Maternal obesity reduces placental autophagy marker expression in uncomplicated pregnancies

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Running Title: Obesity reduces placental autophagy

Abstract

Aim: Obesity has been associated with changes in autophagy and its increasing prevalence among pregnant women is implicated in higher rates of placental-mediated complications of pregnancy such as preeclampsia and intrauterine growth restriction. Autophagy is involved in normal placentation, thus changes in autophagy may lead to impaired placental function and development. The aim of this study was to investigate the connection between obesity and autophagy in the placenta in otherwise uncomplicated pregnancies.

Methods: Immunohistochemisty and western blot analysis were done on placental and omental samples from obese (BMI≥30kg/m2) and normal weight (BMI<25kg/m2) pregnant women with singleton pregnancies undergoing planned Caesarean delivery without labour at term. Samples were analyzed for autophagic markers LC3B and p62 in the peripheral, middle, and central regions of the placenta and in omental adipocytes, milky spots, and vasculature.

Results: As pre-pregnancy BMI increased, there was an increase in both placental and fetal weight as well as decreased levels of LC3B in the central region of the placenta (p=0.0046). Within the obese patient group, LC3B levels were significantly decreased in the placentas of male fetuses compared to females (p<0.0001). Adipocytes, compared to milky spots and vasculature, had lower levels of p62 (p=0.0127) and LC3B (p=0.003) in obese omenta and lower levels of LC3B in control omenta (p=0.0071).

Conclusions: Obesity leads to reduced placental autophagy in uncomplicated pregnancies; thus, changes in autophagy may be involved in the underlying mechanisms of obesity-related placental diseases of pregnancy.

Key Words: pregnancy; obesity; placenta; autophagy; omentum

1. Introduction

The prevalence of obesity in North America continues to rise, and this remains true for women of childbearing age [1, 2]. In Canada, approximately one third of pregnant women are either overweight or obese [3]. Obesity affects an array of metabolic processes and is associated with an increased risk of pregnancy complications, including preeclampsia, hypertensive disorders, intrauterine growth restriction (IUGR), gestational diabetes and stillbirth [4, 5]. One of the metabolic processes affected by obesity that has been found to play a role in pregnancy is autophagy [6, 7].

Autophagy is an intracellular process whereby damaged organelles and proteins within the cytoplasm are recycled in order to promote cellular homeostasis, particularly under stress [8]. This process begins with the formation of a phagophore (isolation membrane), which encloses a small quantity of cytosol, forming a double-membraned structure called an autophagosome. The

autophagosome subsequently fuses with a lysosome, resulting in degradation of cytosolic components. Key markers for autophagy include LC3-I, LC3-II, a p62. LC3-I, which remains in the cytosol, is post-translationally modified into LC3-II, which incorporates into the growing autophagosomal membrane and allows for closure of the autophagic vacuole [8, 9]. The ratio of LC3-II/LC3-I directly correlates with the activation of autophagy [9]. P62 (or sequestosome-1), a less specific marker for autophagy, binds to ubiquitinated proteins targeted for degradation and helps deliver them to autophagosomes through interaction with LC3-II. P62 is degraded together with the ubiquitinated proteins resulting in reduced levels in situations of increased autophagy.

The metabolic abnormalities related to obesity are associated with dysregulation in autophagy [10]. Autophagic activity has been shown to increase in subcutaneous adipose and omental tissue from obese subjects [6, 11] and in adipose tissue of patients with type-2 diabetes [12]. Furthermore, impaired autophagy in hepatocytes has been implicated in non-alcoholic fatty liver disease, obesity-related pancreatitis, and type-2 diabetes [10].

Our understanding of the function of autophagy in the placenta is still in its early stages, with fewer than 50 citations in the literature related to the subject [13]. Autophagy plays a role in normal placentation and is upregulated by physiologic hypoxia in the extravillous trophoblast to promote its invasion [14]. *In vitro* studies have demonstrated impaired invasion and vascular remodeling in autophagy-deficient extravillous trophoblast cells [7], suggesting an important role for autophagy in normal placentation and the development of placental-mediated disorders of pregnancy. Recent research has found differences in placental autophagy between normal and abnormal pregnancies [14-16]. For example, LC3 expression is increased in placentas from pregnancies with preeclampsia, intrauterine growth restriction (IUGR) and gestational diabetes [14-18]. More recently, activation of autophagy was demonstrated in the placentas of male fetuses in

obese women compared to normal weight women [19]. Given these findings, it is of clear interest to further explore the dysfunction of placental autophagy in obese women.

The objective of this study was to investigate the association between obesity and autophagy in the placenta in uncomplicated pregnancies. Furthermore, this study aims to determine whether a correlation exists between autophagic activity in the placenta and in the omentum of obese patients, as this could provide insight to the underlying mechanisms of obesity-related placental and metabolic disorders.

2. Methods

2.1 Ethics approval and participants

Ethics approval was obtained from Lawson Health Research Institute located in London, Ontario (REB# 106663). Following admission to the hospital, patients were consented for their involvement as per institutional research protocols at London Health Sciences Centre, Victoria Hospital.

2.2 Placenta tissue collection, processing and sampling

After informed consent was obtained, patients with singleton pregnancies undergoing planned Caesarean delivery without labor at term (>37 weeks gestation) were included in the study and were stratified into controls (non-obese, BMI <25kg/m^2), and obese (BMI ≥30kg/m^2) based on their pre-pregnancy BMI. Women with hypertensive disorders of pregnancy, gestational diabetes or other significant medical conditions predisposing to placental dysfunction (renal, autoimmune disease) and women who were overweight (prepregnancy BMI ≥25 <30 kg/m2) were excluded.

To assess other factors that may affect autophagy, clinical data on patients were collected including age, gestational age, gravidity, parity, medical history, smoking status, gestational weight gain and blood pressure. A standard panel of laboratory investigations used in our centre for preeclampsia diagnosis and screening was drawn on each patient in addition to the routine preoperative investigations required. Following delivery, fetal and placental weights were documented and placentas were examined for gross structural abnormalities and cord anomalies. Photographs were taken for documentation.

Placental tissue samples were taken from three different regions (peripheral, middle and central) across each placenta (Figure 1) as previous evidence has demonstrated significant variations in autophagy across the placenta [13]. All samples were taken within 30 minutes of delivery. Full tissue sections were taken and used for immunohistochemical (IHC) analysis, while smaller samples of placental parenchyma were used for Western blot analysis.

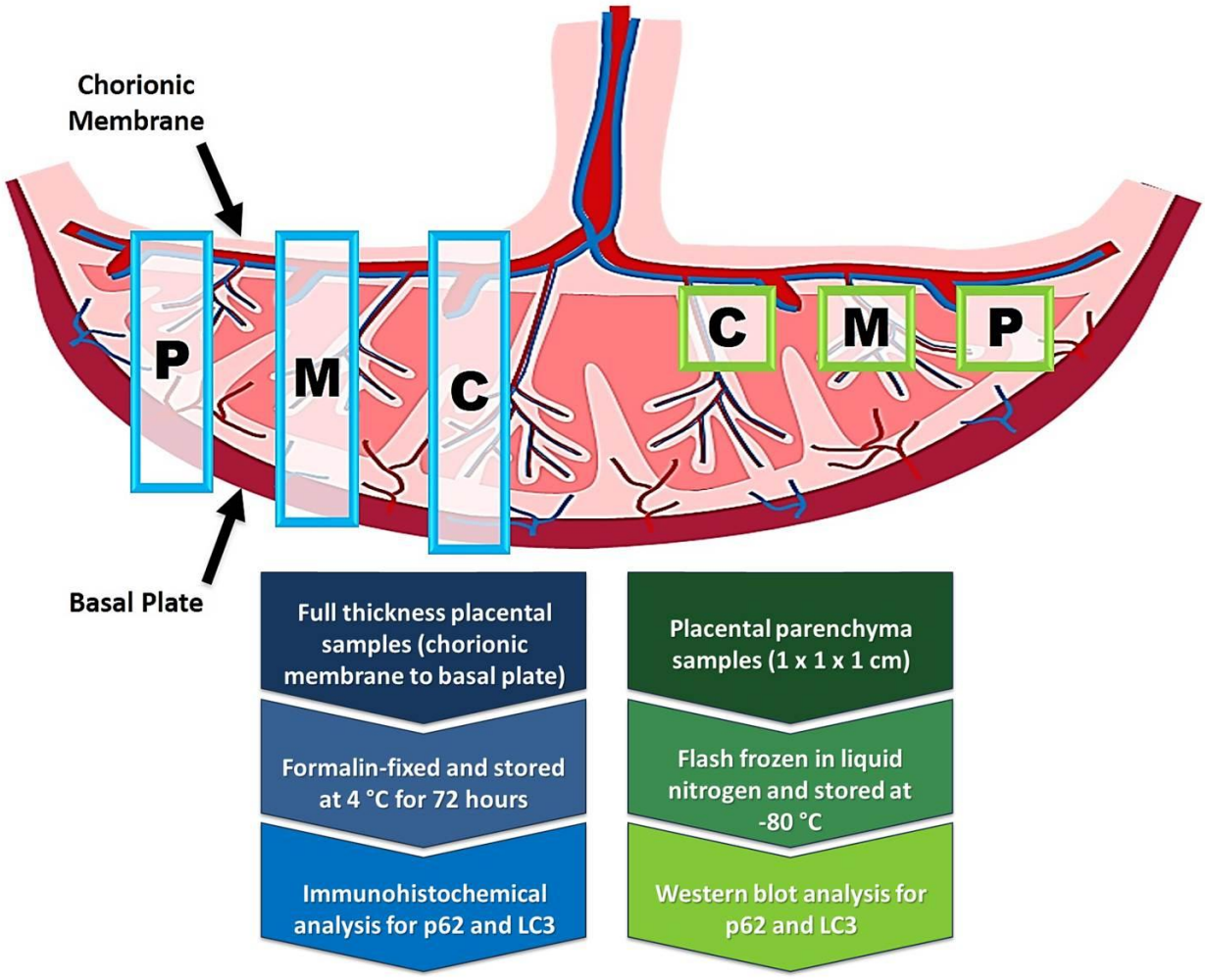

Figure 1 Sample collection diagram. Samples were taken from the placenta in three areas: (C) central, (P) peripheral (approximately 1–2 cm from the placental edge), and (M) middle (between these two sections). At each site, a full tissue sample from the chorionic membrane to

the basal plate was removed and stored immediately in formalin for subsequent histology and immunohistochemistry (IHC). Additionally, at each of the three sites 1 × 1 × 1 cm sections of placental parenchyma containing villous tissue between the basal plate and chorionic membrane were taken, flash frozen in liquid nitrogen and stored at −80C for subsequent protein extraction.

2.3 Western blot analysis

Protein lysates were generated from snap-frozen placental tissue samples stored at –80 °C. Samples were mixed with dry ice pellets, pulverized using a mortar and pestle, homogenized with RIPA lysis buffer [50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate, 1 mM sodium orthovandate, 10 mM sodium pyrophosphate, 10 mM NaF, 1% sodium deoxycholate, betaglyceraldehyde, 1 mM phenylmethylsulfonyl fluoride, and 1x protease inhibitor cocktail (Roche, Laval, QC)], and clarified by centrifugation (15 000 x g for 20 min at 4°C). Protein concentration of each lysate was quantified by Bradford analysis (Bio-Rad Laboratories), and 40 µg of protein was resolved by 13% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane (Roche), and blocked with 5% bovine serum albumin (BSA) in TBST (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20). Membranes were washed in TBST and incubated overnight at 4°C with antibodies (1:1000 in 5% BSA/TBST). Immunoreactive bands were visualized by incubating for 1 h at room temperature with a peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin (1:10 000 in BSA/TBST; GE Healthcare, Chicago, IL) followed by exposure to enhanced chemiluminescence reagent (Luminata Forte Western HRP Substrate, Millipore).

Antibodies against LC3B (#2775, #3868S) and SQSTM1/p62 (#5114S) were obtained from Cell Signaling Technology (Danvers, MA). Antibodies against SQSTM1/p62 (WH0008878M1) and tubulin were obtained from Sigma (Mississauga, ON). Horseradish peroxidase-conjugated anti-rabbit (NA934V) and anti-mouse immunoglobulin (NA931V) were purchased from GE Healthcare (Chicago, IL). The relative intensity of protein signals were detected using the Biorad Chemidoc system (Biorad) and normalized to tubulin by densitometric analysis using Image Lab software (Biorad). Levels of p62, LC3-I, LC3-II, and the ratio of LC3-II/LC3-I were analyzed and compared between obese and control samples.

2.4 Immunohistochemistry

Formalin-fixed paraffin-embedded (FFPE) tissue samples were sent for sectioning and staining with haematoxylin and eosin (Molecular Pathology, Robarts Research Institute, London, ON). Stained slides were scanned using Aperio ScanScope CS (Leica). IHC was performed for LC3B (1:100) and SQSTM1/p62 (1:400) (Molecular Pathology, Robarts Research Institute, London, ON). Staining was detected using Novolink Polymer Detection Systems (Leica, Wetzlar, Germany), and slides were scanned using Aperio ScanScope CS. The placental specimens were evaluated using the semi-quantitative International Remmele Score (IRS) [20]. The IRS is calculated by multiplying the optical staining intensity graded from 0 to 3 (0 = no staining to 3 = strong staining) and the percentage of positively-stained cells graded from 0 to 4 (0 = no staining, 1 = <10% of cells, 2 = 11%–50% of cells, 3 = 51%–80%, 4 = >80% of cells stained). The omental specimens were evaluated using a modified semi-quantitative scoring method [21]. As the omentum is comprised of three morphologically distinct regions (adipocytes, milky spots, and vasculature) [22], an optical staining intensity score from 0 to 3 was given to each

tissue region. The final score for the specimen was then calculated by adding the scores from each of the three tissue regions, for a maximum score of 9 and a minimum score of 0. Two blinded reviewers (Matthew Cohen, Emily Guo) graded all placental and omental specimens and scores were averaged.

2.5 Statistical Analysis

Data are presented as the mean ±SD and were analyzed and plotted using GraphPad Prism 7 for Mac OS X and Windows (GraphPad Software, Inc., La Jolla, CA, USA). Clinical data from the two patient groups was compared using two-tailed unpaired t-tests. Data from all western blot studies were statistically analyzed using the two-tailed unpaired t-test or a one or two-way ANOVA followed by Tukey's test. IHC scores of placental samples were analyzed using the Mann-Whitney U test, and IHC scores of omental samples were analyzed using the two-tailed unpaired t-test. Statistical significance was accepted at $p<0.05$ for all comparisons.

3. Results

From November 2015 to June 2016, 30 subjects were recruited: 15 cases with pre-pregnancy BMI $\geq 30 kg/m^2$ and 15 controls with normal pre-pregnancy BMI of $<25 kg/m^2$. The pre-pregnancy BMI of the case group was significantly higher than the control group (42.3 vs. $21.1 kg/m^2$, $p<0.0001$). Patient clinical characteristics, laboratory data, and fetal weights are presented in Table 1.

Table 1: Clinical characteristics and laboratory values

	Control: NW BMI <25 kg/m^2 (n=15)	**Case: OB BMI >30 kg/m^2 (n=15)**	**p-value**
Age (years)	31.7 (±4.7)	29.7 (±4.0)	0.5973
Primiparous	1 (7%)	2 (13%)	0.5500
Gestational Age (weeks +days)	38+5 (37+4 - 39+3)	39+1 (37+5 - 40+0)	0.9105
Pre-pregnancy BMI (kg/m2)	21.4 (±2.0)	42.3 (±7.6)	< 0.0001*
Gestational Weight gain (kg)	13.2 (±5.4)	7.3 (±6.9)	0.3548
Birth Weight (g)	3170 (±403)	3795 (±718)	0.0065*
LGA Infants	0 (0%)	5 (33%)	0.0160*
Placenta Weight (g)	592 (±111)	762 (±164.3)	0.0026*
Birth Weight: Placenta Weight	5.4 (±0.2)	5.0 (±0.1)	0.2821
Male Neonates	7 (47%)	8 (53%)	0.7199
Venous Cord pH	7.293 (±0.05)	7.287 (±0.08)	0.0426*
Hemoglobin (g/L)	118.1 (±11.0)	119.2 (±9.9)	0.7165
Glucose (mmol/L)	4.32 (±0.5)	4.59 (0.5)	0.8268
AST (U/L)	20.2 (±4.6)	19.8 (±6.7)	0.1608
Creatinine (µmol/L)	53.1 (±8.4)	49.6 (±8.7)	0.8724
Urate (µmol/L)	289 (±58.2)	273 (±67.1)	0.6069

Note: *Data are presented as mean (±SD), mean (range) or n (%)*
* indicates a significant difference between case and control ($p<0.05$)

3.1 Clinical and laboratory data analysis

Birth weight was significantly higher in the elevated BMI group with a mean difference of 625 ±213gm (p=0.0065; Table 1). This was associated with a significantly higher placental weight in pregnancies with elevated BMI with an average difference of 169.7 ±51.2gm (p=0.0026; Table 1). However, the fetal-placental weight ratio, a measure of placental efficiency, was not statistically significant, although there was a trend toward decreased placental efficiency in the elevated BMI group (Table 1).

3.2 Placental autophagy

IHC confirmed the presence of autophagy proteins, LC3B and p62, in various cell types within the obese and control placenta samples (Figure 2). Semi-quantitative scoring of LC3B staining revealed decreased autophagy in obese *versus* control placentas in the central region (IRS score 3.3± 0.7 vs. 4.7± 1.3, p=0.0046). This difference, however, was not found in the peripheral or middle regions of the placenta.

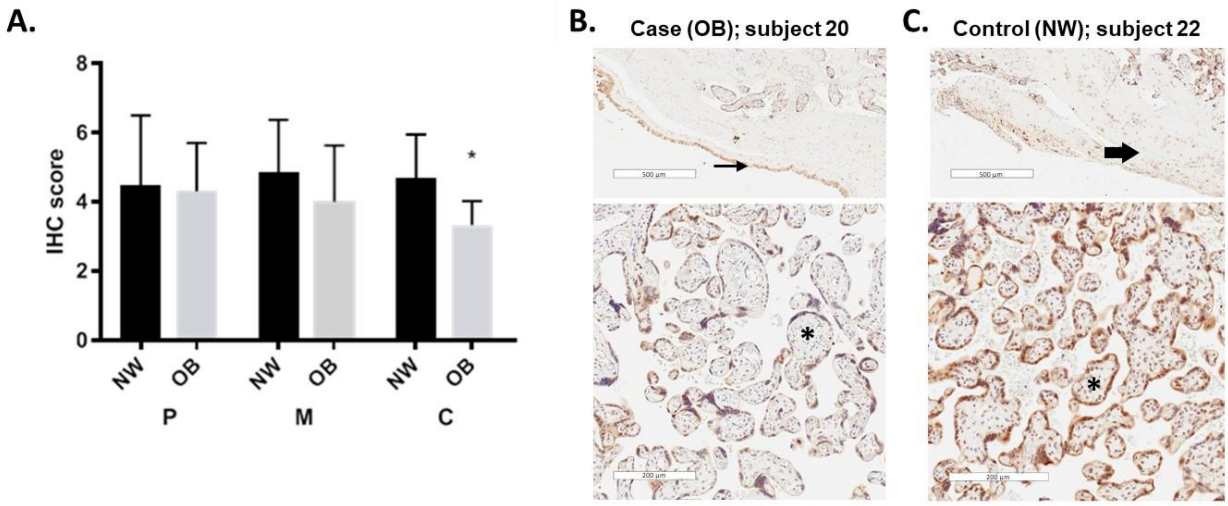

Figure 2 ICH analysis of LC3B in three regions of the placenta from obese and normal weight patients. (a) Immunohistochemistry (IHC) scores of LC3B in the peripheral (P), middle (M), and central (C) regions of obese and control placental samples. Scores were determined by semiquantitative International Remmele Score (IRS). Data represents mean ± SD (Mann–Whitney U test; *P < 0.05). (b, c) Representative IHC of LC3B in the central portion of the placenta from the (b) obese (subject 20) and (c) control (subject 22) groups. Medium-power (bottom) and high-power (top) fields of the (b) fetal and (c) maternal sides of the placenta. Images are representative of LC3B expression in the amniotic membrane (thin arrow), maternal decidua (thick arrow), and chorionic villi (asterisk) in the obese group.

Western blot analysis found no significant differences in autophagy between control and obese placentas in any of the regions sampled even when the scores from each region were combined as a total value within each placenta (Figure 3).

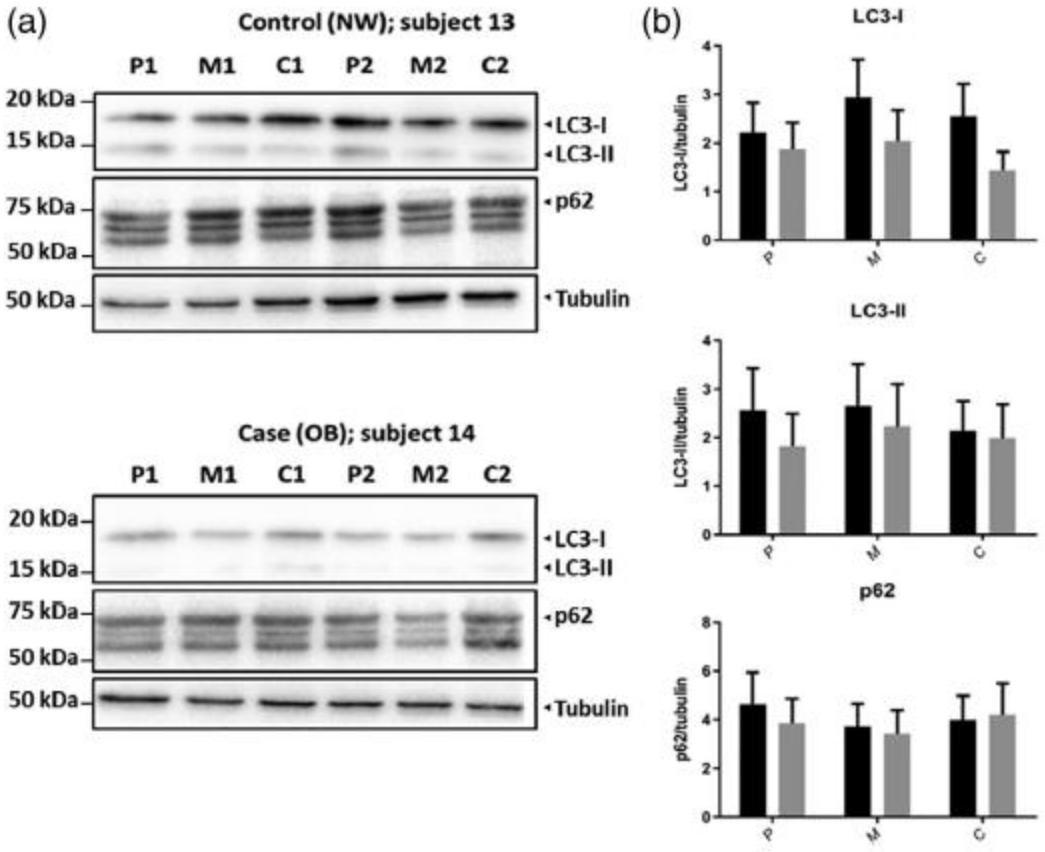

Figure 3 Western blot analysis of autophagic markers in three regions of the placenta from obese and normal weight patients. (a) Representative western blots illustrating LC3-I, LC3-II, and p62 expression in the peripheral (P), middle (M), and central (C) regions the placenta in obese and control. Numbers indicate lysate replicate. (b) Quantification of LC3-I, LC3-II, and p62 expression (mean ± SD) in the three regions of the placenta (P, M, and C) from obese and control patients (two-way ANOVA).

There were significantly decreased IHC scores for LC3B in the placentas of male fetuses/neonates compared to females within the obese patient group (IHC score 3.6 ±1.4 vs. 5.4± 1.1, $p<0.0001$; Figure 4). There were no differences in IHC scores between sexes in the

normal weight control group. However, we did not observe any sex-specific difference by western blot analysis of corresponding protein lysates (data not shown).

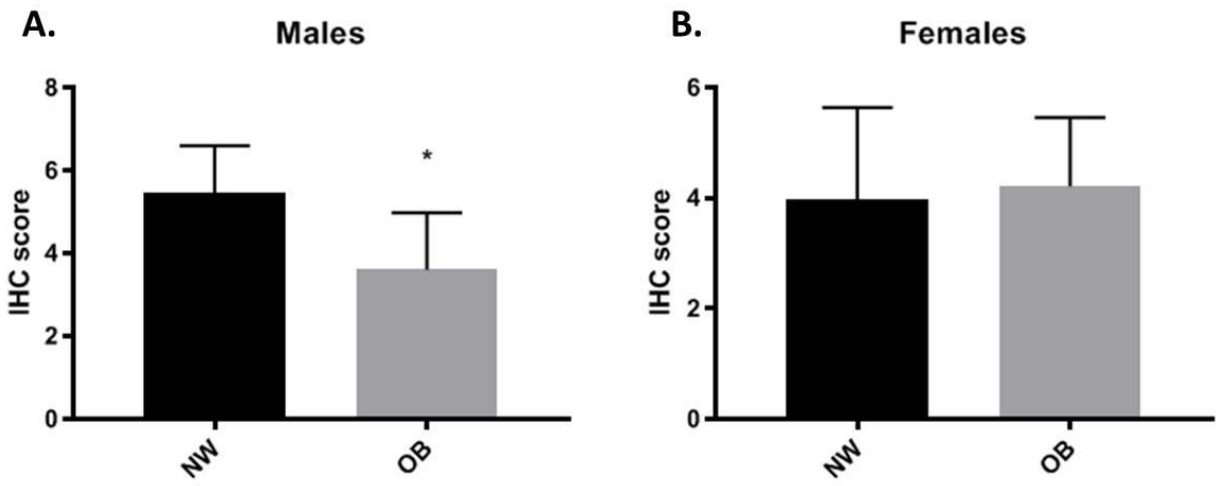

Figure 4 Immunohistochemistry (IHC) analysis of LCB3 in placentas from male and female fetuses in obese and normal weight patients. IHC scores for LC3B in placentas of (a) male and (b) female fetuses of obese and normal weight patients. Data are presented as mean ± SD (Mann–Whitney U test; *P < 0.05).

In both western blot analysis and IHC there were no statistically significant difference in autophagy (as determined by LC3) between peripheral, middle and central samples from the placenta in obese and control specimens.

3.3 Autophagy in the omentum

IHC confirmed the presence of both LC3B and p62 in adipocytes, milky spots and vasculature within the omental specimens (Figure 5). Levels of p62 were significantly lower in adipocytes compared to milky spots and vasculature within obese omenta ($F(2, 42) = 4.851$,

p=0.0127). Levels of LC3B were significantly lower in adipocytes compared to milky spots and vasculature within obese (F (2, 42) = 6.700, p=0.003) and control omenta (F (2, 39) = 5.638, p=0.0071).

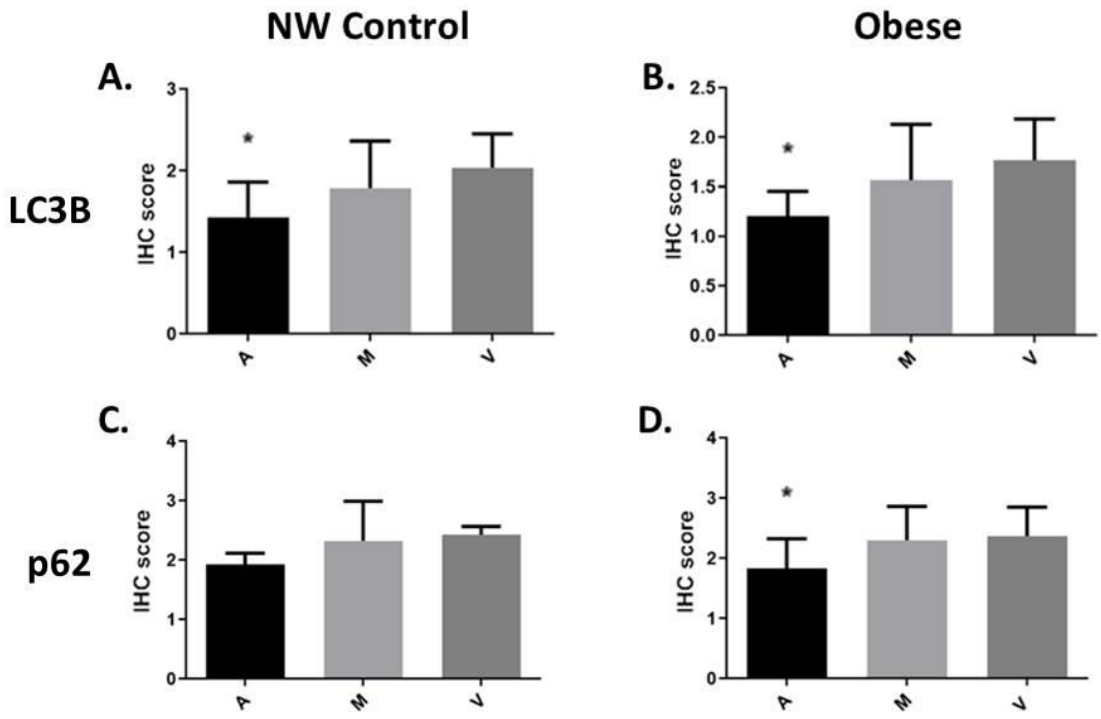

Figure 5 Immunohistochemistry (IHC) analysis of autophagic markers in three cell types within the omentum from obese and normal weight patients. IHC scores of (a, b) LC3B and (c, d) p62 in adipocytes (A), milky spots (M), and vasculature (V) from (a, c) normal weight control and (b, d) obese omenta. Data are presented as mean ± SD (unpaired t-test, two-tailed; *P < 0.05).

Quantitative scoring of IHC staining revealed no significant differences in expression of autophagic markers between omenta of obese and control patients, even when the scores from

each tissue type were combined to generate a total score within each omental specimen (Figure 6).

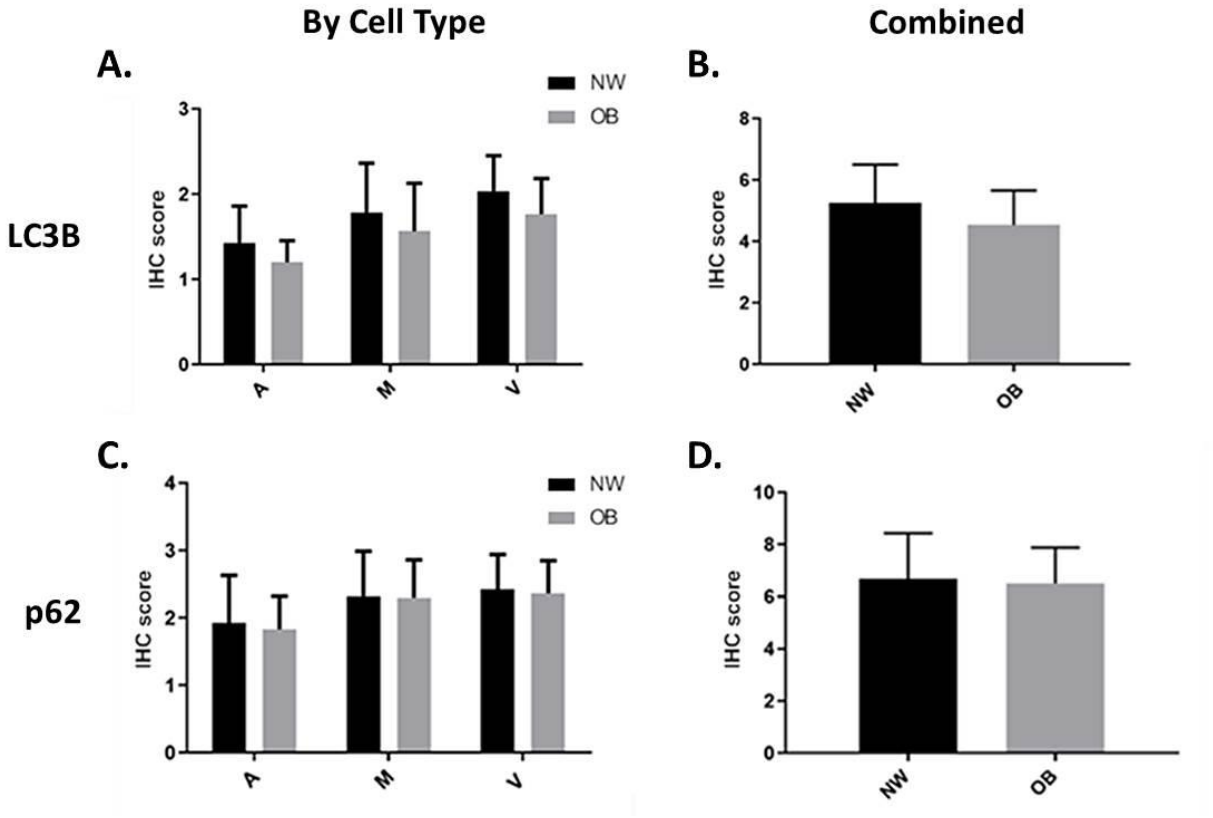

Figure 6 Immunohistochemistry (IHC) analysis of autophagic markers in the omentum from obese and normal weight patients. (a, c) IHC scores of (a) LC3B and (c) p62 in obese and normal weight control samples in omental adipocytes (A), milky spots (M), and vasculature (V). (b, d) Combined IHC scores (A + M + V) for (d) LC3B and (d) in normal weight control and obese omental samples. Data are presented as mean ± SD (unpaired t-test, two-tailed).

4. Discussion

Previous literature has demonstrated that pathological states of pregnancy, such as gestational diabetes, preeclampsia, and IUGR are associated with the activation of placental

autophagy above baseline [15-19]. Autophagy has also been found to be dysfunctional in various tissues of obese patients outside of pregnancy, including the liver and visceral adipose tissue, and appears to play an important role in non-alcoholic fatty liver, type 2 diabetes, and the chronic inflammatory state of obesity [6, 10, 12]. The aim of the present study was to investigate placental and omental autophagy in otherwise healthy obese pregnant women. Exclusion of obese pregnant women with signs of cardiometabolic disease or placental dysfunction allowed us to better elucidate the potential impact of obesity-induced derangements on the process of autophagy in placenta.

The results of this study provide evidence that autophagy is impaired in the placentas of obese pregnant women compared to normal weight controls. Analysis of LC3 and p62 IHC scores demonstrated significantly decreased autophagy in the central region of the placenta. Toward the middle and peripheral regions, there were trends toward decreased autophagy using both IHC and western blot but these did not reach statistical significance. Our findings are consistent with previous studies that found significant decreases in autophagy in the central region of the placenta, around the umbilical cord insertion in women with Caesarean section deliveries, compared to the periphery of the placental disk [13]. This gradient of decreasing autophagic activity across the placenta may be attributed to regional variation of placental blood supply and oxygenation, thus impacting levels of cellular stress.

Findings from this study suggest a decrease in autophagic activity in the placenta of obese patients, which is in contrast to previous literature that has indicated increased autophagy in association with obesity-related diseases of pregnancy, such as preeclampsia and IUGR [15-18]. As obesity has also been associated with changes in autophagic activity in adipose tissue outside of pregnancy [6, 10, 12], we also investigated levels of LC3 and p62 in omental tissue to explore a

potential correlation between omental and placental autophagy during pregnancy. However, we demonstrated no significant change in autophagy in obese omenta. Additionally, IHC scores for LC3B and p62 were not significantly different between obese and control omenta when analyzing each tissue cell type separately (adipocytes, milky spots, vasculature) or combined. Within the omentum, LC3B expression was lower in adipocytes than in milky spots and vasculature suggesting a potential difference in the pathophysiology and mechanisms underlying autophagy and stress adaptation in adipose tissue during pregnancy.

Unlike findings from previous literature, which suggest that the physiological conditions of obesity are associated with enhanced autophagy in omental tissue [6], our study found no difference in autophagy within the omentum of obese patients. Notably, our study population had no signs of cardiometabolic health abnormalities, regardless of their BMI. It is becoming increasingly evident that BMI alone is not an accurate measure of an individual's cardiometabolic health, and thus, the obese women in our study are still within the window of metabolically healthy obesity [23], while dysfunctional hyperactivation of autophagy may be seen in individuals with pathologies indicative of poor metabolic health.

In addition to exploring the difference in autophagic activity between obese and normal weight placentas and omenta in pregnancy, we also compared autophagy differences between fetal sexes. Analysis of IHC scoring noted significantly reduced autophagic activity in placentas of male fetuses compared to that of females in the obese patient group. This finding was found to contrast a recent study, by Muralimanoharan *et al*, demonstrating enhanced autophagy in placentas of male fetuses of obese pregnancies [19]. However, their study also revealed higher levels of p62 in male placentas, suggesting probable autophagic dysfunction in placentas of male fetuses, which supports the results of our own study. These results are compatible with the

growing body of research describing the lesser ability of the male fetus to adapt to external stressors [24, 25].

It is predicted that pregnancy itself induces metabolic changes in adipose tissue, thereby altering autophagic activity and regulation [26]. Therefore, the pathophysiology underlying autophagy in adipose tissue of pregnant compared to non-pregnant obese women must also be considered, especially in obese patients with complications of pregnancy including IUGR, gestational diabetes, and preeclampsia. It is suspected that the inability to utilize autophagy as a homeostatic response to stressful stimuli may heighten the obese patient population's susceptibility to placentally-mediated diseases of pregnancy. This would explain previous findings of increased autophagy in obesity-related pregnancy diseases and why decreased autophagy may predispose obese patients to these disease states.

The results from our study are somewhat limited due to small sample sizes and a semi-quantitative immunohistochemical scoring system. However, despite the small study population, differences in autophagic activity were still observed between otherwise healthy obese and control patients. The advantages of our study are that all of the tissue samples were obtained from unlaboured women, ensuring that differences in autophagy between subjects were not due to the labour process itself. In addition, our study population included only uncomplicated pregnancies, which highlights that differences in autophagy exist even in metabolically healthy obese pregnancies without overt pregnancy complications.

The grading system for omental autophagy is not as well-defined as it is for placental autophagy. Nonetheless, potential differences in autophagic activity were still seen between obese and control omental tissue samples, though a larger sample size may be required for these differences to reach statistical significance. In the future, a more in-depth clinical pathology

assessment of placental and omental tissue will be useful to explore a possible direct connection between obesity and autophagic activity.

The results from this study demonstrate a reduction in placental autophagy, indicated by decreased measures of LC3 and p62, in obese placentas compared to normal weight controls. This reduction was also found in the placentas of male fetuses compared to their female counterparts in obese mothers. Contrary to what was anticipated, autophagic activity within the omentum did not significantly differ between obese and normal weight pregnant patients. Our findings suggest pathophysiological changes within the obese population that may explain the underlying mechanism for obesity-related placental diseases of pregnancy. Future studies of placental and adipose tissue autophagy in pregnancies with comorbidities such as gestational diabetes and hypertensive disorders of pregnancy are necessary in order to determine whether autophagy is further impaired in these situations. Furthermore, correlation with placental pathology, inflammatory markers, and metabolites will be key in furthering our understanding of the role of obesity in placental dysfunction.

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