the increasing number of individuals who now undergo SG, as well as repeat biomarker studies in a larger cohort study. Our prediction, based on these biomarker studies, would be that SG may not be associated with elevated future CRC risk compared with individuals with excess body weight-related elevated CRC risk, who do not undergo bariatric surgery. However, in the continuing absence of a prospective validation of rectal mucosal parameters as CRC risk biomarkers, a long-term cohort study of SG patients using CRC outcomes is essential. A direct biomarker or CRC risk comparison between SG and other restrictive procedures has yet to be carried out in order to explore potential discrepancies in colorectal outcomes between the different "restrictive" surgical approaches. One possible explanation for differences between outcomes after various "restrictive" procedures is that SG involves gastric resection, which affects hormonal (ghrelin) and adiopkine (adiponectin) profiles differently from gastric banding (16,17).

It should be noted that, although the %EWL after SG was similar to that observed in our previous RYGB study (9), the absolute BMI of patients undergoing SG was significantly higher than the RYGB study (9). This makes direct comparison between the two datasets more difficult but it is unlikely to confound the lack of an increase in mucosal biomarkers in the SG patients as the higher absolute postoperative body weight in SG patients might be expected to be associated with elevated biomarker values compared with RYGB patients. Similar differences in clinical factors such as diabetes and NSAID use between the obese patients and nonobese controls were observed in our previous RYGB study (9). Therefore, we do not believe that these clinical factors are likely to have confounded the biomarker data herein.

Importantly, our nonobese controls did not receive any bowel preparation, unlike previous studies (9). We have confirmed our earlier data (9) that rectal crypt biomarkers are elevated in morbidly obese individuals compared with nonobese controls. The lack of bowel preparation allowed a definitive comparison of mucosal pro-inflammatory gene expression in morbidly obese individuals and nonobese controls avoiding potential effects of bowel preparation on mucosal gene expression. The lack of any difference in mucosal transcript levels between morbidly obese patients and nonobese controls argues against the presence of local, mucosal inflammation associated with elevated systemic inflammatory biomarkers in obesity (4). However, it should be noted that the study was small predisposing to possible type II statistical error and that the control group

contained 9 (45%) overweight individuals (BMI 25.0-29.9 kg/m$^2$), which could have masked any relationship between BMI and mucosal gene expression in our study.

The increase in *MIF* transcript levels that was observed in rectal mucosa after SG is consistent with the small degree of *MIF* induction observed at a similar time-point after RYGB (10). In RYGB patients, MIF mRNA levels then increased dramatically at three years after surgery (10). It will be important to determine whether a similar phenomenon occurs during longer term follow-up of SG patients.

This is the first study to investigate differences in PBMC NF NF$\kappa$B DNA binding between morbidly obese patients and nonobese controls, as well as in morbidly obese patients following SG, as a surrogate biomarker of systemic inflammation and/or "classical" M1 tissue macrophage activation. Although PBMC NF$\kappa$B "activity", as measured by Rel-A DNA binding, was higher in morbidly obese patients compared with nonobese controls, in keeping with the previous observations (18), there was no decrease following significant surgical weight loss. This is consistent with the lack of change in serum TNF$\alpha$ levels, but is not consistent with the observations that PBMC NF$\kappa$B binding is reduced following modest calorie-restriction- and exercise-induced weight loss (19). In addition, Goyenechea and colleagues have also reported that Rel-A mRNA levels are reduced in obese individuals who maintain low calorie diet-induced weight loss (20). It is possible that, despite significant post-SG EWL, the PBMC NF$\kappa$B "activity" level may reflect residual, postoperative excess body weight. It should also be noted that our protocol included *ex vivo* PBMC culture in order to provide an internal positive control for PBMC activation, unlike previous studies (18-20).

The level of PGE-M, the stable tetranor metabolite of PGE$_2$, in urine is established as the best indicator of systemic PGE$_2$ synthesis (21,22). However, there has been no previous study of the relationship between body weight and urinary PGE-M level (22). We did not find any evidence for elevated PGE$_2$ synthesis in morbidly obese subjects, nor any change in urinary PGE-M levels after SG.

In summary, we have confirmed that rectal mucosal biomarkers of future CRC risk are increased in morbidly obese patients compared with nonobese controls. However, SG is not associated with a further increase in these biomarkers, unlike the other common bariatric procedure RYGB (9,10). Future CRC risk after SG requires careful evaluation and comparison with alternative bariatric procedures. **O**

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