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**Endothelial function in youth: a biomarker modulated by adiposity-related
insulin resistance.**

Key Words: Endothelial Function, Inflammatory Markers, Insulin Resistance

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Abstract:

Objective To investigate the physical and metabolic determinants of endothelial dysfunction, an early marker of subclinical atherosclerosis, in normal weight and overweight adolescents with and without type 2 diabetes mellitus (T2DM).

Study design A cross-sectional study of 81 adolescents: 21 normal weight, 25 overweight with normal glucose tolerance (overweight-NGT), 19 overweight with impaired glucose regulation (overweight-IGR), and 16 with T2DM underwent evaluation of reactive hyperemia index (RHI) and augmentation index at heart rate 75 bpm (Alx-75) by peripheral arterial tonometry; oral glucose tolerance test, lipid profile, and hyperinsulinemic-euglycemic clamp to measure insulin sensitivity; DXA scan and abdominal MRI for percentage of body fat and abdominal fat partitioning.

Results Participants across tertiles of RHI (1.2 ± 0.02 , 1.5 ± 0.02 and 2.0 ± 0.05 , $p < 0.001$) had similar age, sex, race, lipid profile and blood pressure. BMI z-score, percentage body fat, abdominal fat, and HbA1c decreased, and insulin sensitivity increased from the 1st to 3rd tertile. RHI was inversely related to percentage body fat ($r = -0.29$, $p = 0.008$), total ($r = -0.37$, $p = 0.004$), subcutaneous ($r = -0.39$, $p = 0.003$) and visceral ($r = -0.26$, $p = 0.04$) abdominal fat. Alx-75 was higher (worse) in the lower RHI tertiles ($p = 0.04$), was positively related to percentage body fat ($r = 0.26$, $p = 0.021$) and inversely related to age, insulin sensitivity and inflammatory markers (TNF- α and PAI-1).

Conclusions Childhood obesity, particularly abdominal adiposity, is associated with endothelial dysfunction manifested by worse reactive hyperemia and higher Alx. Insulin resistance appears to mediate this relationship.

Abbreviations List:

Augmentation Index (Alx)

Augmentation Index at heart rate 75 (Alx-75)

Glucose disposal rate (Rd)
Insulin sensitivity per Fat Free Mass (IS_{FFM})
Impaired glucose regulation (IGR)
Intima media thickness (IMT)
Pulse wave velocity (PWV)
Peripheral arterial tonometry (PAT)
Reactive hyperemia index (RHI)
Subcutaneous abdominal fat (SAT)
Type 2 diabetes (T2DM)
Visceral abdominal fat (VAT)

The process of atherosclerosis occurs along a continuum and has its origins in the adolescent years as documented by several cohort studies (4-6). There is a relationship between obesity and central arterial stiffness in youth with and without T2DM(7-9). Aortic pulse wave velocity (PWV) was inversely related to insulin sensitivity and inflammatory markers in these obese adolescents. Intima media thickness (IMT) was found to be higher in youth with T2DM compared to lean and obese peers (10) and was related to HbA1c measure of glycemia (7). These findings support the importance of identifying early biomarkers of vascular function that are easily obtainable and applicable in large scale studies to detect children at higher risk for cardiovascular abnormalities.

Endothelial dysfunction is an early manifestation of subclinical atherosclerosis and results in abnormal regulation of the vessel tone (8, 11). Evaluation of the change in pulse wave amplitude during reactive hyperemia using peripheral arterial tonometry (PAT) is a non-invasive validated method to evaluate endothelial function in the microvasculature (12). The reactive hyperemia index (RHI) derived from this measurement correlates well with brachial artery ultrasound measured flow mediated

dilation(12) and with the coronary blood flow in response to acetylcholine, a direct measure of coronary endothelial function(13). RHI predicts CV events in prospective studies in adults (14, 15). In addition, the augmentation index (Alx), a measure of peripheral arterial stiffness, can be derived from PAT (16). The determinants of RHI and Alx and the effect of obesity related insulin resistance vs. hyperglycemia on these measures of endothelial function in youth have not been comprehensively evaluated. In this study, we hypothesized that: obese youth have evidence of endothelial dysfunction compared with normal weight peers, and that this endothelial dysfunction is mediated by lower insulin sensitivity and/or inflammation and is exacerbated by hyperglycemia. Therefore, we investigated the relationship of endothelial function (RHI and Alx) to body composition, insulin sensitivity, glycemia and circulating inflammatory markers in normal weight and overweight adolescents with and without abnormalities in glucose metabolism.

METHODS

Pubertal adolescents (n=81), 12-19 years of age (mean: 15.5 ± 0.2 years), 35 males and 46 females were recruited via advertisements in the community and from Texas Children's Hospital (TCH) clinics. The study population consisted of 21 normal weight controls (BMI: <85th percentile for age and sex) including 12 males and 9 females, and 60 overweight adolescents (BMI $\geq$ 85th percentile for age and sex). The overweight adolescents included 25 (7 males, 18 females) with normal glucose tolerance (overweight-NGT), 19 (9M, 10F) with impaired glucose regulation (overweight-IGR) and 16 (7M, 9F) with T2DM. IGR and T2DM were diagnosed according to the American Diabetes Association (ADA) criteria(17). Participants were not involved in any regular physical activity or weight reduction programs. Youth with T2DM were required to be in adequate glycemic control (HbA1c<8%) on metformin (n=14) and /or lifestyle therapy (n=2) and to discontinue metformin 24 hrs prior to PAT testing and 48 hrs prior to clamp

studies, as before(7, 18). Participants with T2DM had an average HbA1c of $6.2 \pm 0.16\%$ (range 5.2-7.5%), and a mean duration of diabetes 25.8 ± 6.2 months (range 0-89 months). Exclusion criteria included presence of disease other than T2DM or medication that would interfere with glucose metabolism, positive pregnancy test in females, history of smoking, and diagnosis of dyslipidemia or hypertension. Some data from 53 of the participants were included in a previous manuscript (18). All studies were approved by the institutional review board of Baylor College of Medicine and were performed at the Children's Nutrition Research Center (CNRC), TCH. Informed consent/assent was obtained from all participants and their parents prior to any study-related procedures after a detailed explanation of the study.

Anthropometrics, body composition and abdominal fat distribution

Height was measured on a fixed-wall stadiometer (Holtin Ltd.) to the nearest centimeter three times and then averaged. Weight was measured in light clothing to the nearest 0.1 kg on a balance scale. Puberty stage was assessed in all participants by a pediatric endocrinologist using Tanner staging criteria. Blood pressure (BP) was measured using a Welch Allyn Spot Vital Signs LXi, Welch Allyn Inc, Skaneateles, NY. Body composition including percentage body fat, fat mass, and fat-free mass, was determined by a dual-energy X-ray absorptiometry (DXA) scan (Delphi-A V12.1, Hologic, Bedford MA). Abdominal fat distribution was obtained by magnetic resonance imaging (MRI) scan at L4-L5 intervertebral space.

Fasting measurements and oral glucose tolerance (OGTT) test

Fasting blood sample was obtained for determination of total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides (TG), HbA1c, and circulating inflammatory markers as detailed below. Each participant underwent a 2-hour oral glucose tolerance test (OGTT) (1.75 g/kg of dextrose [max 75 g]) after an overnight fast (minimum 8 hours) to verify glucose tolerance status.

Hyperinsulinemic-euglycemic clamp

In vivo insulin sensitivity was evaluated during a hyperinsulinemic-euglycemic clamp, after 10-12 hours overnight fast as before (7, 18). Briefly, intravenous insulin (Humulin; Lilly, Indianapolis, IN) was infused at a rate of 40 mU/m²/ min in lean individuals and 80 mU/m²/ min in obese individuals to suppress hepatic glucose production.(19) Plasma glucose was clamped at 100 mg/dl (5.5 mmol/liter) with a variable-rate infusion of 20% dextrose based on plasma glucose determinations every 5 min.

Endothelial Function Studies

Assessment of endothelial function was performed by peripheral arterial tonometry (PAT) using Endo-PAT (Itamar, MA)(18, 20) in fasting state, in a quiet, temperature-controlled room. Briefly, the index fingers are placed into pneumatic probes and pulse wave is recorded. After five minutes equilibration period, a BP cuff placed on the non-dominant test arm is inflated to suprasystolic pressure for 5 minutes (the non-occluded arm serving as a control). Thereafter the cuff is rapidly deflated to allow for reactive flow-mediated hyperemia. The reactive hyperemia index (RHI) is calculated by an automated algorithm as the ratio of the pulse wave amplitude during reactive hyperemia relative to baseline.(20) The augmentation index (AIx) is an estimate of pulse wave reflection and a measure of arterial stiffness. AIx is calculated as the difference between the early (P1) and late (P2) systolic peaks of the pulse wave relative to the early peak wave (P2–P1/P1) expressed as percentage (16). Because AIx is affected by the heart rate (HR), values are adjusted to a standard HR of 75 beats per minute (AIx-75) (21).

Biochemical measurements

Plasma glucose was measured with a glucose analyzer (YSI, Yellow Springs, OH); Insulin levels were measured by electrochemiluminescence immunoassay (Elecsys 2010, Roche Diagnostics, Indianapolis, IN). HbA1c was measured using Tina-quant HbA1c immunoassay from Roche and lipids were measured using the standards of the

Centers for Disease Control and Prevention, via Labcorp, Inc. Leptin, tumor necrosis factor- α (TNF- α), and plasminogen activator inhibition (PAI-1) concentrations were determined by MAGPIX (milliplex MAP) immunoassay (EMD milipore Corporation, Billerica, MA).

Calculations

The insulin-stimulated glucose disposal rate (Rd) is equivalent to the rate of glucose infusion during the last 30 min of the euglycemic clamp, and expressed per kg and per kg of metabolically active fat-free mass (mg/min/kg_{FFM}). Insulin sensitivity is calculated as Rd divided by the steady state insulin levels and expressed per kg and per fat-free mass (mg/min/kg_{FFM} per μ U/ml) (7).

Statistical analyses

The distribution of the different variables was examined and the appropriate parametric or nonparametric test was applied. Analysis of variance (ANOVA) followed by post-hoc Bonferroni correction was used for 3 group comparison of continuous variables. Kruskal-Wallis test was used for nonparametric variables. Pearson or Spearman correlation tests were used to examine bivariate relationships as appropriate. Multiple regression analysis was used to examine multivariate relationships. All analyses were performed using SPSS (SPSS 22; Chicago, IL). Data are presented as mean $\pm$ SEM. P value $\leq$ 0.05 was considered statistically significant.

RESULTS

Given that normative values for RHI have not been established in youth and to better understand the determinants of decreased vascular reactivity, we evaluated the participants' physical and metabolic characteristics according to RHI tertiles (Table). RHI

increased from the 1st to the 3rd tertile (1.2 ± 0.02 , 1.5 ± 0.02 , and 2.0 ± 0.05 , $p < 0.001$) consistent with better endothelial function in the highest tertile. All subjects were pubertal (Tanner III-V) with no statistically significant difference among the 3 tertile groups with respect to age, sex, height, tanner stage or ethnic-racial distribution. BMI, BMI z-score and percentage body fat decreased with increasing RHI tertile (Table). Total, subcutaneous (SAT) and visceral (VAT) adipose tissue decreased with increase in RHI tertile, and were significantly lower in the 3rd tertile compared with the 2nd and 1st tertiles (Figure 1). Subjects in the 3rd RHI tertile had significantly lower HbA1c than those in 2nd RHI tertile. This difference persisted after adjusting for ethnicity ($p = 0.008$). There was a difference in the proportion of altered glucose tolerance status among the RHI tertiles ($p = 0.028$). Only 12.5 % of youth with T2DM were in the 3rd RHI tertile, and 61.9% normal weight subjects were in the 3rd tertile of RHI, with majority of overweight-NGT and overweight-IGR subjects in the 1st and 2nd RHI tertiles (76% and 63.1% respectively). The three tertiles didn't differ with respect to traditional CVD risk factors: systolic and diastolic BP, total, LDL or HDL-cholesterol or triglycerides (Table). Alx-75 (higher value indicating greater arterial stiffness) was higher in the lower RHI tertiles compared with the 3rd tertile (Table). Leptin decreased with increase in RHI tertiles ($p = 0.009$) with no significant difference in TNF- α or PAI-1 among tertiles of RHI.

Relationship of RHI and Alx to age and adiposity measures

RHI positively correlated with age ($r = 0.23$, $p = 0.04$). This relationship was no longer significant after adjusting for sex, tanner stage and height. RHI was inversely related to BMI ($r = -0.3$, $p = 0.019$), BMI percentile ($r = -0.3$, $p = 0.006$) and BMI-Z score ($r = -0.3$, $p = 0.005$). RHI was also inversely related to fat mass ($r = -0.3$, $p = 0.005$), percentage body fat ($r = -0.3$, $p = 0.008$), total ($r = -0.4$, $p = 0.004$), subcutaneous ($r = -0.4$, $p = 0.003$) and visceral abdominal fat ($r = -0.3$, $p = 0.04$). Alx-75 was inversely related to age ($r = -0.38$; $p = 0.001$). The relationship of age to Alx-75 persisted after controlling for sex and tanner

stage ($r=-0.31$, $p=0.007$) but was attenuated after adjusting for height ($r= -0.24$, $p= 0.04$). Alx-75 was positively related to percentage body fat ($r= 0.26$, $p= 0.02$) and tended to correlate with SAT ($r= 0.2$, $p= 0.08$).

Relationship of RHI and Alx to insulin sensitivity and inflammatory markers

Insulin sensitivity and IS_{FFM} increased with increase in RHI tertiles (Figure 2). The difference in insulin sensitivity and IS_{FFM} among tertiles did not change after adjusting for ethnicity.

RHI was positively related to insulin sensitivity ($r= 0.24$, $p= 0.04$) and inversely related to leptin levels ($r= -0.24$, $p= 0.03$). Al-75 was inversely related to insulin sensitivity ($r= -0.3$; $p= 0.032$) and positively related to TNF- α ($r= 0.3$, $p= 0.01$) and PAI-1 ($r= 0.2$, $p= 0.04$) (Figure 3).

In multiple regression analyses with age, sex, race-ethnicity, HbA1c (or glucose tolerance status), SBP and insulin sensitivity as independent variables and RHI as dependent variable, insulin sensitivity ($\beta= 0.32$, $p= 0.01$) and age ($\beta= 0.22$, $p= 0.06$) were the significant contributors to the variance in RHI ($R^2= 0.2$, $p= 0.02$). With Alx-75 as the dependent variable in the same model, insulin sensitivity ($\beta= -0.3$, $p= 0.03$) and age ($\beta= -0.3$, $p= 0.009$) were the significant contributors to the variance in Alx-75 ($R^2= 0.24$, $p= 0.007$). In the same model, TNF-alpha ($\beta= 0.29$, $p= 0.01$) further contributed to the variance in Alx-75 as dependent variable ($R^2= 0.36$, $p< 0.001$) but not to RHI. Including adiposity measures in the models (BMI or percentage body fat or abdominal fat) as independent variables instead of insulin sensitivity, the adiposity measures become the main determinants of RHI and Alx-75. Total cholesterol, LDL-cholesterol did not contribute further to the significance of the regression models.

DISCUSSION

Our study demonstrates that endothelial-dependent reactive hyperemia and peripheral vascular stiffness are significantly impaired in relation to obesity in children. This endothelial dysfunction appears to be modulated by insulin resistance related to total body fat and abdominal adiposity.

Worse endothelial function in relation to adiposity in children is consistent with adult studies demonstrating an inverse relationship between RHI and BMI (13, 22). Our results are also consistent with recent reports of worse RHI in obese compared with normal weight adolescents (23-25). In contrast, others reported no difference in RHI between normal weight and obese children (26). However, the latter study evaluated children of a wide age range (8-18 years) and Tanner stage, unlike our study which evaluated all pubertal children within a narrower age range (12-19 years). This may explain the discrepancy in the findings as Tanner stage (27, 28) and age (25, 27, 28) have been reported to be related to the RHI-PAT measurement. Similar to others, we observed the positive effect of age on RHI and Alx, but no relationship to Tanner stage (likely because all our participants are pubertal). The age(29) and pubertal stage(27) effects are attributed to possible maturation of the vasculature that occurs with age and pubertal development and doesn't reach adult level till late adolescence. The relationship between Alx-75 and age was partially dependent on height in our study. It is thus likely that the higher Alx-75 in younger children is related to a shorter return time of the pulse wave (shorter aortic length) in younger children as recently demonstrated (30). In addition to a physiologic effect of age, our study indicates an adverse effect of body fat and abdominal adiposity on RHI and Alx. The significant inverse relationship between abdominal fat (including subcutaneous and visceral fat) with vascular reactivity is consistent with reports of an inverse relationship between RHI and waist circumference, a surrogate measure of abdominal fat, in pediatric(25) and adult studies(22, 31), and supports the contribution of ectopic abdominal fat deposition to CVD risk(32). Of

concern, youth in the lowest RHI tertiles in our study had a mean RHI value of 1.2, lower than the 1.35 cutoff value found to predict coronary artery endothelial dysfunction in adults (13).

Insulin resistance has been proposed to mediate the effect of adiposity on vascular function. Insulin has vasodilator effects under physiologic condition via stimulation of nitric oxide (NO) production (33). However, in states of insulin resistance, insulin mediated NO-production is impaired and production of endothelin 1, a vasoconstrictor, is increased (34). Our results are consistent with the role of insulin resistance in mediating endothelial dysfunction in obese youth. Insulin sensitivity was lowest in individuals with the worst reactive hyperemic response and emerged as a significant determinant of the variance of both RHI and Alx-75, independent of the traditional CVD risk factors of lipids and BP. This is in concordance with our previous report of the role of insulin sensitivity as a determinant of central aortic PWV (7). This is in contrast to a study showing lower mean PAT ratio in obese compared with normal weight adolescents with no relationship to insulin resistance (25). The difference may be related to the use of an indirect measure of insulin sensitivity (Homeostasis Model Assessment) in that study, in contrast to the use of the gold standard hyperinsulinemic-euglycemic clamp measure of insulin sensitivity in our current study.

A role of hyperglycemia in early endothelial dysfunction as manifested by RHI is suggested by findings of lower HbA1c in the 3rd RHI tertile group and by the finding of a greater proportion of youth with T2DM in the lowest RHI tertile. The evidence of a stronger relationship was likely obscured by the good glycemic control and short duration of T2DM in our youth. In support of this proposal, adults with T2DM had lower RHI values compared with those without diabetes, with an inverse relationship between RHI and glycosylated hemoglobin (35). The importance of glycemic control for a healthy

endothelial function is also supported by findings of lower RHI scores in adolescents with type 1 diabetes compared with controls (36, 37).

Circulating inflammatory markers may provide biomarkers to assess endothelial health. Leptin was inversely related to RHI, consistent with a proposed role of leptin in NO-mediated vasodilator function, and the association of hyperleptinemia with vascular dysfunction (38). Similar to our previous report of a relationship between aortic PWV with inflammatory markers (7), we identified a positive relationship between TNF- α and PAI-1 and peripheral arterial stiffness as reflected by Aix-75. There was no relationship between these inflammatory markers and RHI, a finding also observed in adults (22). This suggests that different measures of vascular function are affected differently by inflammation.

Our current study is limited by the absence of an objective assessment of physical activity. We didn't measure LDL particle size or oxidized LDL which may show relationships to endothelial function and will need to be evaluated in future work. In addition, our youth with T2D were maintained on metformin. Although discontinued 24 hours prior to the EndoPAT testing, metformin may have a positive effect on endothelium-dependent blood flow (39), thus preventing the full appreciation of the effect of dysglycemia on RHI. However, it is not practically feasible to recruit adolescents with T2DM who are not on medical therapy to control glycemia.

Longitudinal studies are needed to evaluate whether measuring in-vivo endothelial function non-invasively in youth using this method may lend itself well to CVD risk estimation and to assess the effectiveness of intervention strategies. The present findings highlight the importance of targeting prevention and intervention efforts to high risk obese children in order to prevent the development of overt CVD in early adulthood.

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Figure Legends:

Figure 1- Relationship of RHI tertiles to Total Abdominal Fat (A), Subcutaneous Abdominal Fat (B) and Visceral Abdominal Fat (C). Post hoc significant differences (a: 1st vs. 3rd, b: 2nd vs. 3rd RHI tertile). 13 subjects (5 normal weight, 3 overweight-NGT, 3 overweight-IGR and 2 T2DM) had missing abdominal fat data due to a technical error. As such, abdominal fat data was available for 24 subjects in the 1st tertile, 21 in the 2nd tertile and 23 in the 3rd tertile.

Figure 2- Relationship of RHI tertiles to Insulin sensitivity (A) and Insulin sensitivity per fat-free mass (B). Post hoc significant differences (a: 1st vs. 3rd, b: 2nd vs. 3rd RHI tertile).

Figure 3- Relationship Alx-75 to PAI-1 (A) and TNF- α (B).

Table 1: Participants' characteristics across tertiles of Reactive Hyperemia Index (RHI)

	1 st tertile	2 nd tertile	3 rd tertile	P-value
Anthropometrics and Body Composition				
Age (years)	15.1 $\pm$ 0.4	15.2 $\pm$ 0.4	16.2 $\pm$ 0.3	ns
Male/Female	11/15	13/14	11/17	ns
AA/H/White	6/6/14	13/2/12	9 /6/13	ns
NW/OW-NGT/OW-IGR/T2DM	2/11/7/6	6/8/5/8	12/6/7/2	0.028
Height (cm)	164.6 $\pm$ 1.7	166.0 $\pm$ 1.5	166.4 $\pm$ 1.7	ns
BMI (kg/m²)	30.9 $\pm$ 1.3	30.4 $\pm$ 1.3	26.7 $\pm$ 1.1	0.010 ^a
BMI Z-score	1.8 $\pm$ 0.1	1.7 $\pm$ 0.2	1.1 $\pm$ 0.2	0.002 ^{a,b}
% Body Fat (%)	35.3 $\pm$ 1.5	32.7 $\pm$ 1.7	26.7 $\pm$ 2.1	0.004 ^a
Glycemic Markers				
Average Fasting Glucose (mg/dl)	97.1 $\pm$ 2.6	98.7 $\pm$ 2.6	93.8 $\pm$ 3.8	ns
Average Fasting Insulin (μU/L)	26.4 $\pm$ 3.5	25.7 $\pm$ 3.8	19.4 $\pm$ 3.2	ns
HbA1c (%)	5.6 $\pm$ 0.06	5.9 $\pm$ 0.1	5.5 $\pm$ 0.1	0.031 ^b
Cardiovascular Disease Markers				
Total cholesterol (mg/dl)	157.9 $\pm$ 6.1	152.8 $\pm$ 6.5	152.3 $\pm$ 6.4	ns

LDL cholesterol (mg/dl)	88.6 ± 4.9	92.0 ± 5.8	85.3 ± 4.7	ns
HDL cholesterol (mg/dl)	48.5 ± 2.4	42.3 ± 2.1	50.3 ± 2.9	ns
Triglycerides (mg/dl)	103.6 ± 12.7	92.6 ± 9.1	83.2 ± 8.4	ns
SBP (mmHg)	119.0 ± 1.7	120.0 ± 2.03	121.0 ± 1.9	ns
DBP (mmHg)	74.1 ± 1.5	71.6 ± 5.8	73.04 ± 1.6	ns
Leptin (ng/ml)	20.5 ± 2.5	21.4 ± 2.7	13.8 ± 3.0	0.009
RHI	1.2 ± 0.02	1.5 ± 0.02	2.0 ± 0.05	<0.001 ^{a,b,c}
Alx-75	-6.6 ± 1.6	-6.5 ± 1.9	-12.3 ± 1.7	0.04

Results are Mean±SEM; P-value refers to ANOVA or Kruskal-Wallis test (for leptin and

Alx-75) p-values. Post Hoc significant difference p<0.05 in: 1st vs. 3rd RHI tertile (a); 2nd

vs. 3rd RHI tertile (b) and 1st vs. 2nd RHI tertile (c).