Oxidative and DNA damage in obese patients undergoing bariatric surgery: a one-year follow-up study

Running title: Oxidative stress and DNA damage in obese patients

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Abstract

The pathogenesis of obesity and related comorbidities has long been associated with oxidative stress. The excess of adipose tissue contributes to the production of free radicals that sustain both a local and a systemic chronic inflammatory state, whereas its reduction can bring to an improvement in inflammation and oxidative stress. In our work, using the fluorescent lipid probe BODIPY® 581/591 C_{11} and the γH2AX foci assay, a well-known marker of DNA double strand breaks (DSB), we evaluated the extent of cell membrane oxidation and DNA damage in peripheral blood lymphocytes of normal weight (NW) controls and obese patients sampled before and after bariatric surgery. Compared to NW controls, we observed a marked increase in both the frequencies of oxidized cells or nuclei exhibiting phosphorylation of histone H2AX in preoperatory obese patients. After bariatric surgery, obese patients, resampled over one-year follow-up, improved oxidative damage and reduced the presence of DSB. In conclusion, the present study highlights the importance for obese patients undergoing bariatric surgery to also monitor these molecular markers during their postoperative follow-up.

1. Introduction

In recent decades, the prevalence of obesity in both Western and developing countries has drastically increased, reaching the proportions of an unstoppable epidemic.
The etiology of obesity is multifactorial but mainly based on the disruption of the balance between caloric intake and energy expenditure. Patients suffering from this disease show a simultaneous increase in food intake that coupled with physical inactivity, leads to a continued and excessive energy intake. Whenever this condition persists, adipocytes respond by increasing their volume with lipid storages, resulting in excessive expansion of fat mass, particularly visceral fat, with a consequent significant gain of body weight [1].

Apart from a morphological change of the cells, adipocytes also increase the production of hormones with endocrine functions such as leptin and adiponectin, which in normal conditions contribute to maintaining proper energy homeostasis [2–4].
Moreover, the increase in mass establishes a state of hypoxia at the tissue level: this ischemic state triggers a series of inflammatory responses such as the recruitment of immune cells that release pro-inflammatory cytokines and increase localized oxidative stress [5–7].
As a result, the adipose tissue of the obese patient shows a chronic inflammatory state leading to a wide range of comorbidities such as atherosclerotic cardiovascular disease, type II diabetes mellitus, and hypertension [2,4].

Increasing evidence suggests that oxidative stress plays a crucial role as a causative factor of inflammation and complications associated with obesity. Indeed, obesity can induce systemic oxidative stress through the direct generation of ROS [8] but also through related conditions such as hyperglycemia, low antioxidant defense, chronic inflammation, and postprandial ROS generation [9]. Conversely, studies have reported that weight loss of overweight individuals through diet is associated with reduced circulating levels of cytokines and other markers of inflammation [10,11]. Similarly, severely obese patients that underwent gastric bypass surgery could see, along with stable weight loss, a decrease in the number of macrophages in adipose tissue, and in the levels of pro-inflammatory cytokines [12].

High concentrations of free radicals such as those found in obesity have many negative effects on cells. For example, lipid peroxidation by ROS, causes a loss of polyunsaturated fatty acids in cell membranes, reducing their fluidity and altering their permeability [13,14]. At the level of the nucleus, ROS can induce DNA damage either producing single-strand breaks (SSB) or double-strand breaks (DSB). The latter can also be induced by inflammatory factors released by adipose tissue, further enhancing the inflammatory state [15]. As a consequence of DSB, the cell initiates a DNA damage response (DDR) that includes phosphorylation of serine 139 of histone H2AX, hence creating γH2AX foci [16]. This serves as a starting point for chromatin remodeling and the recruitment of additional proteins to the DSB site to repair the lesion [17–19].

To date, the treatment of obesity and its comorbidities represents one of the greatest public health challenges. Evidence from the literature shows clearly that obesity entails a state of chronic low-grade inflammation, where oxidative stress and genomic damage are interconnected with mechanisms that are not yet well understood.
To gain information on the link between these processes, the present work aims to analyze oxidative and DNA damage in normal-weight subjects and in obese subjects before and after their treatment with gastric bypass surgery. Using a specific lipid probe and the γH2AX assay, we detected the presence of cell membrane oxidation and the extent of DSB occurrence in their lymphocytes.

2. Materials and methods

2.1 Study population

The study population consisted of 49 adult subjects affected by severe obesity (BMI > 35) selected for bariatric surgery, and 30 healthy normal-weight subjects (defined NW), recruited at the Obesity and Lipodystrophy Center, Endocrinology Unit, University Hospital, Pisa, Italy. Obese subjects included in the study were subdivided in different groups according to the time point at which blood samples were taken: 11 severely obese patients in pre-operative phase of gastric bypass (PreOp); 14 postoperative patients, 1 month after surgery (PostOp-1); 11 postoperative patients, 6 months after surgery (PostOp-6); 13 postoperative patients, 12 months after surgery (PostOp-12). Table I reports gender, age, weight and BMI of the study population. Subjects included in the study had to respect the following criteria: absence of cancer pathology, viral infections or other conditions capable of altering T cells, except the pathology under investigation; must not be on treatment with drugs known to induce mutations. The study was approved by the ethical committee of Pisa University.

Table 1. Demographic characteristics of the study population. Values represent mean ± SE of each parameter.

Condition	Subjects	Age	Sex		BMI[a] PreOp	BMI[b] PostOp		
			Males	Females		1 month	6 months	12 months
Obese	48	45.54±1.10	9	39	43.89±0.73	39.76±1.66	36.49±2.04	34.01±1.20
Normal weight	30	40.60±2.01	10	20	n.d.	n.d.	n.d.	n.d.

[a]Body mass index of subjects measured before undergoing bariatric surgery.
[b]Body mass index attained by patients 1 month, 6 months or 12 months after bariatric surgery.
n.d.: not determined.

2.2 Cell Culture

Blood samples from each subject were collected by venipuncture in Vacutainer tubes containing lithium heparin as anticoagulant. Cultures were set up by adding 150 μL of whole blood to 2.35 mL of RPMI-1640 (Life Technologies, Monza, Italy) supplemented with 15% fetal bovine serum (FBS) (Life Technologies, Monza, Italy), 1.5% PHA (Life Technologies, Monza, Italy) and 1% antibiotics and antifungals (Euroclone, Milan, Italy). Cultures were maintained at 37°C for a variable time depending on the specific method applied, as described below. For each subject two independent culture per assay were set up.

2.3 Oxidative damage

Oxidative damage was detected using the fluorescent structural analogue of membrane fatty acid BODIPY® 581/591 C_{11} (Life Technologies, Monza, Italy). This molecule is characterized by an emission of fluorescence at 595 nm, but when it is exposed to oxidative environment the fluorescence emission spectrum shifts to 510 nm. After 47.30 h from the start of culturing, 2.5 μL of the probe were added to each culture (5 mg/mL final concentration)

and the tubes were placed at 37°C for a further 30 min in order to promote the fluorescent molecule incorporation inside the membrane of lymphocytes. After the addition of hypotonic KCl solution (0.0075 M), cells were pre-fixed with acetic acid:methanol at 5:3 ratio. Subsequently, lymphocytes were fixed in 100% methanol and washed twice in a solution of acetic acid:methanol in variable ratio depending on the percentage of atmospheric humidity. After that, they were dropped onto clean glass slides, allowed to dry, counterstained with 4 μL of propidium iodide (PI) and then observed through fluorescence microscope (Nikon-Optiphot 2) equipped with FITC and TRICT filters at 1000× magnification. At FICT filter, undamaged cells presented a light brown cytoplasm, while lymphocytes with an oxidised membrane showed a green fluorescence; TRICT filter was used to detect cells regardless of the oxidation status. Oxidative damage was quantified as a percentage of cells with green cytoplasm on the total of 400 cells scored per culture. Data were expressed as the average value of the two cultures.

2.4 Genomic damage

The presence of DSB was assessed in unstimulated cells by harvesting the cultures immediately after set up. Harvesting procedures were the same as previously described, except that cells were washed in a methanol:acetic acid solution (3:1). Detection of γH2AX foci was performed, according to a method previously validated [26], using rabbit polyclonal phospho-histone H2AX antibody (Ser139) (Cell Signaling, Euroclone, Milan, Italy) and DyLight™ 488-conjugated anti-rabbit antibody (Pierce, Euroclone, Milan, Italy) as primary and secondary antibodies, respectively. Briefly, dry slides were washed twice in PBS (1×) and left for 30 min in blocking solution (10% FBS) + 0.3% TRITON® X-100 (Sigma-Aldrich, Milan, Italy). Slides were incubated overnight at 4°C with the primary antibody diluted 1:400 in blocking solution, then washed three times in PBS (1×) and incubated for 2 h at room temperature with the secondary antibody, diluted 1:200 in blocking solution. After three washes in PBS (1×), slides were counterstained with DAPI, 0.4 μg/mL final concentration, in antifade solution. γH2AX foci were detected using the FITC filter after visualization of cell nuclei under a UV filter. Spontaneous genomic damage was evaluated on 800 nuclei per subject (400 per replicate) recording the number of nuclei with at least one γH2AX signal and the total number of signals in each nucleus. The level of γH2AX for each subject was expressed as (1) percentage of positive nuclei, *i.e.* showing at least one fluorescence spot, (2) mean number of foci per nucleus, obtained by counting the total number of foci over all observed nuclei (γH2AX/N foci), and (3) mean number of foci per positive nucleus (γH2AX/N+ foci).). An example of negative or γH2AX positive nuclei is shown in Figure 1.
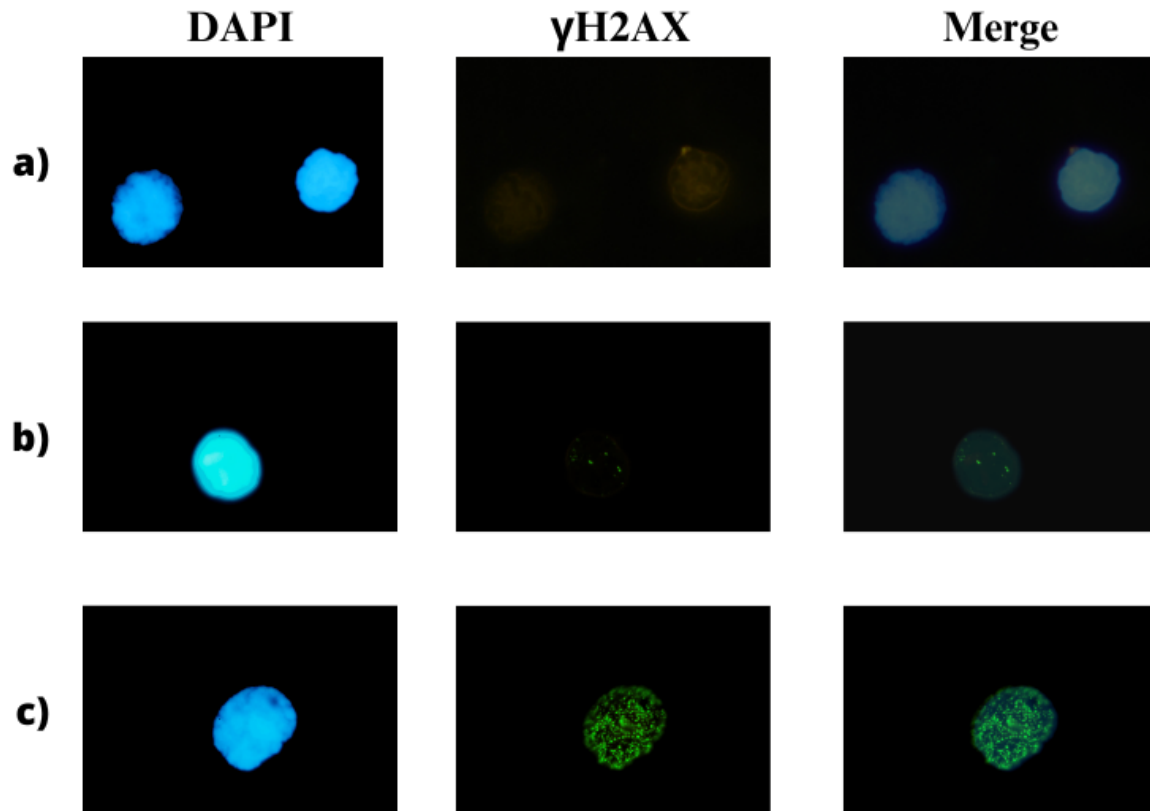
Figure 1. γH2AX foci indicate the presence of DSB in lymphocyte nuclei. Nuclei counterstained with DAPI are visible under the UV filter (left column); γH2AX foci are visualized as green fluorescence signals under the FITC filter (center column); merged images are in the right column. In **a)** two negative nuclei showing no fluorescence signal under each filter. In **b)** a γH2AX positive nucleus showing five green fluorescence signals under the FITC filter or in the merged image. In **c)** a highly damaged nucleus showing a multitude of green fluorescence spots under the FITC filter or in the merged image.

2.5 Statistical analysis

Statistical analyses were carried out by means of multifactor analysis of variance (MANOVA) with the Bonferroni's multiple range test. We compared, for each marker analyzed, the means of controls and obese patients categorized on the basis of their BMI and the time of blood sampling, *i.e.* before bariatric surgery and during the one-year postoperative follow-up (see above Study population) after controlling for chronological age and sex. Data were reported as mean ± standard error (SE), and the differences between groups were considered significant for $P<0.05$. In addition, a regression analysis was performed between the percentage of oxidized lymphocytes or γH2AX positive nuclei and the BMI values of all subjects included in the study. All statistical analyses were performed using the STATGRAPHICS Centurion XV.1 software package (Statistical Graphics Corp., Rockville, MD, USA).

3. Results

The analysis of oxidative and genomic damage was carried out on a group of normal weight (NW) controls and severe obese subjects who underwent bariatric surgery. Blood samples from the obese patients were collected both before surgery, referred as PreOp, and during the one-year post-operative follow-up, referred as PostOp (1, 6 and 12 months).

Concerning the oxidative damage (Figure 2), lymphocytes from obese patients sampled before surgery (PreOp) showed very high oxidation levels (16.74±2.22) compared to those of NW subjects (0.46±0.11). Comparisons within the groups of obese subjects showed a significant difference (P<0.001) between the PreOp (16.74±2.22) and each of the PostOp groups: PostOp-1 (10.05±0.80), PostOp-6 (6.90±0.57), PostOp-12 (5.25±0.58). This indicates that there was a substantial reduction in the frequency of oxidation after bariatric surgery, although the damage maintained still significantly higher (P<0.005) than that of NW controls.
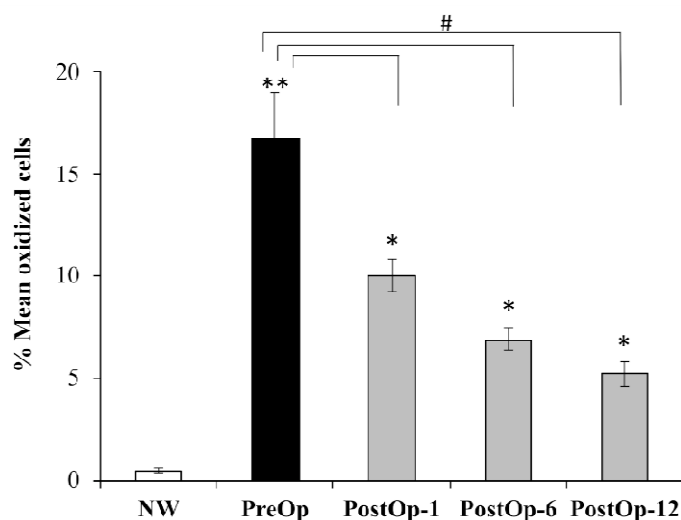
Figure 2. Cell membrane oxidation in peripheral blood lymphocytes of normal weight (NW) and obese patients before bariatric surgery (PreOp) and after surgery (PostOp) during a post-operative follow-up at 1, 6 or 12 months. Cells were analyzed by fluorescence microscopy using the BODIPY® 581/591 C_{11} sensor. Level of damage was expressed as percentage of oxidized cells. Bars represent the mean ± SE of each group (a total of 400 cells was scored for each subject, with independent duplicates). Asterisk indicates a statistical significance with respect to NW group ((**: P<0.001; *: P <0.005). Hash indicates a statistical significance (P<0.001) of PostOp groups compared to PreOp group.

The frequencies of the parameters used to quantify the level of DSB (the percentage of nuclei positive to γH2AX and the average number of γH2AX foci per nucleus or per positive nucleus) in peripheral cells of the study population are reported in Figure 3a, 3b or 3c, respectively. The fraction of nuclei showing γH2AX foci in the PreOp obese group (14.25±1.92) was statistically different (P<0.05) compared to the NW group (0.85±0.14). The frequencies observed in the PostOp groups started to progressively decrease with respect to the PreOp level, even if the statistical significance was reached only after 12 months: PostOp-1 (11.22±1.37), PostOp-6 (9.83±1.73) and PostOp-12 (7.00±1.01, P<0.01). A very similar trend was observed also when DSB are quantified as mean frequency of γH2AX foci per nucleus. In fact, the values of γH2AX foci/N were significantly higher (P<0.05) in the obese

groups (PreOp: 0.234±0.040; PostOp-1: 0.187±0-025; PostOp-6: 0.155±0.038; PostOp-12: 0.118±0.024) than in the NW control group (0.011±0.002). Once again, within obese groups, the only significant difference (P<0.05) *vs*. the PreOp group was seen in the PostOp-12. These results indicate a slower decline of genomic damage after bariatric surgery compared to oxidative stress. In contrast, as shown in Figure 3c, the average number of γH2AX foci per positive nucleus was similar in control group (1.28±0.05) and obese patients, in all analyzed conditions (PreOp: 1.58±0.09; PostOp-1: 1.76±0.13; PostOp-6: 1.44±0.12; PostOp-12: 1.61±0.11). We recorded a significant difference only between NW subjects and PostOp-1 patients (P<0.05) who may have suffered from additional stress after surgery.
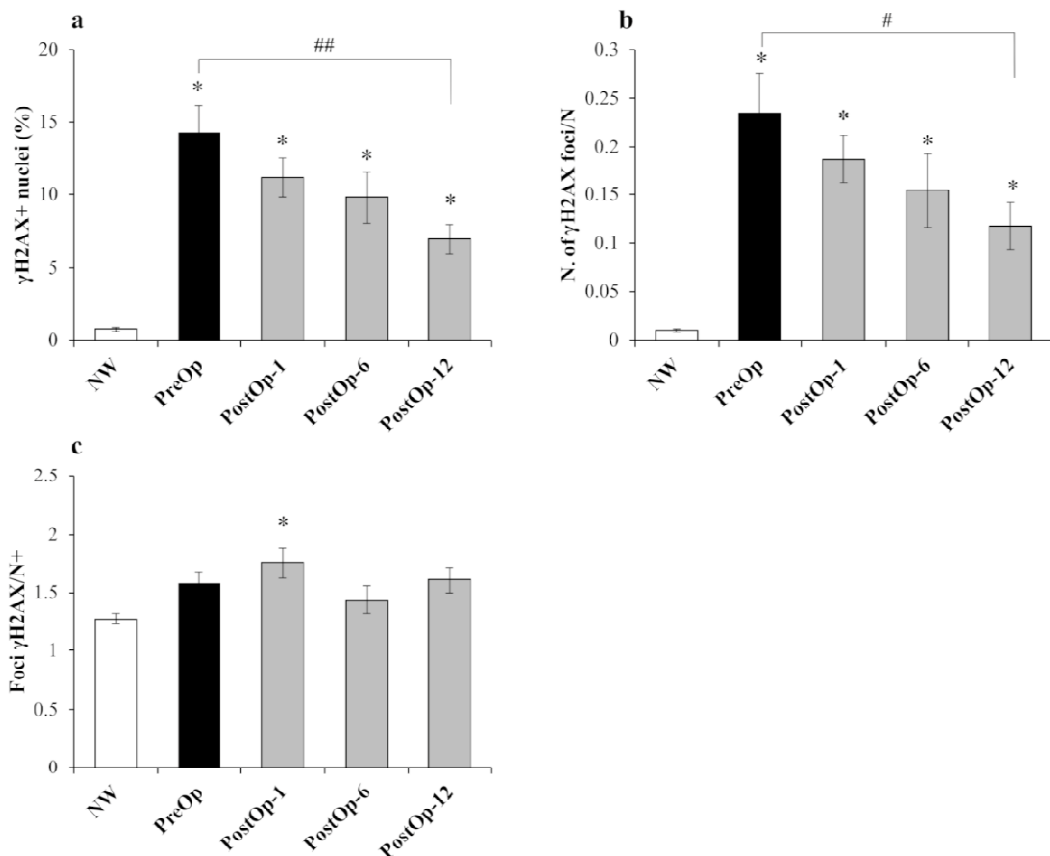
**Figure 3**. Levels of DSB (presence of γH2AX foci) in peripheral blood lymphocytes of normal weight (NW) controls and obese patients before bariatric surgery (PreOp) and after surgery (PostOp) during a post-operative follow-up at 1, 6 or 12 months. (a) Percentage of γH2AX positive nuclei, (b) average number of γH2AX foci per nucleus (both positive and negative), and c) average number of γH2AX foci observed per positive nucleus. Bars represent the mean ± SE of each group (a total of 200 cells was analyzed for each subject, with independent duplicates). Asterisk indicates a statistical significance (P<0.01) with respect to NW group. Hash indicates a statistical significance of PostOp groups compared to PreOp group (##: P<0.01; #: P<0.05).

Hence, we explored the possible relationship between the markers of oxidative and genomic damage and the BMI of subjects measured at the moment of the analysis. Figure 4a and 4b show that the percentage of oxidized cells and γH2AX+ nuclei increased with increasing BMI values (P<0.0001, R2=0.5487 and R2=0.4452, respectively). In other words,

as the patients' BMI decreases, and therefore some weight is lost, the oxidative and DNA damage decrease as well. Given the similar trend, a positive relationship was also found between the percentage of oxidized cells and the proportion of γH2AX+ nuclei. However, most of the variability appears to be only marginally explained by linear regression as it is likely to be driven by other factors that were not considered in this study (data not shown).
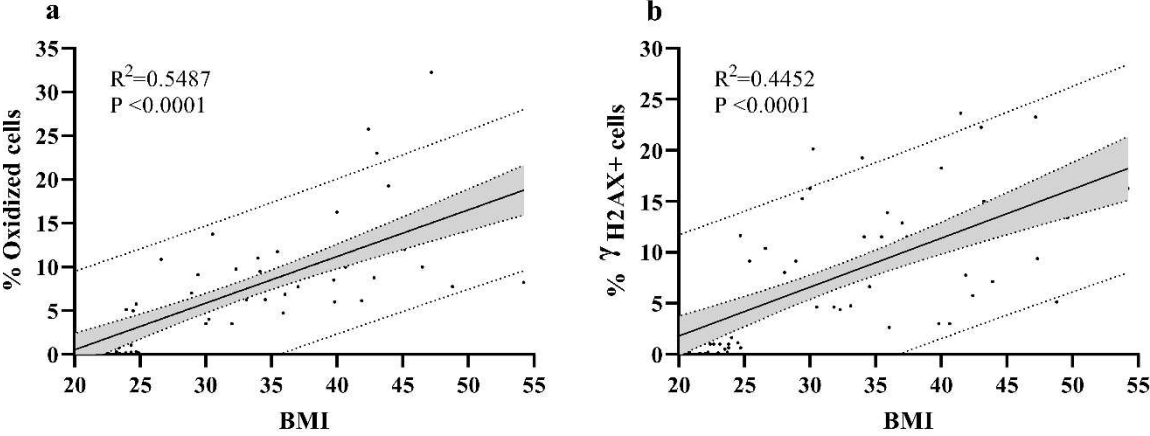
Figure 4. Linear regression analysis of (a) BMI and percentage of cell membrane oxidation, and (b) BMI and percentage of γH2AX positive nuclei. The shaded area represents the 95% confidence interval of the regression line, the dashed line represents the 95% prediction bands.

4. Discussion

Oxidative stress is considered one of the culprits of comorbidities associated with obesity. The main source of ROS in obese patients is the white adipose tissue where the adipocyte hypertrophy leads to the activation of pro-inflammatory signaling pathways and consequently to the massive production of oxidant molecules, with a parallel decrease in antioxidant defenses [8]. Excessive nutrient intake, as commonly occurs with obesity, can overwhelm the mitochondrial respiratory chain and cause mitochondrial dysfunction, leading to ROS formation and exacerbating the inflammatory process [21]. Moreover, other common conditions related to obesity such as hyperglycemia, hyperleptinemia and chronic low-grade inflammation, participate in the generation and maintenance of oxidative stress [22]. All these sources of ROS that the body struggles to suppress, inevitably cause oxidative damage to cells, disrupting cell integrity, impairing tissue functionality and leading to the development of obesity-related complications.

Given the central role that oxidative stress has in obesity, we investigated the membrane lipid oxidation status in peripheral lymphocytes of severely obese patients undergoing gastric bypass surgery. Due to the strong release of pro-inflammatory cytokines, the microenvironment of the adipose tissue promotes the spontaneous formation and the active production of reactive oxygen species. These molecules can induce lipid peroxidation and subsequent changes in membrane permeability, fluidity and integrity, hence triggering severe cytotoxicity [29]. The use of the fluorescent probe BODIPY® 581/591 C_{11} allowed us to observe the presence of an early oxidized state of the cells, prior to the establishment of the actual cellular damage. We observed that oxidation levels were about 36-fold higher in the obese population before surgery compared to the normal-weight controls. Also, we observed a clear decrease in oxidized cells (about 68% reduction) that follows the decrease of BMI going from preoperative condition to the 12-month postoperative follow-up. Prior published studies have evaluated the effect of weight loss after bariatric surgery on oxidative stress levels in very obese patients, reporting that weight reduction achieved after surgery improves all biomarkers of oxidative stress examined [24–26]. Hence, our results, are consistent with previous reports and indicate that reduction of lipid peroxidation is directly related to patients' BMI, confirming, as expected, a strong link between pathologies related to obesity and oxidative stress.

In the cell, the DNA molecule is one of the most sensitive targets to ROS and lipid peroxidation by-products [33]. Since DNA damage contributes to pathologic tissue dysfunction, we investigated whether the presence of an oxidative condition in peripheral lymphocytes of obese patients correlated with DNA damage. Our work highlighted an increase in basal genomic damage of obese patients who had a very high number of DSB in their peripheral cells expressed as both nuclei showing γH2AX-foci and γH2AX foci per nucleus. Interestingly, the levels of the first parameter above mentioned are about 16 times higher in obese PreOp patients compared to normal weight subjects. However, this level decreases by half during postoperative follow-up indicating again a positive effect of BMI reduction on the considered endpoint. The same conclusion can be drawn for the number of foci per nucleus that are 21-fold higher in obese PreOp than normoweight subjects and show a substantial reduction over the three time points of the post-operatory follow-up with 20, 34 and 49% lower values compared to pre-operatory condition. Our results are in line with previous reports where the authors observed that obesity was associated with a higher degree of DNA damage and oxidative stress [34–36]. Moreover, other works have shown that the reduction in adipose tissue is associated with a decrease in the level of DNA damage [37]. Concerning the effects of bariatric surgery, a study performed in 2017 observed an

improvement in oxidative stress and a reduction of DNA damage in severely obese patients, using other techniques compared to the ones presented in this work [38].

This confirms that oxidative damage to cellular components including DNA decreases in response to decreased adipose tissue. Interestingly, the evaluation of damage in terms of the average number of foci per positive nucleus showed no difference between NW subjects and obese patients, with the exception of the measurement carried out in the first month (PostOp-1) where part of the additional stress introduced by the surgical procedure can be still present. This result suggests that even though obesity brings a higher number of cells with DNA damage in the form of DSB, the recruitment of the repair systems is not impaired and the intensity of the damage at the single-cell level is comparable.

The results discussed so far are consistent with the presence of low-grade chronic inflammation, where the abnormal release of adipokines by the white adipose tissue modulates immune cells' response and promotes the release of proinflammatory factors [39]. These factors are active locally contributing to tissue dysfunction but are also released in the bloodstream where they contribute to systemic inflammation and the onset of metabolic syndrome [21,34–36].

Over the years, several clinical studies have shown how post-bariatric surgery promotes weight loss and an improvement in quality of life [46–48]. In patients with obesity who undergo bariatric surgery, weight loss occurs in part through a restrictive mechanism, but also as a result of altered secretion of entero-hormones with regulatory action on energy balance and glucose metabolism [49,50]. In addition to weight reduction, which is still maintained over the long term, there is a dramatic reduction in type 2 diabetes, triglyceride levels, and cholesterol, with a significant increase in the HDL fraction [51]. In general, this type of intervention, when supplemented with an appropriate follow-up program, results in consistent and long-lasting weight loss and almost complete remission of obesity-related diseases.

In the present study, it is important to note that despite the decrease in weight observed one year after surgery, the levels of all the makers analyzed (namely membrane oxidation and DNA damage in the form of DSB) in post-surgery patients are still higher than in normal-weight controls. This is probably due to a reduced, yet still elevated BMI, which means that there is still an excess of adipose tissue, hence a residual source of inflammation. In fact, the reduction in BMI after bariatric surgery was just below the cut-off of 35, which indicates the status of severe obesity. Accordingly to our molecular data, the highest BMI decrease was observed at 1 month Post-Op, (about 10% with respect to the Pre-Op BMI), and then decreased by less at the other two time points (8.2% and 6.8% at 6 and 12 months post-surgery, respectively). Despite this, we observed an overall improvement of all parameters coupled to weight reduction. Indeed, our data confirm that a pronounced inflammatory state leads to the induction and persistence of membrane oxidation and harmful DNA lesions such as DSB in lymphocytes of severely obese patients. Both types of damage in turn enhance inflammatory signaling, which appears to be directly related to body weight. In fact, as the BMI of obese patients decreases after the bariatric surgery, we observed a reduction of oxidative stress and a decrease in DNA damage, although the reduction of genomic damage appears to proceed more slowly.

In conclusion, the present study highlights the importance of long-term monitoring of molecular markers of oxidative stress and DNA damage in these patients for at least one year and probably longer. Long-term monitoring will serve not only to correlate these endpoints with classic pathologies linked to severe obesity, but may also help caregiver prevent the onset of diseases such as cancer that can arise later in life.

Conflicts of interest Declaration: None declared.

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