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# Neutrophil Activation in Morbid Obesity, Chronic Activation of Acute Inflammation

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Recent studies show that morbid obesity is associated with activation of the innate immune response. Neutrophil activation is a fundamental process in the innate immune response. Therefore, the activation state of neutrophils in severely obese subjects and the effect of bariatric surgery on neutrophil activation was evaluated. Neutrophil activation was assessed by measuring circulating concentrations of myeloperoxidase (MPO) and calprotectin in 37 severely obese and 9 control subjects (enzyme-linked immunosorbent assay). Moreover, membrane expression of CD66b on circulating neutrophils was measured using flow cytometry in a group of seven severely obese and six control subjects. Immunohistochemical detection of MPO was performed in adipose and muscle tissue. Plasma MPO and calprotectin levels were significantly increased in severely obese subjects as compared to healthy controls,  $27.1 \pm 10.8$  vs.  $17.3 \pm 5.5$  ng/ml ( $P < 0.001$ ) and  $115.5 \pm 43.5$  vs.  $65.1 \pm 23.1$  ng/ml ( $P < 0.001$ ) for MPO and calprotectin, respectively. In line, CD66b expression was significantly increased in severely obese individuals,  $177.3 \pm 43.7$  vs.  $129.7 \pm 9.2$  (mean fluorescence intensity) ( $P < 0.01$ ). Bariatric surgery resulted in decreased calprotectin, but MPO plasma levels remained elevated. Adipose and muscle tissue did not contain increased numbers of MPO expressing cells in severely obese individuals. These results point out that circulating neutrophils are activated to a greater extent in severely obese subjects. Our data support the finding that the innate immune system is activated in severely obese individuals. Moreover, because neutrophils have a short life span, this indicates that the chronic inflammatory condition associated with morbid obesity is characterized by a continuous activation of the innate immune system.

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## INTRODUCTION

The inflammatory condition associated with obesity is considered to play a major role in the pathogenesis of obesity-related morbidities like cardiovascular disease and type 2 diabetes mellitus (1). Several inflammatory molecules have been shown to be involved. For instance, tumor necrosis factor- $\alpha$  plasma levels are increased in severely obese patients (2,3) and tumor necrosis factor- $\alpha$  knockout mice do not develop insulin resistance after diet-induced obesity (4). Moreover, selective upregulation of I $\kappa$ B kinase- $\beta$ , an inducer of nuclear factor- $\kappa$ B in liver results in decreased insulin sensitivity (5).

So far, most studies have focused on the role of macrophage activation and accumulation in adipose tissue, which has been shown to contribute significantly to insulin resistance (6). However, it appears that there is a more general activation of the immune system. For instance, levels of acute-phase proteins (C-reactive protein and  $\alpha_1$ -acid glycoprotein) are increased in morbid obesity and decreased after surgery-induced weight loss (3). Moreover, circulating levels of endothelial activation

markers are increased in obesity, indicating an activation of endothelial cells (7). Furthermore, the complement system is activated in morbid obesity which decreases after weight loss (8). Collectively, these findings indicate that the immune system is activated in obesity.

Neutrophils represent one of the most prominent components of the innate immune system, displaying strong phagocytic and antimicrobial activity. Their antimicrobial activity is mainly based on specific proteins stored in granules or in the cytoplasm, such as myeloperoxidase (MPO) and calprotectin. MPO is a potent enzyme stored in the azurophilic granules of neutrophils, which is secreted upon neutrophil activation. MPO generates numerous reactive oxidants and radicals, which cause oxidative damage to proteins, lipoproteins, lipids, and DNA of target cells (9). These characteristics of MPO are also important in the relation of MPO with cardiovascular disease. Zheng *et al.* showed that Apolipoprotein-A1, the major lipoprotein of high-density lipoprotein, is a specific target for MPO in atheroma, which

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could lead to plaque instability (10). Moreover, the product of MPO-controlled catalysis of hydrogen peroxide, hypochlorous acid, is thought to play a central role in the etiology of atherosclerosis (11). Calprotectin is a cytoplasmic bacteriostatic protein, which is released upon activation (12). Neutrophil activation is also accompanied by translocation of the glycoprotein CD66b from the secondary granules to the outer cell membrane (13).

Earlier studies have shown that whereas the number of neutrophils remains unchanged in severely obese patients, expression of CD62L, an adhesion molecule on the neutrophil surface, was depressed (14). Expression of other activation markers by neutrophils, such as the Fc receptor CD16 and the adhesion molecule CD11b as well as plasma levels of the activation marker elastase were similar between obese patients and normal weight controls (14,15). Therefore, the current view is that neutrophils are not activated in the severely obese individual. However, it was reported that the total number of circulating neutrophils was increased in obese individuals (16,17). In addition, evidence for a role of MPO in the development of obesity in mice was recently reported. Overexpression of MPO in transgenic mice led to enhanced weight gain on a high fat diet (18). Given these results and the notion that other components of the innate immune system are activated in severely obese subjects, we set out to evaluate the activation state of neutrophils in severely obese subjects. Here, it is reported that, in line with the activation of the innate immune response in morbid obesity, increased numbers of activated neutrophils are circulating in severely obese individuals.

## METHODS AND PROCEDURES

### Subjects and samples

All participants gave written informed consent. The study was approved by the local ethical committee of the Academic Hospital Maastricht.

Plasma levels of the neutrophil activation markers MPO and calprotectin were compared between 37 severely obese individuals and 9 normal weight control subjects (study 1A). Membrane CD66b expression on neutrophils was studied in seven severely obese subjects and compared to CD66b expression on neutrophils of six normal weight controls (study 1B). The effect of bariatric surgery on neutrophil activation was studied in 15 consecutive subjects, admitted to the Department of Surgery of the University Hospital Maastricht (study 2). Characteristics of the different study groups are depicted in [Table 1](#). All subjects were selected to be otherwise healthy according to history, clinical examination, and whenever possible laboratory findings, which means that no overt signs of type 2 diabetes mellitus, hypertension, nonalcoholic fatty liver disease, and other cardiovascular diseases were present. Moreover, patients using anti-inflammatory medication like aspirin, statins, and fibrates were excluded.

For studies 1A and 2, blood samples were collected after at least 8-h fasting using evacuated blood collection tubes containing EDTA and processed as previously described (3). In study 1B, whole blood samples were used. All samples were processed immediately for fluorescence activated cell sorting.

### Immunoassays

Plasma concentrations of MPO and calprotectin were determined using sandwich enzyme-linked immunosorbent assays (HyCult biotechnology, Uden, the Netherlands). All plasma samples were analyzed

in the same run. The intra- and interassay coefficients of variance of the various assays were <10%.

### Fluorescence activated cell sorting analysis

Fluorescence activated cell sorting analysis to detect membrane CD66b expression on neutrophils was performed using the following protocol. Whole blood (100  $\mu$ l) (EDTA) was incubated with a fluorescein isothiocyanate-labeled antibody against CD66b. Phosphate buffered saline and a fluorescein isothiocyanate-labeled isotype antibody were used as negative control. After 30 min of incubation in the dark, erythrocytes were lysed and the vials were centrifuged at 400 g. The supernatant was discarded and the cells were washed twice with phosphate buffered saline–0.1% bovine serum albumin. Finally, cells were resuspended in 1% paraformaldehyde solution and fluorescence activated cell sorting analysis was performed the same day on the FACSCalibur using Cellquest software (Becton Dickinson, Franklin lakes, NJ). Data are expressed as mean fluorescence intensity.

### Immunohistochemistry

Four-micrometer slices of formalin fixed, paraffin embedded visceral adipose (omentum) and muscle (rectus abdominus) tissue were stained for neutrophil infiltration using an antibody directed against MPO. In short, slides were deparaffinized, rehydrated, and incubated for 15 min in methanol–0.6% H<sub>2</sub>O<sub>2</sub> to block endogenous peroxidases. Thereafter, slides were incubated with 10% goat serum in Tris buffered saline–0.1% bovine serum albumin to prevent nonspecific binding of the secondary antibody. After washing with Tris buffered saline, slides were incubated with rabbit anti human MPO (1:1,000). Goat anti rabbit IgG antibody labeled with HRP was used as a secondary antibody. 3-Amino-9-ethylcarbazole was used as substrate for peroxidase. Hematoxylin was used as a counterstaining.

### Reagents and materials

Bovine serum albumin was purchased from Sigma (St Louis, MO). Fluorescein isothiocyanate-labeled antibodies against CD66b (clone 80H3) and the isotype control were purchased from Serotec (Kidlington, England). Polyclonal rabbit anti human MPO was purchased from Dako (Glostrup, Denmark).

### Statistical analysis

All data are expressed as mean  $\pm$  s.d. In studies 1A and 1B, due to unpaired and nonparametrically distributed data, results were compared using the Mann–Whitney test. In study 2, due to paired and nonparametrically distributed data, results were compared using the Wilcoxon sign rank test. A *P* value <0.05 was considered statistically significant.

## RESULTS

### Plasma markers of neutrophil activation are increased in morbid obesity

[Table 1](#) summarizes the patient characteristics of the severely obese and normal weight controls studied.

The plasma levels of the neutrophil activation markers MPO and calprotectin were measured in severely obese and normal weight controls (study group 1A). Both circulating MPO and calprotectin levels were significantly increased in severely obese subjects as compared to healthy controls. The levels of MPO were  $27.1 \pm 10.8$  ng/ml in the severely obese group whereas normal weight controls showed plasma levels of  $17.3 \pm 5.5$  ng/ml (*P* < 0.001). For calprotectin, plasma levels were  $115.5 \pm 43.5$  ng/ml in the severely obese group, whereas plasma levels of normal weight controls were  $65.1 \pm 23.1$  ng/ml (*P* < 0.001) ([Figure 1](#)).

It has been shown that immune activation diminishes during ageing (19). Also immune activation is thought to be more vigorous in women (20). However, no correlation was found between plasma MPO and calprotectin levels and age or sex. Also BMI was not correlated with MPO or calprotectin. In contrast, plasma MPO and plasma calprotectin levels showed a significant correlation ( $R^2 = 0.28$ ,  $P < 0.001$ ), indicating that they represent neutrophil activation (Figure 2).

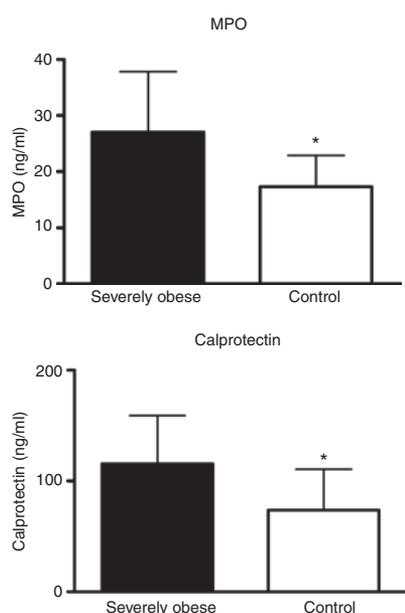
### CD66b expression on neutrophils is increased in severely obese individuals

To further explore the hypothesis that neutrophils are activated in morbid obesity, we assessed the activation marker of circulating neutrophils, CD66b, using flow cytometry. Surface CD66b expression was measured in a smaller group of seven severely obese and six normal weight healthy controls (see for characteristics Table 1, study group 1B). Like MPO and

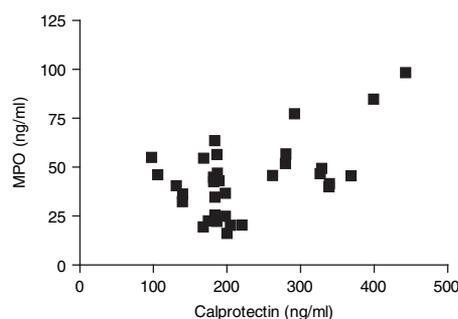
calprotectin plasma levels, CD66b membrane expression was significantly increased in severely obese individuals,  $177.3 \pm 43.7$  vs.  $129.7 \pm 9.2$  ( $P < 0.01$ ) (Figure 3).

### The effect of bariatric surgery on plasma markers of neutrophil activation (study group 2)

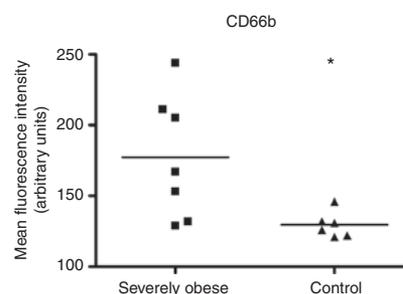
Weight loss after bariatric surgery is associated with decreased plasma levels of inflammatory mediators. Interestingly, MPO levels remained unchanged after bariatric surgery ( $19.7 \pm 4.1$  ng/ml preoperative and  $21.5 \pm 6.8$  ng/ml 2 years postoperative). On the other hand, 2 years after bariatric surgery, calprotectin levels decreased (11 out of 15 patients) significantly from a mean of  $119.6 \pm 31.5$  to a mean of  $93.9 \pm 42.7$  ng/ml ( $P < 0.001$ ). (Figure 4). The outliers of the calprotectin group were not identical with the outliers in the MPO group. Correlation analyses of the change in BMI and  $\Delta$ MPO or calprotectin showed no significant correlation.



**Figure 1** Circulating MPO and calprotectin levels in severely obese and normal weight subjects (study group 1A). The levels of MPO were  $27.1 \pm 10.8$  ng/ml in the severely obese group whereas normal weight controls showed plasma levels of  $17.3 \pm 5.5$  ng/ml ( $P < 0.001$ ). For calprotectin, plasma levels were  $115.5 \pm 43.5$  ng/ml in the severely obese group, whereas plasma levels of normal weight controls were  $65.1 \pm 23.1$  ng/ml ( $P < 0.001$ ). MPO, myeloperoxidase.



**Figure 2** Plasma MPO and calprotectin levels show a significant correlation ( $R^2 = 0.28$ ;  $P = 0.001$ ). MPO, myeloperoxidase.



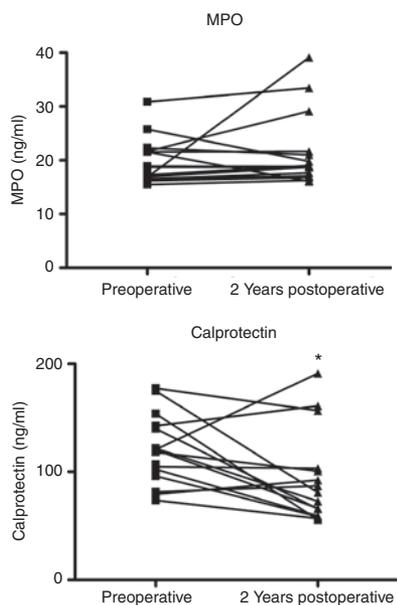
**Figure 3** CD66b expression on the neutrophil cell membrane quantified by mean fluorescence intensity in severely obese and normal weight subjects (study group 1B). CD66b expression was significantly increased in severely obese individuals,  $177.3 \pm 43.7$  vs.  $129.7 \pm 9.2$  ( $P < 0.01$ ).

**Table 1** Characteristics of the study populations

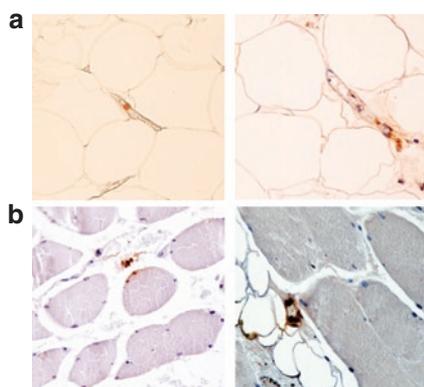
	Study group 1A		Study group 1B		Study group 2	
	Severely obese (n = 37)	Controls (n = 9)	Severely obese (n = 7)	Controls (n = 6)	Preoperative (n = 15)	Postoperative
BMI (kg/m <sup>2</sup> )	46.0 ± 6.0	22.8 ± 3.0	43.8 ± 6.7	22.8 ± 2.6	46.0 ± 4.9	36.2 ± 7.4
Age (year)	37 ± 10	31 ± 11	47 ± 7	44 ± 12	36 ± 10	
Sex (male/female)	5/32	2/7	3/4	3/3	1/14	

Plasma MPO and calprotectin levels were compared between severely obese subjects and normal weight controls in study 1A. In study 1B, CD66b expression was assessed and severely obese subjects were compared to normal weight controls. The effect of bariatric surgery on plasma MPO and calprotectin levels was studied in study 2.

MPO, myeloperoxidase.



**Figure 4** Effect of bariatric surgery on circulating plasma MPO and calprotectin levels. Preoperative and 2-year postoperative plasma levels of MPO and calprotectin were measured (study group 2). Two years after bariatric surgery, MPO levels remained unchanged after bariatric surgery ( $19.7 \pm 4.1$  ng/ml preoperative and  $21.5 \pm 6.8$  ng/ml 2 years postoperative). Interestingly, calprotectin levels decreased (11 out of 15 patients) significantly from a mean of  $119.6 \pm 31.5$  to a mean of  $93.9 \pm 42.7$  ng/ml ( $P < 0.001$ ). MPO, myeloperoxidase.



**Figure 5** Immunohistochemical staining of (a) visceral adipose tissue and (b) muscle tissue of severely obese subjects showing that the MPO positive cells are restricted to the vasculature of these tissues (magnification  $\times 200$ ). MPO, myeloperoxidase.

#### Absence of neutrophils in adipose and muscle tissue

To determine whether the increased neutrophil activation is accompanied by enhanced neutrophil infiltration of adipose or muscle tissue, immunohistochemical staining of MPO was performed. From 17 severely obese subjects, subcutaneous and visceral adipose tissue as well as skeletal muscle was stained for MPO. No MPO positive cells were located outside the vasculature of these tissues (Figure 5). In seven of these patients, plasma levels of calprotectin and MPO were measured as part of study 1A and shown to be increased compared with normal weight controls.

#### DISCUSSION

The inflammatory condition in severely obese subjects is characterized by activation of several components of the innate immune response. This is illustrated by increased plasma levels of acute-phase proteins, endothelial cell activation markers, complement factors, and cytokines derived from activated macrophages. Remarkably little is known about the activation of neutrophils, a central component in innate immunity, in obesity. Thus far, available data suggest that neutrophils are less activated in morbid obesity (14,15). This is in contrast to the overt evidence that the innate immune system is activated in morbid obesity. Therefore, we set out to determine the degree of neutrophil activation using plasma levels of calprotectin and MPO and neutrophil membrane expression of CD66b. MPO and calprotectin are primarily secreted by neutrophils although a minor secretion by activated monocytes cannot be excluded (9,12,21). CD66b on the other hand is specifically expressed on the outer cell membrane of granulocytes and its expression is enhanced upon activation of the cells (22,13) making CD66b a suitable marker for neutrophil activation.

We show increased plasma levels of MPO and calprotectin in severely obese patients, strongly indicating neutrophil activation. The observed increased neutrophil-specific CD66b expression also strongly supports neutrophil activation in morbid obesity. A possible mechanism for the activation of neutrophils could be the increased plasma levels of leptin and tumor necrosis factor- $\alpha$  observed in morbid obesity. Leptin has been reported to activate neutrophils via indirect induction of tumor necrosis factor- $\alpha$  secretion by monocytes (23).

Our findings contrast with the study of Cottam *et al.*, who reported a lower CD62L expression on neutrophils of severely obese individuals as compared to neutrophils of normal weight controls (14). They concluded that neutrophils of severely obese patients are less capable of activation and migration to target tissues rendering such patients more susceptible to inflammatory diseases.

In severely obese patients, enhanced numbers of macrophages have been reported in adipose tissue (6). In this context, the relation of activation of neutrophils with neutrophil infiltration of adipose and muscle tissue was investigated. Positive staining for MPO containing neutrophils in both adipose and skeletal muscle tissue was confined to blood vessels, which was similar in both tissues, indicating that the increased plasma MPO levels originate from other sources.

Interestingly, MPO is associated with cardiovascular diseases, because elevated plasma MPO levels have been reported to predict the presence of coronary artery disease (9). In addition, it has been shown that low-density lipoprotein receptor knockout mice overexpressing MPO, given a high fat diet, had increased aortic lesions and were more obese than their low-density lipoprotein receptor knockout littermates (18). The latter was thought to result from effects of MPO on lipid metabolism.

Interestingly, both plasma MPO levels and obesity are considered independent risk factors for cardiovascular disease. Increased plasma levels of MPO as observed in the present

study could therefore imply an additional risk for obese individuals for cardiovascular diseases. In another perspective, the causative role of MPO in cardiovascular disease and obesity provides opportunities for treatment options aimed at decreasing oxidative damage with antioxidants and related drugs (24), a topic that needs further study.

Bariatric surgery results in marked weight loss as well as decreased plasma levels of several inflammatory mediators (3,7). Therefore, we hypothesized that bariatric surgery would also result in decreased plasma levels of MPO and calprotectin. The decreased plasma levels of calprotectin indeed indicate that neutrophils are less activated 2 years after bariatric surgery. However, similar to earlier observations that several parts of the inflammatory cascade do not decrease after substantial weight loss (3,7), MPO levels remained elevated 2 years after bariatric surgery. This lack of effect of weight loss on plasma MPO levels has also been described for obese subjects (BMI ~33) after both diet-induced as well as exercise-induced weight loss (25). On the other hand, Roberts *et al.* reported that weight loss of obese subjects (BMI ~35) through a combined diet and exercise intervention did result in decreased plasma MPO (26). Thus, weight loss induced by rigorous lifestyle interventions may differentially modulate the inflammatory response as compared with weight loss through surgery. The contrasting effects of weight loss on plasma MPO vs. calprotectin levels in our study may relate to the fact that MPO and calprotectin are subject to different release patterns, due to their different location in the neutrophil.

In summary, we show that increased numbers of activated neutrophils are circulating in severely obese individuals. Bariatric surgery partly reduced neutrophil activation. These findings support the notion that morbid obesity is associated with activation of the innate immune response. Interestingly, the innate immune response is an acute inflammatory response, while obesity is considered a chronic inflammatory status. Therefore, obesity appears to be characterized by a chronic stimulation of the acute inflammatory response.

#### DISCLOSURE

Wim A. Buurman is a shareholder of Hycult Biotechnology (Uden, the Netherlands) that provided the MPO and Calprotectin ELISA kits used in this study. These materials are commercially available worldwide.

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