

# Increased Death of Adipose Cells, a Path to Release Cell-Free DNA Into Systemic Circulation of Obese Women

Maricela Haghiac<sup>1</sup>, Neeta L. Vora<sup>2</sup>, Subhabrata Basu<sup>1</sup>, Kirby L. Johnson<sup>2</sup>, Lorraine Presley<sup>1</sup>, Diana W. Bianchi<sup>2</sup> and Sylvie Hauguel-de Mouzon<sup>1</sup>

Remodeling of adipose tissue is required to support the expansion of adipose mass. In obesity, an increased death of adipocytes contributes to the accelerated cellular turnover. We have shown that obesity in pregnancy is associated with metabolic and immune alterations in the adipose tissue. In this study, we characterized the mechanisms responsible for increased death of adipose cells of pregnant obese women and its functional consequences. We postulated that a higher turnover of dead cells in white adipose tissue of obese women would translate into release of cell-free DNA (cfDNA) into their systemic circulation. Increase in adipose mass of obese compared to lean women results from a lesser number of hypertrophic adipocytes and an accumulation of macrophages in the stromal vascular fraction (SVF). The adipocytes of obese displayed enhanced necrosis with a loss of perilipin staining at the plasma membrane. Apoptosis was prominent in SVF cells with an increased expression of caspase 9 and caspase 3 and a higher rate of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) positive CD68 macrophages in obese vs. lean. Whereas circulating fetal cfDNA concentrations were not changed, there was a twofold increase in circulating glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cfDNA and adipose tissue GAPDH mRNA in obese women. The maternal systemic GAPDH cfDNA was positively correlated with BMI and gestational weight gain. These data suggest that the active remodeling of adipose tissue of obese pregnant women results in an increased release of cfDNA of maternal origin into the circulation.

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## INTRODUCTION

Expansion of the adipose tissue mass involves complex integrated changes in cell structure, endocrine and immune function (1). Increasing evidence suggests that crosstalks between adipose and immune cells play a central role in maintaining adipose tissue homeostasis (2). Obesity-induced remodeling is characterized by the infiltration of macrophages and T cells within the expanded adipose tissue and subsequent recruitment of endogenous immune pathways (3). Subsequent to macrophage activation, a variety of chemokines and cytokines are released both locally and systemically creating a chronic inflammatory environment (4,5). It has been suggested that adipose tissue macrophages regulate the turnover of adipose tissue by phagocytosis and clearance of dead cell debris (6,7). Hence, the death of adipose cells may precede or even trigger the macrophage infiltration and activation (8).

The links between turnover of adipocytes, cell death, and inflammation are being extensively studied. However, it is still not clear if the clustered macrophages, described as crown like structures surrounding dead adipocytes derive from apoptotic or necrotic activation (6,9). Additionally, the clearing mechanisms of the cell debris, whether they are all phagocytosed by macrophages and/or partly exported outside the tissue, have yet to be described. Likewise, the fate of the activated adipose tissue macrophages is not known.

Recently, cell-free DNA has gained attention as a potential biomarker for monitoring of tumor and pathological tissue growth undergoing accelerated apoptosis. Although low levels of cell-free DNA (cfDNA) are detected in the plasma of healthy individuals (10), increased levels have been observed in medical conditions associated with tumor growth (11). Increased release of cfDNA may originate from necrotic and/or apoptotic cell death from rapidly growing or expanding tissues

<sup>1</sup>Department of Obstetrics and Gynecology, MetroHealth Medical Center Case Western Reserve University, Cleveland, Ohio, USA; <sup>2</sup>Mother Infant Research Institute at Tufts Medical Center, Department of Pediatrics, Floating Hospital for Children and Tufts Medical Center, Boston, Massachusetts, USA. Correspondence: Sylvie Hauguel-de Mouzon ([shdemouzon@metrohealth.org](mailto:shdemouzon@metrohealth.org))

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(12,13). Similarities between excessive growth of adipose tissue and that of tumors favors the hypothesis that cfDNA may be generated by remodeling adipose tissue of obese individuals. In support of this concept is the observation that levels of total cfDNA correlate positively with BMI in normal human pregnancy (14).

We have previously shown that adipose tissue of pregnant women with pregravid obesity displays metabolic dysfunction and inflammation associated with macrophage accumulation (5). We postulate that metabolic and immune dysfunction translates into a higher death rate of both adipocytes and macrophages. Therefore, we have investigated the apoptotic characteristics of adipose cells and quantified plasma cfDNA as a potential marker of adipose tissue turnover in pregnancy complicated by obesity.

## METHODS AND PROCEDURES

### Human subjects

The study was approved by the institutional review board of Metrohealth Medical Center, Case Western Reserve University. Written informed consent was obtained before obtaining blood and adipose tissue. Sixteen obese (pregravid BMI >30) and 14 lean (pregravid BMI <25) women with male fetuses were recruited at term (38–40 weeks) before a scheduled cesarean delivery. Women with a multiple gestation, fetal anomalies, IUGR, pre-eclampsia and placenta previa were excluded. Maternal pregravid BMI and metabolic characteristics were obtained from medical records. Maternal peripheral venous blood was collected prior to placement of an intravenous line for hydration, drawn on EDTA and plasma was kept frozen at  $-20^{\circ}\text{C}$ . At delivery neonatal male gender was confirmed and anthropometrics were measured (15; Table 1).

### Adipose cell isolation

At the time of incision, 3–5 g of abdominal subcutaneous adipose tissue was obtained and either flash frozen in liquid nitrogen or immediately processed for cell isolation. Adipocytes were isolated by digestion of adipose tissue with 1 mg/ml collagenase (Worthington Biochemical, Lakewood, NJ) in Hanks buffered solution for 60 min at  $37^{\circ}\text{C}$ . Freshly isolated adipocytes were counted and their diameter measured using a reticule for microscope Olympus BH-2 (Olympus, Center Valley, PA). Cells from the stromal vascular fraction (SVF) were pelleted by centrifugation 20 min at 1,500g. The SVF pellet was resuspended in erythrocyte lysis buffer, centrifuged, resuspended in RPMI medium and counted. One aliquot of SVF cells was immediately plated at a density of  $\sim 1.5\text{--}1.8 \times 10^6$  cells/well in 12-well culture plates (precoated with 1% gelatin) containing complete RPMI medium. The remaining of the pellet was subjected to immunoselection with CD14 coated beads and counting of both positive and negative CD14 cells.

### Histomorphometry and Immunohistochemistry

Formalin fixed paraffin embedded adipose tissue sections were deparaffinized with xylene, rehydrated in alcohol, boiled for 8 min in 1 mmol/l Tris-EDTA with 0.01% Tween and blocked in phosphate-buffered saline containing 4% goat normal serum and 2% bovine serum albumin for

120 min. For immunostaining, the sections were incubated simultaneously with mouse CD68 antibody (1:100 dilution; Abcam, Cambridge, MA) and rabbit perilipin (1:100 dilution, Abcam) overnight at  $4^{\circ}\text{C}$ . Bound antibodies were detected with an Alexa 647-coupled goat anti-mouse antibody for CD68 (1:2,500 dilution) and an Alexa 555-coupled goat anti-rabbit in case of perilipin (1:2,500 dilution) (Invitrogen, Carlsbad, CA). The tissue sections were then incubated with terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) reagents for an additional 60 min at  $37^{\circ}\text{C}$  using In Situ Cell Death Detection kit fluorescein (Roche Applied Science, Indianapolis, IN). For control, the primary antibodies were omitted and the rest of procedure remained unchanged. Sections were mounted using Vectashield hardset (Vector Laboratories, Burlingame, CA). 4,6 diamidino-2-phenylindole dihydrochloride (DAPI) was used for nucleus DNA staining. Immunostaining was visualized using a high resolution fluorescent light microscope Leica DM6000 and images were obtained with Leica Velocity software (Leica Microsystems Nederland BV, Rijswijk, the Netherlands).

### Apoptosis detection assay

Apoptotic cell death was evaluated in cultured stromal vascular cells using *In Situ* Cell Death Detection kit, fluorescein (Roche Applied Science) for TUNEL detection. Plated stromal vascular cells fixed with 4% paraformaldehyde for 60 min at room temperature. The presence of fluorescein as a cell death marker was visualized using a Leica DM6000 microscope and images were obtained with Leica Velocity software. The number of cells that were TUNEL-positive was calculated and averaged across five independent fields from 4–6 independent cell experiments. The percentage of apoptotic stromal vascular cells was calculated as TUNEL-positive cells/total cells  $\times 100$ .

### Lactate dehydrogenase (LDH) measurement

Isolated adipocytes ( $3\text{--}4 \times 10^6$  cells/tube) were incubated for 24 h in RPMI medium containing 1% penicillin/streptomycin and the supernatant was collected by centrifugation at 500 rpm/5 min. Stromal vascular cells were plated ( $1.5\text{--}2 \times 10^6$  cells/well) in RPMI medium containing 1% penicillin/streptomycin and the supernatant was collected after 24 h culture. Supernatants were assayed for LDH using Cytotoxicity Detection kit (Roche Applied Science) using an ELISA reader (Gen5 Epoch; Biotek, Winooski, VT). The concentration of LDH present in the supernatants was normalized to the number of cells used and by using an LDH standard curve.

### Gene expression analysis

Total RNA was isolated from intact adipose tissue and adipose stromal cells using Trizol reagent (Invitrogen). Gene expression was monitored by real-time PCR using a Roche thermal cycler (Roche Applied Science) with Lightcycler Fast-start DNA Sybr Green 1 master mix and primers from Integrated DNA Technologies (Coralville, IA). Specific primers were designed within the 3' coding region of the genes. interleukin-6 (NM\_000600) forward: 5'-taccacaggagaagattcc-3' reverse: 5'-ttttctgccagtgcctctt-3'; caspase 3 (NM\_004346) forward: 5'-ttttcagagggtgctgtg-3' reverse: 5'-cggcctcactggtatttta-3'; caspase 9 (NM\_001229) forward: 5'-ctagtttggccacacccagt-3' reverse: 5'-gcattagcgaccctaagcag-3';  $\beta$ -actin (NM\_001101) forward: 5'-ggacttcgagcaagagatgg-3' reverse: 5'-agcactgtgtgctgctacag3'; GAPDH

**Table 1** Anthropometric parameters of offspring of study subjects at birth (means  $\pm$  SD)

	Gestational age (weeks)	Placental weight (g)	Birth weight (g)	Neonatal fat mass (g)	Neonatal ponderal index
Lean (14)	38.7 $\pm$ 0.6	550 $\pm$ 128	2,948 $\pm$ 194	268 $\pm$ 54	2.58 $\pm$ 0.15
Obese (16)	38.9 $\pm$ 0.7	833 $\pm$ 212	3,885 $\pm$ 495	672 $\pm$ 156	3.14 $\pm$ 0.34
P value	0.47	0.0004	0.0001	0.0001	0.0001

(NM\_002046) forward:5'-gagtcaacggatttggtcgt; reverse: 3'- ttgattttggaggatctcg; L19 (NM\_014763) forward: 5'-agacccagtgagaccaatg, reverse: 3'-gctgtacccttctgctacc). Quantitation of relative gene expression normalized for  $\beta$ -actin was performed by comparative  $C_T$  method and expressed as fold difference between groups.

### cfDNA analysis

DNA was extracted from 400  $\mu$ l of plasma using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) and eluted in 50  $\mu$ l of buffer according to the manufacturer's blood and body fluid protocol. Real-time quantitative PCR amplification was performed as previously described (16,17) to amplify glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and the Y-chromosome sequence *DYS14* as markers of total and fetal DNA, respectively. Analysis was blinded, and a female investigator processed all samples so that there was no risk of contaminating samples with male DNA. The maternal plasma volume from both obese and lean subjects was adjusted for blood volume as previously described (18), as prior experiments in our laboratory suggested an increased blood volume as a function of weight (17).

### Statistical analysis

Analysis were performed on blood and adipose tissue of 16 obese and 14 lean women from a metabolically characterized cohort (5). cfDNA measurements were performed in blood samples of 16 obese and 14 lean women. The number of samples analyzed varied among adipose tissue experiments depending on the total amount of tissue obtained at the biopsy site. Morphology studies were done on biopsies from 15 obese and 10 lean women. SVF cells used in molecular and cellular studies were from 15 obese and 10 lean women with half the samples analyzed for gene expression and the other half analyzed for TUNEL. Differences between dependent variables were examined with one-way or two-way repeated measures ANOVA. Statistically significant mean differences were identified with a Fisher's PLSD *post-hoc* test. Data were analyzed using the Statview II statistical package (Abacus Concepts, Berkeley, CA). Significance was set at  $P < 0.05$ .

## RESULTS

The increased body weight gain in obese pregnant women was associated with hyperleptinemia, hyperinsulinemia, and increased plasma interleukin-6 concentrations, characteristic features of metabolic inflammation (Table 2). The larger adipocyte size (Figure 1a–c) reflected hypertrophy of adipose tissue in obese compared to lean women. Adipose tissue hypertrophy was associated with a lower number of adipocytes but no difference in the total number of cells in the SVF (Figure 1e). However, the SVF of obese women was enriched in CD14 positive cells, staining activated macrophages (Figure 1f). The morphological changes were associated with an increased number of necrotic and apoptotic cells in the adipose tissue of obese compared to lean. Enhanced necrosis was documented

by decreased perilipin staining of adipocytes, a sign of lesser cell membrane integrity (Figure 2) as well as a two- to three-fold higher release of LDH by adipocytes and stromal cells (Figure 3). Increased apoptosis was indicated by a fivefold increased in TUNEL immunoreactivity in nuclei surrounding the adipocytes of obese vs. lean (Figure 2). TUNEL staining was also increased in SVF cells (Figure 4a–e). Costaining of TUNEL with CD68 cells indicated that stromal macrophages represented ~60% of the apoptotic cells (Figure 4f–h). The higher expression of the caspase 3 and caspase 9 genes (four- to sixfold in obese vs. lean) confirmed increased apoptosis in the SVF (Figure 5a). In contrast with the SVF cells, neither caspase 3, caspase 9 nor interleukin-6 expression were modified in the adipocytes of obese (Figure 5b) indicating that apoptosis and inflammation was a primary feature of SVF.

Cell necrosis and apoptosis have been identified as main processes to release free circulating DNA (cfDNA) in plasma. We next quantified cfDNA in plasma of obese vs. lean pregnant women using the sequence for the ubiquitous enzyme *GAPDH*. *DYS14*, a Y-chromosome specific sequence, was used to discriminate between maternal and fetal-placental DNA, since all fetuses were male (14,19). Circulating *GAPDH* cfDNA levels were increased by 1.9-fold ( $P < 0.007$ ) in obese vs. lean women, whereas there was no difference in levels of *DYS14* cfDNA (Figure 6a, upper panel). *GAPDH* gene expression was increased 1.9-fold in obese vs. lean adipose tissue (Figure 6b, upper panel). There was a positive correlation of *GAPDH* cfDNA with BMI and gestational weight gain, indicating an association between increased fat mass of obese women and total cfDNA released into the maternal circulation (Figure 6, lower panel).

## DISCUSSION

Adipose tissue mass expansion relies on increasing the amount of stored triglycerides in preexisting adipocytes resulting in cell enlargement (hypertrophy), on generating new small adipocytes (hyperplasia), or on a combination of both mechanisms (20). The primary findings of this study are that circulating levels of cfDNA of maternal origin are increased in obese compared to lean pregnant women. Additionally, adipose tissue of obese undergoes active necrosis and apoptosis compared to lean pregnant women. In addition to the hypertrophy of mature adipocytes, obesity could also have triggered hyperplasia of a preadipocyte subset which has a high turnover rate in obesity (21). This aspect has not been investigated in the present study.

**Table 2** Metabolic characteristics of study subjects (means  $\pm$  SD)

	Age (years)	Pregavid BMI (kg/m <sup>2</sup> )	BMI at delivery (kg/m <sup>2</sup> )	Gestational weight gain (kg)	Leptin (ng/ml)	IL-6 (ng/ml)	Insulin ( $\mu$ U/ml)	Insulin resistance HOMA index	Smoking status, yes/no
Lean (14)	29.5 $\pm$ 7.1	21.8 $\pm$ 2.8	27.5 $\pm$ 3.0	14.7 $\pm$ 4.4	30 $\pm$ 16	2.5 $\pm$ 1.5	11.6 $\pm$ 3	6.2 $\pm$ 4.2	3/11
Obese (16)	28.8 $\pm$ 5.5	39.2 $\pm$ 6.7	45.7 $\pm$ 7.3	17.1 $\pm$ 6.2	67 $\pm$ 23	4.5 $\pm$ 3.0	31.5 $\pm$ 2	2.3 $\pm$ 1.1	2/14
P value	0.76	0.0001	0.0001	0.05	0.0001	0.002	0.0001	0.004	0.51

HOMA, homeostatic model assessment.

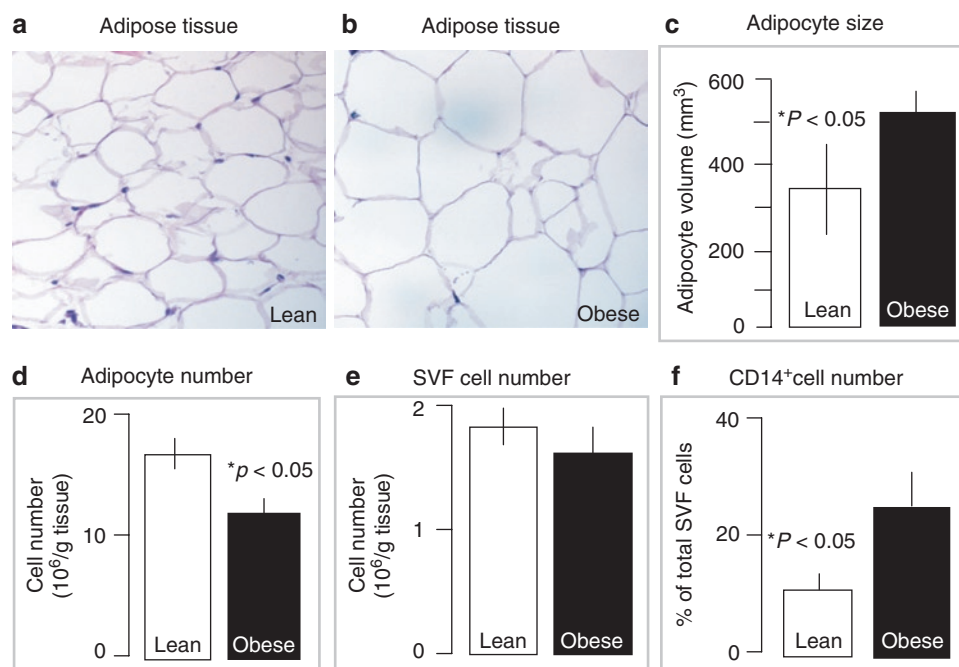


Whereas the total number of SVF cells remained constant, an enrichment in macrophages was observed. The morphometric changes in adipose tissue of obese vs. lean were coupled with increased interleukin-6 gene expression in SVF, systemic inflammation and lowered insulin sensitivity (Table 1), two classic traits of metabolic dysfunction in obesity (5,22,23).

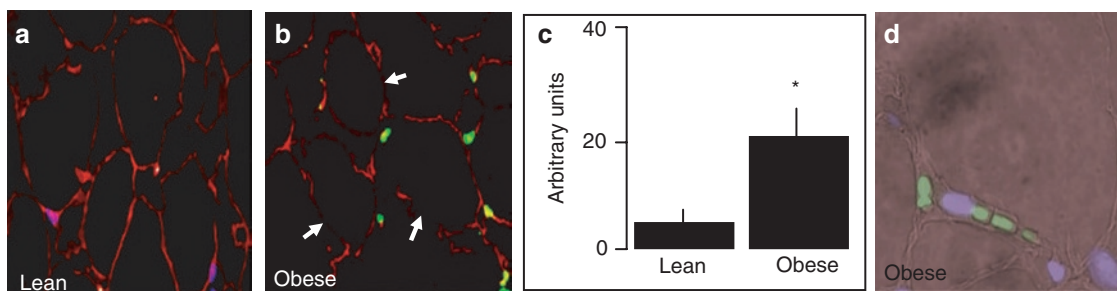
Contrary to the former dogma that adipose tissue is a quiescent depot, it is now well established that there is a permanent cell turnover in white adipose tissue of adult individuals (24,25). The enhanced adipocyte death we have observed in obese vs. lean indicates an active remodeling of adipose tissue in pregnancy. Necrosis was significantly enhanced in adipocytes and stromal cells of lean vs. obese pregnant women.

These observations are consistent with enhanced adipocyte death documented in obese rodents and humans (6). Whereas necrosis mostly occurred in the adipocytes, apoptosis was primarily observed in the cells of the SVF with 60% of the apoptotic cells being macrophages. Additionally, the remaining apoptotic cells could include vascular cells, a stromal cell type actively remodeling to support adipose angiogenesis (26,27). A proangiogenic role of adipose tissue macrophages has been characterized in mice (28). It is thus conceivable that crosstalks between macrophages and endothelial cells favor angiogenesis and tissue expansion.

Scavenging of debris and dead cells is a primary function of resident macrophages that is mandatory for cellular

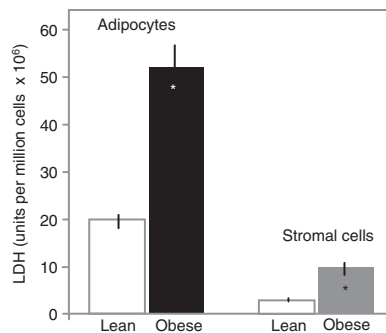


**Figure 1** Morphological changes in adipose tissue in pregnancy with obesity. Histochemical analysis of subcutaneous adipose tissue sections from a representative lean woman (pregravid BMI: 22.4) and one obese woman (pregravid BMI: 35.9). (a, b) The average adipocyte diameter was  $84 \pm 2 \mu\text{m}$  in the lean and  $95 \pm 3 \mu\text{m}$  in the obese women. Magnification  $\times 40$ . Quantification of (c) adipocyte size, (d) number of cells, and (e) stromal vascular fraction are mean  $\pm$  SEM of  $n = 10$  adipose tissue sections from lean (open bars) and 15 obese (solid bars) women. The number of macrophages was estimated as the % of CD14<sup>+</sup> cells isolated from the fraction of stromal vascular cells. Significance: \* $P < 0.05$  in obese vs. lean.



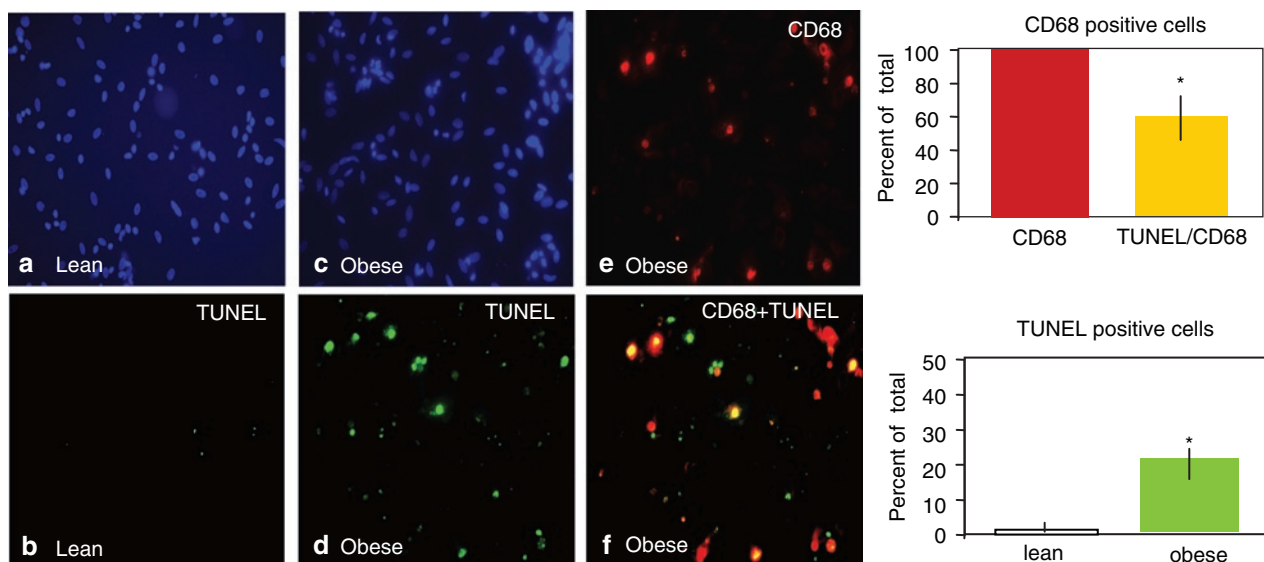
**Figure 2** Histochemical analysis of apoptosis of adipose tissue of obese pregnant women. Adipose tissue sections of (a) lean and (b) obese women were immunostained for characterizing apoptotic nuclei with dUTP-biotin nick end-labeling (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL)) and the adipocyte cell membrane marker perilipin. Perilipin positive cells are stained in red and apoptotic nuclei in green. White arrows indicate sites of ruptured plasma membrane with loss of perilipin staining. (c) Quantification of TUNEL-positive cells from seven lean and seven obese. (d) Higher magnification phase contrast of obese section (b) showing green apoptotic nuclei surrounding an adipocyte. (a, b); original magnification  $\times 40$ . (d) original magnification  $\times 100$ . Significance: \* $P < 0.05$  in obese vs. lean.

maintenance purposes (29). It has been suggested that apoptosis modifies the phenotype of macrophages and helps regulate the turnover of adipose tissue and modifies the phenotype of macrophages (30,31). In line with their housekeeping role, apoptotic macrophages may serve the need for an increased clearance of dead adipocytes from the adipose tissue of obese pregnant women. We propose that increased apoptosis of stromal macrophages would (i) participate to increase adipocyte turnover and (ii) limit excess macrophage accumulation in the SVF. Hence, the increased apoptosis of adipose tissue macrophages may serve the goals of maintaining homeostatic balance and limiting locally induced inflammation associated with obesity (5).

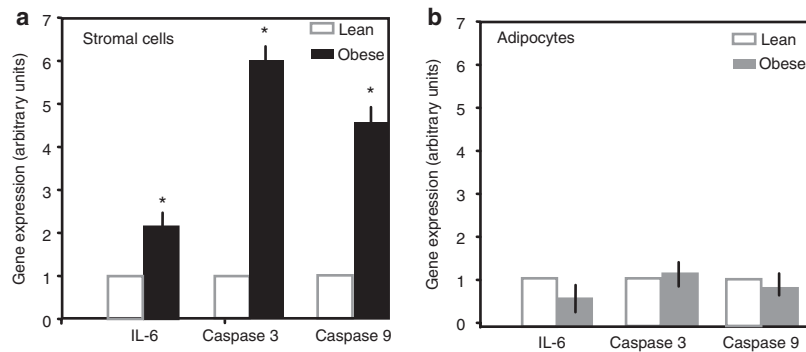


**Figure 3** Biochemical analysis of necrosis in adipose cells of obese pregnant women. Necrosis was estimated by the amount of lactate dehydrogenase released during a 24 h period in the incubation medium of adipocytes and cells of the stromal vascular fraction. Cells isolations were independently performed from adipose tissue of 10 lean and 15 obese pregnant women. Results expressed as mean  $\pm$  SD.

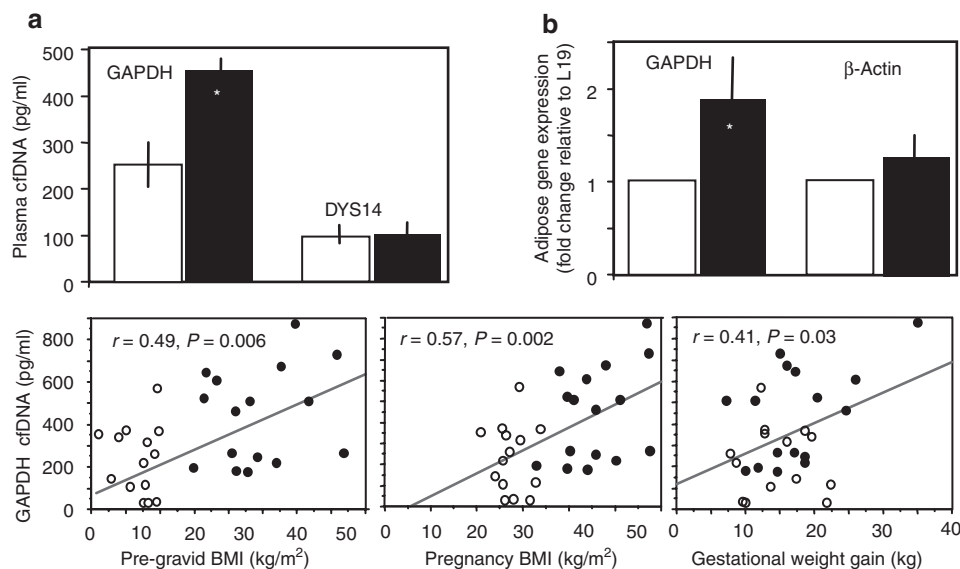
Once activated macrophages are engulfed with lipids or cell debris, they need to undergo phagocytosis and be cleared from the tissue. The DNA of apoptotic macrophages can be digested in lysosomes by DNase 2 before egress the cell (32). However, if the rate of cell death is not properly coupled with macrophage digestion and egress capacity, fragmented DNA may be released as circulating DNA (33). Several pathologies such as myocardial infarction, stroke, cancer, and pre-eclampsic pregnancy are associated with elevated cfDNA concentrations in the circulation (34–36). The physiochemical specificities of circulating DNA have led to the suggestion that it originates from internucleosomal cleavage of DNA occurring during the apoptotic process (37). Our study demonstrates that the concentration of total cfDNA but not placental-fetal cfDNA was increased in obese women. Although the placenta represents an important source of nucleic acids in the form of cfDNA (38). Our data indicate that circulating cfDNA of obese women originates from maternal rather than feto-placental tissues. The increased tissue mass and *GAPDH* gene expression points to adipose tissue as a contributor of circulating cfDNA. The positive correlations between *GAPDH* cfDNA, pregestational, and late gestation BMI further suggest that obesity is a trigger of adipose cfDNA release. Although it remains to be fully established, adipose released cfDNA would be consistent with the enlargement of maternal adipose mass representing 30–40% of total maternal gestational weight gain (39). An obesity-induced release of cfDNA does not preclude that pregnancy-specific changes may also generate cfDNA as suggested in nonpregnant obese women (14,17).



**Figure 4** Histochemical analysis of apoptosis in adipose stromal vascular cells of obese and lean pregnant women. Isolated stromal vascular cells were stained with dUTP-biotin nick end-labeling (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL)) to detect apoptotic nuclei and with the macrophage marker CD68. Positive cells for CD68 were stained in red and nuclei of apoptotic cells were stained in green. Overlay of CD68 and TUNEL staining gives a yellow signal. Dapi was used for counterstaining of nuclei, shown in blue. (a, b) Cells from a representative lean subject (BMI: 21.2). (c–e) cells from a representative obese subject (BMI: 33.5). Quantification of TUNEL-positive cells in lean vs. obese subjects. Quantification of CD68-TUNEL-positive cells expressed as percent of TUNEL-positive cells in obese subjects. Data are from seven independent cell cultures. Seven fields were counted in each cell sample. Original magnification  $\times 40$ .



**Figure 5** Caspase gene expression in adipocytes and stromal vascular cells. Total RNA was purified from freshly isolated cells of (a) stromal vascular fraction and (b) adipocytes from seven obese and seven lean women. Caspase 3, caspase 9, and interleukin (IL)-6 mRNA levels were measured by quantitative reverse transcription (RT)-PCR analysis. Real-time Ct values were normalized to  $\beta$ -actin. Statistical significance: \* $P < 0.05$ ; lean: open bars, obese: solid bars.



**Figure 6** Cell-free DNA concentration in plasma of pregnant women. (a) Upper panel: GAPDH was used as a marker of total cfDNA (maternal and placental-fetal origin) and DYS14 as a marker of placental-fetal cfDNA. (b) GAPDH gene expression in adipose tissue was measured by real-time RT-PCR. Black bars represent data from obese women and white bars from lean women. Lower panel: Total cell-free DNA is expressed as copies of glyceraldehyde-3-phosphate (GAPDH) in pg/ml plasma. Values are mean  $\pm$  SEM after correction for blood volume as detailed in Methods and Procedures section. Filled circles represent data from obese women ( $n = 16$ ) and open circles from lean women ( $n = 14$ ).

Nuclear macromolecules such as DNA and dsRNA play an active role in regulating inflammatory mechanisms through recruitment of toll-like innate immune pathways (3) in normal and tumor growth (40). We have previously documented that immune activation of adipose stromal cells contributes to systemic changes and metabolic inflammation in obese pregnant women (5). Taken together, these observations suggest that release of cfDNA into the systemic circulation of obese women may represent additional inflammatory signals associated with metabolic dysfunction.

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#### DISCLOSURE

The authors declared no conflict of interest.  
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