UNIVERSITÀ POLITECNICA DELLE MARCHE

DIPARTIMENTO SCIENZE DELLA VITA E DELL'AMBIENTE

Corso di Laurea Magistrale in

BIOLOGIA MOLECOLARE E APPLICATA

CELLULE ENDOTELIALI E PLASMI DI BAMBINI OBESI: UN MODELLO INNOVATIVO *IN VITRO* PER LO STUDIO DEI MECCANISMI BIOMOLECOLARI IMPLICATI NELLA DISFUNZIONE ENDOTELIALE NELL'OBESITÀ INFANTILE

ENDOTHELIAL CELLS AND PLASMA FROM OBESE CHILDREN: AN INNOVATIVE *IN VITRO* MODEL FOR THE STUDY OF THE BIOMOLECULAR MECHANISMS IMPLICATED IN ENDOTHELIAL DYSFUNCTION IN CHILDHOOD OBESITY

Tesi di Laurea Magistrale di:

Carola Palmerini

Relatore:

Chiar.mo Prof. Luca Tiano

Correlatore:

Chiar.ma Prof.ssa Assunta Pandolfi

Sessione Luglio 2020

Anno Accademico 2019/2020

TABLE OF CONTENTS

Riassunto

L'obesità infantile è comunemente associata a disfunzione endoteliale, una condizione caratterizzata da alterazioni del segnale insulinico, da una ridotta biodisponibilità di Ossido Nitrico (NO), da aumentato stress ossidativo ed infiammazione cronica.

Recentemente, è stata dimostrata una riduzione della biodisponibilità NO nelle cellule endoteliali derivate da vena di cordone ombelicale umano (HUVECs, *Human Umbilical Vein Endothelial Cells*) in seguito al trattamento con il plasma di bambini prepuberi obesi (OB), rispetto al plasma di bambini normopeso (CTRL, controllo). Tuttavia, i meccanismi alla base di tali alterazioni non sono stati del tutto chiariti.

Pertanto, utilizzando lo stesso modello sperimentale, l'obiettivo principale della presente tesi è valutare se il plasma OB rispetto al plasma di bambini CTRL possa indurre: (i) un aumento della produzione di specie reattive dell'ossigeno (ROS, *reactive oxygen species*); (ii) uno squilibrio tra le vie insuliniche pro- ed anti-aterogenica; (iii) un aumento dell'interazione monociti-endotelio. Inoltre, al fine di studiare il meccanismo coinvolto, si ipotizza di valutare il potenziale coinvolgimento della via di segnale regolata dal complesso 1 bersaglio della rapamicina (mTORC1) e del suo bersaglio a valle, la proteina-chinasi 1 p70-S6 (S6K1), molecole note per essere coinvolte nella modulazione della via di segnale dell'insulina.

I risultati ottenuti indicano che i bambini OB (N = 32, età: 9,2 ± 1,7; *BMI z-score*: 2,72 ± 0,31) presentavano livelli plasmatici di insulina a digiuno più alti e un'aumentata insulino-resistenza (*HOMA-IR*) rispetto ai bambini CTRL (N = 32, età: 8,8 ± 1,2; *BMI z-score*: 0,33 ± 0,75). *In vitro*, le HUVECs incubate per 16 ore con il plasma OB, a differenza dei controlli, mostravano un aumento significativo dei livelli di ROS, dell'esposizione di membrana delle molecole di adesione vascolare e intercellulare (VCAM-1, ICAM-1) e

dell’adesione dei monociti all’ endotelio. Ciò era associato ad uno squilibrio delle vie di segnale insuliniche pro- ed anti-aterogenica, come dimostrato dall'aumento di attivazione della proteina chinasi mitogenica attivata (MAPK) e dalla contemporanea diminuzione dei livelli di fosforilazione del substrato 1 del recettore dell’insulina (IRS1) e della proteina chinasi B (Akt), insieme all’aumento di attivazione di S6K1. In maniera interessante, l’utilizzo della rapamicina, noto inibitore della via di segnale mTORC1-S6K1, ripristinava in modo significativo l'attivazione della via insulinica IRS-1/Akt, suggerendo una regolazione di tipo *feedback* di tale via attraverso S6K1. Nel complesso, tali risultati evidenziano nuovi potenziali meccanismi alla base dell'insorgenza della disfunzione endoteliale nell'obesità infantile.

Abstract

Childhood obesity is characterized by vascular insulin resistance along with altered oxidant-antioxidant state and chronic inflammation, which play a key role in the onset of endothelial dysfunction. Recently, it has been demonstrated a reduced Nitric Oxide (NO) bioavailability in Human Umbilical Vein Endothelial cells (HUVECs) cultured with plasma from obese pre-pubertal children (OB) as compared with plasma from normal-weight children (CTRL). However, mechanisms causing endothelial dysfunction in childhood obesity are still not entirely clear. Thus, the aim of the present thesis is to better clarify the mechanisms responsible for endothelial dysfunction establishment in childhood obesity, using the aforementioned innovative *in vitro* model. More in detail, the effects of HUVECs incubation with OB-plasma on intracellular levels of oxidative stress and on the regulation of pro-and and anti-atherogenic insulin pathways have been investigated. In addition, the potential involvement of mammalian Target of Rapamycin Complex1 (mTORC1)-ribosomal protein S6 Kinase beta1 (S6K1) pathway, known to have a role in regulating the insulin pathway, was also evaluated.

OB-children (N = 32, age: 9.2 ± 1.7; BMI z-score: 2.72 ± 0.31) had higher fasting insulin levels and increased Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) than CTRL-children (N = 32, age: 8.8 ± 1.2; BMI z-score: 0.33 ± 0.75).

In vitro, HUVECs exposed to OB- compared to CTRL-plasma (both 16 hours incubation) exhibited significant increase in Reactive Oxygen Species (ROS) levels, higher vascular and intercellular adhesion molecules exposure, together with increased monocytes-endothelial interaction. This was associated with unbalanced pro- and anti-atherogenic endothelial insulin stimulated signaling pathways, as measured by increased Mitogen Activated Protein Kinase (MAPK) activation and decreased Insulin Receptor Substrate-1

(IRS-1) and protein kinase B (Akt) phosphorylation levels, together with augmented S6K1 activation. Interestingly, inhibition of mTORC1-S6K1 pathway using rapamycin significantly restored the IRS-1/Akt activation, suggesting a feedback regulation of IRS-1/Akt signal through S6K1. Overall, these findings shed light on potential new mechanisms underlying endothelial dysfunction in childhood obesity.

INTRODUCTION

1. CHILDHOOD OBESITY

Definition and assessment. According to the World Health Organization (WHO), overweight and obesity are defined as abnormal or excessive fat accumulation that negatively affect individual health (WHO, Obesity and overweight. Fact sheet April 2020). There is no precise cut-off to distinguish between normal, abnormal and excessive body fat and it is difficult and expensive to directly evaluate the amount of body fat. For these reasons, the accepted clinical standard measure of overweight and obesity is the Body Mass Index (BMI), an indirect measure of body fat mass that well correlates with adiposity. BMI is calculated as weight (kilograms) divided by height (meters) squared (Ogden et al., 2007). However, BMI calculation is enough to identify obesity/overweight condition in adults, while Growth Charts that correct BMI values for age, sex and gender are necessary for weight assessment in children. In fact, growth and sex hormones affect weight/height and body fat distribution in childhood. Weight is usually expressed in percentiles, overweight children have BMI between the 85^{th} and the 95^{th} percentile for age and gender, obese children have BMI greater than the 95th percentile and severely obese children have a BMI greater than the 99th percentile.

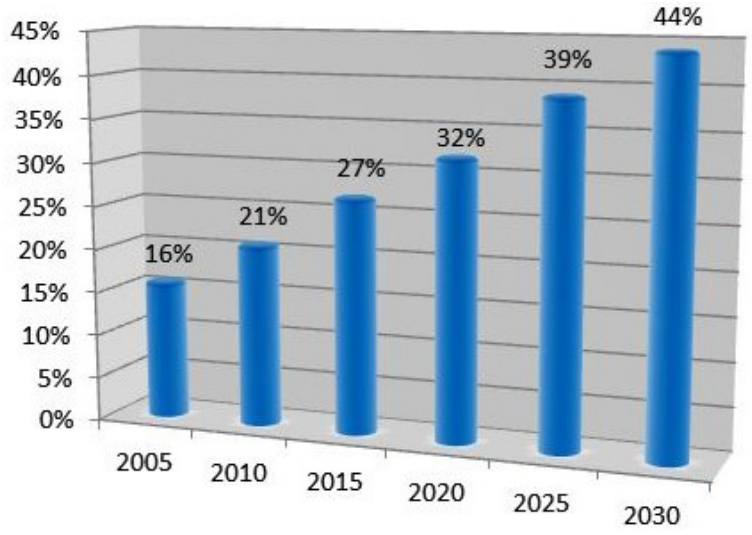
Some authors classify obesity in 3 groups: class I obesity (BMI between the 95th percentile and the 120% of the 95th percentile), class II (BMI between 120% and 140% of the 95th percentile) and class III (BMI higher than 140% of the 95th percentile) (Güngör, 2014; Krebs et al., 2007; Skinner et al., 2015). However, although BMI is a simple and reproducible measurement, it presents disadvantages such as its intrinsic weakness to distinguish between fat and weight gain linked to muscularity (Rothman, 2008). Moreover, it is often necessary to identify very extreme values, that may represent errors in data entry

or measurement but also correctly recorded values that are very high or low. Z-scores (or standard deviation scores) are widely used to quantify a measurement's distance from the mean (Freedman & Berenson, 2017)

Epidemiology. Childhood obesity represents one of the main public health challenges of the 21st century. Recent estimates found that 124 million of children and adolescents aged between 5 and 19 were obese in 2016, an increase of 10-fold than the 11 million classified as obese 40 years ago. The rise was similar among both boys and girls: 6% of girls and 8% of boys were obese in 2016. A further 40 million of children under 5 years were obese in 2016. Hence, one in five children was obese or overweight (Bentham et al., 2017; Di Cesare et al., 2019). In addition, according with data from the International Conference on Childhood Obesity and Nutrition, if this trend continues and without substantial interventions to prevent and to treat childhood obesity, the portion of obese children is predicted to rise to 44% by 2030 (Figure 1).

In last decades, the prevalence of childhood obesity has increased dramatically all over the world, apart from sex, age, nationality, ethnicity and socioeconomic status. In fact, despite obesity seemed to be only a problem related to welfare, the percentage of increase in developing countries was about 30% than that of richer countries. In 2016, Asia presented the highest trend in pediatric obesity. In many low- and middle-income countries is not uncommon to find both undernutrition and obesity as the consequence of an inadequate pre-natal, infant and young child nutrition. A very large epidemiological study of 2017 suggests that with this trend the number of obese children and adolescents will be higher than that underweight by 2022 (Bentham et al., 2017).

Figure 1. Childhood obesity rate

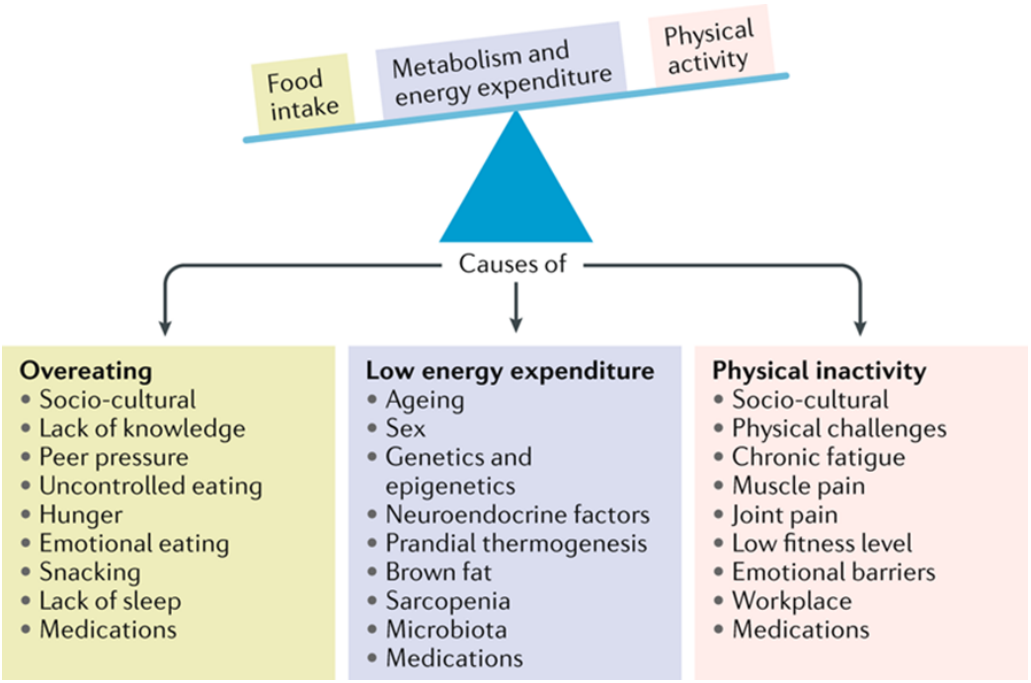


Adapted from the original figure*: 12th International Conference on Childhood Obesity and Nutrition March 18-19, 2019 Rome, Italy.* According with recent estimates, if the increasing trend of childhood obesity seen until 2015 continues, 44% of children and adolescents will be obese by 2030.

Etiology. Obesity is a multifactorial and complex condition that occurs following a chronic imbalance between caloric intake and energy expenditure: calorie excess is stored in adipocytes as triglycerides, causing the adipose tissue growth and consequently, the weight gain (Blüher, 2019; Chooi et al., 2019).

Figure 2 shows the main factors that contribute to the establishment of a chronic positive energy balance, thus favoring the development of obesity.

Figure 2. Factors that cause a chronic positive energy balance



Adapted from original figure: *Bluher M.; Obesity: global epidemiology and pathogenesis; Nat Rev Endocrinol 2019; 15(5):288-298.* Weight gain can result from a combination of increased energy intake, low physical activity and reduced energy expenditure. Several factors can influence the chronic positive energy balance, thus subsequently causing obesity.

First, the modern lifestyle and the urbanization force people to live in an obesogenic environment, that promotes wrong nutrition and sedentary behaviors, favoring the weight gain (Güngör, 2014). Particularly, the consumption of sweetened beverages, sweet snacks, fast foods and large portion sizes result in high calorie intake and has been reported as contributor of obesity in children (Malik et al., 2006; St-Onge et al., 2003). On the other hand, there are factors that predispose to diminished caloric expenditure such as increasing "sit time", time spent watching television, playing videogames and using phones or tablets. Different studies provide evidence of a correlation between time spent in television

viewing and increased body fatness. This link is explained in several ways: TV watching steals time for physical activity, promotes poor diets and unhealthy snacking and interferes with sleep (Robinson, 2001; Rosiek et al., 2015).

The weight gain could be also a consequence of psychological issues. Indeed, infants and children are vulnerable and highly sensitive to external stressors, such as hard language, violence, lack of affection and tend to transform them in anxiety, depression, insecurity and low self-esteem. Consequently, they try to self-medicated and find comfort especially in unhealthy and junk food, developing obesity (Hemmingsson, 2018).

Moreover, obesity can be associated with endocrine diseases, such as hypothyroidism, polycystic ovary syndrome (POS), hypogonadism, Cushing's disease and growth hormone deficiencies (Karam et al., 2007).

Genetics factors also contribute to the establishment of obesity and represent one of the most investigated factors in understanding the mechanisms that cause this disease. Some authors found that people living in "same environment" have an individual variability in body weight and fat mass, suggesting a genetic predisposition to adiposity (Herrera et al., 2011). Monogenic forms of obesity are due to rare single gene mutations that usually affect the key molecules of the leptin-melanocortin pathway that regulate metabolism and appetite. Syndromic forms are also rare and associated with chromosomal abnormalities. There are approximately 30 genetic syndromes of this type, including Prater-Willi or Bardet-Biedel syndrome, whose phenotype generally includes mental retardation, developmental abnormalities and even weight gain. In most cases, obesity is related to the interaction of different genes products that are responsible for hunger-satiety regulation and growth and differentiation of adipose tissue. Thanks to the Genome Wide Association Studies (GWAS), it has been possible to identify more than 100 BMI-associated loci

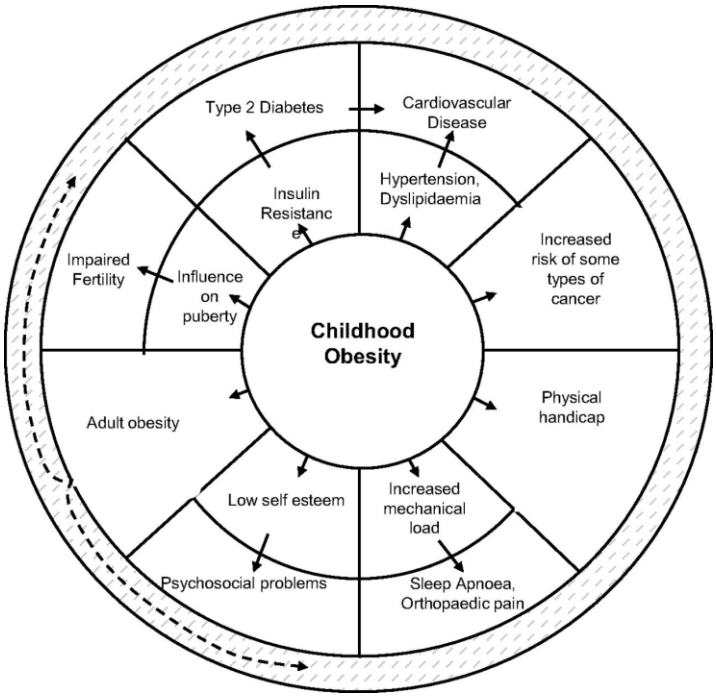
comparing genome of normal weight and obese people. Among them, the fat mass and obesity-associated protein (FTO) gene contribute significantly to obesity causing hyperphagia. Genetic forms of obesity are less than 5% and it is difficult to explain the worldwide rising of obesity only on genetic background (Albuquerque et al., 2017; Herrera et al., 2011). A valid explanation could be found in gene-environment interaction, so there is a great interest in understanding the contribution of epigenetics in the body fat increase (Ataide Lima et al., 2017).

Recently, epigenetic mechanisms on gene expression such as DNA methylation or histone modifications have been identified as one of the major contributors to the development of obesity and other disease. It has been demonstrated that exposure to obesogenic compounds during pregnancy causes epigenetic changes linked to a predisposition to weight gain in the offspring. It is also known that high fat diet can modify the methylation pattern of different genes related with obesity, such as leptin, melanocortin-4 receptor (MC4R) and peroxisome proliferator activated receptor (PPARγ) gene (Janesick & Blumberg, 2012; Thaker, 2017).

Nowadays, there are few data on humans about epigenetics and integrated research on larger groups are needed.

Complications. It is widely accepted that obese children have a greater risk for remain obese in adult age and suffer of various diseases which lead to disability and premature death. Even in childhood, obesity has been linked to both psychological and physical problems and the risk of developing them is proportional with the severity of obesity (Bhadoria et al., 2015). The most important effects of obesity on children health are shown in Figure 3.

Figure 3. Schematic summary of the main childhood obesity complications



Adapted from original figure: *Lakshman R., Elks C.E., Ong K.K.; Childhood Obesity; Circulation (2012); 126(14), 1770–1779*. Comorbidities of childhood obesity are depicted in the outer ring with their intermediate processes in the inner ring. Childhood obesity also increases the risk of adult obesity, which in turn increases the likelihood of those comorbidities.

As regard the psychological impact of obesity in children, several studies found high levels of depression and anxiety in excess weight people, with negative effects on the quality of life, self-esteem and eating attitudes (Değirmenci et al., 2015). Particularly, overweight or obese children and adolescents are often teased or bullied at school, conditions that may contribute to negative emotional consequences, academic failure, depression and low self-esteem (Hong & Espelage, 2012).

Obesity associated comorbidities affect various body areas, like the musculoskeletal, gastrointestinal, pulmonary, endocrine and cardiovascular systems (Lakshman et al., 2012). Childhood obesity increases the risk of multiple musculoskeletal concerns. Compressive forces generated by weight negatively influence the musculoskeletal system development and cause osteo-articular abnormalities in 50% of obese children. Excess weight children present a higher incidence of lower back, shoulder, foot and knee pain because of altered musculoskeletal biomechanics, increased forces on joints and physical inactivity. Moreover, obese children are more likely to develop osteoarthritis as adults (Zdziarski et al., 2015).

Some authors found abnormal sleep patterns in 94% of obese children. Children with obesity experience various sleep breathing disorders, from heavy snoring to obstructive sleep apnoea (OSA). The latter has been associated with increased cardiovascular risk in obese children (Lobstein et al., 2004; Spicuzza et al., 2015). High BMI correlate also with the developing of asthma and worsen established asthma (Papoutsakis et al., 2013; Reilly et al., 2003). Obesity is often associated with nonalcoholic fatty liver disease (NAFLD), the fatty infiltration of the liver in the absence of alcohol consumption. The first stage of NAFLD is bland steatosis but it can transform into nonalcoholic steatohepatitis, cirrhosis and hepatocellular carcinoma. In most children, NAFLD is asymptomatic but the diagnosis can be confirmed with high serum levels of liver enzymes, such as transaminases and the presence of fatty liver observed with imaging techniques (Félix et al., 2016).

Hyperinsulinemia, Insulin Resistance (IR), prediabetes and subsequently increased prevalence of Type 2 Diabetes Mellitus (T2DM) are highly associated with childhood obesity. Obese children that develop T2DM early in life show a more rapid deterioration of glycemic control and appearance of the related complications such as microalbuminuria,

dyslipidemia, and hypertension, as compared with those who develop diabetes in adult age (Pulgaron & Delamater, 2014).

Excess weigh children have also an increased incidence of other cardiometabolic risk factors, ranging from high blood pressure, low levels of high-density lipoprotein (HDL), and elevated levels of triglycerides. The Bogalusa Heart Study found an increase of 8.5-fold and 3.1-8.3-fold of the prevalence of hypertension and dyslipidemia in overweight versus normal weight young people (Perrone et al., 1998). Furthermore, left ventricular hypertrophy, increased left ventricular and left atrial diameter and systolic and diastolic dysfunction are found in obese children (Kumar & Kelly, 2017).

This thesis is an integral part of a research project focused on the mechanisms underlying early vascular alteration in childhood obesity.

2. VASCULAR ALTERATIONS IN CHILDHOOD OBESITY

Evidence on cardiovascular complications in obese children. Obese children are known to develop pro-atherosclerotic vascular alterations early in life as well as increased cardiovascular (CV) morbidity and mortality in adulthood (Cote et al., 2013; Cosimo Giannini et al., 2008). Several evidences indicate that atherosclerosis origins early in life and advances clinically silent before CV events occur, influenced by both intrinsic and external factors, including obesity (Raj, 2012). Several studies have demonstrated the important influence of childhood BMI on future CV health on large cohorts. Owen et al (Owen et al., 2009) found a positive correlation between BMI measured in later childhood and the Coronary Heart Disease (CHD) risk onwards. Bjorge and colleagues (Bjørge et al., 2008) followed a cohort of children with different BMI until the age of 40. They reported a higher risk of death for ischemic heart disease in the group with a BMI >85th percentile, as

compared with healthy weight subjects. These data are in accordance with the findings of Backer JL and colleagues (Baker et al., 2007), which confirmed that higher BMI during childhood is associated with an increased risk of CHD in adulthood. Particularly, they have highlighted a stronger correlation with the increase of the child age in both sexes. Giannini and colleagues (Giannini et al., 2008) demonstrated that early changes in glucose metabolism together with alterations of oxidative homeostasis present in most of obese children could lead to the increase of carotid Intima Media Thickness (IMT) and early cardiovascular disease (CVD). Moreover, according to the Muscatine Heart Study data, BMI levels in young girls (8-18 years) were associated with carotid IMT in adulthood (Lauer et al., 1997).

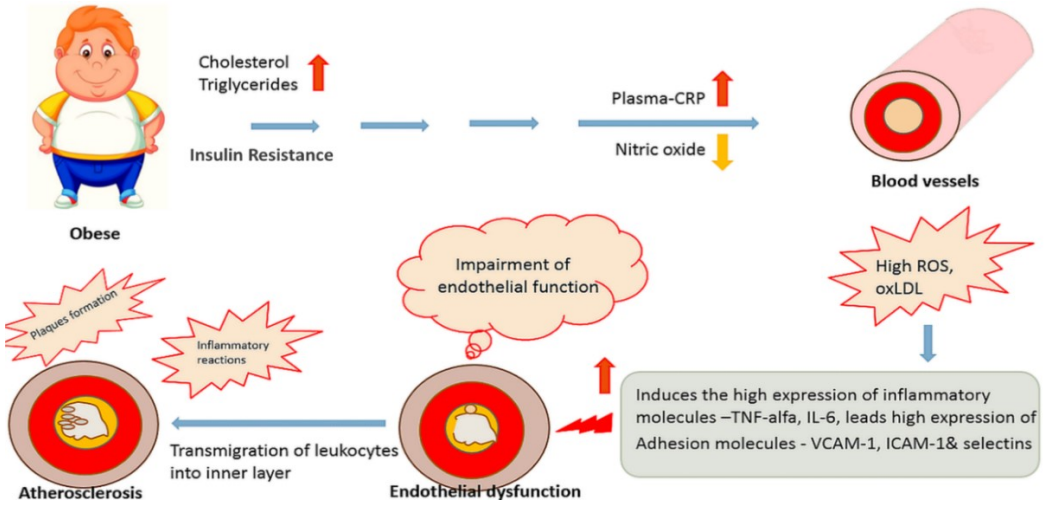
These observations have aroused interest in understanding the underlying mechanisms of atherosclerotic environment establishment in childhood, in order to prevent obesity related consequences in lifetime (McGill et al., 2008).

Obesity increases CVD risk both directly and in an indirect manner. First, the increase in body fat mass causes weight gain that consequently induces structural and functional changes in the CV system, like increased circulating blood volume, increased cardiac output, systemic vascular resistance and a moderate increase in heart rate (Ashraf & Baweja, 2013). Adipose tissue growth creates also an inflammatory environment. In fact, adipose tissue is no more considered as an energy storehouse only, but it has been recognized as an active tissue that produces and releases bioactive adipokines in the circulation that regulate vascular homeostasis, insulin sensitivity, glucose and lipid metabolism and other processes. High circulating levels of pro-inflammatory adipokines such as leptin, tumor necrosis factor α (TNF-α), interleukin 6 (IL-6) and resistin found in obese people induce systemic inflammation, facilitating the atherosclerotic process.

Moreover, indirect consequences on CVD risk are the result of obesity associated comorbidities such as hypertension, dyslipidemia, hyperinsulinemia and Insulin Resistance (IR) (Koliaki et al., 2019)

Inflammation and oxidative stress, together with loss of insulin sensitivity, high blood pressure levels and altered lipid profile are obesity related complications and well-known risk factors for CVD (Chiavaroli et al., 2011; Giannini et al., 2009; Montero et al., 2012). Particularly, they play a key role in the onset of endothelial cells dysfunction (Figure 4), that represents the starting point of the vascular plaque and remains during the progression of the atherosclerotic process until the occurrence of CV events (Michael A. & Guillermo, 2016).

Figure 4. Mechanisms linking obesity with endothelial dysfunction and atherosclerosis



Adapted from original figure: *Nirmalkar K, et al.; Endothelial Dysfunction in Mexican Obese Children, is there A Role of the Gut Microbiota?; 2016; Obes Control Ther 3(1):1-4* Obesity is associated with insulin resistance and high levels of cholesterol and triglycerides, oxidative stress (ROS, reactive oxygen species; oxLDL, oxidized low density lipoprotein), increased levels of different adipokines (IL-6, interleukin-6) and

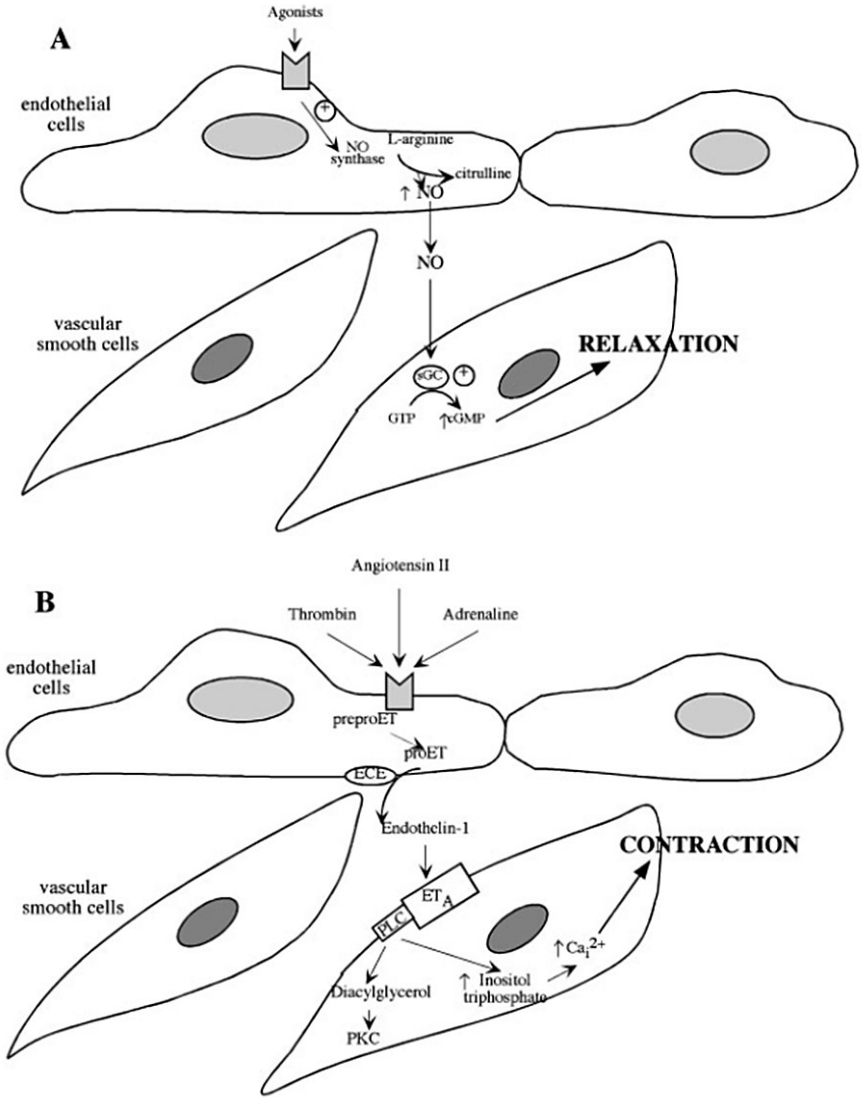
inflammatory markers (CRP, C-reactive protein; TNF α, Tumor necrosis factor α; ICAM-1 intercellular adhesion molecule 1; VCAM-1, vascular adhesion molecule 1), all of which ultimately lead to endothelial dysfunction, favoring increased atherosclerotic plaques formation.

Endothelial function and dysfunction. The vascular endothelium is the monolayer of endothelial cells (EC) that constitute the inner part of arteries, capillaries and veins, being in direct contact with circulating blood components. The endothelium has emerged not only as a mere barrier between vascular environment and underlying interstitial compartments, but it is now considered an endocrine organ (Rajendran et al., 2013). EC regulate the regional blood flow; transport immune cells to foreign materials, infective particles or damaged areas; are responsible for the control of blood fluidity, platelet adhesion and aggregation, leukocyte activation, adhesion, and transmigration; control coagulation/fibrinolysis balance and regulate immune, inflammatory and angiogenic processes. Hence, the endothelium can be considered as a key regulator of vascular homeostasis, integrating several signals that induce changes in the vessel wall phenotype and producing other molecules as response (Krüger-Genge et al., 2019).

Among these, Nitric Oxide (NO) is the key factor in preserving physiological endothelial functions. Gaseous NO is synthesized via the conversion of the amino acid L-arginine into L-citrulline by the nitric oxide synthase isoform found in EC (eNOS). This enzyme, also known as type III NOS, is constitutively expressed in vascular endothelium and it regulate the vascular tone in response to several stimuli, including shear stress and acetylcholine. Once NO is produced by eNOS, it diffuses to the close vascular smooth muscle cell (VMSC) layer and induces an increase in cyclic guanosine monophosphate (cGMP) levels by activating the guanylate cyclase (GC) enzyme. Then, cGMP activates a cGMP-dependent protein kinase that induce an increase of Ca^{2+} levels in the cytosol of VSMCs,

suppressing vasoconstriction and promoting vasodilation (Förstermann & Sessa, 2012; Pandolfi & De Filippis, 2007). Although the main effect of NO production is an increase in vasodilation, it is a pleiotropic molecule that regulate vascular homeostasis also avoiding platelet aggregation and adhesion, controlling the expression of vascular cell adhesion molecules and also inhibiting vSMC proliferation and migration (Hurlimann, 2002). These positive effects in maintaining endothelial functions can be disturbed by several pathological conditions, which are also recognized risk factors associated with the onset and progression of atherosclerosis. These include obesity, hypertension, hypercholesterolemia, insulin resistance, diabetes mellitus and heart disease (Muniyappa & Sowers, 2013; Pandolfi & De Filippis, 2007; Poirier et al., 2006; Tuñón et al., 2007). Moreover, non-modifiable factors such as aging and genetic as well as modifiable factors like smoking, lifestyle and eating habits also contribute to EC activation. In the vascular environment created by these conditions, EC phenotype shift towards a proinflammatory and prothrombotic one, characterized by impaired NO synthesis and/or bioavailability that cause reduced vasodilation and a parallel increase in the production of vasoconstrictors and other molecules that result harmful for vascular health (Marchio et al., 2019; Michael A. & Guillermo, 2016). Among these, the endothelin-1 (ET-1) is the first to counteract the NO action, with its vasoconstrictor properties and ability to induce the release of proinflammatory cytokines such as interleukin (IL) -1, IL-6 and IL-8, (Bourque et al., 2011). NO and ET-1 production and effects on VSMCs are schematic resumed in Figure 5.

Figure 5. Schematic representation of the effect of endothelial NO and ET- 1 release on VSMCs.



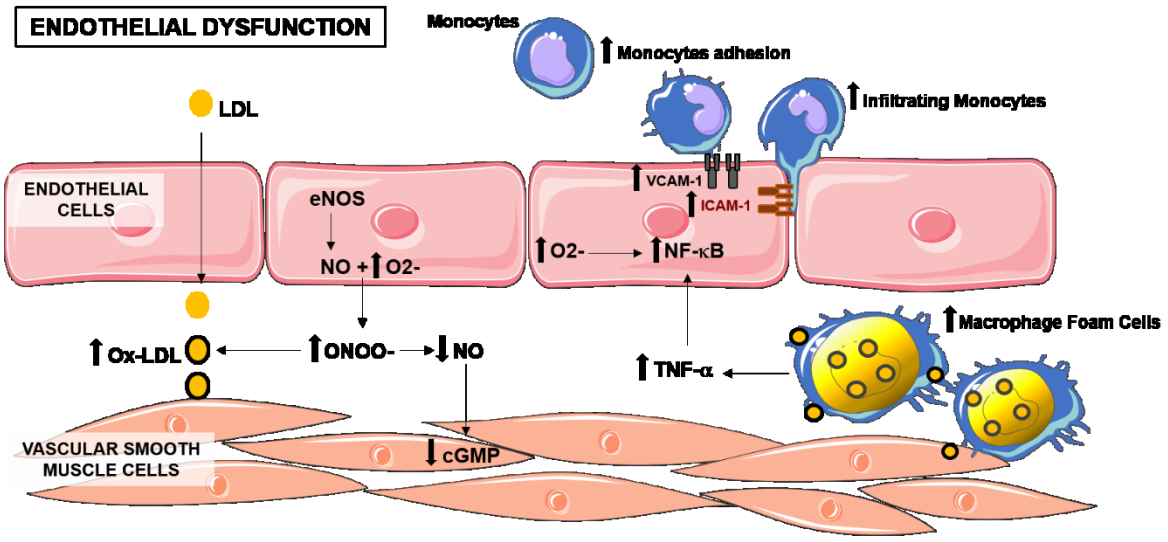
Adapted from original figure: *Michiels C; Endothelial Cell Functions; Journal of cellular physiology 2003; 196:430–443* (A) NO is produced by nitric oxide synthase present in endothelial cells (eNOS) after stimulation by various molecules and conditions: this leads to the induction of cyclic GMP (cGMP) production in vascular smooth muscle cells (VSMCs) and consequently relaxation and vasodilation. (B) Once

produced by EC, ET-1 bind its receptor (ETA, endothelin receptor A) on VSMCs and leads to the activation of several intracellular pathways, inducing contraction and vasoconstriction.

The reduction of NO bioavailability causes the proliferation and migration of VSMCs in the intima and the production of an extracellular matrix that leads to the formation of neointima and fibrous component of the atherosclerosis (Jeremy JY et al., 1999). These dysfunctional endothelial changes are further exacerbated by the increase of oxidative stress and inflammation and their crosstalk (Marchio et al., 2019). Particularly, ROS production contribute in reducing the NO bioavailability through the rapid oxidative inactivation of NO by excess superoxide (O_2^-). Indeed, as schematically described in Figure 6, this reaction leads to the production of more harmful radical species, such as peroxynitrite ($ONOO^-$), associated with permanent ECs damage and consequent pro-atherogenic effect. In addition, the BH4 cofactor is highly sensitive to the oxidation of $ONOO^-$ and the reduction of BH4 also promotes the production of O_2^- by eNOS (referred to as uncoupling of eNOS) (Förstermann & Münzel, 2006). On the other hand, ROS exert their actions through the Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-kB), inducing the synthesis of proinflammatory cytokines, such as TNF-α, which in turn activate NF-kB (Closa & Folch-Puy, 2004). The synergy between ROS and cytokines promotes the synthesis of inflammatory factors from EC and regulates the expression of adhesion molecules such as intercellular adhesion molecules (ICAM-1) and vascular adhesion molecules (VCAM-1), thus allowing neutrophils to transmigrate inside the vessel wall (Jeremy et al., 1999). Moreover, the increased permeability of ECs allows the accumulation in the tunica intima of circulating low density lipoproteins (LDLs), which in a pro-oxidant environment become oxidized LDLs (oxLDLs) more harmful and able of

damaging the endothelium and triggering the inflammatory process (Borén et al., 2020; D. Pietro et al., 2016).

Figure 6. Endothelial dysfunction



Adapted from original figure: *Di Pietro N, et al; Carotenoids in Cardiovascular Disease Prevention; JSM Atheroscler 2016 1(1): 1002.* Under oxidative condition NO may react with O2- to form ONOO-, this leads to the decrease of NO bioavailability leading to endothelial dysfunction, enhanced LDL peroxidation and chronic vascular inflammation. This is associated to lipid accumulation in the arterial wall, NF-κB activation that in turn triggers the up-regulation of VCAM-1 and ICAM-1.

Potential intracellular mechanisms underlying endothelial dysfunction in childhood obesity. Despite it is largely accepted that endothelial dysfunction plays a key role in the development of CVD in obese people, little is known about the underlying mechanisms in obese youth. Gruber and colleagues (Gruber et al., 2008) suggested that juvenile (about 14 years of age) obesity might contribute to atherogenesis via reduced NO bioavailability; Codoner-Franch and colleagues (Codoñer-Franch et al., 2011) reported an increased

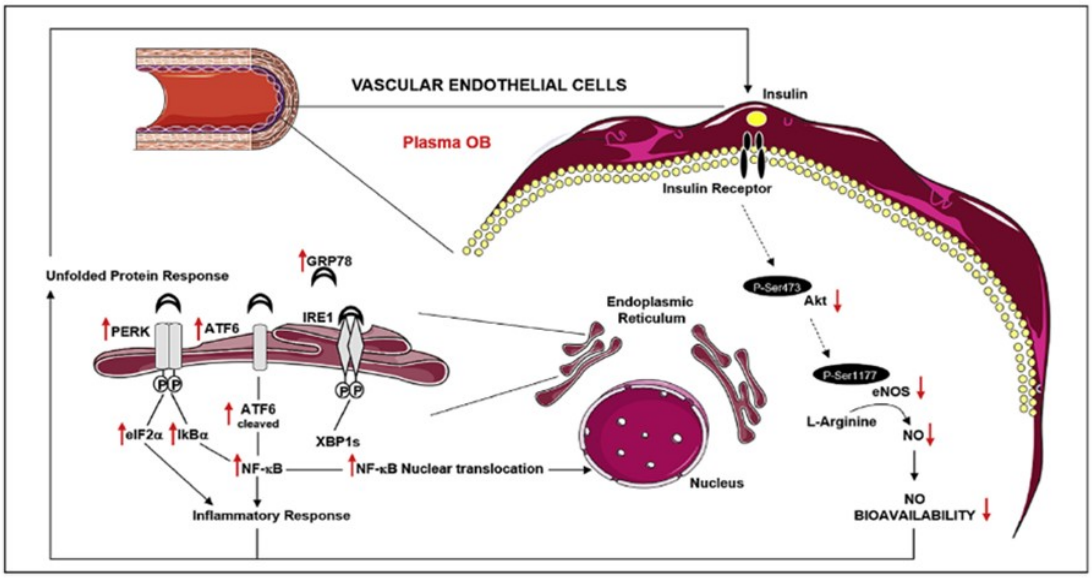
nitrosative stress (high levels of plasma nitrates and nitrites) in severely obese children aged between 7 and 14 years. Despite these *in vivo* studies have examined NO bioavailability in obese children and adolescents, they did not investigate in depth the mechanisms potentially involved.

Recently, Di Pietro and colleagues proved for the first time that plasma from pre-pubertal obese children impairs insulin stimulated NO production and bioavailability in HUVECs. Moreover, this was associated with increased endoplasmic reticulum (ER) stress induction, and increased NF-kB activation, a known key component of the inflammatory response (Figure 7).

Indeed, they found an increased ER stress and consequent Unfolded Protein Response (UPR) activation. In particular, the upstream regulator of ER stress glucose-regulated protein 78 (GRP78) and the two downstream markers protein kinase-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6) together with other markers (translation initiation factor 2 alpha, eIF2α; NF-kB- alpha inhibitor, IkBα) resulted all increased, while inositol-requiring kinase 1 (IRE1) and its downstream target X-box binding protein-1 spilced (XBP1s) resulted not involved in this process. In parallel, an altered insulin stimulated Akt (PKB, phosphokinase B) and eNOS activation and reduced NO bioavailability were also proved. Moreover, these effects were associated to increased NF-kB activation. Interestingly, inhibition of ER stress, by chemical chaperones such as PBA and TUDCA, ameliorates plasma OB-induced insulin resistance and inflammation in HUVECs. Therefore, suggesting an intricate potential loop among ER stress, IR and inflammation in endothelial cells triggered by plasma by obese children. However, although the Authors highlight the potential regulation of one of the anti-atherogenic

branch of the endothelial insulin pathway, this was not deeply analyzed (N. Di Pietro et al., 2017).

Figure 6. *In vitro* effect of plasma from obese children on vascular endothelial cells



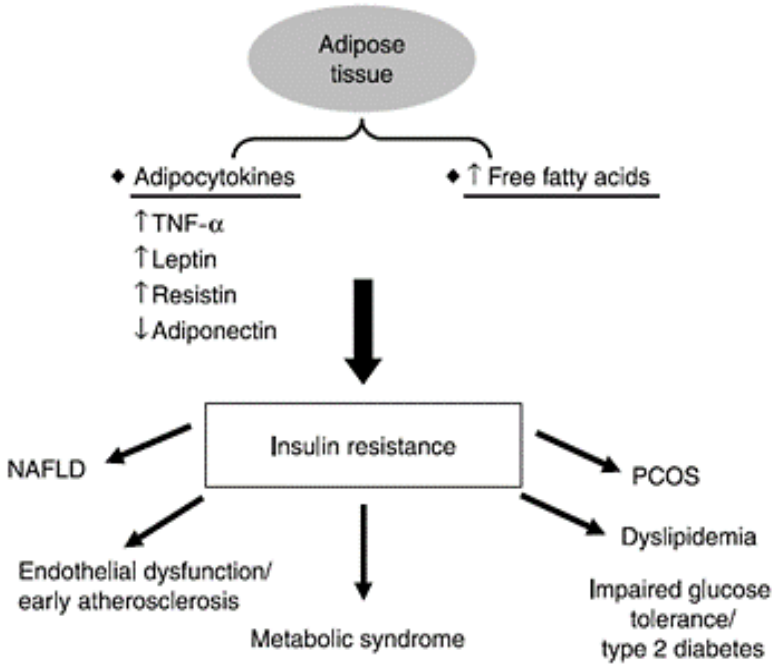
Adapted from the original figure: *Di Pietro N. et al; Plasma from pre-pubertal obese children impairs insulin stimulated Nitric Oxide bioavailability in endothelial cells: Role of ER stress; Molecular and Cellular Endocrinology 2017 52e62*. In this scheme is summarized how plasma from obese children (Plasma OB) affect endothelial function, as already described in the text. Nitric Oxide (NO), endothelial Nitric Oxide Synthase (eNOS), protein kinase B (known as Akt), glucose-regulated protein 78 (GRP78, also called BiP), inositol-requiring kinase 1 (IRE1), double-stranded RNA-activated protein kinase-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6), eukaryotic translation initiation factor 2 alpha (eIF2α), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IkB α), X-box binding protein-1 spilced (XBP1s), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB).

3. EFFECT OF OBESITY-ASSOCIATED INSULIN RESISTANCE IN ENDOTHELIAL DYSFUNCTION

Childhood obesity and insulin resistance. Insulin Resistance (IR) is defined as a clinical condition in which insulin exerts a reduced biological effect in target tissues. IR is linked with the diminished ability of insulin to favor the uptake of glucose by muscles and adipose tissue and to suppress glycogenolysis and glucose output in the liver. IR causes also alterations in protein and lipid metabolism, endothelial cells function and genes expression (Chiarelli & Marcovecchio, 2008).

IR etiology involved both genetic and environmental factor. The genetic forms are usually polygenic and related to several genes' mutations. However, high body fat, perinatal factors, lifestyle and diet as well as ethnicity and gender can influence insulin sensitivity. Obesity represents the major risk factor for the development of IR in youth. (Levy-Marchal et al., 2010). In fact, adipose tissue seems to play a key role in the pathogenesis of IR, by releasing a wide range of molecules (Figure 7) that influence insulin action at different levels.

Figure 7. Obesity-mediated insulin resistance and associated complications



Adapted from the original figure: *Chiarelli F. et al; Insulin resistance and obesity in childhood; European Journal of Endocrinology (2008) 159 S67–S74*. Adipose tissue contributes to Insulin Resistance development by producing elevated free fatty acids, high levels of TNF α (Tumor Necrosis Factor alpha), Leptin, Resistin and low levels of Adiponectin. The establishment of insulin resistance is in turn linked with several pathological conditions often found in obese children, such as NAFLD (Non-alcholic Fatty Liver Disease), endothelial dysfunction and early atherosclerosis, Metabolic syndrome, impaired glucose tolerance and type 2 diabetes, dyslipidemia and PCOS (Polycystic Ovary Syndrome).

Particularly, adipocytes produce free fatty acids (FFAs), which inhibit carbohydrate metabolism via substrate competition and impaired intracellular insulin signaling. Although increased plasmatic FFAs levels have been related to the early onset of IR in childhood obesity, the "endocrine theory" seems to better correlate with IR and obesity in youth. According with this theory, adipose tissue produces a wide variety of hormones and

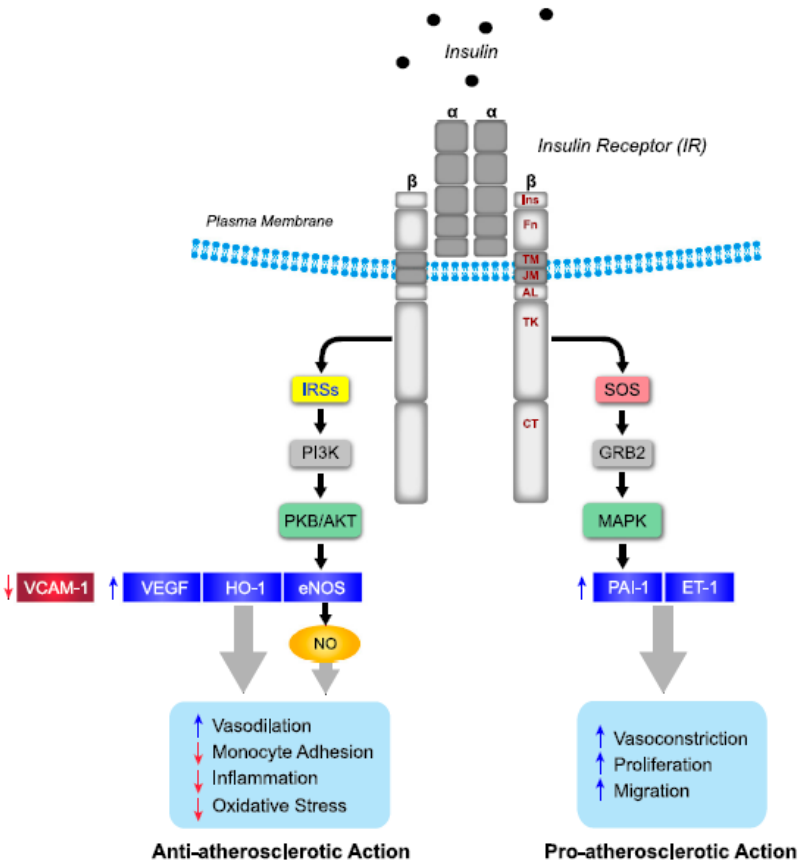
cytokines, the so-called ‘adipocykines’, which have been linked with both adiposity and IR in children and older people (Cañete et al., 2007; Chiarelli & Marcovecchio, 2008). Among these, adiponectin has an important insulin sensitizing effect associated with anti-atherogenic properties and its levels are inversely related to adiposity and IR (Panagopoulou et al., 2008). On the contrary, TNF-α and IL-6 are inflammatory factors whose levels are higher in obesity and IR. Particularly, IL-6 stimulates CRP production in the liver, favoring the systemic pro-inflammatory environment found in obese children (C. Giannini et al., 2009). Furthermore, also leptin and resistin levels are found higher in animal model of IR (Chiarelli & Marcovecchio, 2008; Tagi et al., 2019). Finally, IR condition could be worsened by children diet composition. Indeed, animal and human studies reported that a high energy intake as well as a diet rich in fat and carbohydrates and low in fiber could increase the risk of developing IR (Cañete et al., 2007).

Once IR established, it has several consequences on children health. Among these, an increased prevalence of dyslipidemia, hypertension and NO-dependent vasodilation impairment together with subsequent higher CV risk have been related to IR in obese children and adolescents (Gruber et al., 2008; Júarez-Lopez et al., 2010).

Insulin resistance and endothelial dysfunction. Metabolic changes related to IR condition can interfere with normal vascular homeostasis. In fact, it has been demonstrated that insulin interacts with the vascular wall in several manner. First, endothelium is responsible for the transport of insulin into the peripheral tissues, such as skeletal muscle, adipose tissue, the central nervous system, and others. Moreover, insulin seems to act also on vascular endothelium directly. In fact, like all cells found in blood vessels, ECs have insulin receptors, composed of a hormone binding α subunit and a β subunit, a tyrosine kinase. After insulin binds the receptor subunit α, the β subunit is activated and in turn

phosphorylates on tyrosine residues other substrates (Muniyappa & Sowers, 2013; Potenza et al., 2009). Insulin binding to its specific receptor can induce the initiation of two major pathways: the PI3K/Akt (known as anti-atherogenic pathway) or the MAPK/Erk one (known as the pro-atherogenic pathway) (Figure 8).

Figure 8: Insulin signaling pathways in endothelial cells



Adapted from original figure: *King GL et al; Selective Insulin Resistance and the Development of Cardiovascular Diseases in Diabetes: The 2015 Edwin Bierman Award Lecture; Diabetes 2016; 65(6):1462-71* Insulin's actions in vascular cells are mediated by the activation of either the IRS/PI3K/Akt or the SOS/Grb2/MAPK pathway. The IRS/PI3K/Akt pathway can activate eNOS and regulate the expression of HO-1, VEGF, and VCAM-1, which have anti-atherosclerotic actions. In contrast, the SOS/Grb2/MAPK pathway activates vasocontraction and proliferation, which are pro-atherosclerotic effects. AL, activation

loop; CT, carboxy-terminal tail; Fn, fibronectin type domains; Ins, insert in Fn; JM, juxtamembrane domain; TK, tyrosine-kinase domain; TM, transmembrane domain; IRSs, insulin receptor substrates; PI3K, Phosphoinositide 3-kinase; Akt, phosphokinase B; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; HO-1, hemoxygenase-1; VEGF, vascular endothelial growth factor; VCAM-1, vascular cellular adhesion molecule-1; SOS, Son of Sevenless; GRB2, Growth factor receptor-bound protein 2; MAPK, mitogen-activated protein kinase; ET-1, endothelin-1; PAI-1, plasminogen activator inhibitor 1

The insulin receptor substrates 1 and 2 (IRS-1/-2) phosphorylation is required for the activation of the first mentioned pathway. Once activated, these substrates bind PI3K, essential in insulin-stimulated production of NO: it subsequently activates PDK1 and PKB/Akt, that directly phosphorylates eNOS at Ser1177, inducing NO production and vasodilation. PI3K pathway appears also to counteract the expression of proatherogenic molecules, including plasminogen activator inhibitor type 1 (PAI-1), VCAM-1 and E-selectin. Alternatively, the activated β subunit interact directly with Src homology 2 domain containing transforming protein (Shc) and the growth factor receptor-bound protein-2 (Grb2). This is followed by a cascade of phosphorylation processes that led to activation of G protein Ras, Raf, and MAPKs (MEK1 and ERK1/2). The activation of the MAPK branch of insulin signaling promotes growth, mitogenesis and differentiation, favors the secretion of the vasoconstrictor ET-1, PAI-1 and the VCAM-1 (King et al., 2016; Potenza et al., 2009). Insulin controls vascular homeostasis also by regulating EC expression of hemoxygenase-1 (HO-1), vascular endothelial growth factor (VEGF) and VCAM-1: physiological concentrations of insulin can increase VEGF and HO-1 levels and decrease VCAM-1 exposure, mainly through the PI3K/Akt pathway (Geraldes et al., 2008; Jiang et al., 2003; Kuboki et al., 2000). Furthermore, insulin promote NO production by decreasing the interaction of caveolin-1 (Cav-1), a negative regulator of eNOS activity and

increase its association with eNOS-Hsp90, which promotes eNOS activity. Insulin has also been reported to increase the expression of eNOS in the transcription phase (King et al., 2016).

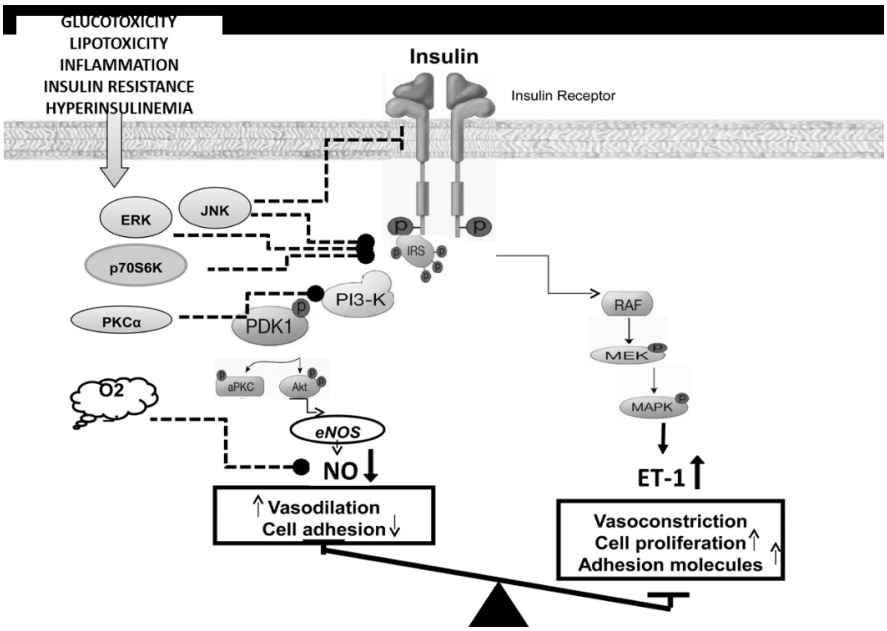
Hence, insulin could exert both antiatherogenic or proatherogenic effects depending on the its level and the activated signaling pathway. Alterations in insulin signaling pathway, as occurs in IR, could lead to the endothelial dysfunction and promote the atherosclerotic progression. Particularly, several experimental and clinical evidence support the concept that defects on PI3K-signaling pathways result in impairment of insulin-mediated endothelial effects. In fact, a reduced NO-mediated vasodilation was found in animal models of metabolic IR with impaired PI3K signaling, in response to insulin (Potenza et al., 2009). Mice lacking endothelial insulin receptor showed diminished eNOS and ET-1 expression, associated with insulin resistance and elevated blood pressure when exposed to a high-fat diet (Vicent et al., 2003). Rask-Madsen et coll. found a decreased insulin effect on phosphorylation of eNOS (Ser1177) and suppression of VCAM-1 levels, in ECs from lung and aorta of an atherosclerotic mouse model (Rask-Madsen et al., 2010). Furthermore, IRS-1 gene deletion in mice causes both IR and impaired endothelium-dependent vasodilation (Abe et al., 1998). In humans, insulin receptor mutation (at Thr1134) was associated with both metabolic and vascular abnormalities (Moller et al., 1990). An IRS-1 genetic polymorphism linked with metabolic IR is also associated with endothelial dysfunction in subjects carrying the point mutation of IRS-1 (Federici et al., 2004). Moreover, Okon EB and colleagues found an attenuated phosphorylation of Akt and eNOS in vessels from patients with IR, as compared with control subjects (Okon et al., 2005). Reduced insulin-stimulated vasodilation together with altered cardiac function are promoted at least by ROS overproduction in EC. Altered expression of catalytic and

regulatory subunits of NADPH complex is implicated in increased ROS production, as demonstrated in both animal and human studies (Furukawa et al., 2017; Silver et al., 2007)

In summary (Figure 9), impaired insulin signaling and the consequent insufficient PI3K-mediated activation of eNOS lead to a reduced NO availability and activity, favored also by ROS production. Moreover, this condition is worsened by MAPK-dependent pathways activation (by compensatory hyperinsulinemia) that induce the overproduction of mediators opposing NO function as ET-1 (Ormazabal et al., 2018).

These events promote a dysfunctional vascular phenotype characterized by greater expression and exposure of cell adhesion molecules ICAM-1, VCAM-1 and E-selectin; acute production of proinflammatory cytokines; increased proliferation of VSMC; increased platelet aggregation; increased leucocytes recruitment, adhesion and infiltration to the vascular walls. This complicated scenario is further worsened by the presence of oxidative stress and inflammation (Marchio et al., 2019; Muniyappa & Sowers, 2013).

Figure 9: Impairment of insulin signaling pathway and endothelial dysfunction



Adapted from original figure: *Muniyappa R. et al; Role of insulin resistance in endothelial dysfunction; Rev Endocr Metab Disord. 2013; 14(1): 5–12*. PI3-kinase branch of insulin signaling regulates NO production and vasodilation in vascular endothelium. MAP-kinase branch of insulin signaling controls secretion of endothelin-1 (ET-1) and adhesion molecule expression in vascular endothelium. Glucotoxicity, lipotoxicity, and various cytokines activate signaling molecules that inhibit PI3K/Akt signaling. eNOS, endothelial nitric oxide synthase; IRS, insulin receptor substrate; MEK, MAPK kinase; PDK, phosphoinositide-dependent protein kinase; PKC, protein kinase C; IRS, insulin receptor substrate; ERK, extracellular signal-regulated kinase; JNK, C-Jun Nterminal kinase; p70S6K, p70 ribosomal S6 kinase; AP-1, activator protein-1; NO, nitric oxide; and ET-1, endothelin-1.

Molecular and pathophysiological mechanisms underlying reciprocal relationships between IR and endothelial dysfunction result in a vicious cycle reinforcing the link between metabolic and cardiovascular disorders. Therapeutic interventions on endothelial function and/or insulin sensitivity improvement seems to ameliorate both metabolic and cardiovascular abnormalities in animal and human model (Kim et al., 2006). Thus, it is important to study these pathological conditions in order to identify the involved signaling pathways or specific key molecule and use them as therapeutic target.

In this thesis, we focused in understanding the potential involvement of the mechanistic/mammalian target of rapamycin (mTOR) in the regulation of the IR related endothelial dysfunction in childhood obesity.

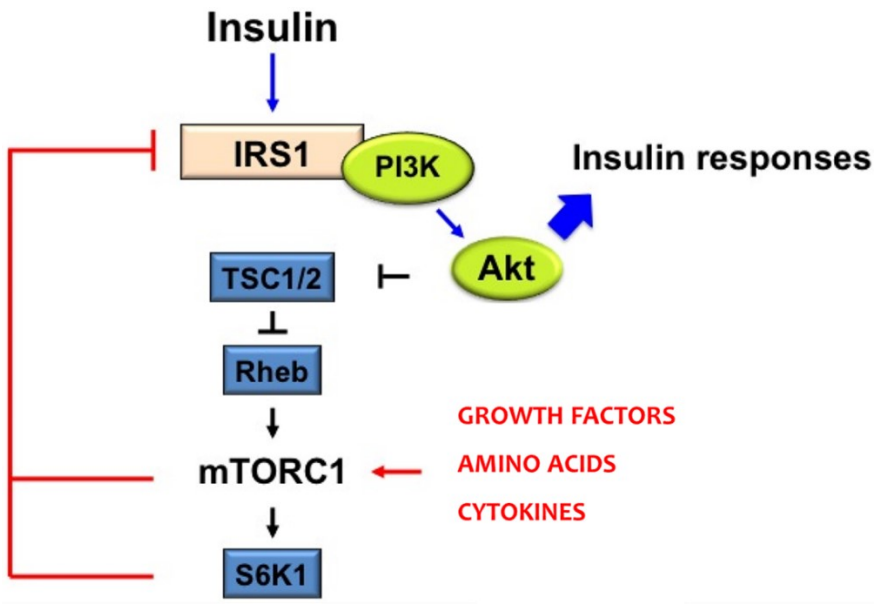
Potential role of mTORC1 in endothelial insulin resistance. mTOR is an evolutionarily conserved serine/threonine protein kinase belonging to the phosphatidylinositol kinase-related kinase (PIKK) family with a key role in mammalian cells growth, proliferation, and survival (Saxton & Sabatini, 2017; Wullschleger et al., 2006). mTOR exists mainly in two macromolecular complexes, the mTOR complex 1 (mTORC1) and mTOR complex 2

(mTORC2) that activate distinct but interconnected pathways with different upstream regulators and downstream target.

More in detail, mTORC1 acts as a hub, which integrates several intracellular and environmental factors such as insulin, cytokines, oxygen, amino acids, growth factors and nutrients and regulates many cellular processes including autophagy, protein translation and ribosomal biogenesis (Kapahi et al., 2010). The mTORC1 is composed of mTOR, regulatory associated protein of mTOR (Raptor) and mLST8 and regulates growth and metabolism via direct phosphorylation of ribosomal p70 S6 kinase (p70S6K) and eukaryotic initiation factor 4E (eIF4E)- binding protein (Sciarretta et al., 2018). mTORC1 is sensitive to Rapamycin, an antifungal macrolide compound, that does not directly inhibit mTOR but binds to its immunophilin, FK binding protein (FKBP12). Rapamycin plus FKBP12 then interact with the kinase in the context of the mTORC1 complex and potently inhibits its activity (Dutcher et al., 2004).

Focusing on mTORC1 regulation by insulin levels, it is known that insulin leads to the mTORC1 activation via the PI3K/Akt signaling pathway. Once activated, mTORC1 phosphorylates and in turn activates S6K1 that seems to create a negative feedback, by blocking the pathway upstream (Figure 10). In fact, it has been reported that the inhibition of S6K1 by rapamycin (known mTOR inhibitor) and the S6K1 deletion in animal models is followed by a metabolic improvement in insulin sensitivity in response to nutrient overload and high insulin levels (Leontieva et al., 2013; Shum et al., 2016; Um et al., 2006).

Figure 10: mTORC1-dependent feedback mechanisms on insulin-stimulated Akt activation



Adapted from original figure: *Yoon MS; The Emerging Role of Branched-Chain Amino Acids in Insulin Resistance and Metabolism; 2016 8(7), 405.* Insulin bind to its receptor and consequently activation of Akt lead to inhibition of TSC1/2 (Tuberous scletotic protein 1-2), known mTORC1 (mammalian target of Rapamycin) inhibitors, inducing mTORC1 and S6K1 (Ribosomal protein S6 kinase beta-1) activation. These two molecules act on IRS-1/PI3K/Akt (Insulin receptor substrate 1, Phosphoinositide 3-kinases, phosphokinase B) pathway with a negative feedback, altering insulin responses. Growth factors, aminoacids and Cytokines also promote mTORC1 activation, favoring impaired insulin signaling.

This loop regulation has been demonstrated to be related with insulin signaling in various tissues, but little is known about mTORC1/PI3K/Akt pathway in endothelium. Interestingly, it has been reported that the S6K1 overexpression in EC negatively regulate IRS-1 through phosphorylation of serine residues causing its consequent degradation, with a feedback loop that leads to IR. Therefore, to maintain a physiological Akt activity with consequent insulin-dependent vasodilation, a modulatory cycle has been assumed that

involves the activation/deactivation of mTOR depending on the activation status of Akt (Villalobos-Labra et al., 2017).

Moreover, mTORC1-S6K1 pathway seems to contribute to endothelial function also by modulating ROS and pro-inflammatory signals. Indeed, as recently demonstrated elevated mTORC1 activity increases ROS generation contributing to reduced endothelial-mediated vasorelaxation. Recently, it has also been proven that mTORC1 contribute to a novel mechanism of atherosclerosis by regulating the activation of nucleotide-binding oligomerization domain-like receptor family, pyrin domain-containing 3 (NLRP3) inflammasome via ROS (Chen et al., 2019)

Based on such evidence, in the present research project -which includes this experimental thesis - it has been hypothesized that mTOR might play a crucial role in the loss of vascular insulin sensitivity observed in childhood obesity, participating to the early onset of endothelial dysfunction along with reduced NO bioavailability, enhanced oxidative stress and chronic inflammation.

AIM

Based on this background, the aim of the present thesis was to better investigate the mechanisms that contribute to the endothelial dysfunction establishment in childhood obesity. For this purpose, an *in vitro* model of HUVECs treated with plasma from obese and healthy weight prepuberal children was used.

Under these experimental conditions oxidative stress levels and exposure of adhesion molecules were evaluated, along with the assessment of the monocyte-endothelial interaction. In parallel, the possible dysregulation between the pro and anti-atherogenic endothelial insulin pathways and the potential involvement of the mTORC1-S6K1 signaling pathway were also evaluated.

MATERIALS AND METHODS

1. Study population

This study involved 32 obese (OB) and 32 normal weight (CTRL) pre-pubertal children, aged 6 to 10 years. OB children were recruited at the Department of Pediatrics, University of Chieti, Italy. The study protocol was approved by the Ethics Committee of the University of Chieti and, in accordance with the Declaration of Helsinki, a signed informed consent was obtained from all subjects taking part in the study.

OB subjects were generally healthy, obese according to the International Obesity Task Force (IOTF) criteria and not on a weight loss diet. Subjects with other chronic diseases (diabetes, endocrine disorders, hereditary diseases, or systemic inflammation) or taking drugs have been excluded. CTRL group involved normal weight children, in good health and without any chronic disease.

All children underwent a complete physical examination, including anthropometric measurements. At the same time, blood samples were taken to evaluate fasting glucose and insulin levels and collected for the *in vitro* studies.

2. Anthropometric and metabolic variables

Height, weight, waist circumference (WC) and BMI were evaluated. Body weight was determined to the nearest 0.1 kg, and height was measured by Harpenden stadiometer to the nearest 0.1 cm. BMI was calculated as the weight in kilograms divided by the square of the height in meters and was converted into standard deviation scores (SDS) using published reference values for age and sex for the Italian population (Cacciari et al., 2006). Pubertal stages were also evaluated, according to Tanner criteria.

Glucose oxidase method was used to determine glucose levels and the two-site immune-enzymatic assay (AIA-PACK IRI; Tosoh, Tokyo, Japan) was used to quantify insulin levels. The Homeostasis Model Assessment of insulin resistance (HOMA-IR) was used as a surrogate index of IR and it was calculated as: [fasting insulin (mU/l) × fasting glucose (mmol/l)/22.5] (Matthews et al., 1985).

3. Plasma collection

At the time of blood collection, all subjects were fasting and free of common infectious diseases. Blood samples were obtained through venous withdrawal and collected into evacuated tubes containing Ethylenediaminetetraacetic acid (EDTA). Then, whole blood was centrifuged at 1,578 *g* for 10 minutes at room temperature (RT) and the plasmatic fraction was aliquoted and stored at -80°C until experimental analysis.

4. Materials and antibodies

Phosphate-buffered saline (PBS), Dulbecco's modified Eagle medium (DMEM), M199 endothelial growth medium, trypsin, ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (BSA), L-glutamine, penicillin-streptomycin, tumor necrosis factor α (TNF-α) and rapamycin were purchased from Sigma-Aldrich (Saint Louis, USA). Fetal bovine serum (FBS) was from Life Technologies (Monza, Italy). Trypsin and EDTA were from Mascia Brunelli (Milan, Italy). CellROX green reagent was purchased from Molecular Probes (Eugene, OR, USA).

Primary antibodies for VCAM-1 and ICAM-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). IRS1, phospho-IRS1 (Ser636/639), Akt, phospho-Akt (Ser473), MAPK and phosho-p44/42 MAPK (Thr202/Tyr204) antibodies were from Cell Signaling Technology (Milan, Italy). S6K1 and phospho-S6K1 (Thr389 + Thr412)

were from Abcam (Cambrige, UK). PE labeled anti-VCAM-1 (phycoerythrin-labeled, CAT. 305806) and FITC-labeled anti-ICAM-1 (fluorescein isothiocyanate-labeled, CAT. 313104) secondary antibodies were from BioLegend (San Diego, CA, USA). Alexa Fluor 488-conjugated secondary antibody was from Invitrogen (Thermo Fisher Scientific, UK).

5. Cell cultures

Umbilical cords were obtained from randomly selected healthy Caucasian mothers delivering at the Hospital of Pescara. All procedures were in agreement with the ethical standards of the Institutional Committee on Human Experimentation and in accordance with the Declaration of Helsinki principles. After approval of the protocol by the Institutional Review Board, signed informed consent was obtained from each participating subject. Primary HUVECs were obtained as described by Di Tomo and colleagues. Briefly, after perfusion of umbilical vein cords with washing solution (physiological saline with 1 % penicillin/streptomycin and 1% amphotericin B) and then with 1 mg/mL Collagenase 1A at 37°C, HUVEC were grown in 1.5% gelatin-coated culture plates in endothelial growth medium composed by DMEM/M199 (1:1) supplemented with 1% L-glutamine, 1% penicillin-streptomycin, 20% FBS, 10 µg/mL heparin, and 50 µg/mL endothelial cell growth factor (ECGF). Primary C-HUVEC were characterized as Von Willebrand factor positive and alphasmooth muscle cell actin negative (Di Tomo et al., 2017).

For all experiments, HUVECs were used between the 3rd and 5th passages *in vitro*. Each experimental set was performed at least in triplicate, using three different cell batches.

6. Experimental protocols

In order to evaluate the intracellular ROS production, VCAM-1 and ICAM-1 membrane exposure, monocytes-endothelial cells interaction and the activation of MAPK, IRS-1, Akt and S6K1, HUVECs were serum starved for 2 hours and then cultured for 16 hours with 0,5 % FBS (baseline condition) and with the addition of 10% pooled OB or CTRL plasma (each pool consisted in three different CTRL or OB plasma). Early activation of S6K1 was also evaluated in HUVECs treated as above but with shorter incubation time of 30 minutes. In some experiments, insulin at concentrations of 40 nM and 100 nM were used, mimicking circulating levels of insulin in CTRL- and OB-children respectively.

Moreover, in order to investigate the potential involvement of mTORC1-S6K1 pathway, HUVECs were pre-incubated with rapamycin, a specific inhibitor of mTOR, at concentration of 100 nM for 30 minutes and therefore for the remaining incubation time (16 hours) with plasma.

7. MTT Assay

The effect of rapamycin on HUVECs viability was assessed by the 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) method. In detail, 1 $*10^5$ cells were seeded in 96-well plates, grown to confluence and stimulated for 16 h with growing doses of Rapamycin (50, 100, 200 and 500 nM), with the medium alone (as a control) or with 0,05 % of dimethylsulfoxide (DMSO, used to prepare Rapamycin solution). Then, 0.5 mg/ml of MTT solution (prepared in phosphate buffer saline, PBS) was added to each well. After 3 h incubation at 37° C, 0.2 ml of DMSO/well was added and maintained for 30 minutes. Crystal solubilization of MTT salt by DMSO adding occurred only in the vital cells. The spectrometric absorbance at 540nm was read using a

microplate reader (SpectraMAX 190, Molecular Devices, Sunnyvale, California, USA).

8. ROS production assessment

The CellROX green reagent was used to evaluate ROS production levels in HUVECs in the experimental conditions described above. This fluorescent probe penetrates the cell and, when oxidized by intracellular free radicals, binds to DNA, emitting a more intense green fluorescence. Briefly, CellROX reagent at a final concentration of 2.5 μM was added to the cells for 30 minutes at 37°C. Samples were analyzed by flow cytometry and detected at 515–530 nm following the manufacturer's instructions.

Hydrogen peroxide (H_2O_2) at 100 μM concentration was added 30 minutes before the assay and was used as positive control.

9. Monocyte adhesion assay

U937 monocytes adhesion to HUVECs was evaluated using a cell adhesion assay, in the basal state and after 16 hours exposure to TNF-α (1 ng/ml), as an inflammatory stimulus. Cells were grown to confluence in 6-well tissue culture plates, incubated with OB- or CTRL-plasma as described in the experimental protocol and U937 cell adhesion was performed. Briefly, U937 (1×10^6 cells) were added to HUVECs under rotating conditions at RT. After 20 minutes, non-adhering cells were removed, and monolayers were fixed with 1% paraformaldehyde. As negative control, anti-VCAM-1 and anti-ICAM-1 antibodies were added to some monolayers 1 hour before the assay.

The number of adherent cells was determined by counting 8 different high-power field (3.5 mm^2). Photographs, showing U937 adhesion to endothelial cells, were randomly chosen from high-power fields taken at half-radius distance from the center of the well in 1 of 3 comparative experiments of same design.

10. Flow cytometric analysis

Activation of key molecules belonging to anti- and pro-atherogenic insulin pathways and S6K1 activation were evaluated in HUVECs, cultured as described in the experimental protocol, by flow cytometry.

Briefly, confluent HUVECs were detached with 0.5% trypsin/0.2% EDTA after 16 hours of incubation and $5*10^5$ cells/sample were resuspended in PBS without Ca/Mg, after a centrifugation at 1500 rpm for 5 minutes. Then, cells were permeabilized (using FACS Lysing and Permeabilizing Solution, BD Biosciences), processed as suggested by the manufacturer's instructions and incubated with primary antibodies against IRS1, phospho-IRS1 (Ser636/639), Akt, phospho-Akt (Ser473), MAPK and phosho-p44/42 MAPK (Thr202/Tyr204), S6K1 and phospho-S6K1 (Thr389+Thr412). The incubation of primary antibody was followed by incubation with the specific FITC-labeled secondary antibody. Moreover, to assess non-specific fluorescence, samples stained with the corresponding secondary antibody alone were used.

In order to evaluate VCAM-1 and ICAM-1 membrane exposure levels, non-permeabilized cells were detached using EDTA 5 mM solution, washed and resuspended in bovine serum albumin (BSA) 0.5%. Cells were pelleted by centrifugation at 800 rpm for 15 min and then incubated at the same time with anti-VCAM-1 PE-conjugate and with anti-ICAM-1 FITC-conjugate, both for 30 min at room temperature.

A total of 1×10^4 events/samples were acquired by fluorescent-activated cell sorting (FACS) Canto II (BD Biosciences, California, USA). All data were analyzed using FACS Diva (BD Biosciences) and FlowJo v.8.8.6 software (TreeStar, Ashland, OR) and expressed as mean fluorescence intensity (MFI) ratio. The MFI ratio was calculated by dividing the MFI of positive events by the MFI of negative events (MFI of secondary

antibody).

11. Statistical analysis

Data were expressed as means ± Standard Deviation (SD) or means ± Standard Error of the Mean (SEM) unless otherwise stated. Not normally distributed variables were log transformed before data analysis. Differences between the two study groups in continuous variables were tested by unpaired t-test. For *in vitro* studies, analysis was performed using the unpaired Student's t-test or the one-way analysis of variance (ANOVA) and Bonferroni post hoc test. P values < 0.05 were considered statistically significant.

RESULTS

1. Clinical and metabolic characteristics

The main anthropometric and metabolic characteristics of the study population are shown in Table 1. OB children presented significantly higher weight, BMI and BMI z-score as compared with CTRL children, but comparable age and pre-pubertal status. Regarding the metabolic parameters assessed, insulin and HOMA-IR were significantly higher in OB- than CTRL-children, while fasting glucose levels were similar.

	Obese children	**Control children**	**p Value**
N	32	32	
Age (years)	9.2 ± 1.7	8.8 ± 1.2	0.28
Height (cm)	140 ± 9	125 ± 6	0.01
Weight (Kg)	59.7 ± 20.4	25.8 ± 5.8	0.00
BMI (Kg/m^2)	26.6 ± 1.6	16.6 ± 2.4	<0.001
BMI z-score	2.72 ± 0.31	0.33 ± 0.75	0.001
Glycemia (mg/dl)	81.5 ± 10.6	77.05 ± 7.4	0.48
Insulin (mU/ml)	17.8 ± 4.8	5.4 ± 2.3	<0.005
HOMA-IR	3.5 ± 2.3	0.8 ± 0.2	0.006

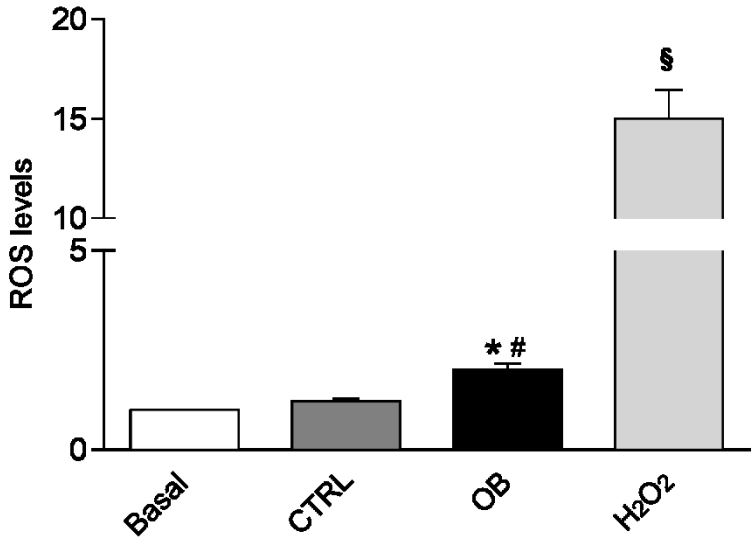
Table 1. **Anthropometric and metabolic characteristics of the study population.**

Data are mean ± SD. BMI, Body Mass Index; HOMA-IR, Homeostatic Model Assessment for Insulin Resistance.

2. Effect of OB-plasma on ROS production

The effect of OB plasma on intracellular ROS levels was first investigated. HUVECs cultured for 16 hours with OB-plasma proved a significant increase in ROS generation (Figure 11) as compared to cells incubated with CTRL-plasma (1.7 folds, $p < 0.05$) and to basal condition (2 folds, $p < 0.05$). H_2O_2 treatment was used as positive control ($p < 0.001$ *vs* Basal, CTRL and OB).

Figure 11. Basal and stimulated ROS production.



HUVECs serum starved were cultured for 16 hrs with 0.5% FBS as baseline condition (Basal) and with the addition of 10% pool of three OB- or CTRL-plasma. H_2O_2 (100 μM) was also employed as positive control. ROS levels were determined using CellROX green reagent and analyzed by flow cytometry. Data are shown as means ± SD of at least six independent experiments (N = 18 OB; N = 18 CTRL). $^{*}p < 0.05$ *vs* Basal; $^{\#}p < 0.05$ *vs* CTRL; $^{\S}p < 0.001$ *vs* Basal, CTRL and OB.

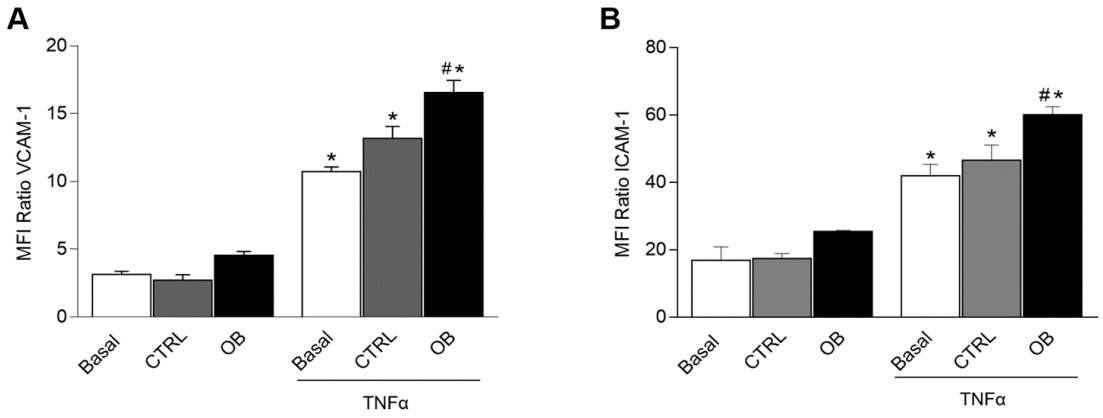
3. Effect of OB-plasma on adhesion molecules membrane exposure

VCAM-1 and ICAM-1 membrane exposure levels were then evaluated in HUVECs incubated with OB- or CTRL-plasma both in basal condition and in the presence of an inflammatory stimulus, such as TNF-α (1 ng/ml).

As expected, TNF-α stimulation increased endothelial VCAM-1 and ICAM-1 exposure compared to baseline conditions, resulting significant for each treatment *versus* its baseline ($p < 0.05$).

Moreover, following TNF-α stimulation, OB plasma significantly increased the levels of VCAM-1 (Figure 12A) and ICAM-1 (Figure 12B) in HUVECs, both compared to CTRL-plasma (1.2 and 1.3 folds, respectively; both $p < 0.05$) and basal (1.3 and 1.4 folds, respectively; both $p < 0.05$). Without inflammatory stimulus, both adhesion molecules levels showed an increasing trend following OB plasma incubation ($p = 0.05$).

Figure 12. Basal and stimulated adhesion molecules membrane exposure.



HUVECs serum starved were cultured for 16 hrs with 0.5% FBS as baseline condition (Basal) and with the addition of 10% pool of three OB- or CTRL-plasma, in the presence or absence of TNF-α (1 ng/ml) as inflammatory stimulus. (A) VCAM-1 and (B) ICAM-1 membrane exposure were evaluated by flow cytometry. The results are expressed as Mean Fluorescence Intensity (MFI) Ratio of surface exposure on the plasma membrane of VCAM-1 and ICAM-1 in non-permeabilized cells (N = 3). Data are shown as mean ±

SD of at least three independent experiments (N = 18 OB; N = 18 CTRL). (A and B) $^{*}p < 0.05$ TNF-α *vs* baseline; $^{\#}p < 0.05$ OB TNF-α *vs* Basal and CTRL TNF-α.

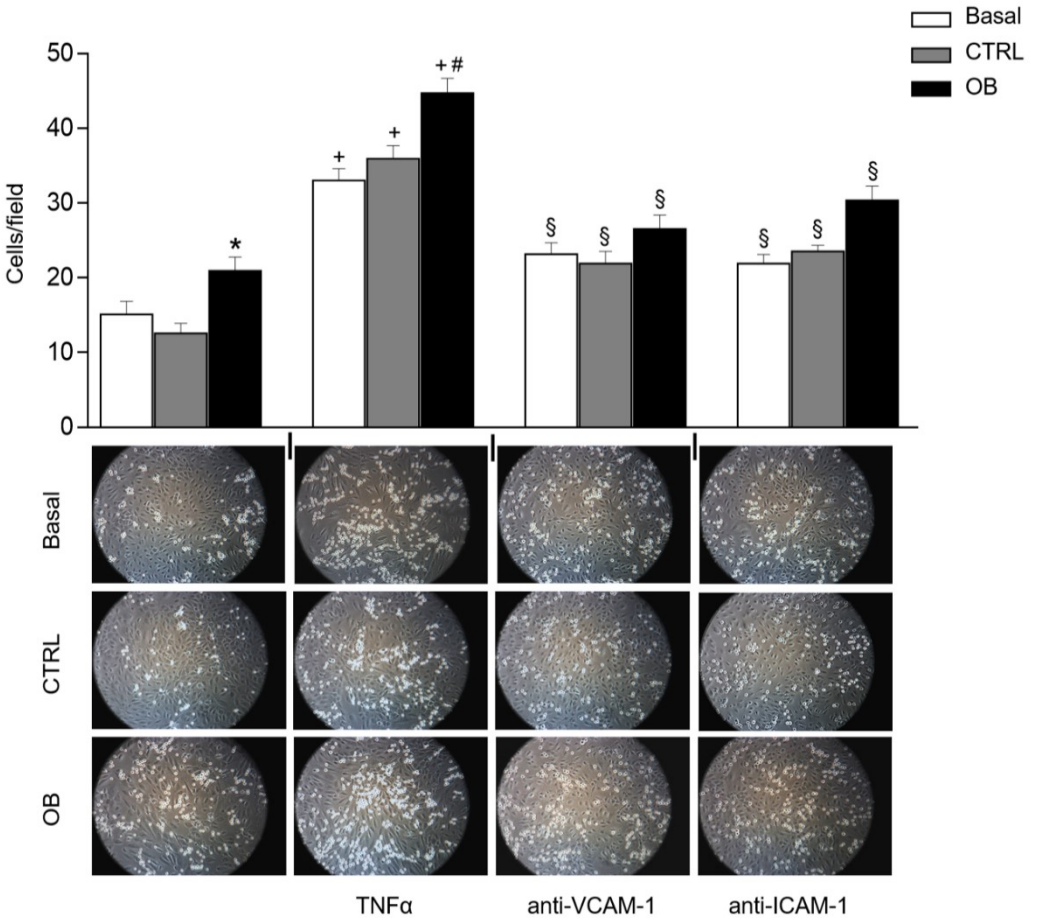
4. Effect of OB-plasma on HUVECs-monocytes adhesion

The increased membrane exposure of VCAM-1 and ICAM-1 is among the key events allowing monocytes adhesion to the endothelium. Therefore, using this *in vitro* model that is close to the *in vivo* pathophysiological state, it has been tested the effect of 16 hrs stimulation with OB- and CTRL-plasma on monocyte (U937) adhesion to HUVECs at baseline and under an inflammatory stimulus (TNF-α, 1 ng/ml).

As shown in Figure 13, OB plasma significantly increased the adhesion of monocytes to HUVECs as compared to CTRL plasma (1.8 folds, $p < 0.05$) and to unstimulated cells (1.5 folds, $p < 0.05$), even in the absence of inflammatory stimuli. As expected, exposure to TNF-α significantly increased the monocytes adhesion in both basal and CTRL- or OB-plasma treated cells compared to their respective basal conditions (2.1, 2.6 and 2.3 folds, respectively; all $p < 0.05$). Interestingly, the number of adherent monocytes was higher in HUVECs exposed to OB-plasma than in basal and CTRL-plasma (1.4 and 1.28 folds, respectively; both $p < 0.05$), both in absence and in presence of TNF-α.

Treating cells with anti-VCAM-1 or anti-ICAM-1 antibodies, at saturating concentrations, resulted in blocking of U937 adhesion to HUVECs, thus confirming that hyper-expression of these molecules on the cell surface was among the main mechanisms of increased U937 adhesion to HUVECs.

Figure 13. HUVECs-monocytes adhesion assay.



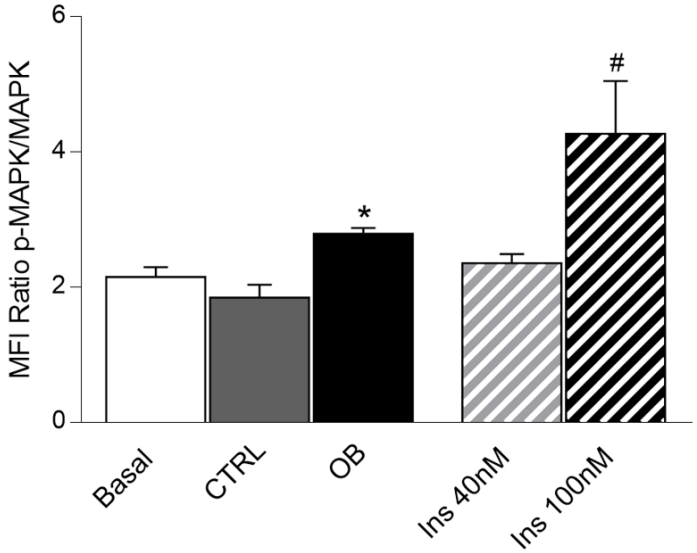
HUVECs serum starved were cultured for 16 hrs with 0.5% FBS as baseline condition (Basal) and with the addition of 10% pool of three OB- or CTRL-plasma, in the presence or absence of TNF-α (1 ng/ml) as inflammatory stimulus. HUVECs were also incubated with anti-VCAM-1 and anti-ICAM-1 antibodies for 1 h before the assay. (Upper panel) Histograms of quantitative data express the number of U937 cells adhering within a high-power field (3.5 mm^2). Each measurement is the mean ± SD of adhering cells from three independent experiments, each consisting of eight counts for condition (N = 12 OB; N = 12 CTRL). (Lower panel) representative images of the adhesion assay. $^{*}p < 0.05$ OB *vs* Basal and CTRL; $^{\#}p < 0.05$ OB TNF-α *vs* Basal and CTRL TNF-α; $^{\dagger}p < 0.05$ TNF-α *vs* baseline; $^{\S}p < 0.05$ *vs* Basal, CTRL and OB TNF-α.

5. Effect of OB-plasma on MAPK activation

In order to evaluate the effect of the OB plasma treatment on the regulation of the two main branches of the vascular insulin signaling pathways, first the activation of MAPK was assessed.

As shown in Figure 14, HUVECs stimulation for 16 hours with OB-plasma significantly increased MAPK phosphorylation levels as compared to cells in basal conditions and exposed to CTRL-plasma (1.2 and 1.5 folds, respectively; both $p < 0.05$). Parallel results were observed treating cells with 40 nM and 100 nM insulin, which are similar to plasmatic insulin levels in CTRL- and OB-children respectively, resulting in a significant increase of MAPK activation following stimulation with 100 nM insulin as compared to 40 nM (1.76 folds, $p < 0.05$).

Figure 14. Basal and stimulated MAPK activation.



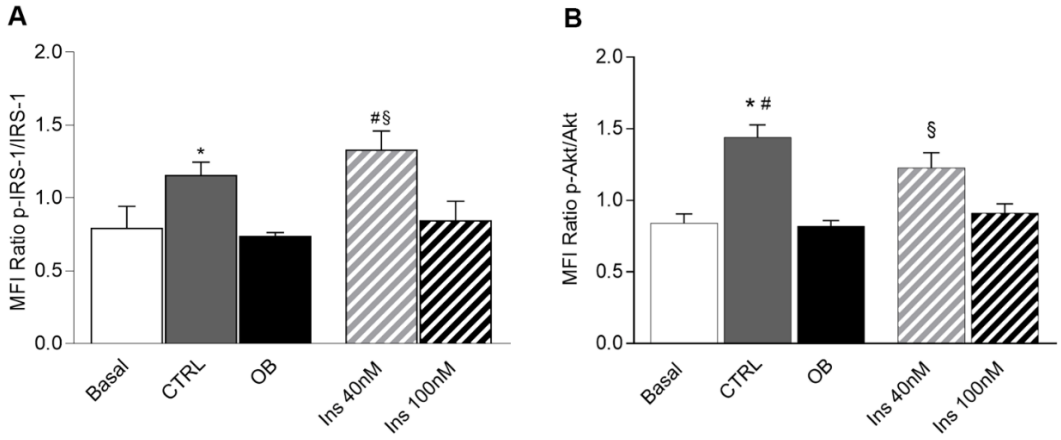
HUVECs serum starved were cultured for 16 hrs with 0.5% FBS as baseline condition (Basal) and with the addition of 10% pool of three OB- or three CTRL-plasma. Insulin (Ins) 40 nM and 100 nM, mimicking insulin plasma levels of CTRL- and OB-children, were also employed. Total levels of MAPK and phosho-p44/42 MAPK (Thr202/Tyr204) were evaluated by flow cytometry. The results are expressed as Mean

Fluorescence Intensity (MFI) Ratio of p-MAPK/MAPK in permeabilized cells (N = 3). Data are shown as mean ± SEM of at least three independent experiments (N = 12 OB; N = 12 CTRL). (A and B) $^{*}p < 0.05$ OB *vs* Basal and CTRL; $^{\#}p < 0.05$ Ins 100 nM *vs* Ins 40 nM.

6. Effect of OB-plasma on IRS-1 and Akt activation

Plasma effects on potential activation of IRS-1 and Akt belonging to the anti-atherogenic branch of the insulin pathway was also evaluated. Interestingly, neither the activation of IRS-1 nor Akt after HUVECs stimulation with OB plasma was observed (Figure 15 A and B, respectively), obtaining comparable values to baseline. On the contrary, incubation with CTRL-plasma significantly increased the phosphorylation levels of IRS-1 as compared to OB-plasma (1.6 folds, $p < 0.05$) and the phosphorylation levels of Akt as compared to baseline and to OB-plasma (1.7 and 1.8 folds, respectively; both $p < 0.05$). As control, stimulation with insulin at 40 nM significantly increased the activation of both IRS-1 and Akt (1.6 and 1.4 folds *vs* Ins 100 nM, respectively; both $p < 0.05$), while using a concentration of 100 nM, as for the OB plasma, neither the phosphorylation of IRS-1 nor Akt were increased.

Figure 15. Basal and stimulated IRS-1 and Akt activation.



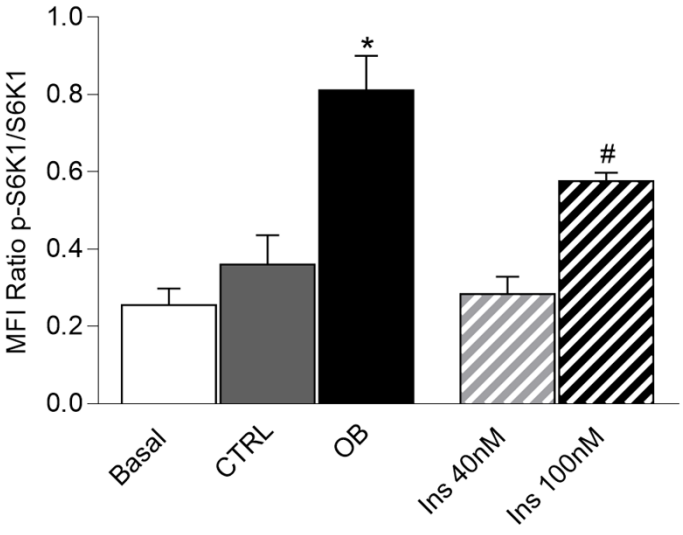
HUVECs serum starved were cultured for 16 hrs with 0.5% FBS as baseline condition (Basal) and with the

addition of 10% pool of three OB- or CTRL-plasma. Insulin (Ins) 40 nM and 100 nM, mimicking insulin plasma levels of CTRL- and OB-children, were also employed. (A) Total levels of IRS-1, phospho-IRS1 (Ser636/639) and (B) Akt, phospho-Akt (Ser473) were all evaluated by flow cytometry. The results are expressed as Mean Fluorescence Intensity (MFI) Ratio of p-IRS-1/IRS-1 or p-Akt/Akt in permeabilized cells (N = 6). Data are shown as mean ± SEM of at least six independent experiments (N = 18 OB; N = 18 CTRL). (A) $^{*}p < 0.05$ CTRL *vs* OB; $^{\#}p < 0.05$ Ins 40 nM *vs* Basal; $^{\S}p < 0.05$ Ins 40 nM *vs* Ins 100 nM. (B) $^{*}p < 0.05$ CTRL *vs* Basal; $^{\#}p < 0.05$ CTRL *vs* OB; $^{\S}p < 0.05$ Ins 40 nM *vs* Ins 100 nM.

7. Effect of OB-plasma on S6K1 activation

Then, the potential involvement of mTORC1-S6K1 pathway in regulating the impaired anti-atherogenic insulin signaling in HUVECs exposed to OB plasma was assessed. To this end, S6K1 activation was evaluated (Figure 16). A significant higher phosphorylation levels was detected in cells after 30 minutes of incubation with OB-plasma as compared to both CTRL-plasma and basal (2.3 and 3.4 folds, respectively; both $p < 0.05$). Same results were obtained with insulin stimulation corresponding to OB- and CTRL-plasma levels, therefore S6K1 resulted activated in cells treated with insulin 100 nM as compared to insulin 40 nM (2.3 folds, $p < 0.05$). Furthermore, in accordance with the experimental protocol used for the study, the S6K1 activity was also measured after 16 hours of stimulation. However, we found no significant changes among the different treatments (data not shown), thus suggesting an early activation of S6K1. This allow us to hypothesize a feedback regulation mechanism of the insulin pathway.

Figure 16. Basal and stimulated S6K1 activation.



HUVECs serum starved were cultured for 30 minutes with 0.5% FBS as baseline condition (Basal) and with the addition of 10% pool of three OB- or CTRL-plasma. Insulin (Ins) 40 nM and 100 nM, mimicking insulin plasma levels of CTRL- and OB-children, were also employed. Total levels of S6K1 and phospho-S6K1 (Thr389+Thr412) were evaluated by flow cytometry. The results are expressed as Mean Fluorescence Intensity (MFI) Ratio of p-S6K1/S6K1 in permeabilized cells (N = 3). Data are shown as mean ± SEM of at least three independent experiments (N = 12 OB; N = 12 CTRL). $^{*}p < 0.05$ OB *vs* Basal and CTRL; $^{\#}p < 0.05$ Ins 100 nM *vs* Ins 40 nM.

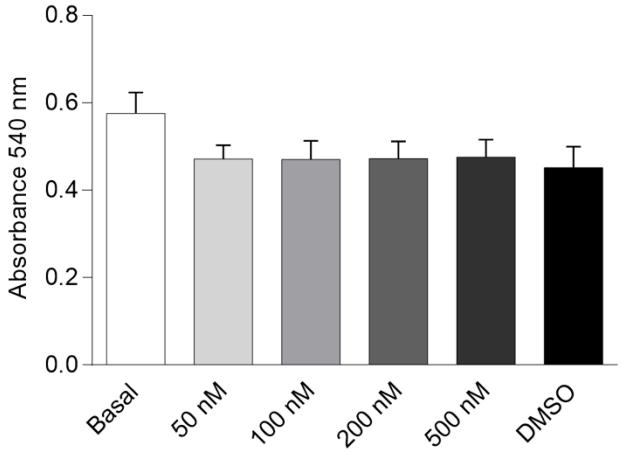
8. Rapamycin effect on OB-plasma regulation of IRS-1 and Akt

To test whether S6K1 activation is responsible for the decrease in IRS-1 and Akt phosphorylation found in this *in vitro* model following OB-plasma stimulation, rapamycin was used as known inhibitor of signaling mediated by mTORC1-S6K1.

The optimal concentration of rapamycin without any effect on HUVECs viability was assessed by treating the cells with 0 - 500 nM range of drug for 16 hours. We did not find significant cell death for all concentrations used (Figure 17) and decided to use 100 nM of

Rapamycin, basing also on the concentrations used in previous studies (Rezabakhsh et al., 2017).

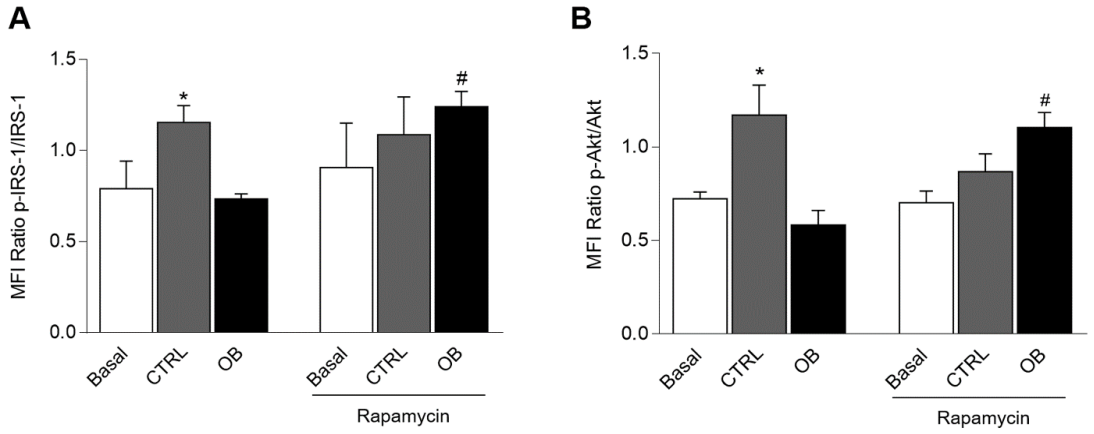
Figure 17. Effect of Rapamycin of HUVECs viability



HUVECs were treated for 16 hrs with 0,50,100,200,500 nm of Rapamycin and with the same concentration of DMSO (Dimetylsulfoxide) used to prepare Rapamycin solution. No differences in cell viability (evaluated by MTT assay) was found. Data are expressed as mean ± SEM of at least three independent experiments.

Interestingly, the use of rapamycin significantly restored both IRS-1 (Figure 18A) and Akt (Figure 18B) phosphorylation levels in OB-plasma exposed HUVECs as compared to cells OB-plasma exposed without rapamycin (1.7 and 1.9 folds, respectively; both $p < 0.05$). Thus, reversing the feedback inhibition of mTOR on IRS-1/PI3K/Akt insulin pathway, which was hypothesized above. While, in cells exposed to CTRL-plasma, where IRS-1 and Akt resulted significantly activated, a slight but not significant decrease in their activation was found under rapamycin stimulation. Finally, the phosphorylation levels of IRS1 and Akt in basal condition were not affected using the mTOR inhibitor.

Figure 18. Rapamycin effect on basal and stimulated IRS-1 and Akt activation.



HUVECs serum starved were cultured for 16 hrs with 0.5% FBS as baseline condition (Basal) and with the addition of 10% pool of three OB- or CTRL-plasma. Rapamycin (100 nM), pre-incubated for 30 minutes and left for the remaining incubation time (16 hours), was also employed. (A) Total levels of IRS-1, phospho-IRS1 (Ser636/639) and (B) Akt, phospho-Akt (Ser473) were all evaluated by flow cytometry. The results are expressed as Mean Fluorescence Intensity (MFI) Ratio of p-IRS-1/IRS-1 or p-Akt/Akt in permeabilized cells (N = 6). Data are shown as mean ± SEM of at least six independent experiments (N = 18 OB; N = 18 CTRL). (A and B) $^{*}p < 0.05$ CTRL *vs* OB; $^{\#}p < 0.05$ OB rapamycin *vs* OB.

DISCUSSION

Obesity is the main risk factor for the development of insulin resistance in childhood, a clinical condition associated with the onset of both metabolic and vascular complications (Chiarelli & Marcovecchio, 2008; Cosimo Giannini et al., 2008). In fact, IR is considered a key link between obesity and CVD since insulin exert its actions in metabolic tissues and in the vascular wall, as described above. Particularly, reduced insulin sensitivity, NO bioavailability, increased oxidative stress and higher inflammation at a vascular level are widely recognized conditions related to endothelial dysfunction and found also in obese children and adolescent (Chiavaroli et al., 2011; Codoñer-Franch et al., 2011; Selvaraju et al., 2019; Tagi et al., 2019).

Several *in vivo* studies have assessed the oxidative and antioxidant status in obese children by proving alterations of some circulating markers such as higher malondialdehyde (MDA), increased oxidized low-density lipoproteins (ox-LDL) levels, lower vitamin E levels and decreased antioxidant capacity (GPx and GSH) (Codoñer-Franch et al., 2012; Mohn et al., 2005; Rowicka et al., 2017). The presence of oxidative homeostasis alterations is often associated with inflammatory states, as demonstrated by the increase in c-reactive protein (CRP) and leptin circulating levels in youth and their proportional relationship with the rate of obesity (Murdolo et al., 2011; Selvaraju et al., 2019; Valle et al., 2005). These data were also confirmed in some studies performed on prepuberal children, which presented alterations in both inflammatory and oxidative status that correlate with the intima-media thickness (IMT), whose increase is related to atherosclerosis and CVD risk (Giannini et al., 2009; Giannini et al., 2008)

Despite these evidences, the intracellular mechanisms underlying endothelial dysfunction in childhood obesity and related to IR are still poorly understood. Of note, previous

published data (N. Di Pietro et al., 2017) using the same *in vitro* system, have shown a reduced insulin-stimulated NO bioavailability associated with increased inflammation and ER stress. Therefore, the present thesis has allowed to broaden previous findings and shed light on new mechanisms that may lead to endothelial dysfunction in childhood.

In detail, our *in vitro* results showed an increase in ROS levels (Figure 11) and enhanced inflammation as demonstrated by the marked increase of VCAM-1 and ICAM-1 exposure on the cell surface following stimulation with OB-plasma in HUVECs co-treated with TNF-α (Figure 12). Similarly, both in the basal state and upon exposure to TNF-α, HUVECs-monocytes adhesion was greater in cell exposed to OB- than CTRL-plasma (Figure 13). Since monocyte-endothelial adhesion represents one of the first steps leading to the formation of atherosclerotic plaque (Čejková et al., 2016), our data support the aforementioned studies that have proven an increase in IMT already in childhood (C. Giannini et al., 2009; Cosimo Giannini et al., 2008). In addition, these results are in line with the increase in the activation of NF-kB found in the same *in vitro* model, a known transcription factor that promote the adhesion molecules production and exposure (Di Pietro et al., 2017)

It should be noted that the increased oxidative stress and inflammation observed in pre-pubertal obese children are often associated with reduced insulin sensitivity, which contributes to further worsening endothelial dysfunction (Giannini et al., 2009; Tagi et al., 2019)

The obese pre-pubertal children belonging to the study population, although normoglycemic, resulted insulin resistant (Table 1) compared to normal weight children. Several evidences indicate that vascular IR may lead to an imbalance between the anti and

pro-atherogenic pathways, supporting the latter (Montagnani et al., 2002; Muniyappa & Sowers, 2013; Pandolfi et al., 2005; Potenza et al., 2009)

Such imbalance was demonstrated by decreased IRS-1 and Akt phosphorylation levels and increased phosphorylation of MAPK in HUVECs treated with OB-plasma (Figures 14 and 15). Interestingly, several *in vivo* and *in vitro* studies showed that the compromised IRS1/PI3K/Akt signal pathway by IR led to a reduced activation of eNOS with a consequent decrease in the production and bioavailability of NO (Consoli et al., 2008; Formoso et al., 2011; Muniyappa & Sowers, 2013; Pandolfi et al., 2005). On the other hand, the increased activation of MAPK under IR condition could, at least in part, account for the increased endothelial exposure of VCAM-1 and ICAM-1 observed in this study and also previously reported by others (Di Tomo et al., 2017; Montagnani et al., 2002; Pandolfi et al., 2005). Diminished NO bioavailability is further favored by the elevated ROS levels, which can react with NO inactivating it and forming more dangerous radical species such as peroxynitrite (Codoñer-Franch et al., 2011; Morgan & Liu, 2011).

Thus, once verified that OB-plasma induces several pro-atherogenic endothelial alterations that are potentially related to each other, the effect of such plasma on possible regulating mechanisms of the anti-atherogenic insulin pathway was assessed. Particularly, the role of mTORC1 and its downstream molecule S6K1 was evaluated, since some studies reported that this complex is involved in the regulation of the IRS-1/ PI3K/Akt insulin pathway (Yoon, 2017).

Our results show a higher activation of S6K1 in HUVECs after a short time treatment (30 minutes) with OB-plasma (Figure 16), suggesting an early activation of S6K1 with a negative feedback on IRS-1 and Akt activation at longer time.

This is in agreement with previous reports stating that serine/threonine phosphorylation

and subsequent proteasomal degradation of IRS-1 are induced by prolonged exposure to insulin in various cell types, where Akt activity, initially stimulated by the ligand, then later decreased, suggesting that IRS-1 degradation acts as an intrinsic feedback inhibition mechanism in the PI3K/Akt signaling (Decker & Pumiglia, 2018; Yoneyama et al., 2018).

In order to confirm the involvement of mTORC1-S6K1 in anti-atherogenic pathway regulation, rapamycin was used. Interestingly, rapamycin treatment significantly restored both IRS-1 and Akt activation in OB-plasma exposed endothelial cells (Figure 18), suggesting that the above described feedback regulation could occur in our *in vitro* model as modulator of the anti-atherogenic insulin pathway.

Conversely, some reports suggest that mTORC1 could directly phosphorylate eNOS (Decker & Pumiglia, 2018) while others indicate a regulation through adenosine monophosphate kinase (AMPK) which, by inhibiting mTOR, increases the PI3K/Akt/eNOS activity leading to higher NO production in HUVECs (Ido et al., 2002). In addition, it has been shown that insulin-dependent MAPK activation inhibits AMPK in rat skeletal muscle cell line, suggesting a functional dependency among MAPK, AMPK and mTOR (Hwang et al., 2013). Thus, the MAPK-AMPK-mTOR pathway regulation could represent an additional mechanism by which, in our cellular model, endothelial insulin sensitivity could be further modulated. Interestingly, other studies show that the increase in ROS is related to higher activity of mTORC1-S6K1 and therefore using rapamycin it was found reduced oxidative stress and increased NO production in HUVECs (Reho et al., 2019; Tzatsos, 2009) This might also be an additional phenomenon involved in the regulation of the endothelial insulin signaling, given that we found increased ROS levels and greater activation of S6K1 following OB-plasma stimulation.

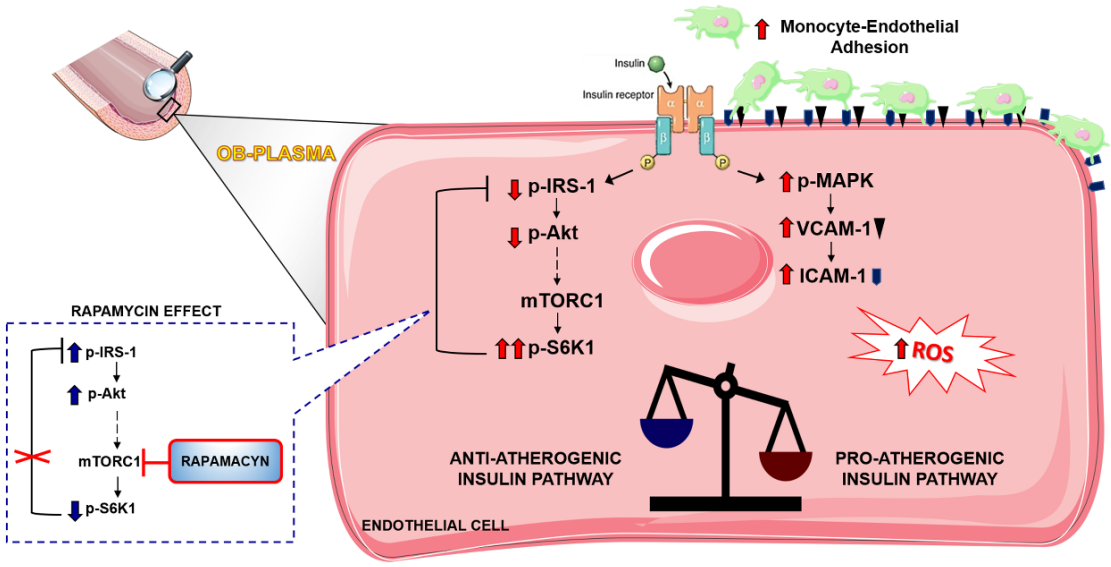
Based on the data discussed, it appears clear that the strength of this thesis is the *in vitro*

model used that mimics the *in vivo* interaction between blood components and vascular endothelial cells. Thanks to the use of plasma, it was possible to investigate HUVECs response without any addition of molecules such as insulin, oxidizing molecules or inflammatory molecules. Furthermore, although HUVECs may not be considered perfectly equal to endothelial cells derived from coronary arteries in terms of metabolic signature and response to stimuli, they have been widely used as a valuable *in vitro* model to study the impact of insulin resistance on the endothelium (Andreozzi et al., 2008; Bacci et al., 2009; Consoli et al., 2008; Formoso et al., 2011; Pandolfi et al., 2005).

Moreover, the limited blood samples availability from children and consequently small quantities of plasma "forced" the main use of flow cytometry as analysis method and the exclusive use of rapamycin as mTORC1 inhibitor.

In conclusion, although further investigation is needed to better clarify the role of mTORC1 and its intricate crosstalk with other signaling pathways, the present thesis provides novel findings useful in designing new potential therapeutic approaches aimed at preventing or reducing the deleterious effects of obesity and insulin resistance on vascular functions starting from childhood.

Figure 19. Effects of plasma from OB children on vascular endothelial cells.



As depicted in the summary scheme, plasma from obese and insulin resistant pre-pubertal children leads to several intracellular signaling pathways alterations in vascular endothelial cells. HUVECs exposed to OB-plasma exhibited increased (⇑): ROS; VCAM-1 and ICAM-1 exposure; increased monocytes-endothelial interaction, one of the first steps leading to the formation of atherosclerotic plaque. This was associated with unbalanced pro- and anti-atherogenic endothelial insulin pathways, as measured by increased (⇑) MAPK activation and decreased (⇓) IRS-1 and Akt phosphorylation levels, and with increased (⇑) S6K1 activation. This allows to hypothesize a feedback regulation of the IRS-1/Akt insulin signaling pathway through S6K1. Interestingly (box on the left), inhibition of the mTORC1-S6K1 pathway using rapamycin significantly restored (⇑) both IRS-1 and Akt activation (blue arrows), thus confirming our hypothesis.

Reactive Oxygen Species (ROS), Vascular Adhesion Molecules-1 (VCAM-1), Intercellular Adhesion Molecules-1 (ICAM-1), phosho-p44/42 Mitogen Activated Protein Kinase (p-MAPK), phospho-Insulin Receptor Substrate-1 Ser636/639 (p-IRS-1), phospho-protein kinase B Ser473 (p-Akt), mammalian Target Of Rapamycin Complex1 (mTORC1), phospho-ribosomal protein S6 kinase beta-1 Thr389+Thr412 (p-S6K1).

REFERENCES

Abe, H., Yamada, N., Kamata, K., Kuwaki, T., Shimada, M., Osuga, J., Shionoiri, F., Yahagi, N., Kadowaki, T., Tamemoto, H., Ishibashi, S., Yazaki, Y., & Makuuchi, M. (1998). Hypertension, hypertriglyceridemia, and impaired endothelium-dependent vascular relaxation in mice lacking insulin receptor substrate-1. *Journal of Clinical Investigation*, *101*(8), 1784–1788. https://doi.org/10.1172/JCI1594

Albuquerque, D., Nóbrega, C., Manco, L., Padez, C., Andreozzi, F., Formoso, G., Prudente, S., Hribal, M. L., Pandolfi, A., Bellacchio, E., Silvestre, S. Di, Trischitta, V., Consoli, A., Sesti, G., Bacci, S., Di Paola, R., Menzaghi, C., Di Fulvio, P., Di Silvestre, S., … Sobrevia, L. (2017). HHS Public Access. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *92*(1), 139–148. https://doi.org/10.1016/j.physbeh.2017.03.040

Andreozzi, F., Formoso, G., Prudente, S., Hribal, M. L., Pandolfi, A., Bellacchio, E., Silvestre, S. Di, Trischitta, V., Consoli, A., & Sesti, G. (2008). TRIB3 R84 variant is associated with impaired insulin-mediated nitric oxide production in human endothelial cells. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *28*(7), 1355–1360. https://doi.org/10.1161/ATVBAHA.108.162883

Ashraf, M. J., & Baweja, P. (2013). Obesity: the “huge” problem in cardiovascular diseases. In *Missouri medicine* (Vol. 110, Issue 6, pp. 499–504). Missouri State Medical Association. /pmc/articles/PMC6179812/?report=abstract

Ataide Lima, R. P., Neto Hayashi, D., de Farias Lima, K. Q., Galdino Gomes, N. I., Ramalho Ribeiro, M., Oliveira Prada, P., & Carvalho Costa, M. J. de. (2017). The Role of Epigenetics in the Etiology of Obesity: A Review. *Journal of Clinical Epigenetics*, *03*(04), 1–5. https://doi.org/10.21767/2472-1158.100075

Bacci, S., Di Paola, R., Menzaghi, C., Di Fulvio, P., Di Silvestre, S., Pellegrini, F., Baratta, R., Marucci, A., Mastroianno, S., Fini, G., Formoso, G., Consoli, A., Perticone, F., Frittitta, L., Pandolfi, A., & Trischitta, V. (2009). ENPP1 Q121 variant, increased pulse pressure and reduced insulin signaling, and nitric oxide synthase activity in endothelial cells. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *29*(10), 1678–1683.

https://doi.org/10.1161/ATVBAHA.109.189191

Baker, J. L., Olsen, L. W., & Sørensen, T. I. A. (2007). Childhood Body-Mass Index and the Risk of Coronary Heart Disease in Adulthood. *New England Journal of Medicine*, *357*(23), 2329–2337. https://doi.org/10.1056/NEJMoa072515

Bentham, J., Di Cesare, M., Bilano, V., Bixby, H., Zhou, B., Stevens, G. A., Riley, L. M., Taddei, C., Hajifathalian, K., Lu, Y., Savin, S., Cowan, M. J., Paciorek, C. J., Chirita-Emandi, A., Hayes, A. J., Katz, J., Kelishadi, R., Kengne, A. P., Khang, Y. H., … Cisneros, J. Z. (2017). Worldwide trends in body-mass index, underweight, overweight, and obesity from 1975 to 2016: a pooled analysis of 2416 population-based measurement studies in 128·9 million children, adolescents, and adults. *The Lancet*, *390*(10113), 2627–2642. https://doi.org/10.1016/S0140-6736(17)32129-3

Bhadoria, A., Sahoo, K., Sahoo, B., Choudhury, A., Sufi, N., & Kumar, R. (2015). Childhood obesity: Causes and consequences. *Journal of Family Medicine and Primary Care*, *4*(2), 187. https://doi.org/10.4103/2249-4863.154628

Bjørge, T., Engeland, A., Tverdal, A., & Smith, G. D. (2008). Original Contribution Body Mass Index in Adolescence in Relation to Cause-specific Mortality: A Follow-up of 230,000 Norwegian Adolescents. *American Journal of Epidemiology*, *168*(1), 30–37. https://doi.org/10.1093/aje/kwn096

Blüher, M. (2019). Obesity: global epidemiology and pathogenesis. *Nature Reviews Endocrinology*, *15*(5), 288–298. https://doi.org/10.1038/s41574-019-0176-8

Borén, J., John Chapman, M., Krauss, R. M., Packard, C. J., Bentzon, J. F., Binder, C. J., Daemen, M. J., Demer, L. L., Hegele, R. A., Nicholls, S. J., Nordestgaard, B. G., Watts, G. F., Bruckert, E., Fazio, S., Ference, B. A., Graham, I., Horton, J. D., Landmesser, U., Laufs, U., … Ginsberg, H. N. (2020). Low-density lipoproteins cause atherosclerotic cardiovascular disease: pathophysiological, genetic, and therapeutic insights: a consensus statement from the European Atherosclerosis Society Consensus Panel Translational medicine. *European Heart Journal*, *41*. https://doi.org/10.1093/eurheartj/ehz962

Bourque, S. L., Davidge, S. T., & Adams, M. A. (2011). The interaction between

endothelin-1 and nitric oxide in the vasculature: New perspectives. In *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* (Vol. 300, Issue 6, pp. 1288–1295). Am J Physiol Regul Integr Comp Physiol. https://doi.org/10.1152/ajpregu.00397.2010

Cacciari, E., Milani, S., Balsamo, A., Spada, E., Bona, G., Cavallo, L., Cerutti, F., Gargantini, L., Greggio, N., Tonini, G., & Cicognani, A. (2006). Italian cross-sectional growth charts for height, weight and BMI (2 to 20 yr). *Journal of Endocrinological Investigation*, *29*(7), 581–593. https://doi.org/10.1007/BF03344156

Cañete, R., Gil-Campos, M., Aguilera, C. M., & Gil, A. (2007). Development of insulin resistance and its relation to diet in the obese child. *European Journal of Nutrition*, *46*(4), 181–187. https://doi.org/10.1007/s00394-007-0648-9

Čejková, S., Králová-Lesná, I., & Poledne, R. (2016). Monocyte adhesion to the endothelium is an initial stage of atherosclerosis development. *Cor et Vasa*, *58*(4), e419–e425. https://doi.org/10.1016/j.crvasa.2015.08.002

Chen, X., Guo, X., Ge, Q., Zhao, Y., Mu, H., & Zhang, J. (2019). ER Stress Activates the NLRP3 Inflammasome: A Novel Mechanism of Atherosclerosis. *Oxidative Medicine and Cellular Longevity*, *2019*. https://doi.org/10.1155/2019/3462530

Chiarelli, F., & Marcovecchio, M. L. (2008). Insulin resistance and obesity in childhood. *European Journal of Endocrinology*, *159*(SUPPL. 1), 67–74. https://doi.org/10.1530/EJE-08-0245

Chiavaroli, V., Giannini, C., de Marco, S., Chiarelli, F., & Mohn, A. (2011). Unbalanced oxidant-antioxidant status and its effects in pediatric diseases. *Redox Report*, *16*(3), 101–107. https://doi.org/10.1179/174329211X13049558293551

Chooi, Y. C., Ding, C., & Magkos, F. (2019). The epidemiology of obesity. *Metabolism: Clinical and Experimental*, *92*, 6–10. https://doi.org/10.1016/j.metabol.2018.09.005

Closa, D., & Folch-Puy, E. (2004). Oxygen Free Radicals and the Systemic Inflammatory Response. *IUBMB Life (International Union of Biochemistry and Molecular Biology:*

Life), *56*(4), 185–191. https://doi.org/10.1080/15216540410001701642

Codoñer-Franch, P., Navarro-Ruiz, A., Fernández-Ferri, M., Arilla-Codoñer, Á., Ballester-Asensio, E., & Valls-Bellés, V. (2012). A matter of fat: insulin resistance and oxidative stress. *Pediatric Diabetes*, *13*(5), 392–399. https://doi.org/10.1111/j.1399-5448.2011.00847.x

Codoñer-Franch, P., Valls-Bellés, V., Arilla-Codoñer, A., & Alonso-Iglesias, E. (2011). Oxidant mechanisms in childhood obesity: The link between inflammation and oxidative stress. *Translational Research*, *158*(6), 369–384. https://doi.org/10.1016/j.trsl.2011.08.004

Consoli, C., Martelli, E., D'Adamo, M., Menghini, R., Arcelli, D., Porzio, O., Pandolfi, A., Pistolese, G. R., Consoli, A., Lauro, R., Ippoliti, A., & Federici, M. (2008). Insulin resistance affects gene expression in endothelium [3]. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *28*(2), 10–12. https://doi.org/10.1161/ATVBAHA.107.152264

Cote, A. T., Harris, K. C., Panagiotopoulos, C., Sandor, G. G. S., & Devlin, A. M. (2013). Childhood obesity and cardiovascular dysfunction. *Journal of the American College of Cardiology*, *62*(15), 1309–1319. https://doi.org/10.1016/j.jacc.2013.07.042

Decker, B., & Pumiglia, K. (2018). mTORc1 activity is necessary and sufficient for phosphorylation of eNOSS1177. *Physiological Reports*. https://doi.org/10.14814/phy2.13733

Değirmenci, T., Kalkan-Oğuzhanoğlu, N., Sözeri-Varma, G., Özdel, O., & Fenkçi, S. (2015). Obezitede psikolojik belirtiler ve ilişkili etmenler. *Noropsikiyatri Arsivi*, *52*(1), 42–46. https://doi.org/10.5152/npa.2015.6904

Di Cesare, M., Sorić, M., Bovet, P., Miranda, J. J., Bhutta, Z., Stevens, G. A., Laxmaiah, A., Kengne, A. P., & Bentham, J. (2019). The epidemiological burden of obesity in childhood: A worldwide epidemic requiring urgent action. *BMC Medicine*, *17*(1), 1–20. https://doi.org/10.1186/s12916-019-1449-8

Di Pietro, N., Marcovecchio, M. L., Di Silvestre, S., de Giorgis, T., Cordone, V. G. P., Lanuti, P., Chiarelli, F., Bologna, G., Mohn, A., & Pandolfi, A. (2017). Plasma from pre-

pubertal obese children impairs insulin stimulated Nitric Oxide (NO) bioavailability in endothelial cells: Role of ER stress. *Molecular and Cellular Endocrinology*, *443*, 52–62. https://doi.org/10.1016/j.mce.2017.01.001

Di Tomo, P., Lanuti, P., Di Pietro, N., Baldassarre, M. P. A., Marchisio, M., Pandolfi, A., Consoli, A., & Formoso, G. (2017). Liraglutide mitigates TNF-α induced pro-atherogenic changes and microvesicle release in HUVEC from diabetic women. *Diabetes/Metabolism Research and Reviews*, *33*(8), 1–12. https://doi.org/10.1002/dmrr.2925

Dutcher, J. P., Motzer, R. J., Atkins, M. B., Figlin, R. A., Kaelin, W. G., Stadler, W. M., Gordon, M. S., & George, D. J. (2004). Mammalian target of rapamycin inhibition. *Clinical Cancer Research*, *10*(18 II), 6382–6388. https://doi.org/10.1158/1078-0432.CCR-050008

Federici, M., Pandolfi, A., De Filippis, E. A., Pellegrini, G., Menghini, R., Lauro, D., Cardellini, M., Romano, M., Sesti, G., Lauro, R., & Consoli, A. (2004). G972R IRS-1 Variant Impairs Insulin Regulation of Endothelial Nitric Oxide Synthase in Cultured Human Endothelial Cells. *Circulation*, *109*(3), 399–405. https://doi.org/10.1161/01.CIR.0000109498.77895.6F

Félix, D. R., Costenaro, F., Gottschall, C. B. A., & Coral, G. P. (2016). Non-alcoholic fatty liver disease (Nafld) in obese children- effect of refined carbohydrates in diet. *BMC Pediatrics*, *16*(1), 1–6. https://doi.org/10.1186/s12887-016-0726-3

Formoso, G., Di Tomo, P., Andreozzi, F., Succurro, E., Di Silvestre, S., Prudente, S., Perticone, F., Trischitta, V., Sesti, G., Pandolfi, A., & Consoli, A. (2011). The TRIB3 R84 variant is associated with increased carotid intimamedia thickness in vivo and with enhanced MAPK signalling in human endothelial cells. *Cardiovascular Research*, *89*(1), 184–192. https://doi.org/10.1093/cvr/cvq255

Förstermann, U., & Münzel, T. (2006). Endothelial nitric oxide synthase in vascular disease: From marvel to menace. *Circulation*, *113*(13), 1708–1714. https://doi.org/10.1161/CIRCULATIONAHA.105.602532

Förstermann, U., & Sessa, W. C. (2012). Nitric oxide synthases: Regulation and function.

In *European Heart Journal* (Vol. 33, Issue 7). https://doi.org/10.1093/eurheartj/ehr304

Freedman, D. S., & Berenson, G. S. (2017). Tracking of BMI z scores for severe obesity. *Pediatrics*, *140*(3). https://doi.org/10.1542/peds.2017-1072

Furukawa, S., Matsuda, M., Furukawa, S., Fujita, T., Shimabukuro, M., & Iwaki, M. (2017). Increased oxidative stress in obesity and its impact on metabolic syndrome Find the latest version : Increased oxidative stress in obesity and its impact on metabolic syndrome. *The Journal of Clinical Investigation*, *114*(12), 1752–1761. https://doi.org/10.1172/JCI200421625.1752

Geraldes, P., Yagi, K., Ohshiro, Y., He, Z., Maeno, Y., Yamamoto-Hiraoka, J., Rask-Madsen, C., Chung, S. W., Perrella, M. A., & King, G. L. (2008). Selective regulation of heme oxygenase-1 expression and function by insulin through IRS1/phosphoinositide 3-kinase/Akt-2 pathway. *Journal of Biological Chemistry*, *283*(49), 34327–34336. https://doi.org/10.1074/jbc.M807036200

Giannini, C., de Giorgis, T., Scarinci, A., Cataldo, I., Marcovecchio, M. L., Chiarelli, F., & Mohn, A. (2009). Increased carotid intima-media thickness in pre-pubertal children with constitutional leanness and severe obesity: The speculative role of insulin sensitivity, oxidant status, and chronic inflammation. *European Journal of Endocrinology*, *161*(1), 73–80. https://doi.org/10.1530/EJE-09-0042

Giannini, Cosimo, de Giorgis, T., Scarinci, A., Ciampani, M., Marcovecchio, M. L., Chiarelli, F., & Mohn, A. (2008). Obese related effects of inflammatory markers and insulin resistance on increased carotid intima media thickness in pre-pubertal children. *Atherosclerosis*, *197*(1), 448–456. https://doi.org/10.1016/j.atherosclerosis.2007.06.023

Gruber, H. J., Mayer, C., Mangge, H., Fauler, G., Grandits, N., & Wilders-Truschnig, M. (2008). Obesity reduces the bioavailability of nitric oxide in juveniles. *International Journal of Obesity*, *32*(5), 826–831. https://doi.org/10.1038/sj.ijo.0803795

Güngör, N. K. (2014). Overweight and obesity in children and adolescents. *JCRPE Journal of Clinical Research in Pediatric Endocrinology*, *6*(3), 129–143. https://doi.org/10.4274/jcrpe.1471

Hemmingsson, E. (2018). Early Childhood Obesity Risk Factors: Socioeconomic Adversity, Family Dysfunction, Offspring Distress, and Junk Food Self-Medication. *Current Obesity Reports*, *7*(2), 204–209. https://doi.org/10.1007/s13679-018-0310-2

Herrera, B. M., Keildson, S., & Lindgren, C. M. (2011). Genetics and epigenetics of obesity. *Maturitas*, *69*(1), 41–49. https://doi.org/10.1016/j.maturitas.2011.02.018

Hong, J. S., & Espelage, D. L. (2012). A review of research on bullying and peer victimization in school: An ecological system analysis. *Aggression and Violent Behavior*, *17*(4), 311–322. https://doi.org/10.1016/j.avb.2012.03.003

Hurlimann, D. (2002). The relationship between the endothelium and the vessel wall. *European Heart Journal Supplements*, *4*, A1–A7. https://doi.org/10.1016/s1520-765x(02)90067-2

Hwang, S. L., Jeong, Y. T., Li, X., Kim, Y. D., Lu, Y., Chang, Y. C., Lee, I. K., & Chang, H. W. (2013). Inhibitory cross-talk between the AMPK and ERK pathways mediates endoplasmic reticulum stress-induced insulin resistance in skeletal muscle. *British Journal of Pharmacology*, *169*(1), 69–81. https://doi.org/10.1111/bph.12124

Ido, Y., Carling, D., & Ruderman, N. (2002). Hyperglycemia-Induced Apoptosis in Human Umbilical Vein Endothelial Cells. *Diabetes*, *51*(22), 159–167.

Janesick, A., & Blumberg, B. (2012). Obesogens, stem cells and the developmental programming of obesity. In *International Journal of Andrology* (Vol. 35, Issue 3, pp. 437–448). Int J Androl. https://doi.org/10.1111/j.1365-2605.2012.01247.x

Jeremy, J. Y., Rowe, D., Emsley, A. M., & Newby, A. C. (1999). Nitric oxide and the proliferation of vascular smooth muscle cells. In *Cardiovascular Research* (Vol. 43). www.elsevier.com/locate/cardioreswww.elsevier.nl/locate/cardiores

Jiang, Z. Y., Zhou, Q. L., Coleman, K. A., Chouinard, M., Boese, Q., Czech, M. P., & Kahn, C. R. (2003). *Insulin signaling through Aktprotein kinase B analyzed by small interfering RNA-mediated gene silencing*. www.pnas.orgcgidoi10.1073pnas.1332633100

Júarez-Lopez, C., Klünder-Klünder, M., Medina-Bravo, P., Madrigal-Azćrate, A., Mass-Díaz, E., & Flores-Huerta, S. (2010). Insulin resistance and its association with the components of the metabolic syndrome among obese children and adolescents. *BMC Public Health*, *10*. https://doi.org/10.1186/1471-2458-10-318

Kapahi, P., Chen, D., Rogers, A. N., Katewa, S. D., Li, P. W. L., Thomas, E. L., & Kockel, L. (2010). With TOR, less is more: A key role for the conserved nutrient-sensing TOR pathway in aging. In *Cell Metabolism* (Vol. 11, Issue 6, pp. 453–465). Cell Metab. https://doi.org/10.1016/j.cmet.2010.05.001

Karam, J. G., Samy, &, & Mcfarlane, I. (2007). Secondary causes of obesity. *Therapy*, *4*(5), 641–650. https://doi.org/10.2217/14750708.4.5.641

Kim, J. A., Montagnani, M., Kwang, K. K., & Quon, M. J. (2006). Reciprocal relationships between insulin resistance and endothelial dysfunction: Molecular and pathophysiological mechanisms. *Circulation*, *113*(15), 1888–1904. https://doi.org/10.1161/CIRCULATIONAHA.105.563213

King, G. L., Park, K., & Li, Q. (2016). Selective insulin resistance and the development of cardiovascular diseases in diabetes: The 2015 Edwin Bierman Award Lecture. *Diabetes*, *65*(6), 1462–1471. https://doi.org/10.2337/db16-0152

Koliaki, C., Liatis, S., & Kokkinos, A. (2019). Obesity and cardiovascular disease: revisiting an old relationship. *Metabolism: Clinical and Experimental*, *92*, 98–107. https://doi.org/10.1016/j.metabol.2018.10.011

Krebs, N. F., Himes, J. H., Jacobson, D., Nicklas, T. A., Guilday, P., & Styne, D. (2007). Assessment of child and adolescent overweight and obesity. *Pediatrics*, *120 Suppl*(December). https://doi.org/10.1542/peds.2007-2329D

Krüger-Genge, A., Blocki, A., Franke, R. P., & Jung, F. (2019). Vascular endothelial cell biology: An update. In *International Journal of Molecular Sciences* (Vol. 20, Issue 18). MDPI AG. https://doi.org/10.3390/ijms20184411

Kuboki, K., Jiang, Z. Y., Takahara, N., Ha, S. W., Igarashi, M., Yamauchi, T., Feener, E.

P., Herbert, T. P., Rhodes, C. J., & King, G. L. (2000). Regulation of endothelial constitutive nitric oxide synthase gene expression in endothelial cells and in vivo - A specific vascular action of insulin. *Circulation*, *101*(6), 676–681. https://doi.org/10.1161/01.CIR.101.6.676

Kumar, S., & Kelly, A. S. (2017). Review of Childhood Obesity: From Epidemiology, Etiology, and Comorbidities to Clinical Assessment and Treatment. *Mayo Clinic Proceedings*, *92*(2), 251–265. https://doi.org/10.1016/j.mayocp.2016.09.017

Lakshman, R., Elks, C. E., & Ong, K. K. (2012). Childhood obesity. *Circulation*, *126*(14), 1770–1779. https://doi.org/10.1161/CIRCULATIONAHA.111.047738

Lauer, R. M., Clarke, W. R., & Burns, T. L. (1997). Obesity in childhood: the Muscatine Study. *Zhonghua Minguo Xiao Er Ke Yi Xue Hui Za Zhi [Journal]. Zhonghua Minguo Xiao Er Ke Yi Xue Hui*, *38*(6), 432–437. http://www.ncbi.nlm.nih.gov/pubmed/9473814

Leontieva, O. V., Paszkiewicz, G., Demidenko, Z. N., & Blagosklonny, M. V. (2013). Resveratrol potentiates rapamycin to prevent hyperinsulinemia and obesity in male mice on high fat diet. *Cell Death & Disease*, *4*(1), e472-7. https://doi.org/10.1038/cddis.2012.202

Levy-Marchal, C., Arslanian, S., Cutfield, W., Sinaiko, A., Druet, C., Marcovecchio, M. L., Chiarelli, F., Amemiyia, S., Berenson, G., Caprio, S., Charles, M. A., Cook, S., Davis, E., Dolan, L., Dunger, D., Fagot-Campagna, A., Flodmark, C. E., Ford, E., Gautier, J. F., … Yajnik, C. (2010). Insulin resistance in children: Consensus, perspective, and future directions. *Journal of Clinical Endocrinology and Metabolism*, *95*(12), 5189–5198. https://doi.org/10.1210/jc.2010-1047

Li, J., Kim, S. G., & Blenis, J. (2014). Rapamycin: One drug, many effects. In *Cell Metabolism* (Vol. 19, Issue 3, pp. 373–379). Cell Press. https://doi.org/10.1016/j.cmet.2014.01.001

Lobstein, T., Baur, L., & Uauy, R. (2004). Obesity in children and young people: A crisis in public health. In *Obesity Reviews, Supplement* (Vol. 5, Issue 1, pp. 4–104). John Wiley & Sons, Ltd. https://doi.org/10.1111/j.1467-789x.2004.00133.x

Malik, V. S., Schulze, M. B., & Hu, F. B. (2006). Intake of sugar-sweetened beverages and weight gain: A systematic review. *American Journal of Clinical Nutrition*, *84*(2), 274–288. https://doi.org/10.1093/ajcn/84.1.274

Marchio, P., Guerra-Ojeda, S., Vila, J. M., Aldasoro, M., Victor, V. M., & Mauricio, M. D. (2019). Targeting early atherosclerosis: A focus on oxidative stress and inflammation. *Oxidative Medicine and Cellular Longevity*, *2019*(Ldl). https://doi.org/10.1155/2019/8563845

Matthews, D. R., Hosker, J. P., Rudenski, A. S., Naylor, B. A., Treacher, D. F., & Turner, R. C. (1985). Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia*, *28*(7), 412–419. https://doi.org/10.1007/BF00280883

McGill, H. C., McMahan, C. A., & Gidding, S. S. (2008). Preventing heart disease in the 21st century: implications of the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) study. *Circulation*, *117*(9), 1216–1227. https://doi.org/10.1161/CIRCULATIONAHA.107.717033

Michael A., G. J., & Guillermo, G.-C. (2016). Endothelial cell dysfunction and the pathobiology of atherosclerosis. *Circulation Research*, *176*(1), 139–148. https://doi.org/10.1016/j.physbeh.2017.03.040

Mohn, A., Catino, M., Capanna, R., Giannini, C., Marcovecchio, M., & Chiarelli, F. (2005). Increased oxidative stress in prepubertal severely obese children: Effect of a dietary restriction-weight loss program. *Journal of Clinical Endocrinology and Metabolism*, *90*(5), 2653–2658. https://doi.org/10.1210/jc.2004-2178

Moller, D. E., Yokota, A., White, M. F., Pazianos, A. G., & Flier, J. S. (1990). A naturally occurring mutation of insulin receptor alanine 1134 impairs tyrosine kinase function and is associated with dominantly inherited insulin resistance. *Journal of Biological Chemistry*, *265*(25), 14979–14985.

Montagnani, M., Golovchenko, I., Kim, I., Koh, G. Y., Goalstone, M. L., Mundhekar, A. N., Johansen, M., Kucik, D. F., Quon, M. J., & Draznin, B. (2002). Inhibition of

phosphatidylinositol 3-kinase enhances mitogenic actions of insulin in endothelial cells. *Journal of Biological Chemistry*, *277*(3), 1794–1799. https://doi.org/10.1074/jbc.M103728200

Montero, D., Walther, G., Perez-Martin, A., Roche, E., & Vinet, A. (2012). Endothelial dysfunction, inflammation, and oxidative stress in obese children and adolescents: Markers and effect of lifestyle intervention. *Obesity Reviews*, *13*(5), 441–455. https://doi.org/10.1111/j.1467-789X.2011.00956.x

Morgan, M. J., & Liu, Z. G. (2011). Crosstalk of reactive oxygen species and NF-κB signaling. *Cell Research*, *21*(1), 103–115. https://doi.org/10.1038/cr.2010.178

Muniyappa, R., & Sowers, J. R. (2013). Role of insulin resistance in endothelial dysfunction. *Reviews in Endocrine and Metabolic Disorders*, *14*(1), 5–12. https://doi.org/10.1007/s11154-012-9229-1

Murdolo, G., Nowotny, B., Celi, F., Donati, M., Bini, V., Papi, F., Gornitzka, G., Castellani, S., Roden, M., Falorni, A., Herder, C., & Falorni, A. (2011). Inflammatory adipokines, high molecular weight adiponectin, and insulin resistance: A population-based survey in prepubertal schoolchildren. *PLoS ONE*, *6*(2), 1–10. https://doi.org/10.1371/journal.pone.0017264

Obesity. (n.d.). Retrieved July 9, 2020, from https://www.who.int/westernpacific/health-topics/obesity

Ogden, C. L., Yanovski, S. Z., Carroll, M. D., & Flegal, K. M. (2007). The Epidemiology of Obesity. *Gastroenterology*, *132*(6), 2087–2102. https://doi.org/10.1053/j.gastro.2007.03.052

Okon, E., Tejerina, T., Mcmanus, B., & Luo, H. (2005). *Compromised Arterial Function in Human Type 2 Diabetic Patients Marfan Syndorme Associated Aortic Aneursym View project Lysosome-SR junctions in vascular smooth muscle View project*. https://doi.org/10.2337/diabetes.54.8.2415

Ormazabal, V., Nair, S., Elfeky, O., Aguayo, C., Salomon, C., & Zuñiga, F. A. (2018).

Association between insulin resistance and the development of cardiovascular disease. In *Cardiovascular Diabetology* (Vol. 17, Issue 1, p. 122). BioMed Central Ltd. https://doi.org/10.1186/s12933-018-0762-4

Owen, C. G., Whincup, P. H., Orfei, L., Chou, Q. A., Rudnicka, A. R., Wathern, A. K., Kaye, S. J., Eriksson, J. G., Osmond, C., & Cook, D. G. (2009). Is body mass index before middle age related to coronary heart disease risk in later life? Evidence from observational studies. *International Journal of Obesity*, *33*(8), 866–877. https://doi.org/10.1038/ijo.2009.102

Panagopoulou, P., Galli-Tsinopoulou, A., Fleva, A., Pavlitou-Tsiontsi, E., Vavatsi-Christaki, N., & Nousia-Arvanitakis, S. (2008). Adiponectin and insulin resistance in childhood obesity. *Journal of Pediatric Gastroenterology and Nutrition*, *47*(3), 356–362. https://doi.org/10.1097/MPG.0b013e31817fcb67

Pandolfi, A., & De Filippis, E. A. (2007). Chronic hyperglicemia and nitric oxide bioavailability play a pivotal role in pro-atherogenic vascular modifications. *Genes and Nutrition*, *2*(2), 195–208. https://doi.org/10.1007/s12263-007-0050-5

Pandolfi, A., Solini, A., Pellegrini, G., Mincione, G., Di Silvestre, S., Chiozzi, P., Giardinelli, A., Di Marcantonio, M. C., Piccirelli, A., Capani, F., & Consoli, A. (2005). Selective insulin resistance affecting nitric oxide release but not plasminogen activator inhibitor-1 synthesis in fibroblasts from insulin-resistant individuals. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *25*(11), 2392–2397. https://doi.org/10.1161/01.ATV.0000185831.13559.a2

Papoutsakis, C., Priftis, K. N., Drakouli, M., Prifti, S., Konstantaki, E., Antonogeorgos, G., Chondronikola, M., & Matziou, V. (2013). Childhood Overweight/Obesity and Asthma: Is There a Link? A Systematic Review of Recent Epidemiologic Evidence. *Journal of the Academy of Nutrition and Dietetics*, *113*(1), 77–105. https://doi.org/10.1016/j.jand.2012.08.025

Perrone, J., Hollander, J. E., De Roos, F., & Berenson, G. S. (1998). Cardiovascular risk factors and atherosclerosis in children and young adults [4] (multiple letters). *New England*

Journal of Medicine, *339*(15), 1083–1084. https://doi.org/10.1056/NEJM199810083391514

Pietro, D., Tomo, D. Di, & Pandolfi, P. (2016). Carotenoids in cardiovascular disease prevention. *JSM Atheroscler*, *1*(1), 1002. https://pdfs.semanticscholar.org/ef52/8a7725a728397cd75cf21c093d0d6f094f4a.pdf

Poirier, P., Giles, T. D., Bray, G. A., Hong, Y., Stern, J. S., Pi-Sunyer, F. X., & Eckel, R. H. (2006). Obesity and cardiovascular disease: Pathophysiology, evaluation, and effect of weight loss: An update of the 1997 American Heart Association Scientific Statement on obesity and heart disease from the Obesity Committee of the Council on Nutrition, Physical Activity, and Metabolism. In *Circulation* (Vol. 113, Issue 6, pp. 898–918). Circulation. https://doi.org/10.1161/CIRCULATIONAHA.106.171016

Potenza, M. A., Addabbo, F., & Montagnani, M. (2009). Vascular actions of insulin with implications for endothelial dysfunction. *American Journal of Physiology - Endocrinology and Metabolism*, *297*(3), 568–577. https://doi.org/10.1152/ajpendo.00297.2009

Pulgaron, E. R., & Delamater, A. M. (2014). Obesity and type 2 diabetes in children: Epidemiology and treatment. *Current Diabetes Reports*, *14*(8), 1–21. https://doi.org/10.1007/s11892-014-0508-y

Raj, M. (2012). Obesity and cardiovascular risk in children and adolescents. *Indian Journal of Endocrinology and Metabolism*, *16*(1), 13. https://doi.org/10.4103/2230-8210.91176

Rajendran, P., Rengarajan, T., Thangavel, J., Nishigaki, Y., Sakthisekaran, D., Sethi, G., & Nishigaki, I. (2013). The vascular endothelium and human diseases. In *International Journal of Biological Sciences* (Vol. 9, Issue 10, pp. 1057–1069). Ivyspring International Publisher. https://doi.org/10.7150/ijbs.7502

Rask-Madsen, C., Li, Q., Freund, B., Feather, D., Abramov, R., Wu, I. H., Chen, K., Yamamoto-Hiraoka, J., Goldenbogen, J., Sotiropoulos, K. B., Clermont, A., Geraldes, P., Dall'Osso, C., Wagers, A. J., Huang, P. L., Rekhter, M., Scalia, R., Kahn, C. R., & King, G. L. (2010). Loss of insulin signaling in vascular endothelial cells accelerates

atherosclerosis in apolipoprotein e null mice. *Cell Metabolism*, *11*(5), 379–389. https://doi.org/10.1016/j.cmet.2010.03.013

Reho, J. J., Guo, D. F., & Rahmouni, K. (2019). Mechanistic Target of Rapamycin Complex 1 Signaling Modulates Vascular Endothelial Function Through Reactive Oxygen Species. *Journal of the American Heart Association*, *8*(9). https://doi.org/10.1161/JAHA.118.010662

Reilly, J. J., Methven, E., McDowell, Z. C., Hacking, B., Alexander, D., Stewart, L., & Kelnar, C. J. H. (2003). Health consequences of obesity. In *Archives of Disease in Childhood* (Vol. 88, Issue 9, pp. 748–752). BMJ Publishing Group. https://doi.org/10.1136/adc.88.9.748

Rezabakhsh, A., Ahmadi, M., Khaksar, M., Montaseri, A., Malekinejad, H., Rahbarghazi, R., & Garjani, A. (2017). Rapamycin inhibits oxidative/nitrosative stress and enhances angiogenesis in high glucose-treated human umbilical vein endothelial cells: Role of autophagy. *Biomedicine and Pharmacotherapy*, *93*, 885–894. https://doi.org/10.1016/j.biopha.2017.07.044

Robinson, T. N. (2001). Television viewing and childhood obesity. *Pediatric Clinics of North America*, *48*(4), 1017–1025. https://doi.org/10.1016/S0031-3955(05)70354-0

Rosiek, A., Maciejewska, N. F., Leksowski, K., Rosiek-Kryszewska, A., & Leksowski, Ł. (2015). Effect of television on obesity and excess of weight and consequences of health. *International Journal of Environmental Research and Public Health*, *12*(8), 9408–9426. https://doi.org/10.3390/ijerph120809408

Rothman, K. J. (2008). BMI-related errors in the measurement of obesity. *International Journal of Obesity*, *32*, S56–S59. https://doi.org/10.1038/ijo.2008.87

Rowicka, G., Dyląg, H., Ambroszkiewicz, J., Riahi, A., Weker, H., & Chełchowska, M. (2017). Total Oxidant and Antioxidant Status in Prepubertal Children with Obesity. *Oxidative Medicine and Cellular Longevity*, *2017*. https://doi.org/10.1155/2017/5621989

Saxton, R. A., & Sabatini, D. M. (2017). Erratum: mTOR Signaling in Growth,

Metabolism, and Disease (Cell (2017) 168(6) (960–976) (S0092867417301824) (10.1016/j.cell.2017.02.004)). *Cell*, *169*(2), 361–371. https://doi.org/10.1016/j.cell.2017.03.035

Sciarretta, S., Forte, M., Frati, G., & Sadoshima, J. (2018). New insight into the role of mTOR signaling in the cardiovascular system. *Circulation Research*, *122*(3), 329–339. https://doi.org/10.1002/9781118729038.ch9

Selvaraju, V., Ayine, P., Fadamiro, M., Babu, J. R., Brown, M., & Geetha, T. (2019). Urinary Biomarkers of Inflammation and Oxidative Stress Are Elevated in Obese Children and Correlate with a Marker of Endothelial Dysfunction. *Oxidative Medicine and Cellular Longevity*, *2019*. https://doi.org/10.1155/2019/9604740

Shum, M., Bellmann, K., St-Pierre, P., & Marette, A. (2016). Pharmacological inhibition of S6K1 increases glucose metabolism and Akt signalling in vitro and in diet-induced obese mice. *Diabetologia*, *59*(3), 592–603. https://doi.org/10.1007/s00125-015-3839-6

Silver, A. E., Beske, S. D., Christou, D. D., Donato, A. J., Moreau, K. L., Eskurza, I., Gates, P. E., & Seals, D. R. (2007). Overweight and obese humans demonstrate increased vascular endothelial NAD(P)H oxidase-p47phox expression and evidence of endothelial oxidative stress. *Circulation*, *115*(5), 627–637. https://doi.org/10.1161/CIRCULATIONAHA.106.657486

Skinner, A. C., Perrin, E. M., Moss, L. A., & Skelton, J. A. (2015). Cardiometabolic risks and severity of obesity in children and young adults. *New England Journal of Medicine*, *373*(14), 1307–1317. https://doi.org/10.1056/NEJMoa1502821

Spicuzza, L., Caruso, D., & Maria, G. (2015). Obstructive sleep apnoea syndrome and its management. In *Therapeutic Advances in Chronic Disease* (Vol. 6, Issue 5, pp. 273–285). SAGE Publications. https://doi.org/10.1177/2040622315590318

St-Onge, M. P., Keller, K. L., & Heymsfield, S. B. (2003). Changes in childhood food consumption patterns: A cause for concern in light of increasing body weights. *American Journal of Clinical Nutrition*, *78*(6), 1068–1073. https://doi.org/10.1093/ajcn/78.6.1068

Tagi, V. M., Giannini, C., & Chiarelli, F. (2019). Insulin resistance in children. *Frontiers in Endocrinology*, *10*(JUN), 1–13. https://doi.org/10.3389/fendo.2019.00342

Thaker, V. V. (2017). GENETIC AND EPIGENETIC CAUSES OF OBESITY. *Adolescent Medicine: State of the Art Reviews*, *28*(2), 379–405. http://www.ncbi.nlm.nih.gov/pubmed/30416642

Tuñón, J., Martín-Ventura, J. L., Blanco-Colio, L. M., Tarín, N., & Egido, J. (2007). Common pathways of hypercholesterolemia and hypertension leading to atherothrombosis: the need for a global approach in the management of cardiovascular risk factors. *Vascular Health and Risk Management*, *3*(4), 521–526. http://www.ncbi.nlm.nih.gov/pubmed/17969382

Tzatsos, A. (2009). Raptor binds the SAIN (Shc and IRS-1 NPXY binding) domain of insulin receptor substrate-1 (IRS-1) and regulates thephosphorylation of IRS-1 at Ser-636/639 by mTOR. *Journal of Biological Chemistry*, *284*(34), 22525–22534. https://doi.org/10.1074/jbc.M109.027748

Um, S. H., D'Alessio, D., & Thomas, G. (2006). Nutrient overload, insulin resistance, and ribosomal protein S6 kinase 1, S6K1. *Cell Metabolism*, *3*(6), 393–402. https://doi.org/10.1016/j.cmet.2006.05.003

Valle, M., Martos, R., Gascón, F., Cañete, R., Zafra, M. A., & Morales, R. (2005). Low-grade systemic inflammation, hypoadiponectinemia and a high concentration of leptin are present in very young obese children, and correlate with metabolic syndrome. *Diabetes and Metabolism*. https://doi.org/10.1016/S1262-3636(07)70167-2

Vicent, D., Ilany, J., Kondo, T., Naruse, K., Fisher, S. J., Kisanuki, Y. Y., Bursell, S., Yanagisawa, M., King, G. L., & Kahn, C. R. (2003). The role of endothelial insulin signaling in the regulation of vascular tone and insulin resistance. *Journal of Clinical Investigation*, *111*(9), 1373–1380. https://doi.org/10.1172/JCI15211

Villalobos-Labra, R., Silva, L., Subiabre, M., Araos, J., Salsoso, R., Fuenzalida, B., Sáez, T., Toledo, F., González, M., Quezada, C., Pardo, F., Chiarello, D. I., Leiva, A., & Sobrevia, L. (2017). Akt/mTOR Role in Human Foetoplacental Vascular Insulin

Resistance in Diseases of Pregnancy. *Journal of Diabetes Research*, *2017*. https://doi.org/10.1155/2017/5947859

Wullschleger, S., Loewith, R., & Hall, M. N. (2006). TOR signaling in growth and metabolism. In *Cell* (Vol. 124, Issue 3, pp. 471–484). Cell Press. https://doi.org/10.1016/j.cell.2006.01.016

Yoneyama, Y., Inamitsu, T., Chida, K., Iemura, S. I., Natsume, T., Maeda, T., Hakuno, F., & Takahashi, S. I. (2018). Serine Phosphorylation by mTORC1 Promotes IRS-1 Degradation through SCFβ-TRCP E3 Ubiquitin Ligase. *IScience*, *5*, 1–18. https://doi.org/10.1016/j.isci.2018.06.006

Yoon, M. S. (2017). The role of mammalian target of rapamycin (mTOR) in insulin signaling. *Nutrients*, *9*(11). https://doi.org/10.3390/nu9111176

Zdziarski, L. A., Wasser, J. G., & Vincent, H. K. (2015). Chronic pain management in the obese patient: A focused review of key challenges and potential exercise solutions. *Journal of Pain Research*, *8*, 63–77. https://doi.org/10.2147/JPR.S55360