Published in final edited form as:

Obesity (Silver Spring). 2019 January ; 27(1): 87–93. doi:10.1002/oby.22354.

Oxidized HDL, Adipokines and Endothelial Dysfunction: A Potential Biomarker Profile for Cardiovascular Risk in Women with Obesity

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Abstract

Objective: High BMI predicts adverse cardiovascular outcomes and positively correlates with increased levels of adipokines. The relationship among BMI, IL6, TNF α , adiponectin and oxidized high density lipoprotein (Ox-HDL) with circulating endothelial cells (CECs) and endothelial progenitor cells (EPCs) has not been well studied. We have described elevated CEC levels in both humans and mice with obesity and diabetes. We have shown that Ox-HDL is a potent driver of adipogenesis *in vivo* and *in vitro*. In this study, we examined elevated BMI in two groups of women studied in Brooklyn, NY and Huntington, WV, respectively.

Methods: 26 females with obesity and 5 lean controls without overt cardiovascular disease were enrolled, 13 from West Virginia and 13 from Brooklyn, New York. Cytokine levels, EPC and CEC were determined.

Results: females with obesity had elevated levels of Leptin, IL6, and Ox HDL, increased CEC and decreased EPC and adiponectin levels (all $p < 0.01$). The Ox- HDL levels were higher in women from Brooklyn vs Huntington ($p < 0.01$), possibly from higher TNF α levels in Brooklyn or higher adiponectin levels in WV. 75% of the variance in Ox-HDL levels could be predicted in this population ($p < 0.01$).

Conclusions: Our study reveals a unique inflammatory biomarker profile in females with obesity.

Keywords

Female morbid obesity; endothelial cell dysfunction; CEC; TNF α ; Ox-HDL; leptin

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Conflict of Interest

All authors agree that there are no disclosures.

INTRODUCTION

Epidemiological studies demonstrate that obesity is a serious medical problem which strongly associates with chronic diseases such as the metabolic syndrome, diabetes mellitus, hypertension, dyslipidemia and cardiovascular disease [1–3]. Over the past 20 years, The United States has experienced a continuous increase in the prevalence of obesity, paralleled by a growing incidence of type II diabetes (T2D) [1–3]. Approximately 35% of the adult population in the United States is classified as obese (body mass index, BMI, >30). Appalachian areas of the United States are some of the worst affected regions. Thirty-four percent of West Virginians were obese in 2012, making it among the states most affected by obesity in the nation [3]. Studies have shown a greater rate of mortality due to cardiovascular disease in women than in men, thus making Appalachian women a unique population of study [4,5]. Brooklyn is the most populous borough in New York City and one of the most diverse in cultural identity. Forty percent of people living in Brooklyn were born in other countries. Metabolic derangements related to obesity, decrease of adiponectin and its associated complications have been attributed to an increase in oxidative stress [6–8]. Scherer has shown that oxidative stress causes endothelial cell dysfunction and death, a key early event in the development of cardiovascular disease.

Endothelial progenitor cells (EPCs) are bone marrow-derived cells that migrate and differentiate into mature endothelial cells, playing a significant role during re-endothelialization and neovascularization of injured endothelium. Endothelial repair and regeneration, key to restoration of vascular function, depends to a great extent on the availability of circulating EPCs. Reduced number of circulating endothelial progenitor cells predicts future cardiovascular events and hypertension [9,10]. Notably, functional properties and absolute numbers of circulating EPCs are severely impaired in patients suffering from cardiovascular disease, resulting in decreased endothelium repair capacity [11,12].

An altered imbalance between apoptosis and regeneration of endothelial cells is indeed a major mechanism in the development and progression of vascular disease. 20-HETE is considered a novel regulator of EPC function associated with angiogenesis *in vitro*, suggesting that an increase in 20-HETE may play a role in EPC function [13].

Reactive oxygen species in general, and Ox-HDL in particular, may play a role in vascular dysfunction and adiposity. Obesity-derived oxidative stress is considered a major risk factor in the development of endothelial dysfunction and vascular disease, which is accompanied by a concomitant increase in the vasoconstrictor 20-HETE. Indeed, there is strong evidence supporting an association between obesity and augmented circulating levels of 20-HETE [14,15]. It has been reported that 20-HETE blocks insulin-dependent phosphorylation of endothelial nitric oxide synthetase (eNOS) and nitric oxide (NO) production [16] and that 20-HETE-derived COX-2 dependent 20-OH-PGE₂ can increase adiposity, adipocyte enlargement, and the release of inflammatory cytokines [17]. In addition, 20-HETE and EPC dysfunction are augmented in patients with hypertension and cardiac events [18]. Hypertension is a contributor to 20-HETE-driven diabetes mediated kidney injury [19,20]. Abnormalities in 20-HETE production resulted in impairments in renal function and in the development of hypertension in mice and humans [21–24]. 20-HETE has been found to

induce angiotensin-converting enzyme and Ang II, an effect that was abolished by the 20-HETE antagonist 20-hydroxyeicosa-6(Z), 15(Z)-dienoic acid (20 HEDE)(REF HERE). Moreover, 20-HETE-dependent vascular dysfunction and hypertension are associated with upregulation of the renin-angiotensin system and Ang II [21,22] More recently it has been shown that the 20-HETE antagonist 20-SOLA and its active compound 20-HEDE [25] reverse the hypertensive effects of 20-HETE presumably by blocking the binding of 20-HETE to its receptor [25]. In addition, Ox HDL is a potent inducer of adipogenesis resulting in activation of Ang II and 20-HETE in obese females [26]. In addition, Ox-HDL was a more potent adipogenic than either Ang II or 20-HETE [27]. Thus, we speculate that an increase in plasma Ox-HDL and Ang II, may represent a novel marker profile of vascular dysfunction in obesity. Furthermore, we speculate that Ox-HDL would correlate with elevation of circulating endothelial cells as evidence of early endothelial cell dysfunction [28].

High-Density Lipoprotein-Cholesterol (HDL-C) levels are typical markers for cardiovascular disease (CVD) in general and coronary artery disease (CAD) in particular. Dysfunctional HDL results mostly from oxidation to Ox-HDL. Furthermore, generation of lipid peroxidation, i.e. F2-isoprostane from arachidonic acid, may propagate free radical damage via the covalent modification of biomolecules. Both Ox-HDL and isoprostane are potent drivers of adipogenesis [26]. Thus, it is of clinical interest to examine levels of ox-HDL and in obese women. The present studies are an extension of previous efforts aimed at establishing an inflammatory biomarker profile that can identify obese females at risk for cardiovascular disease. The identification of new biomarkers to prevent and/or attenuate disease progression is crucial in the treatment of obesity. The present studies were driven by the hypothesis that the chronic inflammatory state associated with obesity, in the absence of diabetes or hyperglycemia, would be manifested as increased levels of inflammatory adipokines, oxidized-HDL (ox-HDL) and Ang II. Since these factors are linked to endothelial cell dysfunction and injury, their increased circulating levels are expected to be positively correlated with increased number of CECs. The objective of the present study was to compare the obese populations of women in Appalachia and Brooklyn, and to determine whether correlations exist between their BMI and a number of parameters known to be linked to development of cardiovascular disease, including inflammatory cytokines, EPCs, CECs, ox-HDL, leptin and Ang II levels.

MATERIALS AND METHODS

Study Subjects:

Between June 2013 and November 2017, 26 female subjects with normal to morbidly obese BMIs and 5 lean controls from Cabell Huntington Hospital, WV and NYP Brooklyn Methodist Hospital, Brooklyn, NY, were considered for enrollment in the study. Height, weight, waist circumference and blood pressure were measured using a standardized protocol. The subjects were given a physical exams, BMI was calculated by dividing weight (Kg) by the square of the subjects' height (m) and waist circumference was measured midway between the lowest rib and the iliac to the nearest 0.1cm. Subjects (19) with overt cardiovascular disease were excluded as they failed to meet the enrollment criteria. A total

of 31 subjects (5 lean controls and 26 women with obesity) with no cardiovascular disease met the criteria and were enrolled. They were divided into non-obese (5 patients with BMI<30) and 26 obese patients with BMI>30. The study was approved by the Ethics Committee of the Cabell Huntington Hospital, WV and the Institutional Review Board of NYP Brooklyn Methodist Hospital, NY. All subjects signed a written informed consent.

Blood Samples and Cytokine Measurements:

After overnight fasting, venous blood was drawn from an antecubital vein to measure serum levels of inflammatory cytokines, Ox-HDL, Adiponectin, Leptin and EPC testing (blood was drawn in heparinized tubes). Serum samples were frozen at -80°C before analysis. TNF α , IL-6, adiponectin and Ox-HDL levels were determined using ELISA.

Isolation of Circulating Endothelial Cells:

A 10 ml sample of peripheral blood was obtained and used for CEC and EPC experiments. One ml of blood was incubated with 100 μl of anti-CD146 coated 45 μm Dyna-beads (1.4×10^8 beads/ml) overnight at 4°C in a Dynal mixer (Dynal, Lake Success, New York) at 50 rpm. Cells bound to anti-CD146 coupled beads were separated from blood in a Dynal magnet, washed (3 washings using phosphate buffered saline and 0.1% bovine serum albumin and repetitive mixing for 5 minutes in the Dynal mixer at 4°C), and dissolved in 100 μl buffer. Side by side assays were performed with Dynabeads coated with human antibodies against mouse IgG but without an anti-endothelial antibody to check for nonspecific binding to the Dynabeads. The cells were mixed with acridine and visualized by light and fluorescence microscopy.

Isolation of mononuclear cells and EPC colony formation:

Peripheral mononuclear cells (PMNCs) were fractionated using Ficoll density-gradient centrifugation. Isolated PMNCs were re-suspended in CFU-Hill Medium (Stem Cell Technologies, Vancouver, Canada) and plated on 6-well plates, coated with human fibronectin at a concentration of 5×10^6 cells per well. After 48 hours, the non-adherent cells were collected and replated onto fibronectin-coated 24-well plates. EPC colonies were counted using an inverted microscope 7 days after plating. An EPC colony was defined as a cluster of at least 100 flat cells surrounding a cluster of rounded cells, as previously described [11] . A central cluster alone without associated emerging cells was not counted as a colony. Results are expressed as the mean number of colony-forming units (CFUs) per well.

Statistical Analysis:

All statistical analysis was performed with the open source program R. Analysis of variance was performed and differences were further investigated with an unpaired t-test using the Holm-Sidak correction for multiple comparisons. Correlations between cytokine values were studied using the Pearson coefficient and displayed as heatmaps. Network analysis of these correlations was performed using the R package gplots. Graphical presentation of boxplots with superimposed scatterplots was performed using the R package ggplot2. Three dimensional surface and scatter plots were produced with the R package plot3D. Note that in

some figures, there was superposition of data points; in other words, if one counts “circles”, some graphs have less than the total number of subjects. This was a consequence of using symbol size that allowed for easy readability. That said, all subjects had data obtained for all measurements and all data were included in the statistical analysis. Significance was reported at the $p<0.05$ and $p<0.01$ levels.

RESULTS

The effect of obesity was profound on most of the measurements studied. First and foremost, both the Brooklyn and Huntington obese subjects had significantly higher BMI values than those seen in the lean controls as expected ($p<0.01$, figure 1a), using gg plot 2 package of R. Although there was a trend toward a higher BMI in patients from Brooklyn compared with Huntington, it was not statistically significant. Subjects with obesity also higher levels of leptin ($p<0.01$, figure 1b) and IL-6 ($p<0.05$, figure 1c), lower levels of adiponectin ($p<0.01$, figure 1e), and higher levels of oxidized HDL ($p<0.01$, figure 1f). Although there was a trend toward higher levels of TNF α , this did not achieve statistical significance (figure 1d). Subjects with obesity from Huntington had higher levels of oxidized HDL than obese subjects from Brooklyn ($p<0.01$, figure 1f).

Predicting oxidized HDL levels, BMI gave the best prediction of any other variable with an R^2 values of 68%. Performing stepwise multiple regression, adding TNF α improved the prediction to $R^2=76\%$. These data are summarized in figures 2a and 2b, the two dimensional plot made using the gg plot 2 package. The 3 D plot was made with the plot 3D package of R.

Examining the EPC and CEC values, both were increased in subjects with obesity as shown in figures 3a and 3b. Again, BMI was the best single predictor (figure 3c) with an R^2 value of 53%, but the addition of other variables did not improve upon the prediction made by BMI alone.

Examining the overall correlations among variables, we found that significant positive associations existed amongst BMI, IL6 and oxidized HDL levels with a significant association between EPC and IL-6. Although there were trends between other measurements, they did not achieve statistical significance (figure 4a), this plot being made with the correlation function from the Psycho package in R. (panel A) As we examined possible networks amongst the different measurements, we see that there is a network involving BMI, IL6 and oxidized HDL with several other groupings defined by positive or negative associations (figure 4b). This plot was performed with the cor function and the heatmap.2 function from the gplots package.

DISCUSSION

Three major observations were made in the course of the present studies. First, female subjects with morbid obesity exhibited a high inflammatory state, as indicated by increased levels of TNF α , IL-6, leptin, Ox-HDL and CEC's when compared with a group without obesity. As expected, functional EPC's decreased in women with obesity compared to subjects without obesity. Although one would expect that the high levels of Ox-HDL and

CECs might predict CV disease, the present study was restricted to subjects that specifically did NOT demonstrate overt CV disease.

It is well established that TNF α exerts a strong stimulatory effect on ROS generation, and that the crosstalk between TNF α and ROS is especially important in setting the inflammatory state associated with chronic obesity [27,29–31]. Our findings provide evidence of chronic oxidative stress in women with BMI>30, as manifested by increased circulating levels of ox-HDL [28,32,33], a potent driver of adipogenesis and oxidative stress [26]. As seen in Figure 4C, Ox-HDL is responsible for the cascade of events that results in endothelial cell dysfunction. In addition, increased TNF α and decreased adiponectin increases insulin resistance, mitochondrial dysfunction and consequent cardiovascular disease. Figure 4 C shows the additive and possibly synergistic effect of these variables on endothelial cell dysfunction. The fact that these findings were accompanied by increases in inflammatory cytokines and, concomitantly, by markedly reduced adiponectin levels, which increases insulin resistance, ROS and inflammatory cytokines, suggests that this might represent an inflammatory biomarker profile in women with obesity at increased risk of cardiovascular disease. Indeed, the high levels of CEC's are indicative of early vascular dysfunction. Larger cohorts of both female and male patients need to be studied to determine whether Ox-HDL and the Ox-HDL/HDL ratio can more readily identify a majority of patients at risk for CAD.

An interrelation between the heme-oxygenase system and circulating levels of ox-HDL has been previously established. In both mice and humans with diabetes, HO-1 deficiency can lead to increased levels of ox-HDL [11,12,34–36]. In obese mice the HDL mimetic L4F induces expression of HO-1 [37] and reduces adiposity while increasing adiponectin levels and improving insulin sensitivity [38]. In addition, upregulating HO-1, regardless of the mechanism, has been shown to be effective in reducing adiposity and improving vascular function [34,39–43]. Whereas data is not available at this time, it would be of interest to gather insight on the status of the HO system in the cohorts of women with obesity analyzed in this study.

More importantly, we observed a significant positive association between the increase in Ox-HDL and Ang II levels in morbidly obese subjects. In addition, in this group of patients the levels of CEC's were increased, suggesting the presence of early endothelial stem cell dysfunction. We also showed that the number of EPC colonies was reduced in these patients with obesity. A possible explanation for this is that an uncoupled state of eNOS exists, likely due to secondary superoxide formation due to high oxidative stress [44]. Fogelman's group has shown that HDL, in the absence of inflammation, maintains the anti-inflammatory state through a complement of antioxidant enzymes. In the presence of inflammation these enzymes may be inactivated and create an inflammatory HDL [45]. This impaired pro inflammatory HDL has been described in other inflammatory states, but to our knowledge, this has not been described in the chronic inflammatory state of obesity in the absence of diabetes. In addition, we have correlated the pro inflammatory HDL levels with increased circulating endothelial cells and decreased endothelial progenitor cells, suggesting cause and effect.

In summary, the present study shows a unique and novel inflammatory biomarker profile in females with morbid obesity that includes increased TNF α , IL-6, ROS and Ox-HDL, increased circulating CEC's and EPC dysfunction long before any manifestations of coronary disease occur. While some subtle differences existed between subjects from Brooklyn and Huntington, the general trends were observed across these different populations. We expected more profound differences between the more homogeneous population of West Virginia and the more heterogeneous population of Brooklyn, but this was not the case. Geography itself also appeared to play no role. If confirmed in a prospective study which include cardiovascular outcomes, our findings may allow advanced prediction of such cardiovascular outcomes and allow for targeted, early intervention.

Acknowledgments

This work was supported by National Institutes of Health grant, HL109015 (JIS), HL 139561 (NGA) and Marshall Health (ET, NGA, and JIS). All authors have read and agree with the manuscript as written. We thank Mrs. Jennifer Brown and Mr. Shawn DiPace for their editorial assistance in preparing the manuscript.

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What is already known about this subject? Please remember to also include this between the title page and structure abstract in your paper.

1. Circulating Endothelial cells (CEC) are associated with early endothelial cell dysfunction
2. Ox-HDL increases in obesity. This renders the HDL incapable of preventing the oxidation of LDL
3. Inflammatory adipokines increase with obesity and decreases adiponectin

What does this study add? Please remember to also include between the title page and structured abstract in your paper.

1. Increased circulating endothelial cells (CEC) in obesity, coupled with decreases in endothelial progenitor cells(EPC) suggests endothelial cell sloughing and decreased repair, ie early endothelial cell dysfunction in obese women
2. Ox-HDL and inflammatory adipokines are associated with endothelial cell dysfunction.
3. This paper correlates the Ox-HDL and the inflammatory cytokines with increased CEC and decreased EPC in 2 separate but very different populations of obesity. We propose that this could be a biomarker profile to identify obese women at risk of cardiovascular disease.

Figure 1:

(a-f) Data obtained is expressed as boxplots with superimposed data points comparing lean controls and obese subjects from Brooklyn and Huntington; these plots were made with the ggplot2 package of R. Data shown involves BMI (panel a), Leptin (panel b), IL-6 (panel c), TNFa (panel d), Adiponectin (panel e) and Oxidized HDL (Ox.HDL, panel f). * $p < 0.05$, ** $p < 0.01$ vs. control, # $p < 0.05$, ## $p < 0.01$ vs Brooklyn obese.

Figure 2.

(a-b) Linear regression analysis assessing the relationship between Ox.HDL and BMI (panel 2a) and Ox.HDL and BMI + TNFα (panel 2b). In panel 2a, a regression line with accuracy estimates is shown whereas in panel 2b, a regression plane is demonstrated. In both graphs, data points are shown which refer to lean controls (black), Brooklyn NY obese subjects (red) and Huntington WV obese subjects (green). The two dimensional plot was made with the ggplot2 package and the 3D plot was made with the plot3D package of R.

Figure 3:

(a-c) Data regarding EPC and CECs is shown. In panels a and b, the distribution of these data are shown as boxplots with superimposed data points. ** $p<0.01$ vs control and ## $p<0.01$ vs. Brooklyn. In panel c, we show a regression line between the BMI and CECs with data groups colored black, red and green as described in figure 2 above.

Figure 4.

(a-c) Panel a: correlation plot of all measurements made in this study. Size of dot and intensity of color (blue or red) indicate degree of correlation. An X through the dot implies that the correlation did not achieve statistical significance at the $p < 0.05$ level following Holm's correction for multiple comparisons. This plot was made with the correlation function from the Psycho package in R. Panel b shows a heatmap derived from the same data set and some network analysis of the correlations. This plot was performed with the cor function and the heatmap.2 function from the gplots package. Figure 4 C The scheme shows the effect of Ox-HDL on oxidative stress, increased CEC sloughing and EPC dysfunction. In addition, it shows the effects of TNF, ROS (and subsequent crosstalk) and decreased

adiponectin on insulin resistance and mitochondrial dysfunction that has an additive and deleterious effect on vascular dysfunction.

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