Vascular Reactivity and Proinflammatory Cytokines in the Obese Children

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The aim of this study was to evaluate the relation between inflammatory markers and the preatherosclerotic marker - flow mediated dilation (FMD). Thirty obese children (10-16 years old) and twenty controls were involved. The plasma inflammatory markers: CRP, fibrinogen, leptin, TNF-α, IL-6 were measured. Ultrasounds were used for FMD measurement, chemiluminescence for monocyte respiratory burst (RB), ELISA for C peptide and spectrophotometry for usual parameters. In the obese children versus the lean ones, the FMD was lower ($p<0.001$), the plasma values for TNF-α were similar (1.68 pg/mL vs 1.54 pg/mL), while plasma IL-6 was increased (4.01 pg/mL vs. 2.02 pg/mL, $p<0.05$). These cytokines were negatively correlated with FMD ($r=-0.42$, $p<0.05$) and positively with RB ($r=50$, $p<0.05$). The FMD was negatively correlated ($p<0.05$) with the values for diastolic blood pressure ($r = -0.47$), waist circumference ($r = -0.55$), uric acid ($r = -0.47$) and atherosclerotic index ($r = -0.37$). In conclusion, in the obese children, inflammation, dyslipidaemia, blood pressure and oxidative stress act in a cluster reducing the elasticity of the vessel walls.

Keywords: childhood obesity, FMD, inflammation

In obese subjects, the adipose tissue, especially the visceral fat is infiltrated by macrophages. Adipocytes, but mainly macrophages from the adipose tissue secrete proinflammatory cytokines like TNF-α, IL-6. TNF-α acts more as an autocrin and paracrin hormon, while high plasma IL-6 can stimulate the hepatic synthesis of acute phase proteins, augmenting inflammation [1,2]. The mRNA expression of IL-6 and TNF-α is higher in the adipose tissue of overweight and obese children versus normal weight ones[3]. TNF-α stimulates lipolysis through activation of extracellular signal-related kinase and elevation of AMPc, controlling the gatekeeper proteins on the surface of the lipid droplets [2]. Like TNF-α, IL-6 stimulates lipolysis and inhibits lipoprotein lipase and both cytokines contribute to the high plasma levels of triglycerides. The imbalance of adipocytokines (high leptin and resistin versus low adiponectin), of cytokines (TNF-α, IL-6, IL-1 versus low IL-10) contribute also to the chronic inflammatory status in obesity [4].

Vascular dysfunction is present in the first stages of atherosclerosis development. Flow-mediated dilation FMD is a subclinical marker of atherosclerosis, estimating the increase in brachial arterial diameter in response to brief arterial occlusion [5] and it has a predictive value for future cardiovascular events [6]. The recruitment and the activation of monocytes are present in the first stages of atherosclerosis [7]. Also, under certain stimuli, monocytes undergo an oxidative burst during which free radicals, as superoxide anion, are released and the increased oxidative stress contributes to the vascular dysfunction [8].

In childhood obesity, the inflammation, insulin resistance and dyslipidaemia increase the risk of cardiovascular disease in adults, leptin was proposed as a strong predictor of overweight status among children [9] and interleukin-6 and CRP levels are associated with the development of type 2 diabetes mellitus [10].

The aim of this study was to evaluate the relation between inflammatory markers and the preatherosclerotic marker, flow mediated dilation (FMD).

Experimental part

Materials and methods

A total of 30 overweight and obese children (obese group, 12 boys and 18 girls, 10-16 years old) and 20 healthy lean children (control group, 9 boys and 11 girls, 10-16 years old) were enrolled. Children under chronic medication, with acute or chronic inflammation were excluded. All subjects were non-smokers. The study protocol was approved by the Ethical Commission of Grigore Alexandrescu University Hospital, Bucharest and a written informed consent was obtained from each parent.

Clinical characteristics

Anthropometric measurements: weight, height, waist circumference (WC) were assessed. The BMI was calculated as the ratio between weight (kg) divided by square height (m^2). Overweight is defined as 85-95th BMI percentile and obesity as ≥ 95th BMI percentile.

Biochemical measurements

Fasting blood samples were taken. The usual plasma variables were measured by using an automatic analyser HITACHI and kits with standard methods from Diasys (Germany). Inflammatory markers TNF-α, IL-6 and leptin were determined by ELISA methods. Cayman ELISA kits, no. 589201 and no. 583361 were used for TNF-α and IL-6, respectively, following the manufacturer's guidelines. EIA-2395 kit for leptin and EIA 1293 kit for C peptide (a surrogate marker of insulin resistance) were purchased from DRG Instruments GmbH, Germany. Low-density lipoprotein-cholesterol (LDL-C) was calculated according to the Friedewald equation [11] and HOMA-IR (homeostatic model assesement-insulin resistance) was calculated according to Matthews DR formula: Glicemia(mg/dL) x insulin(IU/L)/405 [12]. Respiratory burst RB was measured by chemiluminiscent method. For this method, peripheral blood mononuclear cells isolated by density centrifugation were resuspended in phosphate-buffered saline and dark-adapted luminol was added. Spontaneous chemi-

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luminescence for 15 min was measured. The added phorbol 12- Myristate 13-Acetate (PMA; final concentration 5.4 μmol/L) initiated the respiratory burst and the maximum chemiluminescence peak was recorded (Luminoskan Ascent® 392, LabsystemsEx-Thermo Electro Corporation). Chemiluminescence production was expressed as the Relative Chemiluminescence Units over time (RLUX60min). All reagents for RB measurements were purchased from Sigma Chemical Co., St. Lou, USA [13].

The source of variation between the lean children and the obese children was assessed by the unpaired Student t-test. The Pearson correlation coefficient was calculated and the threshold for significance was set at $p < 0.05$.

FMD measurements by ultrasounds

Before testing FMD, with at least 12 h, the subjects were asked to refrain from antiinflammatory drugs, caffeine and herbal supplements. An informed written consent to participate was given by all the subjects involved in the study. The brachial artery was identified and measured on longitudinal images, between lumen-intima interfaces of the anterior wall and the posterior wall by using an ultrasound equipment, General Electric Logiq 500 Pro Ultrasound from Soma Technology. The measurements were done at the onset of the R-wave, according to the electrocardiogram. The brachial artery diameter (BAD1) was measured 2-3 cm above the antecubital fossa. Then, a cuff of a sphygmomanometer was inflated on the forearm, above systolic pressure with 50mm Hg, for 5 min. BAD2 was measured in the 45-60 s interval after cuff deflation. Arterial FMD represents the maximal percent of basal vessel diameter after reactive hyperemia and is expressed in %. The formula for FMD is [(BAD2-BAD1)/ BAD1] 100% [14].

Results and discussions

Clinical data are shown in the table 1.

We demonstrated that TNF-α was correlated ($p<0.05$) with IL-6 ($r=0.77$), with RB ($r=0.49$), and with FMD ($r=-0.43$), while IL-6 was correlated with CRP ($r=0.46$), with RB ($r=0.52$) and with FMD ($r=-0.40$). The FMD was negatively correlated ($p<0.05$) with the values for the diastolic blood pressure ($r = -0.47$), waist circumference ($r = -0.55$), atherosclerotic index ($r= -0.37$) and uric acid ($r = -0.47$).

Calculated Pearson correlations (r value, for $p<0.05$):

Nowadays, more researchers embrace the Inflammatory Hypothesis of Atherothrombosis and

Table 1

COMPARISON OF CLINICAL DATA BETWEEN GROUPS

Parameters	Control (n=20)	Obese children (n=30)	*p*
Age (years)	12.6± 4.9	13.4 ± 5.8	Ns
Gender (M/F)	9/11	12/18	Ns
Weight (kg)	42.8 ± 10	75.7 ±16.02	<0.001
Height (m)	1.52± 0.04	1.54 ± 0.07	Ns
BMI (kg/m^2)	18.15 ± 1.4	28.9 ± 2.2	<0.001
WC (cm)	62.06 ± 4.6	92.3 ± 15.9	<0.001
SBP mmHg	114.6 ± 8.5	133.2± 13.1	<0.01
DBP mmHg	54.3 ± 4.9	69.3 ± 8.02	<0.01
Cholesterol mg/dL	122.5± 20	158.4± 27	<0.03
Triglycerides mg/dL	53.7±30..3	106.6±54.3	<0.001
HDL-C mg/dL	75.3±6.6	43.5±12.9	<0.05
LDL-C mg/dL	36.7± 10.4	97.7±25.6	Ns

BMI-body mass index, WC-waist circumference, SBP-systolic blood pressure, DBP-diastolic blood pressure; p value represents the ttest result of comparison the obese versus the control groups

Table 2

BIOLOGICAL PARAMETERS IN THE STUDIED GROUPS

Parameters	Control (n=20)	Obese children (n=30)	*p*
Glycemia mg/dl	67.6 ± 3.06	87.6±9.3	<0.05
HOMA-IR	1.93±0.59	4.52±2.01	<0.0001
ALT(UI/L)	11.6± 3.5	28.1± 10.5	<0.01
Creatinine mg/dL	0.58±0.08	0.7±0.9	<0.05
Uric Acid mg/dL	4.1 ± 1.5	6.5± 1.8	<0.02
CRP (mg/L)	5.8±0.24	6.9±1.01	<0.05
NADPH oxidase (RLU)	0.38±0.03	0.61±0.06	<0.01
FMD%	17.5±0.72	9.7 ±0.9	<0.001
IL-6 (pg/mL)	2.02±0.4	4.01±1.1	<0.05
TNF-α (pg/mL)	1.54±0.4	1.68±0.3	Ns
Leptin (ng/mL)	13.15±2.2	20.3± 8.9	<0.01
C peptide (ng/mL)	1.1±0.5	1.94±0.9	<0.05
Fibrinogen mg/dL	250±27	372±87.2	<0.01
Atherosclerotic index	2.54±0.7	3.68±0.9	<0.001

ALT – alanine aminotransferase, NADPH Oxidase represents respiratory burst, HOMA-IR homeostatic model assessment-insulin resistance

Table.3
CALCULATED SIGNIFICANT CORRELATIONS

correlations	Uric acid (mg/dL)	Waist circumference (cm)	Fasting C peptide (ng/mL)	HOMA-IR	Atherosclerotic index	DBP (mmHg)	TNF-α (ng/mL)	IL-6 (ng/mL)	NADPH oxidase (RLU)
FMD %	-0.47	-0.55	-0.40	-0.42	-0.37	-0.47	-0.43	-0.40	-
IL-6	-	-	0.41	0.43	-	-	0.77	-	-
TNF-α	-	-	-	0.38	-	-	-	0.77	0.49

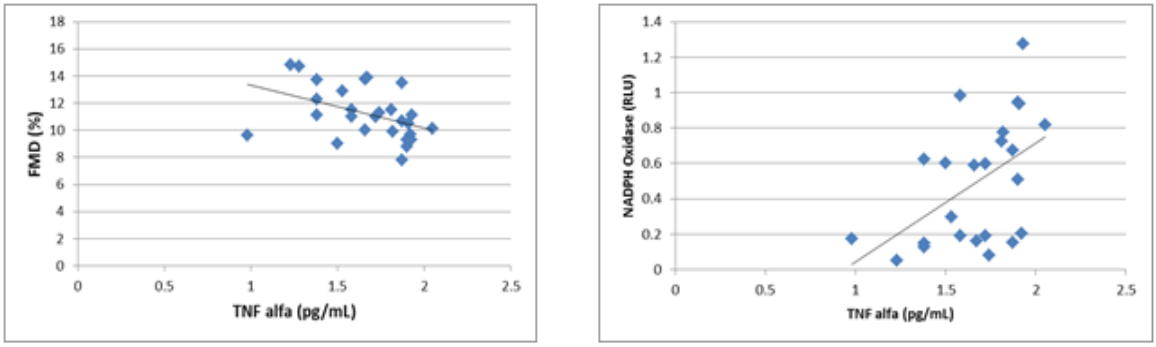

Fig.1. Correlations for TNF-α with FMD (left) and with Respiratory Burst (right)

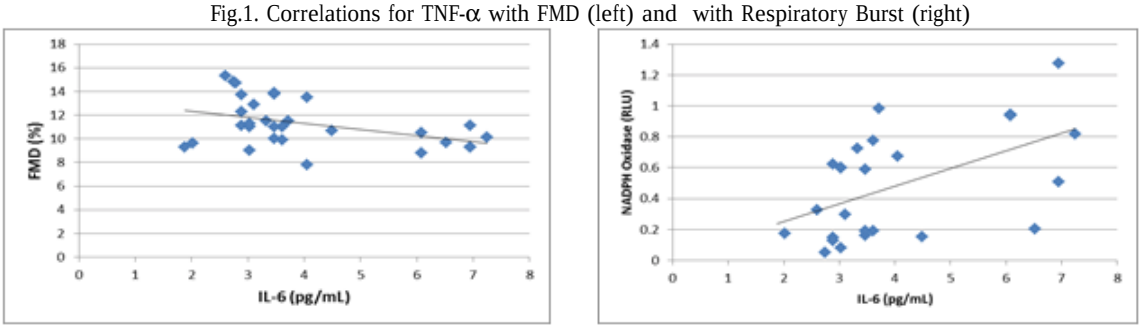

Fig.2. Correlations for IL-6 with FMD (left) and with Respiratory Burst (right)

consider atherosclerosis a chronic inflammatory disease [15, 16]. The process of atherosclerosis starts at an early age and it was described in obese children [17- 23]. FMD is a subclinical marker of atherosclerosis, it can be easily measured, is a non-invasive method suitable for teenagers and adolescents and is used more in research work and less in clinical practice [23]. The inflammatory markers like leptin, IL-6, CRP, fibrinogen are known to affect endothelial function either via direct or indirect mechanisms such as reducing NO production and stimulating inflammation-oxidative stress pathways [24, 25].

It was demonstrated that disturbed flow and high plasma levels for cytokines like IL1, IL8, TNF-α and ox LDL are triggers which can activate endothelial cells via NFkB. The secretion of adhesion molecule by the activated endothelial cells will be increased and more monocytes and proinflammatory cells will be recruited in the subendothelial space. The monocytes/macrophages, the smooth muscle cells and the activated endothelial cells act in cluster, increasing the oxidative stress and inflammation in the vessel wall [26].

The adipocytokine leptin controls food intake, influences growth, immunity and adapts the organism to obesity status. Its secretion from adipose tissue is higher in obesity and it stimulates the proliferation of monocytes and macrophages and the production of cytokines IL-6 and TNF-α, being a proinflammatory marker [27].

In our study, there weren't differences for the plasma TNF-α levels between obese versus lean children, but the obese children had higher plasma levels for IL-6 and leptin and lower FMD. Our results are in accordance with the most of the published data on this subject [28-30, 4].

Our study shows that cytokines are negatively correlated with FMD and positively with RB (fig.1 and fig.2) and underlines the link between inflammation and vascular dysfunction. In a study done in obese diabetic patients it was demonstrated a stronger correlation between TNF-α and RB, and this relation highlights the relation between oxidative stress and inflammation [31].

In obesity, the inflammation acts in tandem with insulin resistance. In our study we found negative correlations between FMD and the surrogate markers of insulin resistance, the C peptide and HOMA-IR. On the one hand insulin has anti-inflammatory effects and on the other hand, the cytokines like TNF-α and IL-6 are involved in insulin resistance by phosphorylation of the IRS at the serine residues. Also, there is a link between C peptide and vascular pathology [32]. In diabetic patients high values for C peptide were associated with macrovascular complications [33] and when C peptide is in excess, it produces undesirable effects, activating proinflammatory pathways.

The crosstalk between insulin resistance and inflammation can be realized by many pathways and one example is by FFA via activating JNK. The saturated FFA promotes inflammation by binding to the TLR-4 (Toll Like Receptor-4) and also, by increasing the synthesis of ceramide (via stimulating sphingomyelinase) which activates the TNF-α [34].

In a recent study, the researchers demonstrated that triglyceride-rich lipoproteins or remnants are responsible for inflammation associated with dyslipidaemia [35].

Overweight adolescents with increased CRP and IL-6 plasma levels have higher plasma saturated fatty acids and lower n-3 PUFAs concentrations versus lean adolescents[36]. In obese adolescents, low serum concentrations of polyunsaturated fatty acids were measured and they were associated with inflammation and vascular dysfunction. Intakes of total fat and antioxidant vitamins are determinants of subclinical inflammation in obese children [3]. High intake of long-chain PUFAs, especially Omega3 fatty acids are recommended in childhood obesity to prevent metabolic syndrome [37]. In obese adolescents the treatment with Omega 3 fatty acids improved the response to the reactive hyperaemia, reduced the level of monocytes and lymphocytes, IL-6 and TNF-α concentrations, improved the vascular function and the inflammation [38, 39]. In a recent study, done on adults with dyslipidemia, the researchers demonstrated, by using the FMD values, an impairment of endothelial function. After six months of treatment with Atorvastatin, an improvement of FMD was observed [40]. The beneficial effect of Statins may be due to many mechanisms and some of them are based on the antiatherosclerotic and on the antiinflammatory properties of these drugs [41]. In adults with essential hypertension and with coronary artery disease, it was demonstrated that the carotid intima media thickness, another marker of atherosclerosis, was correlated with the CRP values. This study underlines the importance of the systemic inflammation on vascular function [42]. Also, the importance of the tandem :oxidative stress and systemic inflammation, which influence the vascular function, was demonstrated in an experimental study, done on Wistar rats treated with oxytocin as an antioxidant [43].

In our study, the FMD was negatively correlated with blood pressure values, waist circumference, uric acid and atherosclerotic index. The negative correlation of FMD with uric acid is not a paradox if we take into consideration that uric acid can act as prooxidant when it has high levels. It can decrease nitric oxide bioavailability and increase lipid oxidation as it was demonstrated on cultured mouse adipocytes [44].

Conclusions

In this study we demonstrated that FMD was lower in the obese children versus the lean ones and negatively correlated with both blood pressure values (systolic and diastolic), with both cytokines (IL-6 and TNF-α) and with respiratory burst. These results are in consensus with the inflammatory hypothesis of atherosclerosis and underline that in the obese children the basic premise of inflammation in preatherosclerosis is present.

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