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DOCTORAL THESIS

SUPPORTIVE TREATMENT OF VASCULAR DYSFUNCTION IN PEDIATRIC SUBJECTS WITH OBESITY: THE OBELIX STUDY

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ABSTRACT

INTRODUCTION: Overweight and obese children develop abnormal endothelial cell dysfunction with increased vasomotor tone and inflammation. Curcumin, resveratrol, zinc, magnesium, selenium, soy, folic acid, and Vitamin D have shown potential beneficial effects on endothelial function. We test, among overweight and obese pediatric subjects, the effects on the endothelium of a combination of curcumin, resveratrol, zinc, magnesium, soy, folic acid, selenium, and Vitamin D.

METHODS: 48 subjects (6-17 years) were randomized into two groups (placebo Vs treatment) and attended three visits at 0, 3, and 6 months (+/- 15 days). Endothelial function was assessed through the Laser Doppler Flowmetry technique using "post occlusive release hyperemic test" (PORH) for estimation of Delta Flow (DF) and Hyperemic Area under Curve index (Hyperemic AUC index) and "heat provocation test" (HPT) to measure Delta Flow HPT" (DFHPT).

RESULTS: DF, Hyperemic AUC index and DFHPT represent recognized parameters for estimating endothelial dysfunction in both pediatric and adult age. Significant DF difference was noted at time-6-month in both groups ($p<0.001$). Overall time trend was significantly different between baseline, time-3-months, and 6-months both in Placebo ($p<0.05$) and Treatment ($p< 0.001$) groups and their comparison ($p < 0.001$). No differences were noted in Hyperemic AUC index (3 and 6 months), while there were significant differences in time trends of Treatment ($p< 0.001$) and Placebo ($p<0.05$) groups and their comparison ($p < 0.001$). DFHPT difference between groups was significant at 3 months and 6 months ($p<0.05$). The overall time trend was significant exclusively in "Treatment group" between time-3-month and 6-months ($p<0.05$). Correlation with anthropometrics was found for DF and BMI ($r=0.677$ at 6 months; $p<0.05$), as well as for Hyperemic AUC index and males ($r= 0.348$, $p<0.05$), while DFHPT showed no correlation.

CONCLUSIONS: Curcumin, resveratrol, zinc, magnesium, soy, folic acid, selenium, and Vitamin D appear to be promising in enhancing endothelial function by improvement of both DF in post-occlusive release hyperemia test and DF in the heat provocation test, potentially lowering the risk of developing cardiovascular diseases in overweight and obese pediatric subjects.

SECTION 1

- Background -

Childhood obesity

Obesity is a chronic condition characterized by excess body fat [Canoy, 2011; Puma, 2022]. It results from a long-term energy imbalance when daily energy intake exceeds expenditure [Canoy, 2011; Puma, 2022]. The increase in the prevalence of obesity in the past few decades appears to be primarily due to environmental changes that promote excessive food intake and discourage physical activity [Canoy, 2011; Puma, 2022]. Obesity is a complex multifactorial condition affected both by genetic and non-genetic factors and their complex interactions [Thomas-Eapen, 2021; Han, 2010]. Almost all childhood obesity is deeply influenced by environmental factors, either a sedentary lifestyle or a caloric intake that exceeds needs [Plachta-Danielzik, 2012; Ayala, 2021]. Environmental factors play a significant role in causing obesity and are important targets for intervention because they are potentially modifiable [Plachta-Danielzik, 2012; Ayala, 2021]. Increased intake of high-glycemic-index foods, sugar-containing beverages, and the spread of unhealthy dietary habits such as eating junk food, eating big portions, reducing time spent with family during meals, and poor nutritional value of school meals are all determinants for the development of pediatric overweight or obesity [Plachta-Danielzik, 2012; Anderson, 2010]. In fact, more evidence suggests that the consumption of artificially sweetened beverages, including fruit juice, is an essential contributor to the development of obesity in some individuals [Malik, 2013; de Boer, 2013]. Moreover, there is a correlation between early consumption of energy-dense foods and the prevalence of early overweight and obesity in children aged 1–5. On the other hand, the decline of physical activity in favor of television, computers, and video games has all been considered as causal influences on the rise in obesity [Ayala, 2021; Anderson, 2010]. Primary obesity is the most common form of obesity. On the other hand, secondary obesity involves different forms of obesity. These forms can be induced by specific endocrine (Cushing syndrome, hypothyroidism, and growth hormone deficiency), hypothalamic (panhypopituitarism, trauma, tumor, or inflammatory diseases), iatrogenic (steroid medication, psychoactive drugs, particularly olanzapine and risperidone, antiepileptic drugs and chemotherapeutics) or genetic causes (i.e., Prader-Willi and Bardet- Biedl syndromes) [Valerio, 2018]. In the diagnosis of obesity, anamnesis represents the first step. It involves the evaluation of possible treatable causes and potential comorbidities [Poorolajal, 2020; Williams, 2020; Puma, 2022]. The history should include the age of onset and some information about the child's eating and exercise habits. [Puma, 2022] The age of onset allows us to distinguish between overfeeding and

genetic causes of overweight since syndromic obesity often begins before two years of age. [Puma, 2022] Information about dietary and activity history may identify potential areas for intervention. [Puma, 2022] The dietary history should include information about the intake of foods rich in calories with poor nutritional value and eating patterns (timing, content, and location of meals and snacks), while activity history should investigate time spent performing physical exercise (school physical education (frequency, duration, and intensity, after-school, and weekend activities). [Poorolajal, 2020; Casazza, 2013] Family history should include information about obesity in first-degree relatives (parents and siblings). Obesity in one or both parents is a good predictor of a child's obesity persistence into adulthood [Dietz, 2005; Blair, 2007; Reilly, 2005]. The examination of the overweight child or adolescent should consider the assessment for dysmorphic features, which may suggest a genetic syndrome. Moreover, the evaluation of fat distribution, stature, height velocity, and a general physical examination must be considered [Dietz, 2005; Krebs, 2007]. The distribution of excess body fat may help distinguish between the aetiologies of obesity. [Puma, 2022] The excess fat in obesity derived from over-eating or overfeeding is usually concentrated on the trunk and periphery [Valerio, 2018]. In contrast, the "buffalo-type" distribution of body fat may suggest Cushing syndrome, although this fat distribution is sometimes also seen in physiologic obesity [Valerio, 2018]. Abdominal obesity (also called central, visceral, android, or male-type obesity) is associated with a lot of comorbidities, including metabolic syndrome, polycystic ovarian syndrome (PCOS), and insulin resistance [Gungor, 2014]. Measurement of waist circumference may help identify patients at risk for these comorbidities. [Puma, 2022] Assessment of stature and height velocity allows us to distinguish between exogenous obesity and obesity secondary to genetic or endocrine abnormalities [Styne, 2017]. Exogenous obesity is usually associated with high stature, so children with obesity are tall for their age [Styne, 2017]. By contrast, most endocrine and genetic causes of obesity are primarily associated with short stature [Taylor, 2012]. Height velocity may slow down in children with endocrine causes of obesity, including hypothalamic lesions. In addition, children affected by Prader-Willi syndrome are often short for their genetic potential or fail to have a pubertal growth spurt [Dietz, 2005]. A useful (but imprecise and unvalidated) screening tool for a possible endocrine cause of obesity is the combination of a weight above the 95th percentile for age and sex but a height below the 50th percentile, considering parental height [Styne, 2017; Taylor, 2012]. A comprehensive physical examination should usually last at least 30 minutes and should be complete. For example, striae and ecchymoses are typical manifestations of Cushing syndrome; striae

are much more likely to derive from the rapid accumulation of subcutaneous fat [Lau, 2013]. Moreover, acanthosis nigricans may underline T2DM or insulin resistance. Hirsutism may present in PCOS and Cushing syndrome [Lau, 2013; Sinha, 2007]. Abdominal tenderness may reveal a gallbladder disease [Huang, 2013]. Hepatomegaly could be caused by NAFLD [Krebs, 2007; Huang, 2013]. Undescended testicles, small penis, and scrotal hypoplasia may suggest Prader-Willi syndrome [Dietz, 2005]. Delayed or absent puberty may occur in hypothalamic-pituitary neoplasia, Prader-Willi syndrome, Bardet-Biedl syndrome, leptin deficiency, or leptin receptor deficiency [Green, 1989; Karra, 2008]. Many methods used to measure body adiposity directly are only sometimes available in clinical practice. Consequently, obesity is typically measured through the relationship between weight and height, which provides an accurate estimate of body fat [Canoy, 2011]. The body mass index (BMI) is a mathematical formula of the weight-for-height index; it is calculated by dividing body weight in kilograms by height in square meters (kg/m2). BMI shows a strict correlation with body adiposity and represents the universally accepted measure for overweight individuals and subjects with obesity [Gungor, 2014]. Like in adults and children from two years old, BMI is a recognized measure of overweight and obesity. [Gungor, 2014] BMI varies with age and gender during a lifetime. It typically rises during the first months after birth, falls after the first year, and increases again around the sixth year of life [Canoy, 2011]. Thus, a given BMI value is plotted on reference charts to obtain a BMI percentile for age and sex [Canoy, 2011]. The BMI percentile indicates the relative position of the child's BMI compared to a historical reference population of children of the same age and sex [Canoy, 2011] Gender-specific BMI-for-age percentile curves are typically used to define overweight and obesity [Gungor, 2014]. Children and adolescents with a BMI over the 85th but less than the 95th percentile for age and gender are considered overweight. On the other hand, children with a BMI exceeding the 95th percentile are considered obese [Gungor, 2014]. Children and adolescents with a BMI exceeding the 99th percentile are considered severely obese [Thomas-Eapen, 2021; Kansra, 2021]. The calculated BMI can sometimes be inaccurate because it does not quantify total body adiposity. It does not distinguish between fat tissue and muscle nor predict body fat distribution [Gungor, 2014]. Therefore, it may overestimate adiposity in a child with increased muscle mass, like in the case of an athletic child, and it may underestimate adiposity in a child with reduced muscle mass, like in the case of a sedentary child. [Gungor, 2014; Puma, 2022]

The consequences of childhood obesity

The recent childhood obesity epidemic has raised concern for its possible clinical and public health consequences [Buoncristiano, 2021; Puma, 2022]. Obesity increases the likelihood of developing cardiovascular diseases (CVD), type 2 diabetes mellitus (T2DM), non-alcoholic fatty liver disease (NAFLD), metabolic syndrome, PCOS, asthma, obstructive sleep apnoea syndrome (OSAS), orthopedic complications, psychiatric illnesses, and cancer in adulthood. With the increasing prevalence of pediatric obesity, clinicians have started identifying the same chronic risk factors in the pediatric population [Horesh, 2021]. The adverse consequences of childhood obesity have traditionally been divided into short-term (affecting the child) and long-term (involving the "future adult") **(tab. I)**.

Hypertension
Dyslipidemia
Insulin resistance
Prediabetes
Type-2 diabetes
Non-alcoholic Fatty liver disease
Metabolic syndrome
Cardiovascular diseases
Stroke
Asthma
Cancer
Social and psychological consequences

Tab. I Short and long-term consequences of childhood obesity

Short-term consequences can adversely affect nearly every organ system of children and are represented by hypertension, dyslipidemia, insulin resistance, prediabetes, T2DM, and fatty liver disease. They occur especially during adolescence [Horesh, 2021; Thomas-Eapen, 2021; Nicolaj, 2012; Caprio, 2020].

The most common comorbidity associated with childhood obesity is represented by hypertension. There are several possible pathophysiological pathways to explain why

adiposity is associated with elevated BP and hypertension. The most important factors are related to to the dysfunctional adipocyte and neurohormonal activation of the sympathetic nervous system (SNS). In the obese state, adipocytes are greater in number and size, and increasing amounts of adipokines are secreted. Over time, there is upregulation of pro-inflammatory adipokines. When the pro-inflammatory adipokines overwhelm the anti-inflammatory adipokines, this imbalance leads to adipose tissue dysfunction and a chronic inflammatory state. Many of these adipokines lead to an increase in SNS activity. Renin-angiotensin-aldosterone system (RAAS) activity is also increased. Increased oxidative stress is another significant contributor to obesity-related hypertension. [Brady, 2017] High blood pressure is an independent risk factor for CVD, and its prevalence in young individuals appears to be increasing along with rising obesity rates [Chen, 2008]. The risk of hypertension increases with the severity of obesity in obese children and adolescents [Friedemann, 2012; Skinner, 2015]. Hypertension during childhood tends to persist from childhood to adulthood [Chen, 2008] and often leads to the development of high blood pressure during adult age [Sun, 2007]. Similarly, obesity during childhood also predicts the development of hypertension during adulthood, but that risk disappears if the individual reaches adulthood with normal weight [Juonala, 2011; Buscot, 2018]. So this demonstrates that the early onset of risk factors for adult diseases in obese youth appears to persist into adulthood [Dietz, 1998].

Overweight children and adolescents often also have dyslipidemia. Specifically, in the obese state, there is increased inflammation with macrophages infiltrating the adipose tissue, and there is increased free fatty acid levels. Dyslipidemia, specifically elevated LDL-cholesterol and triglycerides and low HDL-cholesterol is frequently comorbid with obesity. Elevated cholesterol is a known CVD risk factor, but its contribution to elevated BP and hypertension is complex. In addition to causing atherosclerosis, elevated LDL-cholesterol induces chronic inflammation, activates the SNS, and increases RAAS activity. [Brady, 2017] Dyslipidemia is often present in children with a central fat distribution [Dietz, 1998; Harel, 2010]. These children often present high serum low-density lipoprotein (LDL)-cholesterol and triglycerides and low serum high-density lipoprotein (HDL)-cholesterol [Friedemann, 2012; Harel, 2010]. To summarize, childhood risk factors are represented mainly by obesity and dyslipidemia and are good predictors of adult cardiovascular disease [Srinivasan, 1996]. The Bogalusa Heart Study demonstrated that clinically recognized hypertension and dyslipidemia in adolescence usually persist into young adulthood and substantially impact multiple cardiovascular risk

factors, suggesting the importance of primary prevention early in life [Srinivasan, 1996].

Due to the epidemic of pediatric obesity, T2 Diabetes Mellitus (T2DM) has been transformed from a traditionally adult disease to a severe pediatric public health problem affecting children as young as six years old [Caprio, 2020; Aye, 2003]. Specifically, obesity affects the body's ability to use insulin, therefore causing abnormal blood glucose levels. [Galuska, 2020] Impaired glucose tolerance is a relatively common condition in obese children and adolescents, with a reported prevalence ranging from 15% to 20%. It is a significant predictor for the development of T2DM in adulthood [Sinha, 2002; Goran, 2004]. In a United States population-based study involving adolescents from 12 to 19 years old, prediabetes (defined as hemoglobin A1c >5.7 percent) was reported in 3 percent of subjects with class I obesity (BMI ≥95th percentile), 6 percent in those with class II obesity (BMI ≥120 to <140 percent of the 95th percentile), and in 13 percent of those with class III obesity (BMI ≥140 percent of the 95th percentile) [Skinner, 2015]. Even though only a small proportion of obese adolescents have been diagnosed with T2DM, its early diagnosis is mandatory because youth with T2DM appear to have a more rapid progression of diabetes-related complications concerning adult-onset T2DM. Whether prediabetes in an adolescent with obesity will progress to T2DM is not well established. In one small study, 25 percent of youth with prediabetes and obesity moved to diabetes over two years, while almost 50 percent reverted to normal glucose tolerance [Weiss, 2005].

Since the beginning of the past century, metabolic disturbances have tended to cluster together and represent risk factors for atherosclerotic cardiovascular disease in adults. [Gungor, 2014] These metabolic disturbances have collectively been referred to as “syndrome X” or “metabolic syndrome” and include the following key features: abdominal obesity, hyperglycemia, dyslipidemia, and hypertension [Gungor, 2014]. These abnormalities historically typical of adults have also been described in children and adolescents. Insulin resistance seems to be the major driving force of the development of the cardiovascular risk factors characteristic of the syndrome. Other factors such as local inflammation within relevant tissues and surrounding blood vessels feeding them and systemic subclinical inflammation may play a substantial role in the development of MS via inducing vaso-regulatory effects of local lipid deposits around blood vessels, which may contribute both to insulin action and endothelial dysfunction. In the presence of obesity, adipose tissue produces inflammatory cytokines in excess, whereas secretion of adiponectin is reduced highlighting the interplay between obesity and inflammation. The

metabolic syndrome is the result of multiple underlying factors, yet the syndrome identifies individuals at an elevated risk for accelerated atherosclerosis. [Gepstein, 2019] Identifying children at risk of developing metabolic syndrome remains an essential task because of the associated multiple cardiovascular risk factors and the evidence that the clustering of these conditions tends to persist through adulthood [Lee, 2012].

The most common cause of chronic liver disease in children in the US has become non-alcoholic fatty liver disease (NAFLD), along with the increasing frequency of obesity [Huang, 2013]. NAFLD is characterized by fatty liver infiltration not associated with alcohol consumption. NAFLD spans a wide range of severity, from mild steatosis to non-alcoholic steatohepatitis (NASH), that may ultimately result in advanced fibrosis, cirrhosis, and hepatocellular carcinoma. The prevalence of NAFLD is approximately 7 percent among children and adolescents but up to 34 percent among obese children (Anderson, 2015). The pathophysiology of childhood NAFLD is multi-factorial and includes complex interactions among hormonal, nutritional, genetic, and environmental factors that may begin in utero. Initially, NAFLD involves hepatic steatosis, which comprises lipid accumulation arising from excessive influx of fatty acids from endogenous fat depots, excess consumption of dietary fat, and hepatic de novo lipogenesis. (Mandala, 2020) Because NAFLD is usually asymptomatic, screening is required for its detection. NAFLD can be diagnosed with non-invasive methods. Specifically, fibrosis and fatty infiltration are routinely detected with ultrasounds or magnetic resonance imaging (Gungor, 2014).

Moreover, childhood obesity impacts children's and adolescent's social and emotional status. Obese children often experience bullying and other difficulties, such as negative stereotypes, discrimination, and social marginalization (Smith, 2020). Discrimination against obese individuals has been found in adolescents and children as young as two years old [Budd, 2008]. Overweight and obese children are often excluded from social activities, especially from competitive activities that are physically demanding, as they tend to be slower than their peers and often present with early shortness of breath. [Puma, 2022] These negative social factors in children contribute to low self-esteem, low self-confidence, and a negative self-body image and can also affect their academic performance [Bacchini, 2015]. All the above-mentioned adverse effects of being overweight and obesity can sometimes devastate children and adolescents [Budd, 2008]. The social consequences of obesity may contribute to persistent difficulties in weight control [Bacchini, 2015]. Overweight and obese children tend to protect themselves from

society by retreating to safe places, such as their homes, where they may seek comfort in food [Sahoo, 2015]. In addition, overweight children tend to have fewer friends than normal-weight children, which results in fewer social interactions and play, and, therefore, more time spent doing sedentary activities [Sahoo, 2015; Niehoof, 2009].

Long-term adverse effects of childhood obesity are now well established. Anyway, not all obese children will become obese adults. However, most of them present this risk. [Avila, 2003] Childhood obesity typically persists into adult age, particularly in children with an obese parent [Gordon- Larsen, 2010]. Among obese 6-year-old children with an obese parent, almost 50 percent become obese as adults, and among obese 10-to-14-year-old children with an obese parent, 80 percent remained obese as adults [Liberali, 2020]. Adult obesity is associated with an increased risk of several important health conditions, including heart disease, type 2 diabetes, and cancer. Therefore, if children have obesity, their obesity and disease risk factors in adult age are likely to be more severe [Mc Mullen, 2014; Bass, 2015]. Specifically, being overweight and obese during childhood and adolescence result in adverse outcomes regarding chronic disease risk in adulthood [Horesh, 2021]. Childhood obesity tends to persist into adulthood, and so do the cardiovascular effects of obesity. Mossberg [1999] demonstrated that excessive overweight during adolescence was associated with higher-than-expected morbidity and mortality in adult life. This evenience seems more probable for males than females Therefore, a strong link between childhood obesity and childhood overweight is persistently associated with increased risk of type-2 diabetes, hypertension, CHD, and mortality in adulthood [Horesh, 2021]. Data on a possible relationship between stroke outcomes, adult-onset asthma, or cancer are inconclusive [Park, 2012; Mc Mullen, 2014; Horesh, 2021].

The endothelial dysfunction in the context of childhood obesity

The endothelium is the natural inner lining of the vessels known to regulate and coordinate the vascular and organ integrities [Dhananjayan, 2015]. The layer is composed of the tunica intima, media, and adventitia. Specifically, tunica intima consists of endothelial cells. Tunica media is formed by the vascular smooth muscle cells (VSMC). [Kwaifa, 2020] Tunica adventitia is the elastic lamina of terminal nerve fibers around connective tissues. Usually, the endothelium regulates vascular homeostasis through coordinating blood flow, distribution of nutrients, hormones, and other macronutrients, and migration and proliferation of VSMC. Specifically, VSMC reduces vascular tone, regulates cellular and vascular adhesions, controls coagulation and fibrinolysis activities, inhibits leukocyte adhesions, and modulates inflammation and angiogenesis [Kwaifa, 2020]. The bioactive substances secreted by endothelial cells regulate the structures and functions of the blood vessels through the balance between inflammatory and anti-inflammatory factors, oxidative and anti-oxidative agents, proliferative and antiproliferative agents of VSMCs, dilations, and contractions of the vasculature, blood coagulation, and fibrinolytic system [Mussbacher, 2019]. The secretion and release of endothelial-derived vasodilating and vasoconstricting factors are balanced under normal physiological conditions [Kwaifa, 2020].**(fig.1)**

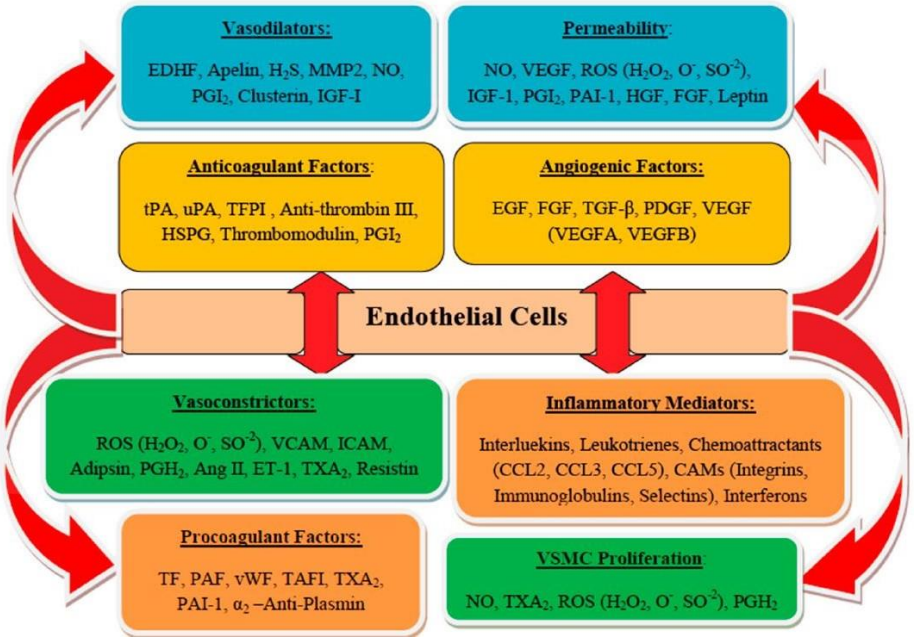

Fig.1. Functions of endothelial cells and role of vascular endothelium [Kwaifa, 2020]

It is known that obesity increases the risk of the child developing cardiovascular disease in adult age [Tirosh, 2011; Bruyndonckx, 2016]. Endothelial dysfunction is the primum movens in the pathogenesis of atherosclerosis, appearing long before clinical symptoms arise. So, it can be qualified as a surrogate endpoint for cardiovascular disease risk [Bruyndonckx, 2016]. In obesity-induced atherosclerosis, the delicate balance between vasodilating and vasoconstricting factors is not respected, further promoting the progression of vascular endothelial dysfunction and, consequently, organ damage [Sena, 2013]. Specifically, endothelial dysfunction is usually characterized by the imbalance in the secretion and release of vasoconstriction and vasodilation agents. [Chhabra, 2009] **(tab. II)**

Vasodilators
Nitric oxide
Bradykinin
Prostacyclins
Endothelium-derived hyperpolarizing factor
Histamine
Substance P
Serotonin
Vasoconstrictors
Angiotensin II (AII)
Thrombin
Serotonin
Prostaglandin H2
Arachidonic acid
Endothelin (ET-1)
Thromboxane A2
Growth Promoters
Platelet derived growth factor (PDGF)
Basic fibroblast growth factor (PGF)
Insulin-like growth factor – I (IGF-I)
Endothelin (ET1)
Angiotensin

Growth Inhibitors
Nitric oxide
Transforming growth factor I (TGFB)
Prostacyclins
Bradykinin
Heparin sulfate
Adhesion molecules
Endothelial leukocyte adhesion molecule; (ICAM)
Intercellular adhesion molecule
Vascular cell adhesion molecule (VCAM)
Thrombolytic factors
Tissue-type plasminogen activator
Thrombomodulin
Plasminogen activator inhibitor-1 (PAI-I)

Tab. II. Endothelium derived molecules involved in the vascular homeostasis [Chhabra, 2009]

This event predisposes the endothelium towards prothrombotic and proatherogenic factors. Specifically, the defective endothelial physiological properties result in the production of pro-oxidation mediators. The loss of endothelial balance brings in endothelial dysfunction and atherosclerosis [Sena, 2013]. Specifically, the essential mechanisms attributed to the progression of endothelial dysfunction in obesity are represented by elevated levels of triglycerides and LDL inflammatory factors and oxidative stress radicals. In addition, an imbalanced hemodynamic activity is involved. **(tab. III)**

Elevated levels of LDL
Elevated levels of triglycerides
Increased oxidative stress radicals
Elevated levels of inflammatory factors
Imbalanced hemodynamic activity

Tab. III. Fundamental factors attributed to the progression of endothelial dysfunction

The inflammation of the endothelium environment is the initial stage of vascular dysfunction,progressing to vascular disease related to obesity [Kwafa, 2020]. Some factors promote atherogenesis in obesity: hyperglycemia, LDL, Ang II. These factors facilitate the activity of NF-kB and MAPKs in endothelium leading to the stimulation of proinflammatory cytokines, chemokines, activation of iNOS, growth factors, increased synthesis of ICAM-1 and VCAM-1, and other enzymes [Xia, 2014]. These responses stimulate the production of interleukins, including IL-18 and IL-1. These interleukins facilitate the progression of inflammation through the activation of proinflammatory signaling complexes of the inflammasomes and oligomerization domain-like receptor family pyrin domain containing 3 (NLRP3) [Rathinam, 2016]. **(fig.2) (fig.3)**

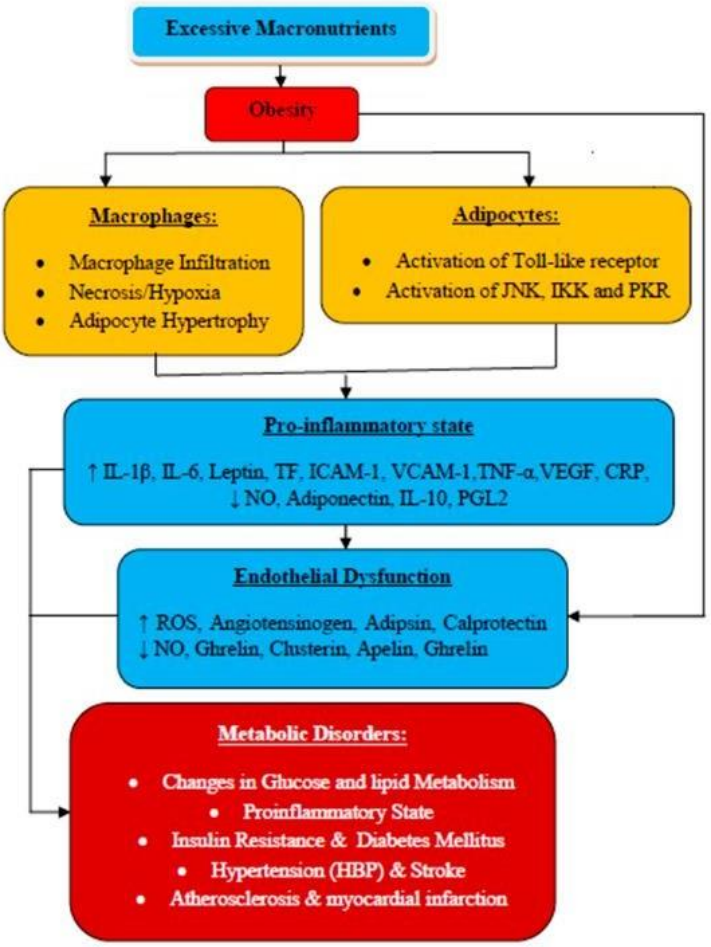

Fig.2. Linking mechanism between obesity, inflammation and endothelial dysfunction [Kwaifa, 2020]

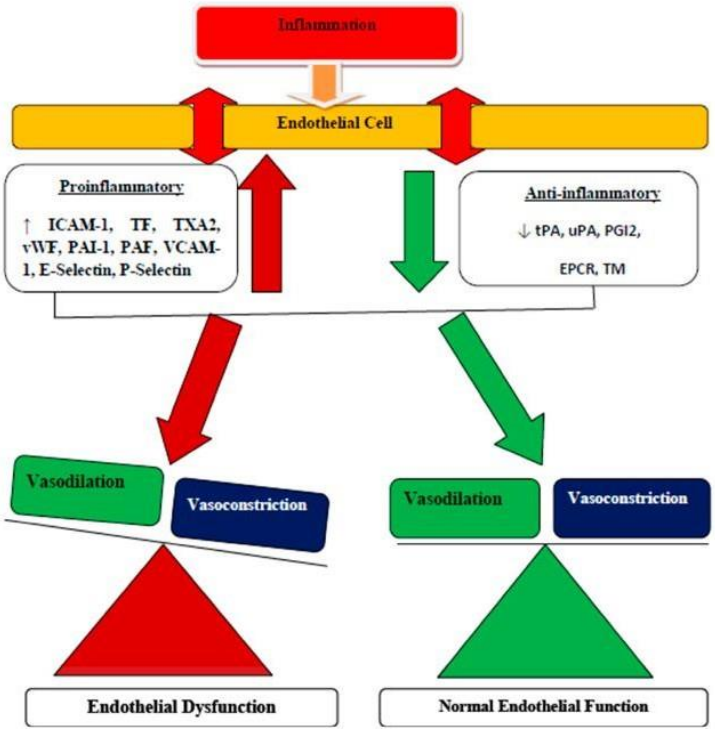

Fig. 3. Mechanisms of endothelial dysfunction associated with inflammation [Kwaifa, 2020]

The primary role of the endothelium is strictly controlling the blood vessel diameter [Bruyndonckx, 2016]. The endothelial nitric oxide synthase is activated in endothelial cells. It produces nitric oxide, which diffuses into the vessel wall to fine-tune vasodilation [Kwaifa, 2020; Dimmeler, 1999] in response to stimuli for increased blood flow demand. Nitric oxide is considered a key regulator of endothelial function, but many other factors are involved [Kwaifa, 2020; Flammer, 2010]. Endothelium close is near the blood circulation. It exposes endothelial cells to many damaging factors [Bruyndonckx, 2016]. These factors harm endothelial cells. It causes endothelial dysfunction, that is, an imbalance between vasodilating and vasoconstricting substances produced by endothelial cells [Deanfield, 2007]. Damage to endothelial cells can also upset vascular smooth cell function in obese children [Ciccone, 2011]. Endothelial dysfunction is referred to macrovascular endothelial dysfunction, and it is demonstrated in the significant conduit arteries of obese children [Tounian, 2001]. The dysfunction of small resistance vessels is related to microvascular endothelial dysfunction and precedes the development of macrovascular endothelial dysfunction [Montero, 2012; Montero, 2014; Jung, 2009]. Multiple cardiovascular risk factors are present in childhood obesity

and negatively affect endothelial function [Aggoun, 2007]. So, endothelial dysfunction represents an excellent surrogate marker for early cardiovascular disease because it summarizes the cumulative burden of risk factors [Bruyndonckx, 2016]. In obese children, the risk factors involve both classical cardiovascular risk factors and cytokines and signaling molecules, including micro-RNA (miRNAs) [Bruyndonckx, 2013; Bruyndonckx, 2016]. **(fig.4)**

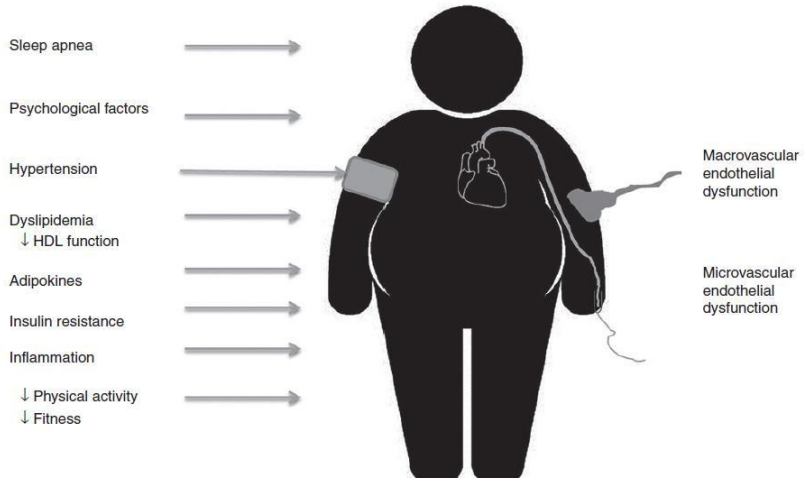

Fig.4 Determinants of obesity-related endothelial dysfunction in obese children [Bruyndonckx, 2016]

Hypertension during adolescence can lead to severe vascular endothelial dysfunction in adult life [Juonala, 2006; Falaschetti, 2020]. Obesity is strongly associated with hypertension in prepubertal children [Bruyndonckx, 2016]. Specifically, obese prepubertal children demonstrate a better functional capacity of their endothelium than normal-weight normotensive children [Charakida, 2006]. Obese children develop an early vascular adaptive response to increased blood flow demands [Bruyndonckx, 2016]. Radtke et al. [2013] demonstrated it by performing a cold pressure test to measure the change in blood pressure in response to stress in children without known cardiovascular risk factors. Children with an increased risk of hypertension had greater endothelial capacity. The endothelium can elicit an adaptive response to stress in obese children. It could also explain why six months of exercise training does not improve endothelium-dependent flow-mediated dilation of the brachial artery in these subjects [Farpour-Lambert, 2009], while many studies have demonstrated positive effects of training on

endothelial status in children after puberty [Dias, 2015]. It is reasonable to think that the impact of hypertension on endothelial dysfunction is influenced by pubertal development. Dyslipidaemia is another factor in impaired endothelial function in obese children [Bruyndonckx, 2016]. Elevated LDL cholesterol is rarely observed in obese children but is often seen in obese adults [Bruyndonckx, 2013]. HDL cholesterol is associated with a reduction in cardiovascular risk in adult populations [Castelli, 1986]. Moreover, HDL's actions include its anti-oxidative and anti-inflammatory properties [Bruyndonckx, 2016], and its function is impaired in childhood obesity [Matsuo, 2013]. HDL is less capable of stimulating endothelial nitric oxide synthase activity and, thus, endothelial function in childhood obesity [Muller, 2013]. In addition, HDL is a significant carrier of miRNAs [Omran, 2012]. miRNAs are small noncoding RNA molecules regulating the expression of protein-coding genes. Uncovering miRNAs relating to the first signs of endothelial maladaptation could allow earlier identification of obese children at increased cardiovascular risk [Omran, 2012]. In addition, Riedel et al. [2015] demonstrated that exercise could thrive HDL to induce a more proangiogenic miRNA profile in endothelial cells in adults. This evidence is not certain for children. On the other hand, it is known that physical activity strongly correlates with endothelial function in children aged 5-10 [Abbott, 2002]. Anyway, no correlation between an endothelial function with physical inactivity is observed in adolescents [Radtke, 2013]. It is essential to underline that the adipose tissue is an endocrine organ, and adipocytes secrete a vast array of cytokines called adipokines [Kershaw, 2004]. Macrophages invade hypertrophic adipose tissue. It results in the upregulation of adipocyte adhesion molecules. This process leads to the diapedesis of monocytes. It initiates a vicious circle of adipogenesis and inflammation [Hajer, 2008]. A lot of adipokines have a direct effect on endothelial function. [Park, 2001; Torigoe, 2007]. Moreover, childhood obesity and inflammation lead to oxidative stress because they lower the antioxidant capacity and induce the generation of reactive oxygen species [Araki, 2010, Codoñer-Franch, 2011]. Sleep apnoea is frequent in obese children [Verhulst, 2008] and is involved in the impairment of endothelial function [Li, 2013]. The link between childhood obesity, sleep apnoea, and inflammation is related to the serum levels of pentraxin-3, a biomarker of cardiovascular risk [Garlanda, 2011]. A relationship between endothelial dysfunction and insulin resistance causes a vicious cycle, leading to renal, metabolic, and cardiovascular diseases [Kim, 2006]. Psychological psychosocial distress is frequent in childhood obesity [Bruyndonckx, 2016]. Moreover, it is known that scores for depression, anger, and anxiety are negatively correlated with endothelial function also in healthy children [Osika, 2011]. It is necessary

to step up and improve our understanding of the mechanism of childhood obesity-related endothelial dysfunction to prevent an increase in the prevalence of the cardiovascular disease. Its assessment through some non-invasive methods can represent a useful tool in children helping realize translational and clinical studies.

The instrumental assessment of Endothelial Dysfunction in Childhood Obesity

There are no clear recommendations regarding assessing the structure and function of the vascular system in children. Many techniques were developed, deepening the structure and function of the arteries and endothelial function [Skrzypczyk, 2017]. Regarding the function and structure of the arteries, carotid intima-media thickness (cIMT) deepens arterial structure. On the other hand, arterial function can be assessed through arterial stiffness [Skrzypczyk, 2017]. Finally, endothelial dysfunction can be evaluated through flow-mediated dilation, peripheral arterial tonometry, and laser Doppler flowmetry techniques [Skrzypczyk, 2017].

The laser Doppler flowmetry will be deepened because it is the subject of this doctoral thesis.

Laser Doppler flowmetry

In children, the endothelial function can be assessed through the laser Doppler flowmetry (LDF) technique. [Urbina, 2008] **(fig. 5)**

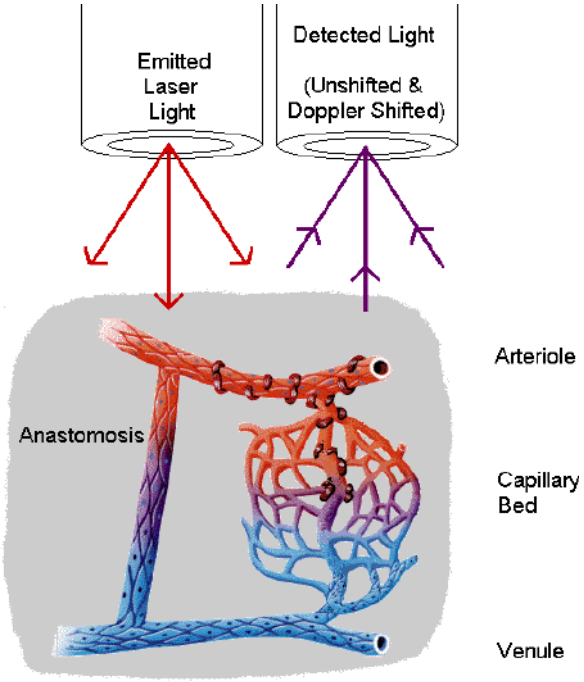

Fig. 5. Basic principles of LDF [http://www.biopac.com/Manuals/laser-doppler-flow.pdf]

It monitors the perfusion by measuring microvascular red blood flow using the Doppler principle [Leahy, 1999]. Specifically, the laser Doppler signal is proportional to the velocity and the number of moving blood cells in the skin [Hedval Kallerman, 2013]. LDF determines the magnitude of perfusion at rest (resting flow), at occlusion (biologic zero discharge), and peak post-flow occlusion **(fig. 6)**.

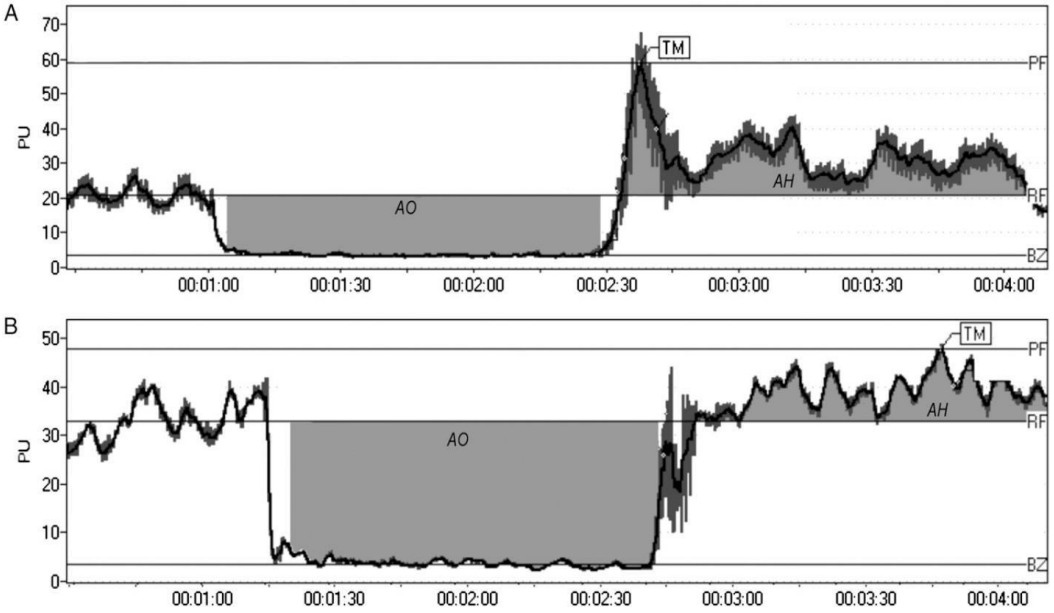

Fig. 6. Results of representative cuff occlusion tests. A: normal endothelial function. B: abnormal endothelial function. AH: area of hyperemia; AO: area of occlusion; BZ: biologic zero; PF: peak flow; PU: perfusion unit; RF: rest flow; TM: time to peak flow after the occlusion. Time unit is represented by minutes and seconds. [Wahlberg, 1995]

All measurements are extrapolated to the baseline perfusion. The analysis of reperfusion kinetics is based on the measurements over time. Therefore, a lot of parameters can be extrapolated. Tmax seems to be the best index of the endothelial function because it indirectly determines post-occlusion hyperemic response [Wahlberg, 1995] LDF has adequate reproducibility when performed in a controlled environment with a standardized protocol [Hedval Kallerman, 2013]. Cutaneous blood flow is measured in conventional perfusion units (PU) using a specific system (Periflux PF 6000, Perimed, Stockholm, Sweden) equipped with a thermostatic LDF probe with an effective surface

of 0.95 cm2 on the volar surface of the left forearm [Leahy, 1999; https://www.perimed.it/content/periflux- 6000-laser-doppler/; Accessed 22/10/2022]. **(fig. 7)**

Fig. 7. Periflux 6000® [https://www.perimed.it/content/periflux-6000-laser-doppler/; Accessed 22/10/2022]

LDF probes are maintained at a constant temperature of 33ºC throughout the measurement. During this test, the child must stay on a bed with the upper extremity positioned at 45°. A cuff was placed around the medium third of the forearm to occlude the radial and ulnar arteries. At the same time, the hand was gently immobilized, and the probe was positioned over the volar aspect of the arrow at the 1st finger distal metacarpal surface to minimize the occurrence of motion artifacts. All tests were performed with a laser Doppler sensor. As NO-dependent mechanisms are involved in the prolonged phase of vasodilation caused by local heating and reactive hyperemia, another application of LDF is assessing the local hyperthermia response, heating the probe to 42ºC for 5 minutes [Kellogg, 2009]. Using this approach, the abnormal endothelial function has been shown in pediatric patients with type 1 diabetes [Shah, 2015]. Moreover, it was demonstrated that obese children without comorbidities have significantly impaired microvascular endothelial function [Hedval Kallerman, 2013].

Potential role of antioxidants in the context of Endothelial Dysfunction in Childhood Obesity

Systemic inflammation and oxidative stress are exacerbated in several chronic diseases. It is known that the onset of obesity at pediatric age is associated with developing other cardiovascular risk factors. [Stoppa-Vaucher, 2012] It is associated with a proinflammatory and prothrombotic state. [Muras 2010; Stoppa-Vaucher, 2012]. Hypertension represents a known consequence of childhood obesity. It derives from the alterations of the endocrine determinants, such as corticosteroids and adipokines, altered sodium homeostasis, sympathetic nervous system activity, inflammation, oxidative stress, and endothelial dysfunction. [Mocnik, 2021] Morandi et al [2020] demonstrated that high systolic blood pressure and pulse pressure are related to systemic oxidative status in childhood obesity. Specifically, serum total anti-oxidant capacity (TAC) was measured through the use of a commercial kit (Sigma-Aldrich). Starting from the prediction of systolic blood pressure using TAC, z-BMI, and diastolic blood pressure, this cross-sectional study demonstrated that serum TAC levels and z-BMI were inversely related with systolic blood pressure and pulse pressure. Moreover, low-grade systemic inflammation and endothelial activation could influence elevated blood pressure early in life in childhood obesity [Syrenicz, 2006]. Additionally, T-helper cells are activated before the onset of clinical indicators of target organ damage in childhood obesity [Övünç Hacıhamdioğlu, 2015]. In addition, an increased risk of developing dyslipidemia, hepatic cholestasis, diabetes mellitus, and metabolic syndrome is related to the excess fat in childhood obesity [Aburawi, 2019]. Specifically, metabolic syndrome has been associated with a higher incidence of cardiovascular disease and all-cause mortality during adulthood [Al-Shorman, 2017]. Cardiovascular risk is also increased by a high waist circumference in children affected by obesity and metabolic syndrome [Jung, 2009]. In addition, childhood obesity is related to endothelial dysfunction. It predisposes to multi- organ disease involving cardiovascular, pulmonary, muscular, and nervous system [Radman,2020; Sorop, 2017] **(fig. 8) (fig. 9)**

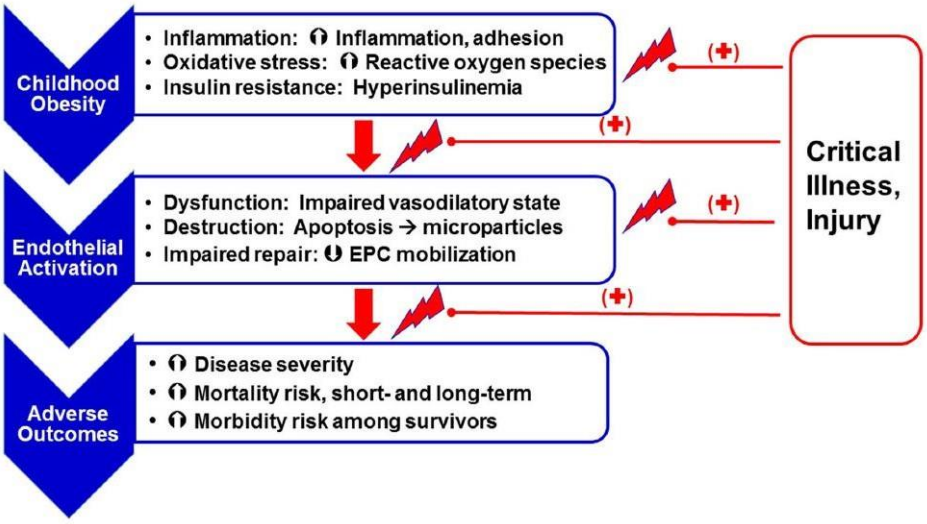

Fig. 8. Factors involved in the chronic endothelial stress and adverse outcomes followingcritical illness in obesity. [Radman, 2020]

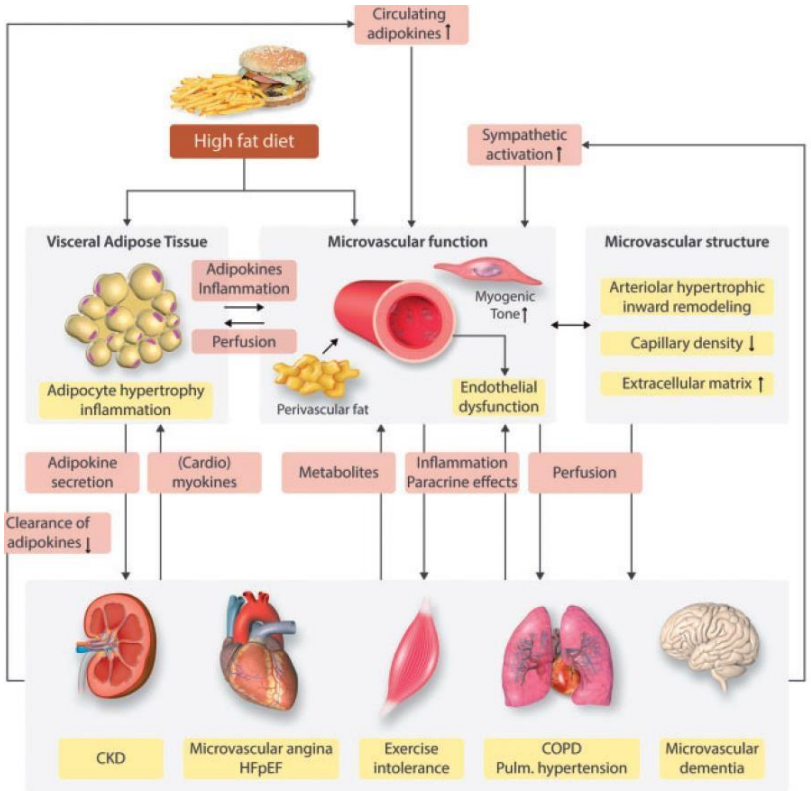

Fig. 9. Proposed mechanisms of obesity-related microvascular dysfunction predisposing tomulti-organ disease. [Sorop, 2017]

Anyway, childhood obesity is related to an increased oxidative stress [Hertis-Petek, 2022]. It is difficult to measure it. In fact, there are not standardized and demonstrated markers. The measurements of protein and lipid oxidation products represents a possibility [Codoñer Franch, 2012]. Specifically, the oxidized low-density lipoprotein concentration is elevated inchildhood obesity before the detection with instrumental methods [Okur, 2013]. **(fig. 10)**

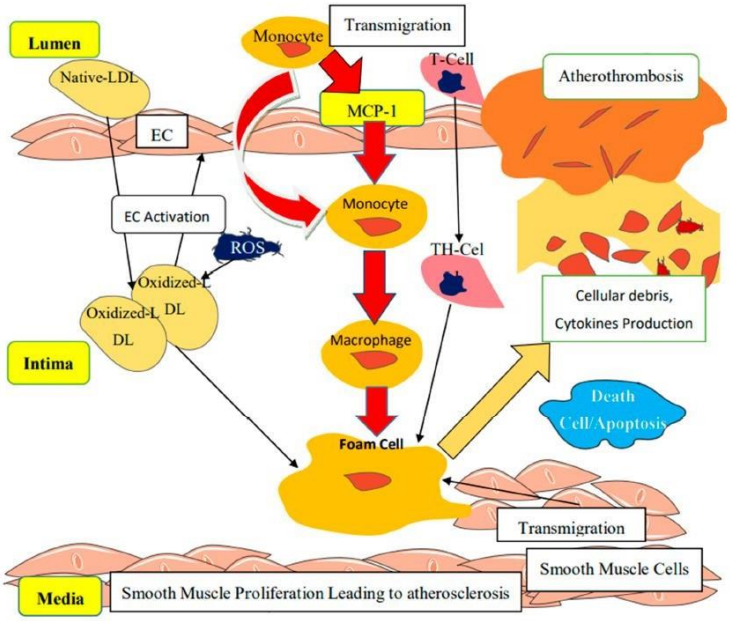

Fig. 10. Molecular Mechanisms Linked to the Progression of Atherosclerosis [Kwaifa,2014]

The measurement of plasma antioxidants represents another potential tool. [Otani, 2013] **(fig. 11)**

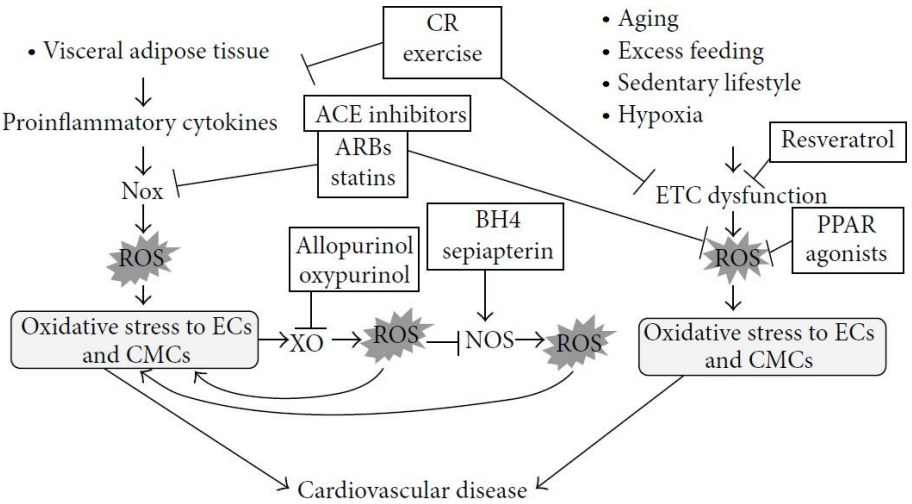

Fig. 11. Potential strategy of the site-specific antioxidative therapy to potentially prevent cardiovascular disease [Otani, 2013]

Thiol/disulphide is impaired in obesity, indicating its contribution to oxidative stress and inflammation [Elmas, 2017].

Nitric oxide represents another potential laboratory instrument. Specifically, it increased with fat accumulation and translated into higher values of cardiometabolic risk markers in children [Correia-Costa, 2016].

In addition, it is demonstrated that polyamines, derived from arginine (precursor of nitric oxide), are higher in childhood obesity [Codoñer-Franch, 2011].

Chemerin, a chemoattractant protein, is over-expressed in obesity, suggesting an association between endothelial activation markers and inflammatory status and obesity [Niklowitz, 2018]. It was also associated with increased systolic pressure in obese children [Wójcik, 2020].

It is demonstrated that catestatin, adipokines (including leptin), and interleukin-6 play a role in the inflammatory, atherogenic, and insulin-sensitizing systems. These factors are related to the development of cardiovascular complications of obesity, even in children and adolescents [Hertis-Petek, 2022].

Hypovitaminosis D is related to obesity. Specifically, increased oxidative stress, inflammation, and endothelial activation markers are demonstrated in children affected by obesity and vitamin D insufficiency [Codoner-Franch, 2012]. Vitamin D supplementation in children affected by overweight or obesity is controversial. It did not

affect arterial endothelial function measures, stiffness, or systemic inflammation. At the same time, it reduces blood pressure and fasting glucose concentration and improves insulin sensitivity. Therefore, its role in lipid profile is controversial [Rajakumar, 2020; Pecoraro, 2022].

Improving the antioxidant defense through fat volume reduction decreases oxidative stress [Hertis-Petek, 2022]. Children with cardiovascular risk have increased oxidative stress and reduced antioxidants [Kapiotis, 2006]. So, antioxidant supplementation can potentially contrast oxidative stress, and its use could positively affect cardiovascular health.

Potential useful antioxidants in the context of Endothelial Dysfunction in Childhood Obesity

The visceral fat accumulation in childhood obesity is associated with chronic oxidative stress and excessive production of proinflammatory adipokines. These events contribute to low-grade chronic inflammation and a pro-oxidant state. Consequently, an antioxidant supplementation could potentially reduce the oxidative state and improve endothelial function [Hertis-Petek, 2022].

Curcumin

Curcumin is a scavenger of reactive oxygen species. Its role enhances the activity of antioxidants. Curcumin is the most active component of the curcuminoids extracted from Curcuma longa L. It protects against cellular inflammatory responses and oxidative stress in endothelial damage and vascular complications [Sun, 2016; Kokaadem, 2017]. Specifically, curcumin supplementation significantly increased flow-mediated dilatation. [Hallajzadeh, 2019] Promising results in inflammatory responses and inhibition of advanced glycation end product-induced oxidative stress and endothelial cell damage was demonstrated [Kheirandish-Gozal, 2010].

Resveratrol

It is demonstrated that resveratrol has an antioxidant effect and beneficial effects on endothelial function. [Breuss, 2019] It increases NO synthesis, and this event plays an antioxidant function on the endothelium [Van der Spy, 2009; Das, 2006; Xia, 2017]. It is contained in red wine. Its consumption is positively related to some processes involved in vascular dysfunction [Van Bussel, 2018]. Specifically, its supplementation can significantly increase flow-mediated dilatation. [Abkari, 2019]

Zinc

Zinc has antioxidant-like properties in activated endothelium cells [Hennig, 1999]. Zinc

is a protective and critical nutrient for maintaining endothelial integrity [Hennig, 1999]. It is also demonstrated that zinc deficiency is associated with a reduced vasodilator response in animal models [Mendes Garrido, 2018]. Its role seems to be related to the attenuation of tumor necrosis factor-mediated activation of the upregulation of inflammatory cytokines in endothelial cells [Connell, 1997]. Moreover, humans zinc supplementation improves diabetic endothelial dysfunction [Liu, 2020]. Besides, there is a relationship between prenatal and neonatal zinc deficiency and vascular dysfunction. [Mendes Garrido 2018; Liu 2020] About obesity, the accumulation of visceral fat is associated with a state of chronic oxidative stress and excessive production of proinflammatory adipokines, which contributes to a low-grade chronic inflammation state that can be attenuated with zinc supplementation [Abdali, 2015]

Magnesium

Magnesium is involved in the endothelium function [Lopez Juonor, 2019]. Magnesium supplementation significantly improved flow-mediated dilation and pulse wave velocity [Marquez, 2020]. Moreover, the hard water's magnesium content seems to protect against cardiovascular diseases [Gianfredi, 2017]. In addition, oral Mg supplementation could improve endothelial function for at least six months in unhealthy, overweight, or older individuals [Marques, 2020]. About obesity, visceral fat accumulation is associated with chronic oxidative stress and excessive production of proinflammatory adipokines, which contributes to a low-grade chronic inflammation state that can be attenuated with magnesium supplementation. [Oliveira, 2017]

Selenium

Selenium is involved in endothelial function [Lopez Juonor, 2019]. Specifically, selenium and seleno protein are associated with endothelial cytoprotection [Lopez Juonor, 2019] and a role in endothelium activation biomarkers [Zhang, 2016]. Selenium seems to promote the activities of a vitamin group of enzymes that form free radicals preventing damage to cells and tissues. [Harthill, 2011] The association between selenium deficiency and hypercholesterolemia promotes endothelial dysfunction [Raij, 1993]. Moreover, there is an association between selenium deficiency and increased risk of morbidity and

mortality [Lopes, 2019]. Selenium supplementation has been shown to significantly reduce the risk of cardiovascular mortality in patients with hypertension, diabetes, and ischemic heart disease [Alehagen, 2018]. About obesity, the accumulation of visceral fat is associated with a state of chronic oxidative stress and excessive production of proinflammatory adipokines, which contributes to a low-grade chronic inflammation state that can be attenuated with magnesium supplementation [Oliveira, 2017]

Vitamin D

Among several functions, Vitamin D deficiency is associated with endothelial dysfunction [Kim, 2020]. Specifically, it shows anti-inflammatory effects through the release of IL-6 and suppression of TNF-alpha [Ebihara, 1996]. In addition, vitamin D reduces cellular damage from oxidative stress and stimulates the Nrf2 pathway of intranuclear signal transduction, which facilitates the synthesis of anti-inflammatory cytokines. [Lan, 2014] Vitamin D insufficiency is associated with endothelial dysfunction and increased arterial stiffness [Al Mheid, 2011]. In addition, vitamin D levels are inversely associated with increased arterial stiffness in a normative aging population [Gialluria, 2013]. Its supplementation is associated with decreased vascular dysfunction in patients with chronic kidney disease [Dou, 2019] and improved NO-dependent arteriolar vasodilation in obese adults [Mahmoud, 2019].

Soy and Flavonoids

Soy shows antioxidant properties and could have a role in quenching reactive oxygen species. [Rizzo, 2020] Flavonoids are extracted from Bidens bipinnata and are known for their antipyretic, anti-inflammatory, and antirheumatic effects in Chinese medicine. These antioxidants have their effect by inhibiting the production of inflammatory cytokines. A possible therapeutic role in Henoch–Schönlein purpura was described [Bo, 2012].

Folic acid

Folic acid has potent antioxidant, cardiovascular, anticancer, and neuroprotective effects [Asbani, 2021]. Its antioxidant activity is mediated through multiple mechanisms, with a reduction of ROS formation. Anyway, there are controversial data on folic acid supplementation's antioxidant capacity. [Asbani, 2021] Weak evidence suggests that folic acid supplementation causes a significant rise in serum concentrations of glutathione and total antioxidant capacity but seems not to affect NO. Due to its function as a cofactor for glutathione-related enzymes or its antioxidant properties in combination with other dietary antioxidants, folic acid could improve oxidative stress status [Senorer, 2019].

Other antioxidants

Other antioxidants can potentially contribute to the diminution of the oxidative state and improve endothelial function in selected conditions.

Other antioxidants are represented by polyphenols, vitamin C, vitamin E, vitamin K, coenzyme Q-10, L-arginine, Melatonin, and Alpha-Lipoic Acid [Hertis- Petek, 2022].

Polyphenols have antioxidant activity, neutralizing free radicals by donating an electron or hydrogen atom. (Tsao, 2010) It is demonstrated that long term consumption of diets rich in plant polyphenols offer protection against development of cancers, cardiovascular diseases, diabetes, osteoporosis and neurodegenerative diseases. (Pandev, 2009)

Vitamin C provides protection against oxidative stress-induced cellular damage by scavenging of reactive oxygen species, and by protecting proteins from alkylation by electrophilic lipid peroxidation products. (Traber, 2011) It protect cellular components against oxidative damage caused by toxic free radicals and other reactive oxygen species (ROS) that are involved in the development of various types of chronic diseases. (Mescic Macan, 2019)

Vitamin E is a potent chain-breaking antioxidant that inhibits the production of reactive oxygen species molecules when fat undergoes oxidation and during the propagation of free radical reactions. It seems to be effective against cancer, ageing, arthritis, cataracts, platelet hyperaggregation, which can lead to atherosclerosis (Rizvi, 2014)

Vitamin K seems to thave an anti-inflammatory effect by suppressing nuclear factor κB (NF-κB) signal transduction and a protective effect against oxidative stress by blocking

the generation of reactive oxygen species. High vitamin K status can exert a protective role in the inflammatory and mineralization processes associated with the onset and progression of age-related diseases. (Simes, 2019)

Coenzime Q10 can exercite its antioxidant activity protecting cells and tissues involved in the innate and adaptive immune response. It play an important role in immunological cytotoxicity against pathogens, through the production of reactive oxygen species (ROS) by macrophages. It seems to be effective in cardiovascular diseases, obesity and viral infections. (Sifuentes-Franco, 2022)

L-arginine acts as antioxidant through the stimulation of GSH synthesis and activation of Nrf2 pathway, leading to the up-regulation of antioxidant factors. (Liang, 2018)

Melatonin has lipophilic antioxidant and free radical scavenging action. Specifically, melatonin-induced signal transduction through melatonin receptors promotes the expression of antioxidant enzymes as well as inflammation-related genes. Melatonin also exerts an immunomodulatory action through the stimulation of high-affinity receptors expressed in immunocompetent cells. It can be have a potential role in treating oxidative stress- and/or inflammation-related disorders, such as obesity, cardiovascular diseases, immune disorders, infectious diseases, cancer, neurodegenerative diseases, as well as osteoporosis and infertility. (Ferlazzo, 2020)

Alpha-Lipoic acid is an antioxidant because it plays an essential role in mitochondrial dehydrogenase reactions, Alpha-Lipoic acid administration has been shown to be beneficial in a number of oxidative stress models such as ischemia-reperfusion injury, diabetes (both alpha-lipoic acid and dihydrolipoic acid exhibit hydrophobic binding to proteins such as albumin, which can prevent glycation reactions), cataract formation, HIV activation, neurodegeneration, and radiation injury. (Packer, 1995)

SECTION 2

- Experimental Study -

(Pecoraro L, Zoller T, Atkinson RL, Nisi F, Antoniazzi F, Cavarzere P, Piacentini G, Pietrobelli A. Supportive treatment of vascular dysfunction in pediatric subjects with obesity: the OBELIX study. Nutr Diabetes. 2022 Jan 10;12(1):2. doi: 10.1038/s41387-021-00180-1. Erratum in: Nutr Diabetes. 2022 Jan 20;12(1):5. PMID: 35013093; PMCID: PMC8748969.)

BACKGROUND

Pediatric overweight and obesity are traditionally characterized by excess body fat [Greydanus, 2018], an independent cardiovascular risk factor that could lead to type 2 diabetes, hypertension, insulin resistance, and reduced endothelial function development [de Meneck, 2018]. The link between excess body fat, endothelial dysfunction, and insulin resistance is related to the fact that endothelium-dependent vasodilatation is impaired in proportion to insulin resistance and other adiposity-related indices [Avogaro, 2005]. Bussey and colleagues in 2016 found reduced nitric oxide (NO) production and increased inflammation in perivascular adipose tissue (PVAT) of obese mice compared to non-obese controls. The same study found significantly improved PVAT anticontractile function after weight loss by reduced adipose inflammation and increased NOS availability [Bussey, 2016]. Similar results were previously stated by Ketonen et al. in 2010; they found impaired endothelium-dependent vasodilation in response to acetylcholine in obese mice receiving a high-fat diet compared with mice receiving a normal fat diet. Differences between the obese and control group markedly reduced after the introduction of caloric restriction in the obese group [Ketonen, 2010]. Moreover, it is very well known that adipose tissue is a key regulator of inflammation with the secretion of pro-inflammatory cytokines (i.e., adipokines) that play a role in influencing glucose metabolism and endothelial function [Montero, 2012]. Children who are overweight or obese develop abnormal endothelial cell dysfunction and arterial intima-media thickening with increased vasomotor tone and inflammation [Ciccone, 2011; Bruyndonck, 2016]. This may lead to atherosclerotic plaque formation [Kim, 2016]. The endothelium contributes to blood pressure and flow regulation by releasing NO and other compounds that contribute to vasodilation or vasoconstriction [Sorop, 2017; Sowka, 2021]. On the other hand, the interaction between endothelium and adipokines suggests a role for adipokines in vascular homeostasis and, ultimately, in the mechanisms for the development of cardiovascular diseases [Sorop, 2017; Schinzari, 2017; Vanhoutte, 2017; Kheirandish-Gozal, 2010]. Also, a healthy endothelium prevents platelet aggregation, the proliferation of vascular smooth muscle cells, adhesion, and subsequent diapedesis of leukocytes through the vascular wall [Bruyndonckh, 2013]. The endothelium plays a unique role in vascular homeostasis that is maintained by endothelium-derived biomolecules with different functions (i.e., vasodilation, vasoconstriction, growth promoter, growth inhibitor, adhesion molecules, thrombolytic factors) [Bruyndonckh, 2013]. Endothelium-dependent damage arises from metabolic abnormalities of glucose

metabolism that lead to vascular dysfunction [Bruyndonckh, 2013]. Endothelial dysfunction is characterized by abnormal vasodilator response, and increased arterial stiffness is associated with an increased risk of cardiovascular events [Bruyndonckh, 2013] and is present in pediatric subjects with obesity [Sowka, 2021]. The major goal of obesity therapy in children should be reducing the long-term risks of cardiovascular diseases. Since damaged endothelium is so involved in the development of later risks of morbidity and mortality, it may be helpful to monitor and eventually treat endothelium status to prevent long-term risk factors [Sun, 2016; Kocaadem, 2017; van der Spy, 2009; Das, 2006; Xia, 2017]. Among different therapies, curcumin showed promising results in inhibiting advanced glycation end-product-induced oxidative stress and inflammatory responses in endothelial cell damage [Kheirandish-Gozal, 2010]. Curcumin is the most active component of the curcuminoids extracted from Curcuma longa L. It has been demonstrated to protect against cellular inflammatory responses and oxidative stress in vascular complications and endothelial damage [Sun, 2016; Kocaadem, 2017]. Another antioxidant, resveratrol, has shown beneficial effects on endothelial function since it can increase NO synthesis that, in vivo, plays an antioxidant function in the endothelium [van der Spy, 2009; Das, 2006; Xia, 2017]. Animal models showed a relationship between prenatal and neonatal zinc deficiency and vascular dysfunction [Mendes Garrido Abregú, 2018; Liu, 2020], keeping in mind that zinc has antioxidant-like properties in activated endothelium cells [Hennig, 1999]. Magnesium, an essential mineral for human health, plays a role in endothelium function and participates in vascular calcification [Marquez, 2020]. Magnesium supplementation significantly improved flow-mediated dilation and pulse wave velocity [Lopez Juonor, 2019]. Recent findings showed the active role of selenium in endothelial function25. Specifically, selenium and seleno protein are associated with endothelial cytoprotection [Marquez, 2020], having a role in endothelium activation biomarkers [Zhang, 2016]. Among several functions, Vitamin D is associated with endothelial dysfunction [Kim, 2020], showing anti-inflammatory effects through the suppression of TNF- alpha and the release of IL-6 [Ebihara, 1996].

To the best of our knowledge, we did not find information regarding studies done in pediatrics looking at endothelium dysfunction treatment. In light of these findings, using a double-blind, randomized control study with a rigorous approach, we tested the effects on the endothelium of a combination of curcumin, resveratrol, flavonoids, zinc, magnesium, selenium, soy and vitamin D in a cohort of pediatric subjects with obesity.

METHODS

Participants selection

In this study we recruited 48 children aged 6-17 years who were obese as defined by a BMI higher than the 95 percentiles for age based on the CDC standard[29]. Children with genetic syndromes or cardiovascular diseases were excluded from the study. The study was approved by the local Ethical Committee (OBELIX: code CE 5384, 2019). Informed written consent for study participation was collected from legal caregivers of each participant and from participants older than 10 years during the first visit. Participants were asked to attend 3 visits at 0, 3 and 6 months (+/- 15 days).

Randomization

This study was a double-blind randomized control study done with a rigorous approach. Using a computer-generated randomization schedule, study supplement and placebo were randomized (1:1) into 70 batches (each consisting of 6 packs containing 30 tablets a pack) and each was given a unique identification number. The coordinator of the study maintained the randomization list. Study physicians, other study personnel, and parents or legal guardians were blinded to the batches of medication and to the identification. Subjects who satisfied the inclusion for the study were assigned an identification number (linked to a batch) in sequential order. Since the randomization list was made before batch assignment and later preserved in a closed envelope that made it unavailable for the entire duration of the study, neither study physicians or patients could know subjects belonging to placebo or treatment group. Subjects took one tablet per day orally starting day one after the visit and continuing for the 6-month duration of the study. A number of tablets not taken equal to or greater than 2 tablets/month was considered not adherence to the study (drop out).

Supplements characteristics:

Treatment tablet composition (Auxilie® Immuplus, Envicon Medical, Verona, Italy): Vitamin D3: 25,00 mcg, Folic acid: 90,00 mcg, Selenium: 55,00 mcg Magnesium: 300,00 mg, Zinc: 7,00 mg, Curcuma (Meriva®): 100,00 mg; Polygonum dry extract: 20,41 mg (of which Resveratrol: 20,00 mg), Soy dry extract: 37,50 mg.

Placebo tablet composition: Saccharose, fructose, aroma, anti-agglomerate agents: fatty acids magnesium salts, silicium dioxide, colorant: riboflavin 5-sodium phosphate; sweetener: steviaglycoside, sucralose, neosperidin DC.

Both tablets (treatment and placebo) were similar in form, colour and flavour.

Anthropometric measurements and habits

Height (cm) and weight (kg) were measured for each child at every visit. BMI (kg/m^2) was calculated as raw value and as Z-score for age. At the first visit we conducted an oral interview with both parents and children, and we collected information regarding sport/exercise practices and dietary habits.

Endothelial function

Endothelial function was assessed using two methodologies: a "post occlusive release hyperemic test" (PORH) and a "heat provocation test" (HPT). Subjects laid on a bed with theupper extremity positioned at 45°, a cuff was placed around the medium third of the forearmin order to occlude the radial and ulnar arteries. The probe was positioned over the volar aspectof the hand at the 1st finger distal metacarpal surface and the hand was gently immobilized to minimize the occurrence of motion artefacts. All tests were performed with a laser Doppler sensor (Periflux 6000 System integrated with a thermostatic 457 probe, Perimed, Sweden).

Post occlusive release hyperaemia (PORH)

Once cutaneous blood flow over the area became stable, basal values were recorded for 2 minutes, then the pressure within an inflatable cuff placed at the forearm and connected to a computer-controlled manometer was raised to 200 mmHg for 3 minutes. Using a computer- controlled pressure release to allow for consistent deflation times, the cuff was rapidly deflated and the laser Doppler measured hyperemic responses over the next 2 minutes. Commercially available software (Perimed, Järfälla, Sweden) allowed for

unbiased estimatesof Delta Flow (DF), Hyperemic Area under Curve (Hyperemic AUC), and Hyperemic AUC index.

Delta Flow (DF)

The flow variation from Resting Flow to Peak Flow (maximal arterial flow achieved after abrupt cessation of occlusion) is called Delta Flow (DF). DF was computed at baseline, after 3 months and after 6 months in each group. Delta Flow was compared between the two groupsand its temporal trend was evaluated.

Hyperemic Area under Curve (Hyperemic AUC) and Hyperemic AUC index

Hyperemic AUC is the difference between area under the hyperaemia zone and area under the rest flow zone expressed in perfusion units multiplied by time. We also chose to compute an index named "Hyperemic AUC index" in order to better reflect the real value of AUC in relation to the resting flow of each participant. AUC index= AUC /RF.

Heat Provocation Test (HPT)

After the PORH test we wait at least 2 minutes to proceed with the "heat provocation test" (HPT) in order to re-establish basal blood flow under the probe. The HPT consisted of recording resting blood flow in the forearm for two minutes, heating the forearm by raising the temperature of the probe to 44° Celsius, and recording the hyperaemic response induced by the heat expressed as a percentage difference in perfusion units (PU) above resting flow. This was defined as "Delta Flow HPT" (DFHPT).

Statistical analysis

Qualitative variables were expressed as percentages and 95% confidence intervals (95% CI),and quantitative variables as means SD or medians and interquartile ranges (IQR) depending on whether the variables were normally distributed. The Kolmogorov-

Sminorv test was usedto ascertain the normal distribution. Student's t test and ANOVA or Mann Whitney test, Wilcoxon signed-rank test and Kruskal-Wallis test were used as appropriate to the data to compare distributions of Delta Flow, Hyperemic AUC index and DFHPT increase between Control and Treatment groups at fixed time intervals (0, 3 and 6 months) and to evaluate the trend of changes over time within each group. The association between each of these Perifluxparameters at baseline (T0) and some relevant population characteristics such as BMI, gender,ethnicity, dietary fat intake, sport type and hours per week was tested using Pearson's r correlation coefficient or Spearman's correlation coefficient as appropriate. MedCalc Statistical Software version 17.6 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org) and GraphPad Prism version 6.00 for Mac (GraphPad Software, La Jolla California USA, http://www.graphpad.com) were used to perform the analyses and α was set at 0.05.

RESULTS

Forty-eight subjects were recruited and submitted to a questionnaire, medical examination, anthropometric evaluation and measurement of endothelial function. Twenty-one subjects dropped out. Nine subjects were not compliant with tablet prescription (2 or more tablets nottaken each month), whilst 12 patients did not attend 3- and 6-month follow up due to personal reasons (mainly young subjects who found the 3 minutes cuff occlusion too annoying to tolerate). It was not possible to make some drop out analysis involving these subjects because they drop out before the second visit (at 3 months). Consequently, it was not possible to evaluate the parameters related to the endothelial function over the time for these subjects. Among the patients who completed the study, 16 patients took antioxidant supplementation, 11 took placebo. No one reported adverse effects. The characteristics of all subjects at baseline are shown in **Table IV**.

	n	**%**
Patients enrolled	48	
Drop-outs	21	43,75
Age (years)	12.85±3.04	
Gender (male)	25	52,08
Weight (kg)	76.90	[66.0 - 92.5]

BMI	30.89±5.22	
BMI z-score	0.61	[0.22 - 1.12]
Ethnicity		
Caucasic	41	85,42
North African	4	8,33
Moroccan	3	6,25
Sport		
sport type score§§	3	[2 - 3]
hours/week	3	[1 - 4]
Dietary fat intake		
low	23	47,92
medium	23	47,92
high	2	4,17
Drugs		
D-vitamin	8	16,67
Methylphenidate	1	2,08
Montelukast	1	2,08
None	38	79,17

Table IV. Baseline Characteristics of the Study population. Data are expressed as number (N) and percentage or mean±SD or median [IQR] as appropriate. §§Sport type = (dynamicscore)*(static score).

The characteristics and homogeneity of the subjects who completed the study are shown in **Table V.**

	Control		**Treated**	
N	**11**		**16**	
Age (years)	11.4	[9.2 - 14.7]	12.8	[11.2 - 15.3]
Gender (male)	7	63,6	9	56,3
Weight (kg)	70.7	[65.0 -	76.3	[66.9 - 90.0]

		113.0]		
BMI	30.4	[27.0 - 40.1]	30.6	[27.8 - 36.0]
BMI z-score	0.8	[0.1 - 1.7]	0.6	[0.2 - 0.9]
Ethnicity				
Caucasic	7	63,6	13	81,3
North African	3	27,3	1	6,3
Moroccan	1	9,1	2	12,5
Sport				
sport type score§§	3.0	[2.0 - 3.5]	2.5	[0.0 - 3.0]
hours/week	2.5	[1.0 - 4.0]	2.0	[0.0 - 4.0]
Dietary fat intake				
low	6	54,5	7	43,8
medium	4	36,4	7	43,8
high	1	9,1	2	12,5

	Control		**Treated**	
Periflux baseline data				
Resting Flow	103.0	[42.0 - 176.0]	89.0	[47.5 - 137.3]
Biological Zero	8.0	[8.0 - 13.0]	9.0	[7.0 - 12.0]
Peak Flow	201.0	[146.0 - 274.0]	209.0	[159.5 - 232.3]

ΔFlow	79.0	[48.0 - 107.0]	117.0	[70.3 - 143.5]
HA Hyperemic Area	3015	[1745 - 4108]	4336	[2489 - 8194]
HA index	27.9	[10.0 - 77.3]	49.4	[20.6 - 133.7]
Pre heat Laser Doppler	122.0	[38.0 - 179.0]	84.0	[50.0 - 123.0]
Post heat Laser Doppler	232.0	[132.0 - 278.0]	210.0	[187.3 - 278.0]
Laser Doppler increase (%)	118.0	[20.0 - 219.5]	244.3	[71.9 - 397.1]

Table V. Characteristics of Completers: Placebo versus Treatment groups. Data are expressed as Median [IQR] or Number and percentage as appropriate. §§Sport type = (dynamic score) *(static score). AUC: Area Under the Curve; HPT: heat provocation test.

"Treatment group" and "Placebo group" were compared at baseline and were homogeneous with no significant difference in anthropometric and endothelial function parameters.

Delta flow (DF)

"Treatment group" and "placebo group" did not show differences in post Occlusive Reactive Hyperemic delta flow at baseline and time-3-months **(Table VI).** On the other hand, a significant difference was present at time-6-months (78.5 [72.0 - 94.0] vs 63.0 [53.0 - 78.0], respectively treatment and control, $p<0.001$). Overall time trend of "Treatment group" and "placebo group" was detected and compared with Kruskal-Wallis test, showing a significant difference in the flow time trend between baseline,

time-3-months and time-6-months both in“Placebo group” ($p<0.05$) and “Treatment group” ($p<0.001$) **(Fig. 12)**.

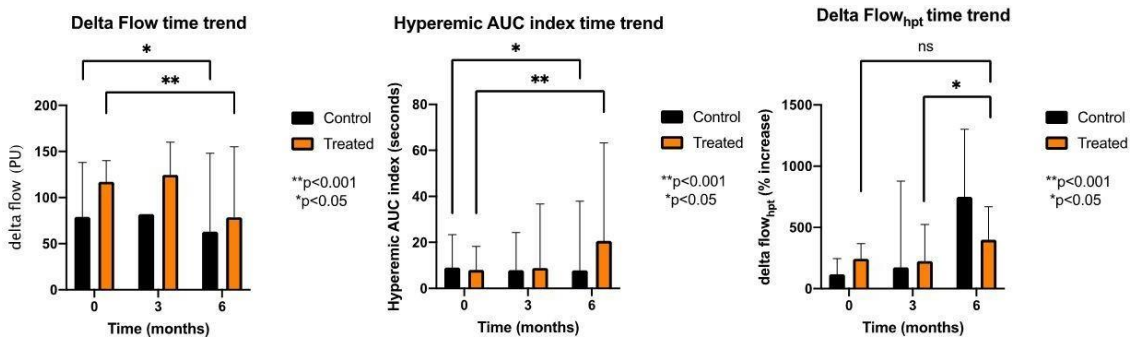

Fig. 12. Time trend variation of Periflux data.

Moreover, the comparison between the two groups was highly significant ($p<0.001$). Regarding the correlation between anamnestic-anthropometric data and endothelial function parameters at baseline, the flow variation from Resting Flow to Peak Flow (Delta-Flow, DF) showed a significant correlation with BMI (Spearman's r= 0.355, $p<0.05$). The strength of thiscorrelation increases with time; specifically, the correlation between BMI and DF was stronger at time-6-months (r=0.678) (**Fig. 13, Tab. VII).**

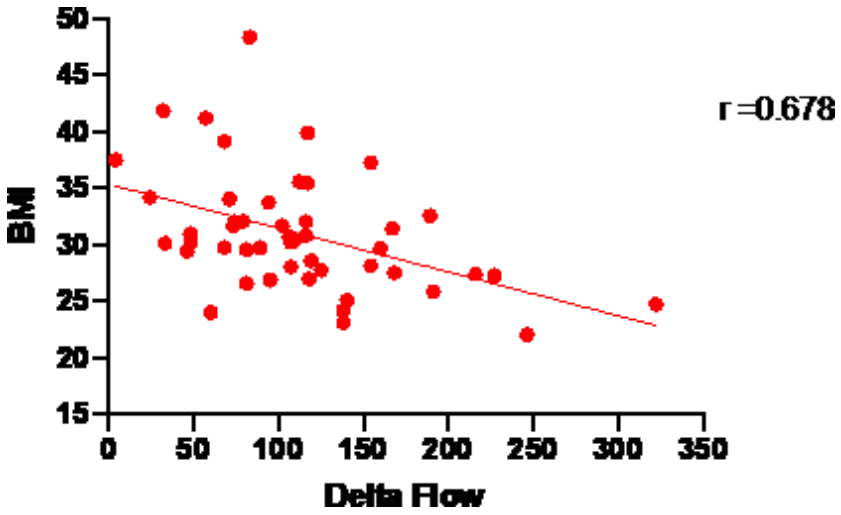

Fig. 13. Correlation between Delta Flow and BMI.

Hyperemic area under curve (AUC) index

"Treatment group" and "placebo group" did not show mutual differences in "Hyperemic AUCindex" at time-3-month and time-6-months (**Tab. VI)**. An overall time trend of "treatment group" and "placebo group" was detected and compared with Kruskal-Wallis test, showing asignificant difference in the Hyperemic AUC index both in "Placebo group" ($p<0.05$) and "Treatment group" ($p< 0.001$) **(Fig. 12)**. In addition, the comparison between the time trends of the two groups was very significant ($p<0.001$). About the relationship between anamnestic and anthropometric data and endothelial function parameters at baseline, the Hyperemic AUC index showed a significant correlation with male gender (Spearman's $r= 0.348$, $p<0.05$) **(Tab.VII)**.

Delta Flow Heat Provocation Test (DFHPT)

"Treatment group" and "placebo group" showed a significant difference between groups in DFHPT when comparing the results of the "Heat provocation test" at time-3-month and time-6-month ($p<0.05$) (**Tab. VI**).

	Post Occlusive Reactive Hyperemia (PORH)							Laser doppler (LD)							
	RF Resting flow (PU)		BZ Biological zero (PU)		PF Peak Flow (PU)		DF ΔFlow	HA Hyperemic Area (PU*sec)		HA index		Pre heat LD		Post heat LD		LD increase (%)	
Basal																	
Control	103.0	[42.0 - 176.0]	8.0	[8.0 - 13.0]	201.0	[146.0 - 274.0]	79.0	[48.0 - 107.0]	3015	[1745 - 4108]	27.9	[10.0 - 77.3]	122.0	[38.0 - 179.0]	232.0	[132.0 - 278.0]	118.0	[20.0 - 219.5]
Treated	89.0	[47.5 - 137.3]	9.0	[7.0 - 12.0]	209.0	[159.5 - 232.3]	117.0	[70.3 - 143.5]	4336	[2489 - 8194]	49.4	[20.6 - 133.7]	84.0	[50.0 - 123.0]	210.0	[187.3 - 278.0]	244.3	[71.9 - 397.1]
3 months																		
Control	58.0	[51.0 - 125.0]	9.0	[7.0 - 13.0]	186.0	[96 - 222.0]	99.0	[51.0 - 148.0]	4202	[1283 - 6140]	49.7	[18.2 - 87.0]	86.0	[32.0 - 136.0]	193.0	[167.0 - 264.0]	174.1*	[32.0 - 807.7]
Treated	74.5	[53.8 - 104.3]	9.5	[6.5 - 11.5]	201.5	[164.8 - 224.0]	124.5	[71.3 - 157.0]	4114	[2328 - 8390]	52.2	[25.8 - 127.9]	77.0	[57.3 - 89.8]	237.0	[212.5 - 310.5]	226.1*	[133.8 - 518.9]
6 months																		
Control	41.0	[21.0 - 70.0]	7.0	[6.0 - 15.0]	116.0	[82.0 - 189.0]	63.0**	[53.0 - 78.0]	2434	[1766 - 3588]	78.2	[16.1 - 141.5]	32.0	[15.0 - 41.0]	164.0	[154.0 - 183.0]	749.0*	[293.0 - 1196.0]
Treated	43.5	[35.8 - 51.8]	7.0	[5.3 - 14.5]	119.0	[113.0 - 176.0]	78.5**	[72.0 - 94.0]	2814	[1674 - 4350]	72.0	[32.5 - 114.5]	40.5	[33.3 - 56.8]	191.5	[163.0 - 211.8]	400.0*	[232.5 - 415.8]

Tab. VI. Periflux data assessing endothelial function. **p<0.001; *p<0.05. Delta Flow

= Peak flow-Resting flow. Hyperemic AUC index = Hyperemic AUC /Resting Flow. PU: perfusion unit; AUC: Area Under the Curve; HPT: heat provocation test.

The overall time trend of "treatment group" and "placebo group" was detected and comparedwith Kruskal-Wallis test. In the "placebo group" there was no significant difference in the DFHPT analysis between baseline, time-3-months and time-6-months. In the "Treatmentgroup", DFHPT analysis did not show a significant difference between baseline and time-3- months. However, there was a significant difference between time-3-month and time-6-month($p < 0.05$) in this group **(Fig. 12)**. Regarding the association between anamnestic and anthropometric data and endothelial function parameters at baseline, DFHPT did not show a significant correlation with anamnestic and anthropometric parameters **(Tab. VII)**.

	BMI		**Gender (male)**		**Ethnicity**		**Sport type score**		**Dietary fat intake**		**Sport hours/week**	
ΔFlow	-0,457**	[-0,661; -0,191]	0,015	[-0,278; 0,306]	0,156	[-0,143; 0,428]	-0,079	[-0,363; 0,218]	-0,116	[-0,407; 0,196]	0,099	[-0,209; 0,389]
AH index	-0,108	[-0,388; 0,190]	0,348*	[0,062; 0,581]	-0,084	[-0,367; 0,214]	-0,079	[-0,363; 0,218]	0,105	[-0,207; 0,397]	0,114	[-0,195; 0,402]
LD increase (%)	-0,213	[-0,476; 0,084]	0,133	[-0,166; 0,409]	0,057	[-0,239; 0,344]	-0,043	]-0,331; 0,252]	-0,339*	[-0,583; -0,037]	-0,147	[-0,430; 0,162]
ΔFlow (6 months)	-0,677**	[0,237; 0,887]										

Tab. VII. Correlation coefficients of Periflux parameters with antrophometric data. Data are expressed as Spearman r coefficient and related 95%CI (95% Confidence Interval); *p<0.05; **p<0.001. Delta Flow = Peak flow-Resting flow. Hyperemic AUC index = Hyperemic AUC /Resting Flow. Ethnicity: Caucasic >North African >Moroccan. PU: perfusion unit; AUC: Area Under the Curve; HPT: heat provocation test.

DISCUSSION

The study's results illustrate the correlation between obesity status and endothelial dysfunction in children, showing that cardiovascular damages begin early in life.

Although the instrument and methodology used in this study are considerably new, we used the most validated parameters, such as delta flow, the hyperemic area under the curve, and delta flow heat provocation test (DFHPT), to estimate endothelial function or dysfunction in adults. Previous studies showed that endothelial dysfunction was associated with adiposity in obese children as well [Bruyndonckx, 2016; Farpour-Lambert, 2009]. Our results showed that delta flow improved significantly ($p<0.001$) in the treatment group compared to the control group. All the enrolled subjects, both treatment and controls, have had some benefits in their endothelial function over time, perhaps because some educational advice was given during the medical assessment. However, in the treatment group, this benefit was constantly and significantly higher ($p<0.001$), implying a role of the supplements in promoting a higher degree of improvement. The correlation between basal delta flow and basal BMI shown in Figure 1 is negative, indicating that delta flow decreases while BMI increases, once again underlining the treatment effect in enhancing endothelial performances despite an increase in BMI. Although we did not find any correlation with dietary fat content, type, and hours spent on physical activities, sports, or ethnicity, we may speculate that a larger sample size and/or different ethnicity could show significant results. It is important to recognize that our population was mostly Caucasian. Regarding physical activity, previous studies showed improvement in arterial stiffness and reduction of abdominal fat, increased cardiorespiratory fitness, and delayed arterial wall remodeling in pre-pubertal obese children [Bruyndonckx, 2016]. We did not find any significant correlation between groups in the area of hyperemia, showing a similar trend of time variation in both groups. The influence of advice by the physician in promoting changes in both groups cannot be excluded. However, the more significant changes in the treated group compared to the control group suggest a treatment effect of the supplements in modifying the "hyperemic area under the curve." Again, a larger sample size or an even longer observational/treatment period might influence future results. Regarding gender, males showed a wider range of hyperemia compared to females. Despite the small sample size, North African subjects showed a higher range of hyperemia, followed by Italo-Moroccan and Caucasians. In contrast with our results, Mueller et al. [2017] found that in females, endothelium function was lower than in men. However, the cohort of adolescents in Mueller et al. [2017] was mainly healthy. Adult men usually develop cardiovascular diseases at a younger age and have a higher propensity to develop coronary heart diseases than women [Shannon, 2020]. Looking at the "delta flow heat provocation test," significant changes between the treated group and control were found after three months

of treatment ($p<0.05$), giving us the impression that the combination of curcumin and resveratrol plus zinc, magnesium, selenium, and Vitamin D require some time to influence endothelium function per se. Regarding the relationship between endothelial function and BMI, a recent systematic review and meta-analysis in adults showed that diet improved endothelium function independently from BMI [Kim, 2014]. Still, we did not find similar results in children using the same approach and measurements. In summary, curcumin, resveratrol, zinc, magnesium, soy, folic acid, selenium, and Vitamin D appear promising in enhancing endothelial function by improving both delta flow in post-occlusive release hyperemia test and delta flow in the heat provocation test. However, the combination effectively acts after three months since the therapy started. The primary mechanism of action of polyphenols was initially thought to lie in their direct antioxidant effects. However, several other possible biochemical and molecular mechanisms have been identified, including various effects within intra- and inter-cellular signaling pathways that govern anti-oxidative properties like nuclear factor E2-related factor 2 (Nrf2) and inflammation pathways, e.g., nuclear factor kappa B (NF-κB), and thus modulating the synthesis of inflammatory mediators including cytokines tumor necrosis factor α, interleukin (IL)-1β, and IL-6 [Fraga, 2018; Hassan, 2019; Cione, 2019]. Furthermore, curcumin and resveratrol have been demonstrated to exert epigenetic regulatory roles, including the inhibition of DNA methyltransferases (DNMTs), regulation of histone modifications via the regulation of histone acetyltransferases (HATs) and histone deacetylases (HDACs), regulation of microRNAs (miRNA), and action as a DNA binding agent [Cione, 2019; Oliveira, 2017]. The accumulation of visceral fat in obesity is associated with a state of chronic oxidative stress and excessive production of proinflammatory adipokines, which contributes to a low-grade chronic inflammation state that can be attenuated with magnesium [Abdali, 2015], zinc [Zulet, 2009], and selenium supplementation as recognized antioxidant trace elements [Crilly, 2012]. The effects we observed have biological plausibility. Daily vegetable consumption was associated with more favorable arterial function [Noad, 2016], and the same was observed in increasing the polyphenol content of the diet via the consumption of fruit and vegetables [McCall, 2009], with a dose-dependent effect [van Bussel, 2018]. Red wine consumption has been shown to positively influence processes involved in vascular dysfunction [Akbari, 2019], and resveratrol, one of the main compounds of the tablets used in our study, is the major polyphenol in wine. A systematic review and meta-analysis of randomized controlled trials documented that resveratrol [Hallajzadeh, 2019] or curcumin [Yamagata, 2019] intervention significantly increased flow-mediated

dilatation. Several studies demonstrated that a high intake of dietary polyphenols inhibits endothelial dysfunction and induces vascular endothelium-dependent vascular relaxation, redox regulation, and nitric oxide production [Hallajzadeh, 2019] and may have a preventive effect against cardiovascular diseases [Al Mheid, 2011]. Moreover, Vitamin D insufficiency is associated with increased arterial stiffness and endothelial dysfunction [Giallauria, 2012]. Vitamin D levels are inversely associated with increased arterial stiffness in a normative aging population, irrespective of the traditional risk factor burden [Mahmoud, 2019]. Its supplementation improved NO-dependent arteriolar vasodilation in obese adults [Dou, 2019] and is associated with decreased vascular dysfunction in patients with chronic kidney disease [Gianfredi, 2017]. Hard water consumption seems protective against cardiovascular diseases, particularly its magnesium content [Marques, 2020]. A meta-analysis suggests that oral Mg supplementation may improve endothelial function for at least six months in unhealthy, overweight, or older individuals [Connell, 1997], as observed in our studied children. In animal models, zinc deficiency is associated with reduced vasodilator response [Mendes Garrido Abregú, 2018], and in humans, zinc supplementation alleviates diabetic endothelial dysfunction [Liu, 2020]. Zinc is a protective and critical nutrient for maintaining endothelial integrity [Hennig, 1999] through attenuation of tumor necrosis factor-mediated activation of upregulation of inflammatory cytokines in endothelial cells [Raij, 1993]. In rats, it has been documented that hypercholesterolemia, a condition commonly found in obese subjects, promotes endothelial dysfunction in the presence of selenium deficiency [Alehagen, 2018], and many studies in humans indicate an association between selenium deficiency and increased risk of morbidity and mortality [Kovacs, 2014]. Selenium supplementation has been shown to significantly reduce the risk of cardiovascular mortality in patients with diabetes, hypertension, and ischemic heart disease [Van der Spuy, 2009]. According to the study results, looking at the day-by-day clinical work and regular physical activity, increased consumption of the correct diet containing nutritive and non-nutritive compounds may improve the quality of life by delaying the development of endothelial dysfunction in pediatric subjects with obesity.

LIMITATION OF THE STUDY

Despite intriguing findings, this study has some limitations. The high drop-out level we had in the study population is in line with the low adherence of obese children to health-behaviour recommendations [Kocaadam, 2017], and this fact may also be responsible for the partial effect we observed with the nutraceutical supplementation. Moreover, the study has not considered the potential impact of growth on our subjects. It is well known that the laser doppler flowmetry technique used to evaluate the endothelial function and, ultimately, cardiovascular risk in adults is used only in research, and no clinical studies can be found. We did not find any guideline or protocol to diagnose endothelial dysfunction utilizing this technique. Another important point that needs to be considered is the time during the day of the vascular assessment. We know that fasting and "circadian rhythm" may influence results per se, and we did not have the possibility to take measurements at the same time of the day, although we tried very hard to standardize the time of measurements. Starting from the consideration that environmental interventions (such as increasing portion sizes, increasing consumption of fast food and soft drinks, limited compliance with physical education requirements in many schools, limited access to healthy foods in low-income neighborhoods) could influence the results of the study, we deepened this aspect in all visits of the study. Anyway, these data were referred by our patients and their parents and could not be controlled fully.

SECTION 3

- Conclusions -

Treatment of childhood obesity is always an issue. Deepening endothelial function should be a fundamental tool to evaluate the obese child in a 360 degree view. Since there is a high prevalence of inflammation in obese subjects leading to several problems, including endothelial dysfunction and related cardiovascular complications [Van der Spuy, 2009], the assessment of the endothelial function represents a promising tool to act a prevention of these comorbidities. Moreover, substances with antioxidant, anti-inflammatory, angiogenic, and platelet aggregation properties such as resveratrol, curcumin, zinc, selenium, soy, magnesium, folic acid, and vitamin D [Kocaadam, 2017] may have protective effects to improve endothelial dysfunction, thus lowering the risk of developing cardiovascular diseases [Carr, 2000; Bruyndonckx, 2016]. In conclusion, it is possible to speculate that multicomponent supplementation with minerals, vitamin D, and functional food-derived factor that resembles but not is a substitute for a healthy diet may help to improve vascular dysfunction in obese children.

SECTION 4

- Appendix -

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