Microcirculation, Adiposity, and Traditional and Emerging Cardiovascular Risk Factors in Prepubertal Children

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Purpose: Previous studies have shown that microvascular dysfunction (MD) is associated with a number of cardiovascular risk factors, including obesity. Few studies have assessed microvascular reactivity in children, and in most of these, results were confounded by the effects of puberty. Our aim was to establish whether MD is already present in obese prepubertal children.

Methods: This cross-sectional study included 52 obese, 18 overweight, and 28 eutrophic children, with a mean ± standard deviation age of 7.44 ± 1.22 years. We evaluated cardiovascular risk factors and nutritive microvascular function by using nailfold dynamic videocapillaroscopy and determined functional capillary density (FCD), red blood cell velocity at resting conditions (RBCV) and at peak ($RBCV_{max}$), and time to reach peak velocity during the post-occlusive reactive hyperemic response following 1 minute ischemia.

Results: On univariate analysis, differences in microvascular reactivity were not observed among the groups. Obese and overweight children had significantly higher scores than eutrophic children for the following parameters: body mass index, waist circumference, waist-to-height ratio, mean arterial pressure, homeostasis model assessment for insulin resistance, levels of insulin, leptin, glucose, triglycerides, total cholesterol, uric acid, and C-reactive protein. Multivariate analysis demonstrated the association between metabolic, anthropometric, and microvascular variables, stratified according to the degree of adiposity and body fat distribution.

Conclusions: Univariate analysis did not show any difference in microvascular reactivity between groups but, by testing these variables by multivariate means, we noticed a common and direct variation between cardiovascular/metabolic risk factors and microvascular reactivity occurring early in life.

Freeform/Key Words: obesity, microcirculation, children, cardiovascular risk

The incidence of obesity is increasing worldwide, not only in adults but particularly in children, to the extent that obesity must be viewed as one of the most serious public health

issues of current times [1, 2]. The outlook toward nutrition in developing countries has significantly transformed as a result of economic, social, and demographic changes occurring over the past decades and changes in population lifestyle and diet. Studies have found that in Brazil, one-third of children aged 5 to 9 years are overweight and 14.3% are obese [3]. A recently published longitudinal study has shown that overweight 5-year-old children are four times more at risk for becoming obese adolescents [4], placing obesity as an ongoing risk factor during growth for a number of obesity-related conditions, including cardiovascular disease.

Obesity during childhood is associated not only with adulthood obesity but also with dyslipidemia, hyperinsulinemia, hypertension, atherosclerosis, and, of note, increased mortality [5–7]. It is well known that atherosclerotic lesions can be observed early in life in at-risk groups. Endothelial dysfunction is one of the earliest signs of atherosclerotic disease [8], appearing long before cardiovascular events. Testing vascular reactivity is one of the chosen ways to measure endothelial function, and impaired vascular reactivity was present in selected high-risk groups of children [9, 10], although these results remain controversial.

The transport of nutrients to tissues and the removal of cellular waste is the most purposeful function of the circulation, and this process occurs within the microcirculation. The small arterioles control blood flow to each tissue area, and local conditions within the tissues themselves control the diameters of the arterioles. Thus, each tissue generally controls its own blood flow in relation to its needs. Microvascular morphology and hemodynamics can be studied noninvasively in humans by nailfold videocapillaroscopy [11], and studies of cutaneous microcirculation have demonstrated that microvascular dysfunction (MD) at this level is related to several cardiovascular risk factors [12–16]. Very few studies have assessed MD in obese prepubertal children [17–21].

The aim of this study was to establish whether MD is already present in overweight and obese children during the prepubertal stage.

1. Materials and Methods

Fifty-two obese (26 girls/26 boys), 18 overweight (13 girls/5 boys), and 28 eutrophic or lean prepubertal children (14 girls/14 boys) aged between 5 and 10 years (mean ± SD, 7.44 ± 1.22 years) were consecutively selected from our pediatric endocrinology outpatients unit at the Pedro Ernesto University Hospital assessed in a cross-sectional study. It took about 1 month from the beginning of recruitment period until the last day of examination, and the timeframe of the study was 24 months. Exclusion criteria included puberty, regular use of any medication, hypertension, heart disease, renal and blood diseases, presence of any acute or chronic inflammatory/infectious diseases, and presence of diabetes mellitus. The study was approved by the local ethics committee, and an informed assent was obtained for all children; their parents provided written informed consent prior to their inclusion in the study.

Height and weight were measured with minimal clothing and without shoes. Weight was measured to the nearest 0.1 kg, using the Filizola scale (São Paulo, SP, Brazil). Height was measured to the nearest 0.1 cm, using a Tonelli stadiometer (Tonelli, Criciúma, SC, Brazil). Body mass index (BMI) was calculated as weight in kilograms divided by height squared in meters. Degrees of body mass defined groups as eutrophic [eutrophic controls (C); ≥−2 to <1 standard deviation score (SDS)], overweight (OW group; >1 to <2 SDS), and obese (OB group; ≥2 SDS) groups according to the SDS for BMI-for-age and were calculated by using the World Health Organization AnthroPlus software [22]. Waist circumference was measured using a flexible tape, to the nearest 1 mm, at the midpoint between the lower rib and the pelvic bone, and the waist-to-height ratio was calculated. The waist-to-height ratio has been validated as a measure of central adiposity in children [23–25] and the proposed cutoff is 0.5 [26]. The pubertal stage of the subjects was assessed according to Tanner staging [27, 28]. Systolic blood pressure and diastolic blood pressure were measured on the right arm, in a sitting position, using a manual sphygmomanometer (Tycos; Welch Allyn Company, Arden, NC) and adequate sized cuffs. Mean arterial pressure was calculated as follows: mean arterial pressure = diastolic blood pressure + [(systolic blood pressure – diastolic blood pressure)/3].

Blood samples were obtained following an overnight fast of at least 10 hours. Laboratory testing included fasting plasma glucose (FPG), insulin, total cholesterol and high-density lipoprotein (HDL) cholesterol, triglycerides, leptin, adiponectin, high-sensitivity C-reactive protein (hs-CRP), and interleukin-6 concentrations, using standard laboratory techniques. Low-density lipoprotein cholesterol concentration was calculated as follows: low-density lipoprotein = total cholesterol − (HDL + triglycerides/5). Biochemical analysis was performed using Konelab equipment, with a BT3000 Winer kit, by testing variables using the following methods: for FPG, the enzimatic colorimetric (oxidase); for cholesterol, the CHOP-POD enzymatic (esterase-oxidase); for triglycerides, the enzimatic colorimetric (esterase-oxidase); and for HDL cholesterol, the colorimetric without precipitation (enzymatic colorimetric; Winterlab, Rosário, Santa Fé, Argentina). Insulin level was measured using the GAMMA-C12 equipment, with a kit employing the Coat-a-Count method, a solid-phase radioimmunoassay marked with [125]I (DPC, Los Angeles, CA). The intra-assay coefficient of variation (CV) ranged from 3.1% to 9.3%, whereas the interassay CV ranged from 4.9% to 10.0%. Leptin and adiponectin were measured by radioimmunoassay, using the GAMMA-C12 equipment with the double antibodies/polyethylene glycol method (Linco Research, St. Charles, MO). For leptin concentration, the intra-assay and interassay CVs were 3.4% to 8.3% and 1.7% to 6.2%, respectively. For adiponectin concentration, these were 3.0% to 6.2% and 6.9% to 9.2%, respectively. Interleukin-6 level was measured by the electrochemiluminescence method (Roche Diagnostics GmbH, Mannheim, Germany) with a sensitivity of 6.01 pg/mL. The hs-CRP level was measured by the turbidimetric method (Bio-Systems SA, Barcelona, Spain), and the intra-assay and interassay CVs were 0.18% and 0.36%, respectively. Insulin resistance was calculated according to the homeostasis model assessment for the insulin resistance (HOMA-IR) equation: FPG (mmol/L) × fasting insulin (mU/L)/22.5. The cutoff point of 2.5 for the HOMA-IR index was used to define insulin resistance [29].

A. Microvascular Assessment

Nailfold videocapillaroscopy was performed by the same observer and analyzed according to a standardized and well-validated method using the fourth finger of the left hand in a temperature-controlled environment (22°C) in the fasting state [30] in the morning. Using the CAPIMAGE software [31], the following microvascular parameters were determined at resting state: a) functional capillary density (FCD), the number of capillaries/mm^2 with red blood cell flux, evaluated with ×250 magnification in a 3-mm area of the distal row of capillaries in three different areas (lateral, central and medial); b) red blood cell velocity at rest (RBCV) and during postocclusive reactive hyperemia (PORH); c) the peak RBCV after 1-minute arterial occlusion (RBCV$_{max}$); and d) the time taken to reach RBCV$_{max}$. The arithmetic average of four measurements of FCD (two on the lateral aspect and two centrally located in the periungual site) resulted in the mean FCD, whereas the average of two measures at the central site resulted in FCD. After quantifying FCD, the magnification was increased to ×680, and the other variables were assessed before (at rest) and during PORH. First, a pressure cuff (1-cm wide) was placed around the proximal phalanx and connected to a mercury manometer. Baseline RBCV was measured three times, and the intra-assay CV was 17.1%. During PORH, each variable was tested once. The nailfold videocapillaroscopy examination was repeated on nine subjects on different days, and the interassay CV of the tested functional parameters ranged from 2.0% to 9.0%.

B. Statistical Analysis

Univariate and multivariate analyses were performed using Graphpad Prism 5 software (GraphPad Software, San Diego, CA) and Statistica version 8.0 (StatSoft, Tulsa, TX). Variables were tested for their distribution (normality, kurtosis, skewness, and homoscedasticity). Univariate intergroup comparisons were performed by analysis of variance or Kruskal-Wallis tests according to whether the tested variables followed a normal or nonnormal distribution, respectively. *Post hoc* Bonferroni and Dunn tests were then performed.

The statistical power of the sample size of 95 participants, used in the discriminant function analysis, was tested according to the estimated to the multivariate analysis of variance family of global effects design tests. This sample size grants an actual statistical power of 0.82404 (critical F = 1.3361200; Pillai V = 0.40). Therefore, the sample size used was responsible for a statistical power above 80%, which was considered satisfactory to our analysis.

Biological systems, as many processes in nature, are inherently multivariate, therefore, we took a multivariate approach to determine which variables discriminate between the groups and applied canonical discriminant function analysis. This analysis determines how well it is possible to distinguish groups from a multivariate data set. The variation of a specific physiological parameter may influence other parameters, and their synergistic action can generate results not easily detectable through a univariate analysis. All normally distributed variables are expressed as mean ± SD or as median [first to third quartiles]. The level of significance adopted was 0.05.

2. Results

Ninety-eight children were included in the analysis. Table 1 shows the clinical and anthropometric characteristics. As predicted, OW and OB groups had higher BMI, weight, waist circumference, waist-to-height ratio, mean arterial pressure, FPG, insulin, HOMA-IR, leptin, triglycerides, total cholesterol, uric acid, and hs-CRP levels. According to adiposity status, the comparison among the three groups (C vs OW vs OB) did not show any significant difference in nutritive microvascular reactivity at rest or during PORH (Table 2).

Our main objective was to assess microvascular function in prepubertal children according to the degree of adiposity; however, to further investigate whether other variables could affect microvascular reactivity, we also stratified by sex, insulin resistance (HOMA-IR cutoff >2.5), and waist-to-height ratio (cutoff 0.5). Univariate analysis (data not shown) did not indicate any differences in microvascular function tested through dynamic nailfold videocapillaroscopy.

Multivariate analysis has shown that the association of waist-to-height ratio, HDL, hs-CRP, mean FCD, glucose, adiponectin, and $RBCV_{max}$ allowed greater discrimination between groups, according to the degree of adiposity. The canonical discriminant function analysis obtained from the seven aforementioned variables (Wilk's λ = 0.21064) produced two canonical roots that allowed significant discrimination between the groups (obtained from the square Mahalanobis distance $P < 0.00001$). Root 1 explained 87.9% of variance between the groups, whereas root 2 explained only 12.1% of variance. At root 1, the combined influence of waist-to-height ratio, glucose, and hs-CRP levels followed the expected pattern according to the BMI SDS, whereas the combined influence of HDL and $RBCV_{max}$ followed the opposite pattern (Table 3). P values for the squared Mahalanobis distance between the groups according to the BMI SDS (*i.e.*, significant distances between C and OW, C and OB, and OW and OB) were all at $P < 0.00001$. Figure 1 shows the resulting bivariate distribution of canonical roots and the distance between groups according to the BMI SDS. Multivariate analysis also showed that children with increased waist-to-height ratio had higher levels of leptin, mean blood pressure, insulin, total cholesterol, uric acid, and hs-CRP and lower RBCV and $RBCV_{max}$ (Fig. 2). These results suggest that excessive central adiposity was associated with markers of cardiovascular disease risk in prepubertal children.

3. Discussion

The skin is a well-established locus for research on microcirculation, and the study of cutaneous microcirculation is regarded as a valid model for the investigation of microvascular function more generally [32]. Several studies have demonstrated the relationship between cutaneous MD and risk factors for coronary disease [13, 15, 16].

Our main goal was to determine whether prepubertal children with excessive adiposity have MD which could not be demonstrated in the study population, by assessing nutritive microvascular function using dynamic nailfold videocapillaroscopy. We did not observe any

Table 1. Clinical, Anthropometric, and Laboratory Variables of Participants, According to the SDS for BMI

Characteristic	C	OW	OB	P Value
No. of participants	28	18	52	
Age (y)	7.39 ± 1.03	7.33 ± 1.46	7.51 ± 1.26	0.8265
Sex (M/F)	14/14	5/13	26/26	0.232
BMI (kg/m^2)	15.64 ± 1.19	19.65 ± 1.14[a]	25.24 ± 3.63[b,c]	<0.001
Weight (kg)	25.33 ± 4.67	31.84 ± 5.71[d]	44.63 ± 9.62[b,c]	<0.001
MAP	63.33 [60.0–63.33]	63.33 [62.50–73.33]	70.0 [63.33–73.33][b]	<0.01
Waist-to-height ratio	0.44 ± 0.02	0.51 ± 0.03[a]	0.60 ± 0.06[b,c]	<0.001
WC (cm)	55.59 ± 3.38	64.72 ± 5.11[a]	80.03 ± 9.88[b,c]	<0.001
FPG (mg/dL)	83.57 ± 6.26	88.83 ± 8.56	87.85 ± 7.64[e]	0.025
Insulin (mUI/L)	2.45 [1.22–4.02]	6.1 [3.47–7.30][d]	8.25 [4.35–13.48][b]	<0.001
HOMA-IR	0.50 [0.25–0.80]	1.16 [0.74–1.76][d]	1.78 [0.97–2.94][b]	<0.001
Triglycerides (mg/dL)	70.0 [54.25–95.0]	76.0 [57.25–92.25]	90.0 [69.50–130.0]	0.0392
Total cholesterol (mg/dL)	146.14 ± 29.31	162.59 ± 28.26	168.80 ± 28.86[f]	0.004
HDL (mg/dL)	45.70 ± 8.66	51.21 ± 8.79[c]	40.99 ± 8.92[e]	<0.001
LDL (mg/dL)	83.98 ± 29.68	95.35 ± 25.72	105.81 ± 26.83[f]	0.004
Uric acid (mg/dL)	3.50 [2.90–3.90]	3.70 [3.07–4.25]	3.95 [3.50–4.70][f]	0.007
hs-CRP (mg/dL)	0.09 [0.06–0.18]	0.12 [0.08–0.43][g]	0.28 [0.14–0.50][b]	<0.001
Leptin (ng/mL)	3.85 [2.05–5.07]	8.06 [5.27–18.18][d,g]	19.55 [10.45–27.35][b,g]	<0.001
Adiponectin (μg/mL)	12.85 [9.26–17.08]	10.92 [8.37–16.45]	10.21 [7.26–15.57]	0.2434
IL-6 (pg/mL)	1.84 [1.50–3.15]	2.38 [1.50–3.64]	2.79 [1.89–3.73]	0.104

Values are presented as mean ± standard deviation or median [first to third quartiles].
Abbreviations: IL-6, interleukin-6; LDL, low-density lipoprotein; MAP, mean arterial pressure; WC, waist circumference.
[a]C vs OW, $P < 0.001$.
[b]C vs OB, $P < 0.001$.
[c]OW vs OB, $P < 0.001$.
[d]C vs OW, $P < 0.01$.
[e]C vs OB, $P < 0.05$.
[f]C vs OB, $P < 0.01$.
[g]OW vs OB, $P < 0.05$.

difference in microvascular reactivity among the tested groups, according to adiposity. Taking the analysis one step further, we stratified the groups according to sex, body fat mass distribution, and insulin resistance and found that microvascular reactivity was the same across the sample population.

Few studies have evaluated vascular reactivity in children, and these have largely involved patients at different pubertal stages. Bhangoo *et al.* [17] studied the effect of puberty on endothelial function in healthy children and adolescents by peripheral arterial tonometry and found a greater vasodilatatory response with pubertal advancement, most likely due to the onset of sex steroids action. Importantly, the rates of the reactive hyperemia response in the

Table 2. Comparison of Microvascular Reactivity Between Controls, Overweight, and Obese Prepubertal Children

Characteristic	C	OW	OB	P Value
Functional capillary densities (n/mm^2)				
FCD$_m$	5.50 [4.82–7.27]	7.05 [5.25–9.35]	6.85 [5.45–7.65]	0.107
FCD$_c$	6.10 [4.80–7.55]	7.40 [6.15–9.72]	7.40 [5.00–8.80]	0.192
RBCV (mm/s)	0.34 [0.30–0.50]	0.33 [0.32–0.33]	0.33 [0.31–0.34]	0.352
RBCV$_{max}$ (mm/s)	0.38 [0.37–0.59]	0.37 [0.37–0.38]	0.37 [0.37–0.39]	0.265
TRBCV$_{max}$ (s)	4.0 [3.25–5.0]	4.0 [3.0–5.0]	4.0 [3.0–5.0]	0.483

Values are presented as median [first to third quartiles].
Abbreviations: FCD$_c$, central functional capillary density; FCD$_m$, mean functional capillary density; TRBCV$_{max}$, time to peak RBCV$_{max}$ during PORH.

Table 3. Factor Structure Matrix

Characteristic	Root 1	Root 2
Waist-to-height ratio	**0.95**	0.17
HDL	**−0.20**	**−0.68**
hs-CRP	**0.15**	**−0.30**
FCD_m	0.07	**−0.39**
Glucose	**0.18**	**−0.31**
Adiponectin	−0.01	0.23
$RBCV_{max}$	**−0.17**	**0.30**
Eigenvalue	2.53	0.35
Proportional accumulated variation, %	87.9	100.0

The sign indicates the direct or inverse correlation between the root and variable. Marked in bold are the highest values according to the root. The higher the value of the variable of the load, the greater the influence of it on the canonical root and, hence, the influence of this variable in the separation of groups.
Abbreviation: FCD_m, mean functional capillary density.

prepubertal children were comparable to those found in adults with cardiovascular disease [17]. These findings may indicate that in prepubertal children, microvessels are only partially responsive during reactive hyperemia and become fully responsive with the onset of sex steroids action. This hypothesis is corroborated by studies assessing vascular reactivity by peripheral arterial tonometry in eutrophic children and adolescents. This increased rate of reactive hyperemia with age suggests that the development of the microvascular response to various stimuli may not be complete until late adolescence [18, 19, 20, 21]. On the basis of
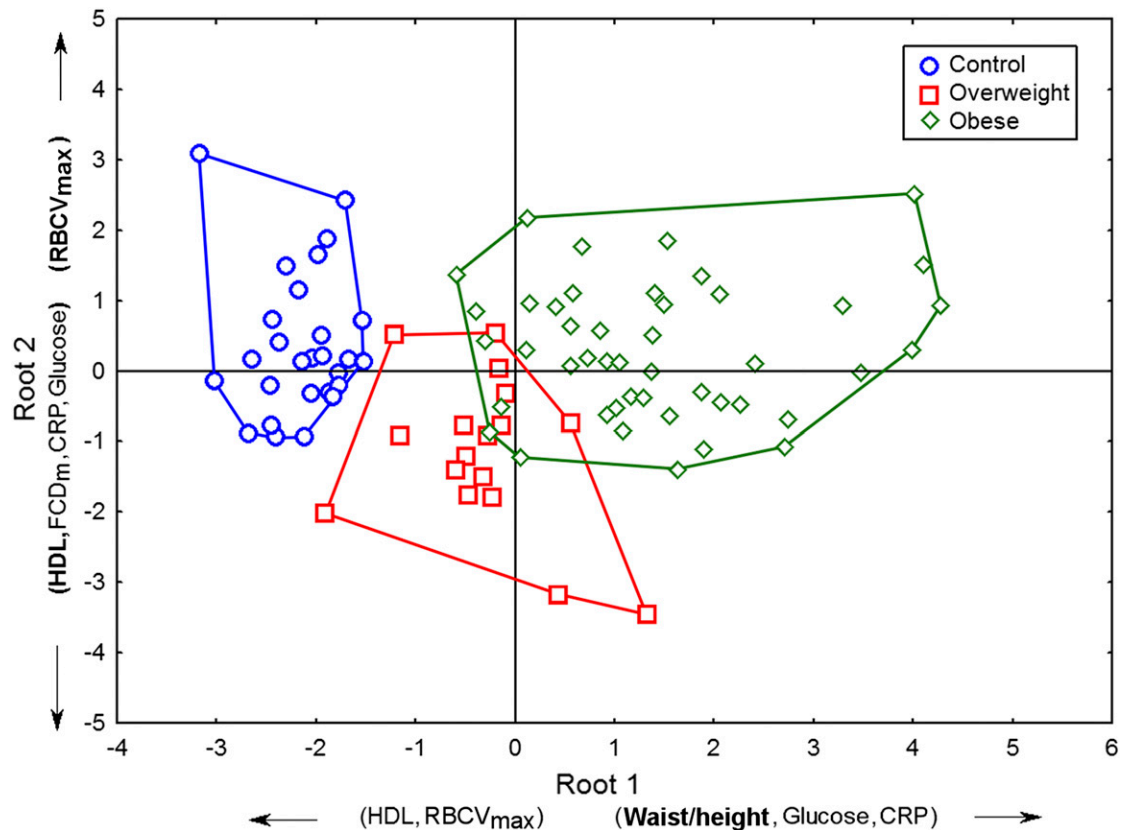

Figure 1. Bivariate distribution of canonical roots 1 and 2. FCDm, mean functional capillary density; PCR, polymerase chain reaction.
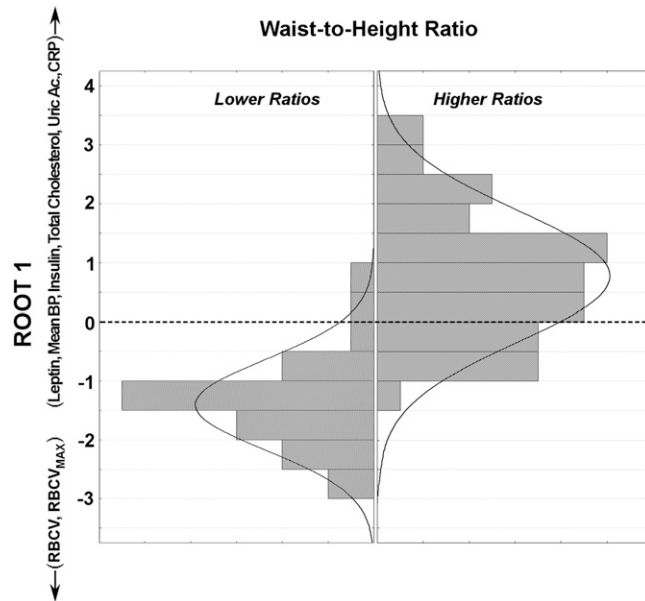
Figure 2. Bivariate distribution according to central adiposity (waist-to-height ratio). Higher ratios represent waist-to-height ratios greater than or equal to 0.5; lower ratios represent waist-to-height ratios less than 0.5. BP, blood pressure; CRP, C-reactive protein; Uric ac., uric acid.

these findings, it appears that there is an association between pubertal stages and vascular maturity, and low levels of reactive hyperemia response in prepubertal children may reflect an immature microvascular function in this population. If this is correct, adiposity status, insulin resistance, and body fat mass distribution do not have sufficient impact to result in differences in microvascular reactivity in the prepubertal period.

A cross-sectional study of nonnutritive cutaneous microcirculation in obese and normal-weight prepubertal children found no difference in blood microflow at rest or during reactive hyperemia; however, the time taken to reach peak reperfusion was longer in obese children [21]. The prolongation of reperfusion time during the reactive hyperemia response is a finding that has been consistently demonstrated in several studies performed by our research group. However, these studies focused on adult subjects with obesity, with and without metabolic syndrome [30, 33, 34], and assessed cutaneous nutritive microcirculation.

Recently, a large prospective study examined macrovascular reactivity in obese prepubertal children (n = 6576) by flow-mediated dilation of the brachial artery. No change in vascular function was found in the first decade of life, and the authors concluded that adiposity in this age group was not associated with macrovascular dysfunction [35]. Although our studies on microcirculation were not prospective and assessed the nutritive microcirculation, our results are nevertheless corroborated by these recent findings.

The adverse effects of excessive adiposity on microvascular function during the first decade of life remain controversial; nevertheless, they may highlight the influence of puberty on the development and maturation of the vascular system.

In our study, as predicted, overweight and obese children had adverse metabolic and inflammatory biomarkers, with higher insulin, HOMA-IR, leptin, glucose, triglycerides, total cholesterol, uric acid, and hs-CRP levels than normal-weight children. Our findings are consistent with existing evidence showing that prepubertal children with excessive adiposity have some surrogate markers of cardiovascular disease risk [36, 37].

In the absence of an established cutoff value, studies in health sciences are performed comparing a control group with a group of subjects considered unhealthy. At this point of the data analysis, prepubertal children did not show any difference in microvascular reactivity. However, using multivariate analysis of clinical, anthropometric, and microvascular variables as a data-mining tool, we noted an association between some of these variables, clearly

separated by previously established categorization of adiposity (SDS BMI; see Fig. 1). It is well known that the impact of metabolic changes resulting from adiposity in the blood vessels is significant, in adults as well as in children, and may promote the development and progression of atherosclerosis. It is important to note that variables allowing for greater discrimination between groups according to the degree of adiposity and body fat distribution included major cardiovascular risk factors, such as excessive central adiposity, HDL, hs-CRP, FPG, leptin, insulin, mean blood pressure, total cholesterol, and uric acid, as well as microvascular variables, such as RBCV, $RBCV_{max}$, and mean FCD. Therefore, despite the absence of differences in microvascular reactivity, when comparing the groups of prepubertal children by univariate analysis based on their degree of adiposity, insulin resistance, and distribution of body fat, a refined multivariate analysis further used to test possible common variations among all tested variables showed that, even in prepubertal children, there is a common and direct variation between cardiovascular/metabolic risk factors and microvascular reactivity occurring early in life.

Despite the cross-sectional design of the study, we could infer that the continued presence of metabolic and cardiovascular risk factors in these children and, specifically, a constant or worsening level of adiposity with age would affect microvascular reactivity. This hypothesis clearly requires validation by long-term studies. Furthermore, taking into account the vascular effects of sex steroids and the potential parallelism between sexual and vascular maturation, the relationship between obesity-related cardiovascular risk factors and the occurrence of MD may become more evident as the state of excess adiposity persists with the progression of puberty. Further studies are necessary to confirm this hypothesis.

Acknowledgments

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