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Obesity

Relationships Among Obesity, Type 2 Diabetes, and Plasma Cytokines in African American Women

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Objective: The principal objective of this investigation was to identify novel cytokine associations with BMI and type 2 diabetes (T2D).
Methods: Cytokines were profiled from African American women with obesity who donated plasma to the Komen Tissue Bank. Multiplex bead arrays of analytes were used to quantify 88 cytokines and chemokines in association with clinical diagnoses of metabolic health. Regression models were generated after elimination of outliers.
Results: Among women with obesity, T2D was associated with breast adipocyte hypertrophy and with six plasma analytes, including four chemokines (chemokine [C-C motif] ligand 2, chemokine [C-C motif] ligand 16, chemokine [C-X-C motif] ligand 1, and chemokine [C-X-C motif] ligand 16) and two growth factors (interleukin 2 and epidermal growth factor). In addition, three analytes were associated with obesity independently of diabetes: interleukin 4, soluble CD40 ligand, and chemokine (C-C motif) ligand 3.
Conclusions: Profiling of inflammatory cytokines combined with measures of BMI may produce a more personalized risk assessment for obesity-associated disease in African American women.

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Introduction

As BMI increases, blood biomarkers of chronic inflammation important for cardiovascular disease, such as C-reactive protein, also increase (1); weight loss usually reduces cytokines. Inflammation has been positively associated with cardiometabolic risk in numerous studies (2). However, minimal work has surveyed comprehensively the cytokine signatures associated with obesity independent of metabolic disease, cytokine signatures associated with metabolic disease independent of obesity, or joint associations. These biomarkers could help stratify disease risks among patients with different comorbidities of obesity. Furthermore, these relationships are understudied in African American women, who experience elevated risk for cardiometabolic complications of obesity, including type 2 diabetes (T2D) and hypertension (3). African American women are expected to experience an obesity prevalence of 70% by 2020 (4). For breast cancer patients, a codiagnosis of metabolic syndrome has been associated with a worse prognosis (5). African American women have higher risks of poor-prognosis triple-negative cancer and breast cancer mortality than white women (6). We recently reported the first compelling evidence that breast cancer mortality is positively associated with T2D in African American women (7).

As the obesity epidemic worsens, the incidence of T2D- and obesity-associated cancer is expected to increase. However, obesity is poorly correlated with metabolic disease and imbalanced pro- and anti-inflammatory cytokines. Histologically distinct features called "crown-like" structures (CLS) of proinflammatory CD68 + macrophages and their product cytokines (8) have been reported in subcutaneous adipose tissue of patients with obesity, and they are associated with cardiometabolic disease. CLS in breast white adipose tissue (CLS-B) (9) have been associated with elevated inflammation (10), aromatase (11), and breast cancer risk (12). The limited number of blood-based proinflammatory cytokines tested in obesity and T2D, for example, interleukin 6 (IL-6) and C-reactive protein, has been associated with abnormal breast adipocytes (9). Cytokines such as IL-6 are functionally significant because they act on cellular

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TABLE 1 Characteristics of African American female subjects with obesity

	ND	T2D	*P*
n	11	28	-
Age, y			
Mean (SD)[a]	57.1 (7.2)	58.9 (7.7)	0.5
BMI, kg/m^2			
Mean (SD)[a]	30.4 (4.0)	36.5 (7.4)	0.002
Medication (%)[b]			
Metformin	0	12 (43)	0.048
Anti-inflammatory (oral)	5 (45)	8 (29)	0.506
Antihypertensive	4 (36)	22 (79)	0.368
Lipid lowering	2 (18)	16 (57)	0.191
Hypertension	6 (54)	21 (75)	0.776

[a]*P* value computed from *t* test.
[b]*P* value from Fisher exact test.

signal transduction pathways to drive dangerous changes in breast cancer progression, as we have recently shown (13).

Here, we hypothesized that more extensive profiling of cytokines in African American women with T2D and obesity might reveal new signatures associated with metabolic risk. We compared African American women with T2D, with and without obesity and overweight, to African American women without T2D.

Methods

Human subjects

The Boston University Medical Center Institutional Review Board approved procedures in accordance with the Declaration of Helsinki. Nonfasting plasma (as specified in Supporting Information Table S1) and histological sections were selected from volunteers drawn from the general community, who donated specimens to the Susan G. Komen for the Cure Tissue Bank (KTB) at the Simon Cancer Center (Indiana University). All subjects had a BMI >25 kg/m^2, and 75% of T2D subjects had comorbid hypertension. Subjects without diabetes but with obesity (ND) by definition did not have T2D, and 54% of subjects had well-controlled hypertension. Yes/No questionnaire-reported health history was obtained, including the histories of T2D, hypertension, hypercholesterolemia, thrombosis, osteoporosis, respiratory disease, stroke, thyroid disease, arthritis, and anxiety/depression. Subject characteristics are summarized in Table 1, and demographic, clinical, and medication information is provided for each subject in Supporting Information Table S1. Histological, biochemical, and molecular methods are described in the Supporting Information.

Cytokine profiling

Plasma from KTB donations was snap-frozen, shipped in cryogenic vials, and maintained at $-80\,^{\circ}$C until assay. Plasma underwent fewer than two freeze-thaw cycles before measurements. Cytokines and chemokines were determined using magnetic bead assays (human cytokine/chemokine panels I, II, and III and testing for insulinlike growth factors 1 and 2 conducted with 23-plex, 25-plex, and 41-plex systems [Millipore Sigma Corp., Billerica, Massachusetts]) with a miniaturization drop array and washer system (Curiox Biosystems, Inc., San Carolos, California). Quantitation was performed on a Luminex MAGPIX instrument using xPONENT 4.2 software to fit standard curves (Luminex Corp., Austin, Texas).

Adipose tissue analyses

Adipocyte area reflects metabolic function; larger adipocytes are typically insulin resistant and metabolically abnormal. However, range of metabolic health has not been previously correlated with breast adipocyte area. Individual adipocytes (≥ 100 cells per subject) were traced by ImageJ, areas were recorded for four fields of view, and histograms of cell size distribution were generated. ND subjects ($n = 11$) were compared with T2D subjects ($n = 28$). To assess CLS-B frequency, 42 samples of hematoxylin and eosin-stained breast adipose tissue from ND and T2D subjects were examined with blinding to metabolic status, and the CLS per section for each subject were enumerated. Adipocyte-associated CD68 was confirmed by mouse monoclonal antihuman CD68 (PG-M1; Dako North America, Inc., Carpinteria, California), with biotinylated goat antimouse immunoglobulin G (Dako) as secondary.

Statistical analysis

Data are shown as means $\pm$ SEM, unless otherwise indicated. Cytokine data were log-transformed, outliers (absolute values >3 SDs) were removed, and data in duplicate were analyzed using mixed effect regression models for repeated measures. Models were

TABLE 2 Cytokine associations

	Regression coefficient	Standard error	*P*
A. Cytokines associated with T2D/ND status[a]			
IL-2	−0.205	0.088	0.020[b]
EGF	−0.546	0.248	0.028
CXCL16	0.179	0.088	0.041
MCP-1/CCL2	0.311	0.156	0.046
CCL16/HCC-4	0.314	0.136	0.020
GRO-1	0.790	0.356	0.027[c]
B. Cytokines associated with BMI independently of T2D[d]			
IL-4	0.510	0.249	0.040
sCD40L	1.142	0.410	0.005
MIP-1α/CCL3	3.127	1.311	0.017[e]

[a]Regression coefficient (log-fold change comparing T2D vs. ND), standard error, and *P* value to test whether the regression coefficient is different from 0.
[b]Adjusted for anti-inflammatory use.
[c]Adjusted for antihypertensive use.
[d]Regression coefficient (log-fold change for a change of one of log [BMI]), standard error, and *P* value to test whether the regression coefficient is different from 0.
[e]Adjusted for metformin and lipid-lowering medication.
CCL16/HCC-4, C-C motif chemokine ligand 16; CXCL16, C-X-C motif chemokine ligand 16; EGF, epidermal growth factor; GRO-1/CXCL1, C-X-C motif chemokine ligand 1; IL-2, interleukin 2; IL-4, interleukin 4; MCP-1/CCL2, C-C motif chemokine ligand 1; MIP-1α/CCL3, C-C motif chemokine ligand 3; sCD40L, soluble CD40 ligand.

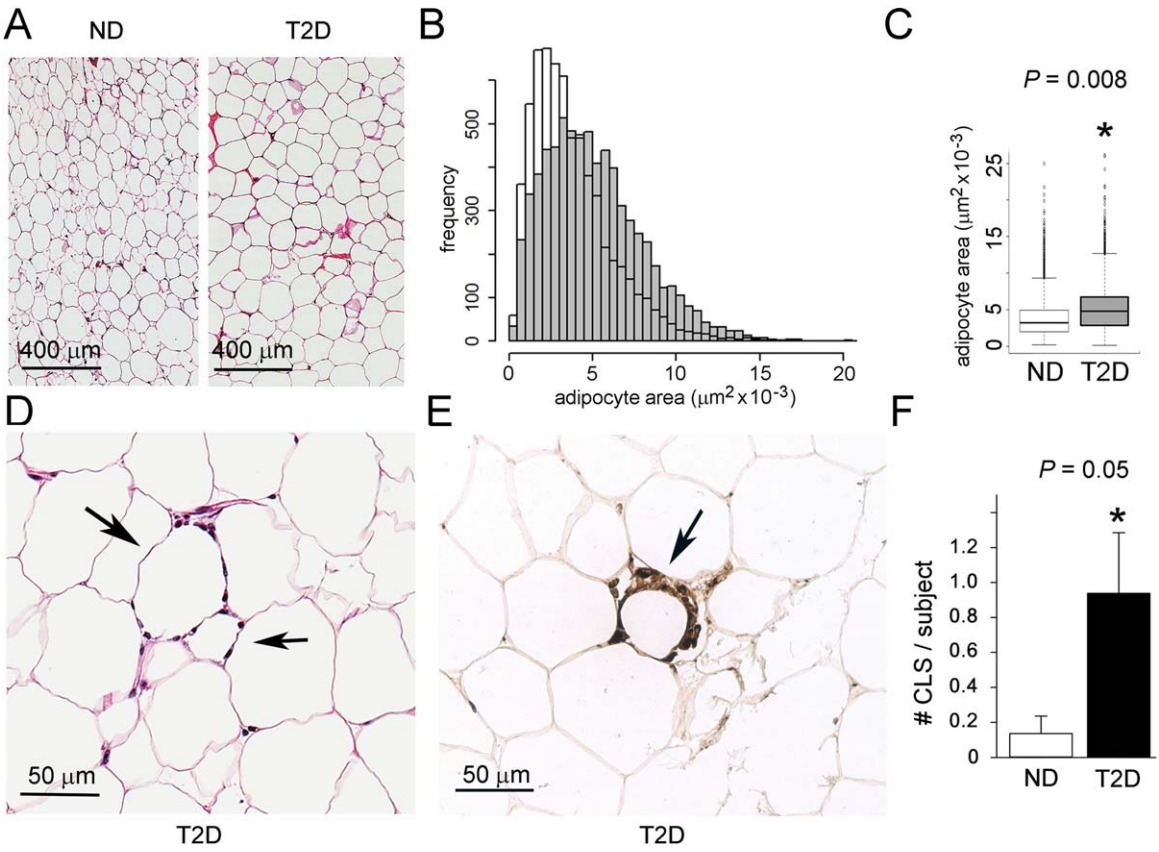

Figure 1 Adipocyte area and CLS frequency in breast adipose tissue of ND and T2D subjects with obesity. (**A**) Micrographs of representative, fixed, H&E-stained paraffin sections of breast adipose tissue from ND and T2D subjects. Magnification was the same for both sections. Scale bar = 400 μm. H&E, hematoxylin and eosin. (**B**) Overlay plot of histograms of cell area distributions, generated from measurements of cell area in a histological section. (**C**) Mean area of adipocytes in breast adipose tissue from ND and T2D groups ($N = 39$ total; 11 were ND subjects and 28 were T2D subjects). The median number of cells measured was 221, with comparison by Student t test. ($P = 0.008$ after adjusting for age and BMI). (**D**) Representative micrograph of H&E-stained breast adipose tissue showing apparent CLS (arrows). (**E**) Representative micrograph of anti-CD68 stain confirms CLS (8) within breast adipose tissue of T2D subject. Scale bar = 50 μm. (**F**) Numbers of CLS in breast adipose tissue sections of 11 ND subjects and 28 T2D subjects with two sections per subject examined. The plot shows the mean for each group. $P = 0.05$ by Student t test, $^*P \leq 0.05$.

adjusted by BMI and all medications. A search strategy was implemented to remove nonsignificant confounders from the fully adjusted model, based on likelihood ratio tests. Results in Table 2 list the cytokine-specific adjustment. Statistical significance was based on $P < 0.05$. Enumerated CLS were compared between T2D and ND using regression for repeated measures, adjusting for age and BMI. Analyses were conducted in R version 3 (The R Foundation for Statistical Computing, Vienna, Austria).

Results

Descriptive statistics

All subjects with overweight (BMI 25.0-29.9 kg/m^2) or obesity (BMI $\geq$ 30 kg/m^2) (Table 1) were classified into one of two groups, T2D or ND; both groups comprised 39 postmenopausal, self-reported African American women without cancer (age range: 44-71 years). Medication history was available for 34 of 39 subjects. ND subjects had a mean BMI of 30.4 kg/m^2 (SD: 4.0) and lacked T2D or metabolic syndrome. T2D subjects had a mean BMI of 36.5 kg/m^2 (SD: 7.4), and 21 of 28 subjects (75%) had comorbid hypertension, based on self-report or medications. Donors were not fasted; thus, plasma adipokines were not assayed, nor were values available for homeostatic model assessment of insulin resistance.

Histological assessment of breast adipose tissue inflammation

Adipocyte dysfunction, including hypertrophy (14), is known to be associated with tissue inflammation, such as CLS (9). Furthermore, breast adipocyte size associates with systemic inflammation, insulin resistance, and increased aromatase (10-12). We expected that T2D subjects would have larger adipocytes with CLS-B present, compared with ND subjects, consistent with blood biomarkers. Representative histological sections are shown in Figure 1A. The area distribution for T2D subjects (Figure 1B, gray) is right-shifted compared to that for ND subjects (Figure 1B, white), indicating that adipocytes in T2D tissue tend to be larger and less healthy than those in ND tissue. Mean adipocyte area (Figure 1C) was significantly larger in T2D than ND subjects ($P = 0.008$). Representative CLS-B is shown in Figure 1D-1E. CLS-B frequency was higher in T2D ($P = 0.05$) (Figure 1F).

Plasma cytokine biomarkers associated with metabolic phenotype

Although insulin resistance is associated with local and systemic elevations of proinflammatory cytokines (15), studies of plasma cytokines in adults with obesity have focused on limited subsets of analytes and have not comprehensively surveyed plasma with multiplex technologies. We therefore systematically quantified a total of 88 cytokines/chemokines to reveal novel associations with T2D. Of six cytokines associated with T2D (Table 2A; Supporting Information Figure S1), four were elevated in T2D patients and two were elevated in ND patients. Known proinflammatory molecules, tumor necrosis factor α and IL-6, were not significantly different between T2D and ND patients. In addition, three cytokines were associated with BMI, independently of T2D (Table 2B; Supporting Information Figure S2). The analysis suggests that the true association among these cytokines is with BMI and that the association with T2D is indirect.

Discussion

Vulnerable populations, such as African American women with obesity, who experience elevated risk from obesity-driven comorbidities such as breast cancer could benefit from more personalized profiling of immunometabolic biomarkers. Some studies in white women (12) have suggested that histological markers of inflammation in breast tissue (16) might help identify at-risk patients, if blood-based cytokines reflect breast risk factors (9). However, this approach has not been extended to African American women, and profiles of cytokines have been limited. Precision medicine approaches to risk management should consider traditionally underserved patients to address needful populations. Noninvasive clinical measures and blood biomarkers should correlate with breast adipose tissue biomarkers for breast cancer incidence or progression. Our ability to identify significant differences in cytokines and chemokines among individuals drawn from a random nonclinical population of subjects recruited at diverse sites across the country has value for traditionally underserved subjects, who might have difficulty with a fasting protocol or reluctance to travel to a hospital for a blood draw for research purposes. We have previously shown that estimates of cancer risk drawn from nonfasting populations are likely underestimates of the true effect of abnormal metabolism (17).

Chronic unresolved inflammation in obesity has previously identified chemotactic and adipokine signaling molecules (18). Different subtypes of obesity can reveal blood biomarkers for stratifying cardiometabolic risk (19,20). Although a novel group of seven cytokines and chemokines was associated with T2D, 81 of the other analytes tested (Supporting Information Table S2) were not significantly correlated with metabolic disease, simplifying the task of developing personalized medicine profiles for high-risk patients. The biomarkers described here can now be examined for utility as prognostic tools and to develop drug targets. C-C motif chemokine 11, soluble CD40 ligand, and interleukin 4 have been previously implicated in obesity; our analysis suggests that soluble CD40 ligand and interleukin 4 are associated with BMI and that T2D mediates the effect. Evidence links chemotactic factors such as C-X-C motif chemokine ligand 16 to breast cancer aggressiveness (21), but functional studies in obesity are needed.

Breast adipocyte hypertrophy and inflammation correlate in white women with elevated aromatase expression and breast cancer risk (10-12). These tissue-specific features are associated with clinical diagnoses such as T2D in obesity and blood biomarkers (1). Here, we have shown that in African American women, T2D was associated with a rightward shift in adipocyte size distribution, elevated CLS-B, and novel blood biomarkers.

We were unable to evaluate risks for progression of T2D or cancer, because of the cross-sectional design, or to obtain measures of regional adiposity to correlate with cytokine and metabolic data. Interventions and recommendations for bariatric surgery that are tailored to patients' inflammatory blood profile could be an important tool for risk assessment in obesity, but this step awaits investigation.

Conclusion

Combining profiling of inflammatory cytokines with measures of BMI may lead to a more personalized risk assessment for African American women with obesity.O

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