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Pathophysiological alterations and cardio-metabolic changes in childhood obesity

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Preface

Aim of this dissertation is to illustrate the results of the research projects carried out during the PhD course within the main research line concerning childhood obesity, carried out at the Pediatrics Unit of the University Hospital of Messina, Department of Adult and Childhood Human Pathology "Gaetano Barresi".

Focus of our research group is childhood obesity, with particular attention to the pathophysiology and obesity-related complications in pediatric age. The main objective of our research is to find biomarkers for the early detection of children and adolescents with an increased risk of developing obesity-related cardio-metabolic (e.g. impaired glucose regulation, dyslipidemia, arterial hypertension) or to diagnose those who already have incipient cardio-metabolic alterations as a prelude to the onset of obesity-related complications. This research has been carried out both by studying the pathophysiological mechanisms underlying complications in children and adolescents, such as oxidative stress pathways and cytokines involved in impaired glucose regulation, and by evaluating the clinical, laboratory and instrumental characteristics of patients in whom it is already possible to diagnose these complications or to identify precocious, preclinical, alterations.

The ultimate goal is to implement the clinical-therapeutic management of children with overweight and obesity to prevent the onset of obesity-related cardio-metabolic complications and to plan a personalized approach in obese children who already present incipient alterations.

This dissertation is structured in two sections reporting the research projects realized and published in international scientific journals concerning the pathophysiological alterations and cardio-metabolic changes in childhood obesity. The first section focuses on results presentation concerning the researches in the pathophysiological field aimed to identify early biochemical biomarkers implicated in obesity-related complications; the second one focuses on the results of researches carried out to characterize the cardio-metabolic profiles of children and adolescents through innovative non-invasive methods.

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CHAPTER 1

Introduction

Childhood obesity has become one of the major health issues worldwide and its prevalence is progressively increasing in both industrialized and developing countries. The World Health Organization (WHO) has coined the term "globesity", a neologism that combines the words "global" and "obesity", in order to define the spread of obesity as a worldwide phenomenon. The increased prevalence of obesity in childhood and adolescence is the result of a complex interaction between genetic, environmental, socioeconomic and behavioral factors (1). A large epidemiological study, taking into analysis 2416 population studies totaling approximately 130 million individuals of various ethnicities, 25% of whom aged between 5 and 20 years, documented a massive and progressive increase in childhood obesity incidence worldwide over the past four decades (2). In particular, the prevalence of obesity in children and adolescents increased about 10-fold from about 4% to nearly 18% between 1975 and 2016, particularly in low- and middle-income countries, promoted by an increased consumption of unhealthful food (nutrient-poor and energy-dense foods) (2). In Europe, the prevalence of childhood obesity is estimated to be between 10 and 40 percent, higher in southern states than in northern Europe (3). Italy is among the European countries with the highest prevalence of childhood overweight and obesity. A 2019 Italian report, "Okkio alla salute", as part of the surveillance program promoted by the Ministry of Health and coordinated by the National Center for Epidemiology, Surveillance, and Health Promotion of the Superior Institute of Health, in a homogenous cohort of children documented a prevalence of overweight and obesity of 20.4% and 9.4%, respectively, higher in southern regions (4).

Childhood obesity is associated with an increased risk to develop a wide spectrum of cardio-metabolic complications, including insulin resistance (IR), impaired glucose regulation (IGR), dyslipidemia, nonalcoholic fatty liver disease (NAFLD), cardiovascular diseases (5, 6). Childhood obesity tends to persist into adulthood promoting the early onset of related complications. Obese children and in particular adolescents are more likely to become obese adults with an increased risk of premature morbidity and mortality due to cardio-metabolic diseases (5-10). The severity of these complications is greater the earlier the obesity onset and more severe the degree of obesity (11).

The Bogalusa Heart Study best highlighted the importance of cardiovascular disease prevention in childhood, demonstrating how the factors involved in the pathogenesis of cardiovascular diseases more common in adulthood, such as atherosclerosis and hypertension, begin to act during childhood (12). In another recent study, in which data from 4 prospective cohort studies (about 5800 individuals) were analyzed and processed, the presence of metabolic syndrome in childhood is a significant

predictor, as early as 5 years of age, of the risk of metabolic syndrome, diabetes mellitus and/or mid-intimal thickening in adulthood (13).

Several of cardio-metabolic obesity-related complications, that have their origins in childhood, are strictly related to the presence of IR and hyperinsulinemia, which represents the most common complication in pediatric obesity. IR plays a crucial role in the relationship between obesity and associated cardiovascular risk (14) and it has been suggested as one of the earliest mechanisms involved in the development of endothelial dysfunction in obese youth (9). Giannini et al. demonstrated that precocious changes in glucose metabolism, detected in obese prepubertal children, could be implicated in the intima media thickness increase (9). IR promotes hepatic triglycerides accumulation, favoring inhibition of lipolysis in adipocytes, promoting the increased uptake and the reduced catabolism of free fatty acid (FFA) in hepatocytes, playing a pivotal role in NAFLD development, the most common liver disease in pediatric age (15). In addition, IR has been directly linked to metabolic syndrome in childhood. Weiss et al. demonstrated that the prevalence of the metabolic syndrome increased significantly with increasing IR in children and adolescents with obesity (16). Pathogenesis of obesity-related cardio-metabolic complications is the result of a complex interaction between several not completely known mechanisms among which subclinical inflammation and oxidative stress play a central role. Investigation of these mechanisms aims at the identification of precocious markers for the early diagnosis of the abovementioned complications and/or able to identify obese children and adolescents at higher cardio-metabolic risk.

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CHAPTER 2

Pathophysiological alterations in childhood obesity

2.1 Could AGE/RAGE-related oxidative homeostasis dysregulation enhance susceptibility to pathogenesis of cardio-metabolic complications in childhood obesity?

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Introduction

Childhood obesity and its correlated comorbidities, such as insulin-resistance (IR), fatty liver, type 2 diabetes, metabolic syndrome, are among the most important health issues worldwide (1, 2). The pathogenesis of adiposity-related cardio-vascular and metabolic precocious alterations is the result of concurrent pathways of inflammation, apoptosis, and oxidative stress (3, 4). The oxidative stress pathways related to advanced glycation end-products (AGEs) generation were well-studied in adults and they were implicated in inflammation, endothelial dysfunction, IR, glucose homeostasis alterations and metabolic syndrome (5–7). AGEs result from non-enzymatic glycation and oxidation of proteins, lipids, and nucleic acids. AGEs and their transmembrane cell receptor (RAGE) have been involved in the pathophysiology of cardiovascular and metabolic diseases (7). Interaction of AGEs with RAGE results in both increased generation of oxygen radicals and increased expressions of pro-inflammatory cytokines (7). Circulating soluble RAGE (sRAGE) is able to interact with AGEs, in order to counterbalance the negative effects of AGEs-RAGE interaction (7). AGEs/sRAGE-ratio has been suggested to be a reliable marker of oxidative state (8) as well as advanced oxidation protein products (AOPPs) (9). In children, the relationships between oxidative stress markers and clinical and biochemical variables considered markers of cardio-metabolic risk are not widely investigated and the results so far available are contrasting.

Purposes of this study are to define factors influencing AGEs, sRAGE, AGEs/sRAGE-ratio, and AOPPs levels and to investigate changes in oxidative balance in a cohort of overweight and obese children and adolescents compared to controls.

Materials and Methods

Subjects

Study population included 41 Caucasian overweight and obese children aged between 5 and 16 years (Group A) and 36 Caucasian, healthy, lean (BMI SD < 1), age and sex-matched controls (Group B). Patients, who referred to the Outpatient Clinic of Pediatric Endocrinology at the University of Messina (Italy), were selected according to the following criteria: BMI ≥ 1 SD according to the WHO growth references (10), full-term birth appropriate for gestational age; no genetic or endocrine pathological causes of obesity; no associated chronic diseases; no chronic pharmacological therapies. This study was approved by Ethics Committee of Messina. Written informed consent was obtained from parents or legal tutors.

Methods

At entry, family history and personal anamnesis were obtained and all subjects underwent physical examination, including staging of puberty and anthropometric assessment (height, weight, waist circumference, BMI) according to standard procedures, as previously described (11, 12). Waist-to-height-ratio (WHtR) was measured in patients of group A. Furthermore, the following diagnostic investigations were performed: (1) serum lipid profile (total cholesterol, HDL, LDL, triglycerides), uric acid, C-reactive protein (CRP), thyroid, kidney and liver function tests were measured, in the fasting state, with routine methods using commercial kits, as previously described (11); (2) oral glucose tolerance test (OGTT) was performed, in patients of group A only, with standard method (1.75 g/kg of body weight, up to a maximum of 75 g), measuring glucose and insulin serum levels at baseline and during OGTT (at 0, 30′, 60′, 90′, and 120′ min); (3) homeostasis model assessment of insulin resistance (HOMA-IR) (13), atherogenic index of plasma (AIP) (14), total cholesterol-to-HDL cholesterol ratio (TC/HDL-ratio) (15), and triglycerides-to-HDL cholesterol ratio (TG/HDL-ratio) (16) were assessed; (4) serum levels of oxidative stress markers (AGEs, sRAGE, and AOPPs) were measured. We considered abnormal levels of triglycerides (TG), total cholesterol (TC), LDL, HDL according to the National Cholesterol Education Panel (17): TC > 170 mg/dl, LDL > 130 mg/dl, HDL < 40 mg/dl, TG > 110 mg/dl. Fasting glucose >100 mg/dl and fasting insulin >15 μUI/ml were assessed as abnormal. To define the other indices of cardio-metabolic risk as abnormal, the following cutoffs were considered: HOMA-IR >2.5 (13), AIP > 0.11 (14), TC/HDL-ratio ≥ 5.6 (15), TG/HDL-ratio >1.25 (16).

Oxidative Stress Markers Assessment

Serum levels of total sRAGE were measured by quantitative enzyme immunoassay technique, the RayBio® Human RAGE ELISA (RayBiotech, Norcross GA), according to the manufacturer's instructions. All assays were done in duplicate. The minimum detectable dose of Human RAGE was determined to be 3 pg/ml. The detection limit of the assay was 10 pg/ml. The intra or the inter-assay

CV were <5 and <10%, respectively. Serum levels of AGEs were measured by spectrofluorimetric detection, as previously described (18). Serum (25 µl, triplicates) was diluted 1:50 with phosphate-buffered saline (PBS) pH 7.4 and fluorescence intensity was recorded (λ emission = 370 nm, λ excitation = 440 nm; spectrofluorimeter Shimadzu, Japan). PBS solution was used as blank. Serum concentration of AGEs was normalized to the total protein amount determined by the Bradford assay and expressed in arbitrary units (AU) per gram of protein (AU/g prot). Serum levels of AOPPs were estimated by spectrophotometric detection. Blood serum (100 µl) or same volume of chloramin T (0–100 µmol/l) for calibration were diluted 1:5 with PBS pH 7.4. Afterward, 25 µl of 1.16 M KI and 50 µl of acetic acid were added to the diluted solutions and absorbance was immediately measured at 340 nm (spectrophotometer Shimatzu, Japan). Concentration of AOPPs was expressed in chloramine T units (µmol eq Cl T/L).

Statistical Analysis

Numerical data were expressed as median and range, categorical variables as absolute frequencies, and percentages. Most of the examined variables were not normally distributed, as verified by Kolmogorov–Smirnov test; consequently, the non-parametric approach was used. Comparison analysis was performed by Mann-Whitney test for numerical variables and by Chi-Square test for categorical variables (sex and pubertal stage). Spearman's correlation coefficient was evaluated for correlation analysis. Univariate and multivariate linear regression models were estimated to assess the possible dependence of sRAGE, AGEs, AGEs/sRAGE-ratio, and AOPPs (dependent variables) on some potential explicative variables such as BMI SD, WHtR, HOMA-IR, TC, TC/HDL-ratio, TG/HDL-ratio, AIP, LDL, HDL, TG, uric acid, CRP. In particular, multivariate model (by stepwise procedure) was estimated considering the following independent variables: BMI SD, HOMA-IR, TC/HDL-ratio, LDL, TG, uric acid, CRP. Statistical analyses were performed using SPSS 17.0 for Window package. A P-value smaller than 0.050 was considered statistically significant.

Results

The two groups were comparable for age, gender, and pubertal stage (all P-values >0.05). Median age of subjects belonging to group A was 10.5 (range 4–16 years); 51% of them were female and 51% were pre-pubertal. No significant gender or pubertal differences were detected in analyzed variables. In group A, 21% of subjects had BMI SD > 2.5, 43% between 2.5 and 2 SD, 36% between 2 and 1 SD. All subjects of group A presented abdominal obesity (WHtR ≥ 0.5; median 0.6/range 0.5–0.69). In group A, total cholesterol >170 mg/dl was seen in 11 patients (26.8%), and in two subjects (4.9%) it was >200 mg/dl. LDL was >130 mg/dl in two subjects (4.9%); HDL was <40 mg/dl

in 10 children (24.4%). Triglycerides were >110 mg/dl in five children (12.2%). Two patients (4.9%) had fasting blood glucose >100 mg/dl. OGTT excluded diabetes in all patients and documented a condition of impaired glucose tolerance in three patients of group A (7.1%) who had normal fasting glucose. Hyperinsulinism was demonstrated in 17 patients (41.5%) at basal and OGTT evaluations. HOMA-IR was >2.5 in 17 subjects (41.5%), AIP was > 0.11 only in 4 (9.7%), TG/HDL-ratio was >1.25 in 27 patients (65.8%); no patients had TC/HDL-ratio ≥5.6.

Comparison analysis demonstrated significant differences of cardio-metabolic risk factors between group A and B (Table 1). Moreover, assessment of oxidative stress markers levels highlighted that sRAGE was significantly lower while AOPPs and AGEs/sRAGE-ratio were significantly higher in group A compared to group B (Table 1 and Figure 1). AGEs serum levels were higher in group A in comparison with group B, but the difference did not reach the statistical significance (Table 1 and Figure 1). Results of correlation analysis between oxidative stress markers and clinical and biochemical variables are reported in Table 2. In particular, BMI SD and TC/HDL-ratio were significantly negatively correlated with sRAGE and positively with AOPPs and AGEs/sRAGE-ratio (Figure 2). Moreover, AOPPs was significantly positively correlated with AGEs ($r = 0.33$, $p = 0.003$) and AGEs/sRAGE-ratio ($r = 0.46$, $p = 0.000$) and negatively with sRAGE ($r = -0.33$, $p = 0.003$); sRAGE and AGEs levels were not significantly related each other ($r = 0.05$, $p = 0.66$). The associations among sRAGE, AGEs, AGEs/sRAGE-ratio, AOPPs, and the main clinical and biochemical variables were analyzed by univariate linear regression analysis (Table 3).

To investigate the independent effect of cardio-metabolic risk factors on oxidative stress markers levels a multivariate stepwise regression analysis was performed (Table 4). BMI SD was a strong predictor of AGEs/sRAGE-ratio, sRAGE, and AOPPs. Furthermore, CRP was associated with AOPPs and AGEs/sRAGE-ratio, and TG were associated with AOPPs and AGEs levels (Table 4).

Discussion

To the best of our knowledge, this is the largest case-control pediatric study to simultaneously evaluate serum levels of sRAGE, AGEs, and AOPPs and to investigate the relationships between oxidative stress markers and clinical and biochemical parameters of cardio-metabolic risk in overweight and obese children and adolescents. The main findings of this study are: (1) in overweight and obese children and adolescents, sRAGE was significantly lower and AOPPs and AGEs/sRAGE-ratio were significantly higher compared to controls. (2) BMI SD was an independent predictor of oxidative stress markers serum levels.

The links between AGEs, sRAGE, and AOPPs and obesity-related cardio-vascular and metabolic risk factors have been extensively investigated in adults. In particular, a significantly higher serum levels

of AGEs and AOPPs and a significantly lower serum levels of sRAGE have been documented among obese adults compared to lean controls (5, 6, 19, 20). Conversely, pediatric studies about the role of oxidative stress biomarkers in obesity showed few and contrasting results. Sebekova et al., in a case-control study involving children and adolescents, reported a significantly lower plasma levels of AGEs in obese, despite higher AOPPs, in comparison with lean counterparts; however, these authors did not document any difference in sRAGE concentration (21). In a cohort of children and adolescents, Accacha et al. found an inverse relation of both sRAGE and AGEs with BMI and percent body fat (22). Other studies confirmed that obesity is associated with lower levels of sRAGE in pre-pubertal cohorts of children (23, 24) and reported that sRAGE was independently associated with liver steatosis (23) and carotid intima-media thickness (24). However, recently, other authors did not find any significant association between markers of obesity and AGEs or sRAGE (25). Other studies reported a significantly higher AOPPs levels in obese children in comparison with controls (18, 26–28). Our findings confirmed the role of AOPPs as a trustworthy oxidative stress biomarker in obese children, showing a significant correlation of AOPPs negatively with sRAGE and positively with AGEs and AGEs/sRAGE-ratio.

In our study, we demonstrated an alteration of oxidative homeostasis expressed by lower serum levels of sRAGE and higher levels of AOPPs in overweight and obese children and adolescents, as documented in adults. Despite the difference of AGEs serum levels between groups did not reach the statistical significance, we demonstrated a significant higher values of AGEs/sRAGE-ratio in overweight/obese children compared to controls. In the assessment of oxidative homeostasis, AGEs/sRAGE-ratio has become increasingly important since it reliably expresses the relationship between oxidant factors, as AGEs and RAGE, and anti-oxidant factors, as sRAGE, glyoxilase-1, and glyoxalase-2 [two key enzymes in anti-glycation defense system (29, 30)], and AGER1, AGER2, AGER3 [receptors of AGEs involved in blocking AGEs–RAGE-mediated intracellular pathways (7)], which should be difficult-to-measure in vivo. An increase in AGEs/sRAGE-ratio would reveal a relative shift to oxidant from anti-oxidant factors (7). In our study, for the first time in a pediatric cohort, a significant higher value of AGEs/sRAGE-ratio among overweight and obese children and adolescents was documented.

Oxidative stress markers levels measurement, in addition to clinical and laboratory parameters evaluation, might be useful in the assessment of precocious cardio-metabolic risk of obese children; however, it is necessary to determine a specific range of risk for AGEs and sRAGE levels, through longitudinal studies, before to introduce these markers in daily clinical practice.

Moreover, in our study a significant association between BMI SD and oxidative stress markers serum levels was documented. At regression analysis BMI SD was an independent predictor of

AGEs/sRAGE-ratio, sRAGE, and AOPPs serum levels, therefore the severity of overweight seems to play an important role in increasing oxidative stress in human organism, even in children. Our results support the hypothesis that overweight, independently from modifications of biochemical markers of cardio-metabolic risk, influences the alteration of the oxidative balance.

Another interesting aspect highlighted in our study was the possible influence of lipid profile alterations in oxidative dysregulation. This relation is supported by both the evidence of an independent association between TG levels and AGEs or AOPPs levels, and the demonstration of a significant association between oxidative stress markers levels and TC/HDL-ratio that is a reliable marker of cardio-metabolic risk (15, 31).

Oxidative stress promotes the inflammation through activation of intracellular cascades that ultimately determine an increased expression of pro-inflammatory cytokines and chemokines, oxygen radicals, cell adhesion molecules and acute phase proteins (7, 32, 33). Moreover, obesity itself contributes to determine a sub-clinical inflammation promoting the production of pro-inflammatory factors involved in the pathogenesis of obesity-related complications (3). According to these evidences, in our study CRP, although unspecific marker of inflammation was strongly related to oxidative stress markers levels and it was an independent predictor of AOPPs and AGEs/sRAGE-ratio. It might be argued that our study has some limitations. First, due to the cross-sectional design of the study, we are unable to verify the causal relationships between oxidative stress markers and cardio-metabolic risk variables that could be clarified in a longitudinal study involving a further enlarged cohort of patients. Second, CRP was the only inflammatory marker considered, rather than more specific ones, as pro-inflammatory interleukins.

In conclusion, our findings suggest the presence of an AGE/RAGE-related and AOPPs-related oxidative homeostasis dysregulation that could enhance susceptibility to oxidative/inflammatory tissues damage in overweight and obese children and adolescents. Severity of overweight, influencing the increase of oxidative stress in human organism and even in children, may contribute to the pathogenesis of long-term cardiovascular and metabolic alterations.

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Tables and figures

Table 1 - Comparison analysis of clinical data and cardio-metabolic risk factors between groups.

	Group A (n = 41)	**Group B (n = 36)**	***P*-value**
Age (years)	10.5 (4 – 16)	10.8 (4.5 – 15.4)	0.09
Male/Female (%)	48.8 / 51.2	33.3 / 66.7	0.17
Pre-pubertal/Pubertal (%)	51.2 / 48.8	48.1 / 51.9	0.08
BMI SD	2.1 (1.7 - 3.4)	0.41 (-1.4 - 1.7)	0.000
TC (mg/dl)	154 (123 - 238)	153 (112 - 193)	0.30
LDL (mg/dl)	88 (51 - 169)	78 (58 - 122)	0.07
HDL (mg/dl)	45 (25 - 119)	56 (35 - 69)	0.002
TC/HDL-ratio	3.42 (1.6 - 5.5)	2.59 (1.8 - 4.8)	0.000
TG (mg/dl)	71 (36 - 228)	60 (41 - 136)	0.01
TG/HDL-ratio	1.5 (0.6 – 6.5)	1.1 (0.7 – 3.6)	0.001
AIP	-0.2 (-0.6 – 0.5)	-0.3 (-0.5 - 0.2)	0.001
HOMA-IR	2.32 (0.7 – 6.3)	1.34 (0.8 - 3.5)	0.003
TSH (µlU/ml)	2.3 (0.6 - 7.98)	2.0 (1.3 - 5.3)	0.76
GPT (U/L)	17 (8 - 69)	14 (6 - 27)	0.000
GOT (U/L)	23 (16 – 37)	23 (17 - 32)	0.41
CRP (mg/dl)	0.2 (0.1 – 1.6)	0.1 (0.1 – 0.9)	0.008
Uric Acid (mg/dl)	4.6 (3.2 - 7.5)	4 (2.9 - 5)	0.000
sRAGE	393.3 (183.3 - 831.3)	558.3 (265.8 - 1132.3)	0.000
AGEs	149 (75.5 - 292)	139.3 (94.1 - 251.1)	0.36
AOPPs	1.6 (0.6 - 4.4)	1.2 (0.8 - 2.3)	0.000
AGEs/sRAGE-ratio	0.4 (0.2 – 1.2)	0.3 (0.1 - 0.6)	0.001

Comparison analysis was performed by Mann-Whitney test for numerical variables (expressed as median and range) and by Chi-Square test for categorical variables (gender and pubertal stage).

Group A: overweight and obese patients (BMI = 1 SD); Group B: healthy matched controls (BMI < 1 SD). *Total cholesterol (TC), Triglycerides (TG), Atherogenic index of plasma (AIP), Homeostasis model assessment of insulin resistance (HOMA-IR), C-reactive protein (CRP), Advanced glycation end-products (AGEs), Circulating soluble AGE receptor (sRAGE), Advanced oxidation-protein products (AOPPs).*

Table 2 – Correlation analysis among oxidative stress markers and cardio-metabolic risk factors (Spearman's test).

		sRAGE	AGEs	AOPPs	AGEs/sRAGE
BMI SD	*r*	-0.52	0.72	0.46	0.42
	p	**0.000**	0.53	**0.000**	**0.000**
WHtR	*r*	-0.2	0.07	-0.01	0.19
	p	0.21	0.65	0.95	0.22
TC	*r*	-0.10	0.08	0.09	0.19
	p	0.38	0.47	0.50	0.09
LDL	*r*	-0.12	0.23	0.16	0.28
	p	0.31	**0.047**	0.20	**0.015**
HDL	*r*	0.30	-0.17	-0.47	- 0.33
	p	**0.008**	0.15	**0.000**	**0.004**
TC/HDL-ratio	*r*	-0.33	0.23	0.47	0.41
	p	**0.004**	**0.044**	**0.000**	**0.000**
TG	*r*	-0.05	0.15	0.52	0.14
	p	0.65	0.23	**0.000**	0.24
TG/HDL-ratio	*r*	-0.18	0.20	0.54	0.25
	p	0.12	n.s.	**0.000**	**0.026**
AIP	*r*	-0.18	0.19	0.54	0.25
	p	0.11	0.09	**0.000**	**0.028**
HOMA-IR	*r*	-0.20	-0.60	0.37	0.13
	p	0.10	0.63	**0.002**	0.28
CRP	*r*	-0.26	0.23	0.39	0.40
	p	**0.02**	**0.044**	**0.000**	**0.007**
Uric acid	*r*	-0.11	0.06	0.08	0.16
	p	0.35	0.58	0.50	0.17

Waist-to-height-ratio (WHtR), Total cholesterol (TC), Triglycerides (TG), Atherogenic index of plasma (AIP), Homeostasis model assessment of insulin resistance (HOMA-IR), C-reactive protein (CRP), Advanced glycation end-products (AGEs), Circulating soluble AGE receptor (sRAGE), Advanced oxidation-protein products (AOPPs).

Table 3 – Statistically significant associations at univariate linear regression analysis.

AGEs		
Predictors	**B**	***P*-value**
TC/HDL-ratio	14.07	0.02
LDL	0.59	0.03
Triglycerides	0.45	0.01
AIP	54.12	0.03
sRAGE		
BMI SD	-67.12	0.000
TC/HDL-ratio	-56.31	0.015
AOPPs		
BMI SD	0.202	0.000
HOMA-IR	0.123	0.03
Triglycerides	0.01	0.000
HDL	-0.012	0.02
TC/HDL-ratio	0.24	0.001
AIP	1.395	0.000
CRP	0.55	0.01
AGEs/sRAGE-ratio		
BMI SD	0.062	0.000
TC/HDL-ratio	0.065	0.008
CRP	0.214	0.002

Total cholesterol (TC), Atherogenic index of plasma (AIP), Homeostasis model assessment of insulin resistance (HOMA-IR), C-reactive protein (CRP), Advanced glycation end-products (AGEs), Circulating soluble AGE receptor (sRAGE), Advanced oxidation-protein products (AOPPs).

Table 4 – Statistically significant associations at multivariate stepwise regression analysis for BMI SD, HOMA-IR, TC/HDL-ratio, LDL, Triglycerides, uric acid, CRP.

AGEs		
Predictors	**B**	***P*-value**
Triglycerides	0.618	0.01
sRAGE		
BMI SD	-73.18	0.000
AOPPs		
BMI SD	0.13	0.02
CRP	0.522	0.008
Triglycerides	0.010	0.000
AGEs/sRAGE-ratio		
BMI SD	0.06	0.008
CRP	0.160	0.03

C-reactive protein (CRP), Advanced glycation end-products (AGEs), Circulating soluble AGE receptor (sRAGE), Advanced oxidation-protein products (AOPPs).

Figure 1 – Box-plot of oxidative stress markers in groups A (overweight/obese) and B (controls).

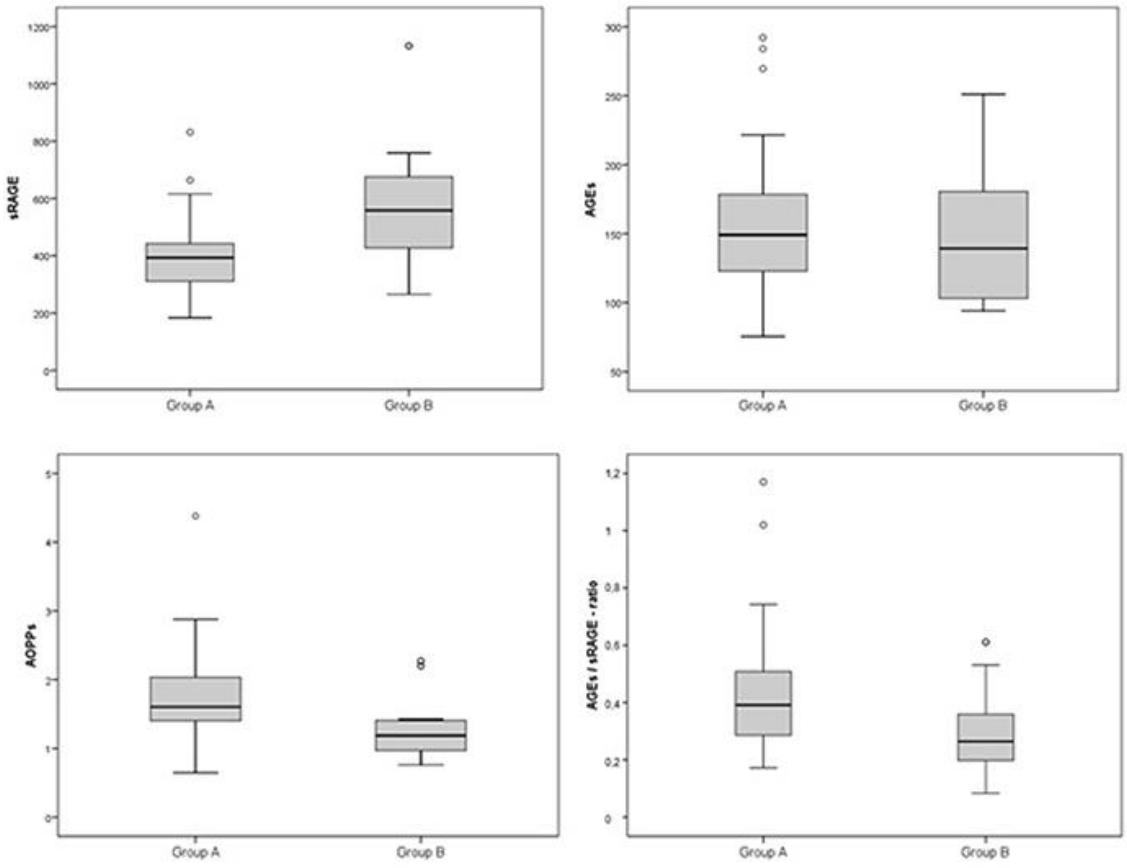
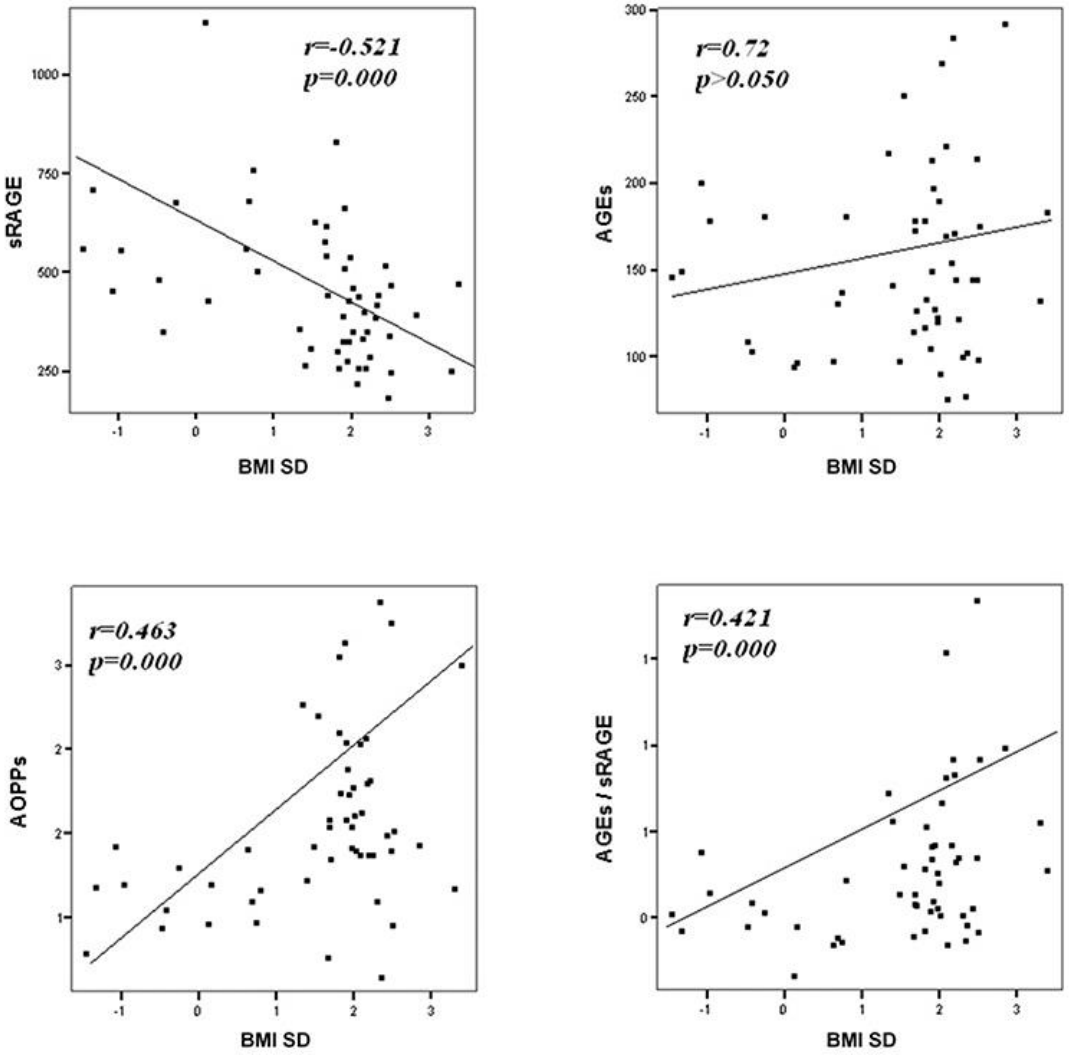


Figure 2 – Correlation between oxidative stress markers and BMI SD.



2.2 Vitamin D affects insulin sensitivity and β-cell function in obese non-diabetic youths.

Corica D, Zusi C, Olivieri F, Marigliano M, Piona C, Fornari E, Morandi A, Corradi M, Miraglia Del Giudice E, Gatti D, Rossini M, Bonadonna RC, Maffeis C. Eur J Endocrinol. 2019 Oct;181(4):439-450. doi: 10.1530/EJE-19-0369.

Introduction

Vitamin D plays a central role in calcium and phosphorus homeostasis and bone metabolism but it might exert also extra-skeletal actions, affecting pancreatic β-cell function (BCF) and glucose homeostasis. Vitamin D circulating levels are mainly influenced by geographic location of residency, season, and ethnicity (1, 2). Hypovitaminosis D is widely diffuse worldwide and is highly prevalent in children and adolescents (1, 3-6). A higher incidence of low total vitamin D levels has been reported in obese compared to the general population, in both adults and children (2, 7-11). In a large cohort of US children Ganji *et al.* reported a prevalence of vitamin D deficiency of 21% in normal-weight children, 29-34% in overweight and obese children, and 49% in severely obese children (12). Several mechanisms have been considered to explain the lower concentration of vitamin D in the obese, including inadequate diet consumption, decreased sun exposure due also to sedentary lifestyle, 25-hydroxy-vitamin D (25-OHD) trapping in adipose tissue, although a combination of these factors is likely (10). Moreover, it has been suggested that, in spite of their low levels of total vitamin D, obese children would have bioavailable vitamin D (BVD) levels similar to those of normal-weight children, due to reduced concentration of vitamin D-binding protein (VDBP) (13).

Consistent evidences suggest that vitamin D plays a central role in glucose metabolism. Vitamin D promotes the release of insulin, both directly by vitamin D receptors localized in pancreatic β-cells, as well as indirectly, by influencing plasma concentration of calcium and, consequently, calcium-dependent release of insulin (14). Furthermore, 1,25-dihydroxy-vitamin D (1,25-OH_2D) promotes the transcription of insulin and its receptor genes, increasing its expression in liver, muscle and fat tissues, and promotes the expression of glucose transporter GLUT4 in muscles and its translocation into adipocytes (15-18). In vivo studies in animals documented that vitamin D deficiency causes an altered release of insulin in response to glucose (19). Furthermore, a protective effect of vitamin D on BCF, through a renin angiotensin system-suppressing action, has been documented in murine models (20). However, a causal effect relationship between hypovitaminosis D and glycemic control, IR or diabetes risk is not completely accepted; indeed, randomized controlled studies, carried out in human cohorts, demonstrated no effect of vitamin D supplementation or maintenance of adequate vitamin D levels in glycemic control and beta-cell function in T2D, and suggested that the association between

vitamin D concentration and T2D might not be causal (21-24). Moreover, notwithstanding a role of vitamin D supplementation in modulation of hepatic insulin resistance (IR) through the effect of vitamin D-regulated pathways on hepatic lipogenesis and gluconeogenesis has been suggested a relationship between IR and vitamin D concentrations is not clearly demonstrated (9, 13, 21, 25, 26). The aim of our study was to test the hypotheses of a direct relationship between vitamin D, both total vitamin D and bioavailable vitamin D (BVD) concentrations, and insulin sensitivity (IS) and BCF in a sample of non-diabetic, overweight/obese children and adolescents.

Material and methods

Subjects

Study population included 122, 12.8±2.3 years old overweight and obese children and adolescents, enrolled at the Pediatric Diabetes and Metabolic Disorders Unity, University Hospital, Verona (Italy). Inclusion criteria were: Caucasian ethnicity, gender- and age-specific BMI cut-off for obesity reported in the WHO growth references (27). Exclusion criteria were: genetic or endocrine causes of obesity, diabetes (American Diabetes Association criteria) (28), either pre-term or post-term birth, associated chronic diseases or chronic pharmacological therapies. The protocol was approved by the Institutional Ethics Committee of Verona (Italy). Informed consent was obtained from children and their parents.

Physical examination

At recruitment, physical examination was performed according to standard procedures, as previously described (29). Weight was measured to the nearest 0.5 kg on standard physician's beam scales, with the child wearing only underwear and no shoes. Height was measured to the nearest 0.5 cm on a stadiometer without shoes, with the child's heels, buttocks, shoulders and head against the vertical wall with line of sight aligned horizontally. BMI was calculated as weight (in kilograms) divided by height (in meters) squared. BMI values were standardized using age and sex-specific median, standard deviation (SD) and power of the Box-Cox transformation (least mean square method) based on WHO growth references (27). Waist circumference was measured to the nearest 0.5 cm while the subjects were standing, after gently exhaling, as the minimal circumference measurable on the horizontal plane between the lowest portion of the rib cage and the iliac crest (30). Waist-to-height ratio (WHtR), an index of body fat distribution, was calculated as previously described (31). Pubertal stage was assessed according to Tanner criteria (32). Subjects were categorized in prepubertal (Tanner stage 1), pubertal (Tanner stage 2-4), post-pubertal (Tanner stage 5). Systolic blood pressure

(SBP) and diastolic blood pressure (DBP) were recorded three times on the right arm in mmHg using a manual sphygmomanometer; for analysis the average of three blood pressure values was used (33).

Biochemical Measurements

Within 15 days from recruitment visit, all patients underwent fasting blood tests for measuring plasma glucose, serum insulin concentration, glycated hemoglobin (HbA1c), lipid profile, liver enzymes and albumin. Plasma glucose was measured with glucose oxidase method and HbA1c by immunoturbinometric assay (Accu-Chek Inform II and Cobas B101, Roche). Insulin and C-peptide levels were measured by enzyme-immunoassay (Mercodia AB, Sweden). Aspartate aminotransferase (AST), alanine aminotransferase (ALT), serum triglycerides, total cholesterol and high-density lipoprotein cholesterol (HDL-c) were measured by standard methods. Low-density lipoprotein cholesterol (LDL-c) was calculated using the Friedewald's equation. Serum albumin levels were measured in a spectrophotometer at 620 nm through colorimetric method (Sclavo Diagnostics International, Italy). Seasonality of blood collection was recorded as summer/fall and spring/winter.

Furthermore, patients underwent a prolonged oral glucose tolerance test (modified-OGTT), performed with standard method (1.75 gr/kg of body weight, up to a maximum of 75 gr) according to the ADA guidelines (28), with sampling at times of -10, 0, +10, +20, +30, +45, +60, +90, +120, +150, +180 minutes for measurements of glucose, insulin and C-peptide. Blood samples were collected in SST II or EDTA tubes and centrifuged at 1300 g for 10 minutes after collection; serum and plasma were stored at -80°C until analyzed. No subjects had diabetes according on ADA definition.

BCF was reconstructed by mathematical modeling, as described by Bonadonna et al. (34). By this method, BCF is described by two parameters: Derivative (or dynamic) Control, i.e. the response of the β-cell to the rate of glucose increase of 1 mmol/L per minute that lasts for 1 min (expression of 1st phase of insulin secretion), and Proportional (or static) Control, i.e. the stimulus-response curve linking of the β-cell to glucose concentration per se (expression of 2nd phase of insulin secretion).

OGTT-derived indices

The following OGTT-derived indices were calculated:

- Homeostasis model assessment for insulin resistance (HOMA-IR) was calculated as [insulin (mU/L) x glucose (mg/dL)]/405, and used as a fasting biomarker of IR (35).

-Homeostasis model assessment for BCF (HOMA-B) was performed as [(360 x insulin (mU/L))/(glucose (mg/dL)-63)] and considered as a measure of BCF (36).

- Insulinogenic index (IGI), assessed as [insulin30 (mU/L) -insulin0 (mU/L)]/[glucose30 (mg/dL)-glucose0 (mg/dL)], was selected as a biomarker of insulin bioavailability in response to oral glucose (37).
- Matsuda index: 10000/[(glucose0' (mg/dL) x insulin0' (mU/L)) x (mean OGTT glucose concentration (mg/dL)) x (mean OGTT insulin concentration (mU/L))]$^{1/2}$; it was used as a postprandial biomarker of IS (38).
- Mathematical modeling of glucose and C-peptide curves following the OGTT was performed and Derivative and Proportional Controls of BCF were computed (39).

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes using salting-out procedures. Genotyping was carried out by a predesigned TaqMan probe (Applied Biosystem, California, USA), according to the manufacturers' protocol, with a >99% positive call-rate. Polymorphism genotyping was performed using 7900 HT Real Time PCR (Applied Biosystem, California, USA). We genotyped two common SNPs (rs4588 and rs7041) in the coding region of the VDBP gene.

Vitamin D measurements

Total vitamin D and parathyroid hormone (PTH) were measured through chemiluminescent method (IDS-iSYS Multi Discipline automated analyzer; IDS iSYS, Immunodiagnostic System, Boldon, UK). Vitamin D deficiency, insufficiency and sufficiency were defined as a 25(OH)D levels < 20 ng/ml, 20-29 ng/ml and ≥ 30 ng/ml, respectively (40). Vitamin D Binding Protein was measured by ELISA method (R&D Systems) (13, 41).

Forty-seven out of 122 subjects were in homozygoses for the two analyzed SNPs. A single genotype–specific binding affinity constant on the basis of the presence of a single VDBP variant according to the equation reported by Powe et al; was applied (41). In these subjects BVD dependent concentration was calculated.

Statistical analysis

Patients' baseline characteristics are reported as mean ± SD. Kolmogorov-Smirnov test was used to assess normal distribution of variables. Skewed variables were transformed (natural log transformed or square root transformed, if and as needed) to correct for non-Gaussian distribution, unless deviations from the Gaussian distribution could not be corrected by transformation. Spearman correlation was applied to normal and skewed distributed variables. Student's *t*-test, Mann-Whitney or chi-squared test were used to detect differences among patients stratified by gender or total vitamin D groups. Multiple regression analyses, using HOMA-IR, HOMA-B, Matsuda index, IGI, Derivative Control or Proportional Control as dependent variables, were run. Two models were calculated: in

the first, total vitamin D was considered as independent variable together with age, gender, pubertal stage, BMI SDS and season of vitamin D measurement, in the second one, total vitamin D was replaced by BVD. Covariates included in regression models were selected as potential confounding factors based on their biological plausibility. A p-value <0.05 was considered as a nominal statistical significance. Benjamini–Hochberg (BH) step-up procedure was applied for multiple test adjustment (42). All analyses were performed using SPSS v.22.0 (SPSS).

Results

A sample of 122 subjects (69 males, 53 females) with a mean age of 12.8±2.3 years and a mean BMI SDS of 2.9±0.8 was recruited. Anthropometric and metabolic characteristics of the study population are showed in Table 1. Males had higher waist circumference (p=0.009), WHtR (p=0.004), 2h-plasma insulin (p=0.039), ALT (p=0.009), AST (p=0.015) and HOMA-B (p=0.033) than females. No other nominal significant differences for others variables between genders were found.

Vitamin D insufficiency (defined as total vitamin D 20-29 ng/ml) and deficiency (defined as total vitamin D < 20 ng/ml) were found in 50% (N=61) and 40.2% (N=49) of children, respectively. No significant differences between genders were found.

Splitting subjects into two groups (group A: subjects with vitamin D deficiency; group B: subjects with normal vitamin D and vitamin D insufficiency), we found nominal significant differences between groups in BMI (p=0.007), PTH (p=0.023), HOMA-IR (p=0.002), HOMA-B (p=0.008), IGI (p=0.041), Matsuda index (p=0.001) and Proportional Control (p=0.035) (Table 2).

None of these differences stayed statistically significant after multiple test adjustment.

Genotyping for two common single-nucleotide polymorphisms in the coding region of the VDBP gene (rs4588 and rs7041), we detected 47 subjects homozygous for rs4588 and rs7041 VDBP polymorphisms: 25 homozygous for Gc1S/Gc1S alleles, 4 for Gc1F/Gc1F and 18 for Gc2/Gc2. Bioavailable vitamin D in this group of 47 subjects was 4.30 ± 0.31 ng/mL.

Correlation analysis

Results of the correlation analyses are reported in Table 3. Total vitamin D was positively correlated with BVD, also after multiple test adjustment (r=0.68, p-adj=0.0009), whereas was nominally correlated with Matsuda index (r=0.28, p=0.002), and negatively with BMI SDS (r=-0.18, p=0.043), HOMA-IR (r=-0.24, p=0.008), IGI (r=-0.25, p=0.007), Derivative Control (r=-0.20, p=0.036), Proportional Control (r=-0.22, p=0.018), and fasting plasma insulin.

Bioavailable vitamin D was positively correlated with Matsuda index (r=0.25, p=0.026), and negatively with BMI SDS (r=-0.29, p=0.041), IGI (r=-0.38, p=0.007), Derivative Control (r=-0.29,

p=0.05), Proportional Control (r=-0.31, p=0.036) and plasma insulin at time 30' (r=-0.43, p=0.026) of the modified-OGTT. Vitamin D-binding protein did not significantly correlate with any other variable except BVD, that remained significant after multiple test adjustment (r=-0.51, p-adj=0.023), and total vitamin D (r=0.17, p=0.045). Total vitamin D and BVD did not significantly correlate with waist circumference, WHtR, systolic and diastolic blood pressure, HbA1c, lipid profile, and plasma glucose concentration during modified-OGTT.

Regression analysis

Multivariate regression analysis (Table 4) showed that total vitamin D, independently from age, gender, BMI SDS, pubertal stage, season of vitamin D measurement, was positively associated with Matsuda index (p=0.020) and negatively with HOMA-IR (p=0.038), HOMA-B (p=0.012), IGI (p=0.042), and Proportional Control (p=0.045). Bioavailable vitamin D, independently from age, gender, BMI SDS, pubertal stage, season of vitamin D measurement, was inversely associated with IGI (p=0.034) (Table 5). None of these associations remained statistically significant after multiple test adjustment.

Discussion

The main finding of this study is the significant association between total vitamin D and IS, IR and insulin secretion indices, adjusting for confounders, supporting the hypothesis of a potential direct effect of vitamin D status on IS, IR and BCF, in non-diabetic, overweight and obese children and adolescents.

Vitamin D affects BCF through several pathways: directly, through interaction with vitamin D receptor localized on β-cells, and indirectly, via regulation of serum calcium concentration and calcium flux through the β cell, therefore influencing both insulin synthesis and secretion (14). Vitamin D promotes also insulin receptor gene transcription and the expression of this receptor in peripheral insulin-target tissues (liver, skeletal muscle, and adipose tissue) (15-18). Moreover, vitamin D would affect insulin levels reducing inflammation-induced β-cell apoptosis by an immune-regulation effect (43). Accordingly, low vitamin D levels seems to be related to defective insulin secretion, reduced glucose homeostasis, and increased risk of metabolic syndrome and T2D (44, 45). Several studies investigated mechanisms through vitamin D could influence β-cell function and insulin sensitivity, different from direct effects on calcium metabolism. In two studies including murine models, a suppressing effect of vitamin D on pancreatic renin angiotensin system, that is involved in islet cell proliferation and apoptosis, seems to determine a protective effect on BCF (20, 46, 47). Particularly, mice with hypovitaminosis D presented increased expression of renin angiotensin system components, impaired β-cell gene transcription and impaired glucose tolerance

(47). Accordingly, isolated murine pancreatic islets, incubated *ex vivo* under high-glucose conditions, exhibited an increase in renin angiotensin system components expression and production, prevented by calcitriol administration that contemporary promoted the β-cell glucose-stimulated insulin secretion (20). Considering the close link between IR and the pathogenesis of steatohepatitis in obese and diabetic patients, vitamin D appears to be also involved in regulation of hepatic insulin resistance and development of nonalcoholic fatty liver disease (NAFLD) (48). Hypovitaminosis D and NAFLD frequently coexist in obese, and growing evidences supported a potentially causative relationship between these two conditions. In murine models, vitamin D shows a protective effect against liver damage and steatosis, by inhibiting lipid accumulation through autophagy and by attenuating oxidative stress (49, 50). Treatment with 1,25-OH_2D seemed to improve abnormal hepatic lipid and glucose production in T2D mice and seemed to reduce, through activation of $Ca2^+$/calcium-calmodulin protein kinase-beta /AMP-activated protein kinase pathways, hepatic triglyceride storage and glucose output under *in vitro* insulin-resistant condition (51). These results support the regulatory action of vitamin D on glucose and lipid metabolism and, consequently, on insulin resistance and sensitivity. Accordingly, hypovitaminosis D was significantly associated with histologically-proven NAFLD and vitamin D seems to be protective against progression from NAFLD to nonalcoholic steatohepatitis (NASH), inhibiting the proliferation hepatic stellate cells, responsible for collagen deposition and fibrosis (52, 53). However, conversely, other studies have not demonstrated an association between vitamin D deficiency and histological characteristics or severity of NAFLD neither positive effect of vitamin D supplementation on hepatic fat content or markers of hepatic injury or insulin sensitivity (54-57). Therefore, on the light of these conflicting results, further prospective randomized clinical trials in humans will be necessary to establish the role of vitamin D concentration and supplementation in NAFLD and in hepatic insulin resistance.

In our cohort of overweight and obese children and adolescents, we found a high prevalence of hypovitaminosis D (90%), according with others (2, 9). However, we found a direct correlation between total vitamin D and BVD, supporting the hypothesis that bioavailability of vitamin D is reduced proportionally to the reduction of total vitamin D. In our study, the first that simultaneously investigated the association between total vitamin D, BVD and IS, IR and BCF in overweight and obese, non-diabetic, children and adolescents, we found that total vitamin D was significant predictor of IS, IR and BCF, in term of both HOMA-B and the 2nd phase of insulin secretion (Proportional Control). These relationships were independent from age, gender, pubertal stage, season of vitamin D measurement, and BMI SDS. Our data confirm previous studies demonstrating an inverse association between total vitamin D and BMI SDS (2, 7, 9). Moreover, for the first time, we found

that also the bioavailable form of vitamin D is inversely associated with BMI SDS, suggesting a direct relationship between the severity of overweight and the degree of hypovitaminosis D.

IR and an altered BCF are two conditions that usually preceded the development of abnormal glucose tolerance and type 2 diabetes (T2D). Our data contribute to support the hypothesis that hypovitaminosis D might be implicated in the pathogenesis of T2D in overweight and obese individuals, who are at risk of glucose metabolism impairment (37, 38). Previous studies in adults provided evidences on the relationships between vitamin D and T2D, sustaining the positive effect of normal vitamin D concentrations in reducing risk of T2D (39-42), as well as the favorable effects of vitamin D supplementation both in reducing the risk of developing IR and T2D, and in improving the glycemic control in patients with T2D (24, 43-45). In a meta-analysis, Parker *et al*. reported a significant association between high levels of vitamin D and a 55% of reduction on the risk of having T2D (58). Deleskong *et al*. reported a positive and significant effect of higher vitamin D concentration in reducing risk of T2D in individuals with prediabetes, but not in those subjects with normal glucose tolerance (59). Chiu *et al.,* in a cohort of healthy glucose-tolerant adults, demonstrated a positive correlation between 25(OH)D and IS, and a negative effect of hypovitaminosis D on BCF, assessed by an independent, negative, relationship between 25(OH)D and plasma glucose during a hyperglycemic clamp (60). Moreover, in a large cohort of children and adolescents, Ganji *et al.* reported an inverse correlation between 25(OH)D levels and HOMA-IR (12). Conversely, other studies, carried out both in children and adults, did not document association between vitamin D levels and IR or glycemic control (9,21,61,62). A causal-effect relationship between hypovitaminosis D and glycemic control, IR or diabetes risk (22) as well as not even favorable effects of vitamin D supplementation on plasma glucose, plasma insulin and IR or BCF were reported (24, 63, 64). These contrasting results suggest that several factors, including genetic background (i.e. VDBP polymorphisms), baseline vitamin D concentration and measurement methods, dose and routes of administration of vitamin D supplementation, influence the activity of vitamin D.

Although available data of the literature are not consistent, the results of our study suggest that, as an attempt to reduce the risk to develop glucose metabolism impairment, a periodic monitoring of vitamin D concentration in overweight and obese children and adolescents may be justified as well as the treatment with 25(OH)D in subjects with vitamin D deficiency or insufficiency (65). Of course, the role of vitamin D supplementation as a potential intervention strategy for the management of IR and T2D in obese children and adolescents needs to be assessed by long-term randomized controlled studies.

The study has some potential limitations: (i) the cross-sectional design of the study, that did not allow to verify the causal relationships between vitamin D levels and IS, IR and BCF; (ii) the lack of data

on dietary intake vitamin D and sun exposure. Nevertheless, the potential impact of vitamin D intake with diet on circulating total vitamin D should be negligible, due to the really modest intake of vitamin D in the diet usually reported by overweight and obese children living the same geographical area (70). Moreover, the season of blood test sampling for measuring total vitamin D was considered in the statistical analysis, as an attempt to take indirectly into account sun exposition, higher in spring and summer and lower in autumn and winter; (iii) ethnicity; this study was conducted in subjects with European ancestry, so that results are not directly exportable to subjects with other ethnic backgrounds; (iv) vitamin D dosage was not detected by gold standard metabolite test (HPLC with MS/MS); (v) serum calcium was not available in our cohort despite no phenotype signs of hypocalcemia were found.

This study has also some strengths: (i) the sample set, including glucose-tolerant, healthy subjects, who were not taking chronic medication, that allows to explore relationships between variables avoiding potential confounders due to co-morbidity, so common in obese adult samples; (ii) the glucose metabolism assessment: in this study, differently from other studies (66-69) in which the relationship between vitamin D and glucose metabolism in children was investigated considering just indices derived from fasting insulin and glucose levels, we also considered indices of IS and BCF derived from a modified-OGTT, a reliable and little invasive test to evaluate glucose tolerance; (iii) vitamin D status assessment: in this study not only the total vitamin D measurement was provided but also BVD was estimated by the VDBP polymorphisms, according to Powe et al. equations (35); (iv) PTH analysis let us establish a significant correlation with total vitamin D in order to exclude that adiposity could be the only cause of hypovitaminosis D.

In conclusion, overweight and obese children and adolescents have a high rate of vitamin D deficiency and insufficiency and hypovitaminosis D is associated with lower BCF and insulin sensitivity.

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Tables and figures

Table 1 Physical and biochemical features of the total sample and comparison analysis according to genders.

Variables	Total	Male	Female	p-value
N	122	69	53	
Age (years)	12.8 ± 2.3	13.0 ± 2.4	12.6 ± 1.9	0.375
Weight (kg)	83.3 ± 25.2	85.1 ± 28.3	80.9 ± 20.6	0.370
BMI (kg/m^2)	31.9 ± 7.0	31.8 ± 7.7	32.0 ± 5.9	0.891
BMI SDS	2.93 ± 0.77	2.97 ± 0.85	2.87 ± 0.66	0.483
Waist circumference (cm)	98.8 ± 15.9	103.2 ± 17.7	93.6 ± 11.6	0.009
WHtR	0.62 ± 0.07	0.65 ± 0.08	0.60 ± 0.06	0.004*
Puberty Status				0.039
Pre-pubertal status n (%)	24 (19.8)	15 (21.7)	9 (17.0)	
Pubertal status n (%)	49 (40.1)	34 (49.3)	15 (28.3)	
Post-pubertal status n (%)	49 (40.1)	20 (29.0)	29 (54.7)	
Season of vitamin D measurement				0.672
Summer/Fall n (%)	59 (48.4)	34 (50)	25 (46.3)	
Winter/Spring n (%)	63 (51.6)	34 (50)	29 (53.7)	
SBP (mmHg)	119.4 ± 13.7	121.1 ± 16.6	117.3 ± 8.6	0.085
DBP (mmHg)	70.2 ± 10.4	70.3 ± 11.8	70.1 ± 8.3	0.679
Triglycerides (mg/dL)	85.9 ± 69.2	82.8 ± 67.7	90.0 ± 71.6	0.370
Total cholesterol (mg/dL)	155.0 ± 31.1	156.1 ± 34.3	153.5 ± 26.4	0.672
HDL-Cholesterol (mg/dL)	44.0 ± 9.9	44.9 ± 9.8	42.7 ± 10.1	0.293
LDL-Cholesterol (mg/dL)	90.0 ± 29.0	92.4 ± 31.7	86.9 ± 25.1	0.351
ALT (UI/L)	31.8 ± 19.8	35.6 ± 20.7	26.7 ± 17.5	0.009
AST (UI/L)	21.8 ± 9.9	23.2 ± 9.6	19.8 ± 9.9	0.015
PTH (pg/mL)	35.2 ± 12.9	34.5 ± 12.7	36.0 ± 13.3	0.526
Albumin (g/dL)	5.29 ± 0.30	5.30 ± 0.27	5.28 ± 0.33	0.538
VDBP (µg/mL)	201.8 ± 22.5	199.6 ± 21.4	204.6 ± 23.9	0.227
Total vitamin D (ng/mL)	19.66 ± 7.8	20.59 ± 7.63	18.45 ± 7.82	0.132
BVD (ng/mL)*	4.30 ± 2.06	4.52 ± 2.23	4.00 ± 1.79	0.408
Fasting plasma glucose (mg/dL)	91.0 ± 7.9	92.0 ± 6.6	89.7 ± 7.2	0.072
2h-plasma glucose (mg/dL)	113.4 ± 19.6	113.8 ± 22.0	112.9 ± 16.1	0.963
HbA1c (%)	5.54 ± 0.35	5.54 ± 0.33	5.53 ± 0.38	0.872
Fasting plasma insulin (mU/L)	23.8 ± 13.2	22.6 ± 13.0	25.3 ± 13.4	0.262
2h-plasma insulin (mU/L)	137.6 ± 116.9	122.5 ± 86.2	156.8 ± 145.7	0.039
HOMA-IR	5.41 ± 3.10	5.21 ± 3.14	5.66 ± 3.05	0.258
HOMA-B	313.8 ± 186.1	282.4 ± 160.2	355.6 ± 210.3	0.033
IGI	3.43 ± 2.28	3.34 ± 2.41	3.54 ± 2.12	0.466
Matsuda Index	2.38 ± 1.76	2.55 ± 2.04	2.17 ± 1.32	0.369
Derivative Control (pmol/m^2 BSA)/(mmol/L/min)	2,252.4 ± 2,049.1	2,351.4 ± 2,474.6	2,132.5 ± 1,383.8	0.669
Proportional Control (pmol/min/m^2 BSA)	135.3 ± 82.4	131.8 ± 86.9	139.6 ± 77.3	0.310

* N=47 subjects (M=28, F =19).

Data are expressed as means ± SD or number and percentage n (%). Differences between gender were tested by Student's *t*-test for normally distributed variables and the Mann-Whitney test for skewed distributed variables or using χ^2-test for categorical variables. Nominal p-values were reported.

BMI, Body Mass Index; WHtR, waist-to-height ratio; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; PTH, parathyroid hormone; VDBP, Vitamin D Binding Protein; BVD, Bioavailable Vitamin D in 47 subjects genotyped for VDBP variants; HbA1c, glycated hemoglobin; HOMA-IR, Homeostasis model assessment for insulin resistance; HOMA-B, Homeostasis model assessment for β-cell function; IGI, Insulinogenic Index.

Table 2 Comparison analysis of physical and biochemical characteristics among subjects with vitamin D deficiency (Group A) *vs* subjects with normal and insufficiency vitamin D (Group B).

Variables	Group A (N=61)	Group B (N=61)	p-value
Age (years)	13.3 ± 2.3	12.4 ± 2.2	0.075
BMI (kg/m^2)	33.6 ± 7.3	30.2 ± 6.2	0.007*
BMI SDS	3.05 ± 0.73	2.81 ± 0.80	0.088
Waist circumference (cm)	100.6 ± 15.2	96.7 ± 16.6	0.306
WHtR	0.63 ± 0.08	0.61 ± 0.07	0.437
Puberty Status pre-puberal/puberal/post-puberal n (%)			0.022
Pre-pubertal status n (%)	10 (16.4)	14 (23.0)	
Pubertal status n (%)	23 (37.7)	26 (42.6)	
Post-pubertal status n (%)	28 (45.9)	21 (34.4)	
Season of Vitamin D measurement Summer-Fall/Winter-Spring n (%)			0.001*
Summer/Fall n (%)	21 (33.3)	38 (64.4)	
Winter/Spring n (%)	42 (66.7)	21 (35.6)	
SBP (mmHg)	120.6 ± 14.9	118.2 ± 12.4	0.394
DBP (mmHg)	71.6 ± 12.4	68.8 ± 7.7	0.180
Triglycerides (mg/dL)	83.40 ± 59.5	88.46 ± 78.2	0.691
Total cholesterol (mg/dL)	153.5 ± 34.3	156.7 ± 27.4	0.600
HDL-Cholesterol (mg/dL)	42.1 ± 9.1	46.0 ± 10.5	0.052
LDL-Cholesterol (mg/dL)	90.8 ± 31.7	89.2 ± 26.1	0.781
PTH (pg/mL)	37.8 ± 12.7	32.5 ± 12.7	0.023
Fasting plasma glucose (mg/dL)	91.2 ± 7.3	90.9 ± 6.7	0.814
2h-plasma glucose (mg/dL)	113.4 ± 19.3	113.3 ± 20.1	0.980
HbA1c (%)	5.51 ± 0.34	5.56 ± 0.37	0.424
Fasting plasma insulin (mU/L)	27.2 ± 13.3	20.3 ± 12.2	0.004*
2h-plasma insulin (mU/L)	166.0 ± 139.9	108.2 ± 77.7	0.007
HOMA-IR	6.20 ± 3.22	4.60 ± 2.78	0.002*
HOMA-B	358.5 ± 189.8	268.3 ± 172.3	0.008
IGI	3.79 ± 2.19	3.06 ± 2.33	0.041
Matsuda index	1.91 ± 1.15	2.87 ± 2.12	0.001*
Derivative Control (pmol/m^2 BSA)/(mmol/L/min)	2392.6 ±1,691.3	2114.6 ± 2,355.6	0.151
Proportional Control (pmol/min/m^2 BSA)	151.5 ± 90.4	119.4 ± 71.0	0.035

Patients were divided in the two groups according to total Vitamin D value. Data are expressed as means ± SD or number and percentage n (%). Differences between gender were tested by Student's *t-test* for normally distributed variables and the Mann-Whitney test for skewed distributed variables or using χ2-test for categorical variables. Nominal p-values were reported. *Significant p-values after further adjustment for multiplicity (by using the Benjamini–Hochberg step-up procedure). BMI, Body Mass Index; WHtR, waist-to-height ratio; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; HbA1c, glycated hemoglobin; HOMA-IR, Homeostasis model assessment for insulin resistance; HOMA B, Homeostasis model assessment for β-cell function; IGI, Insulinogenic index.

Table 3 Spearman correlation analysis among total vitamin D, BVD and clinical and biochemical variables.

Variables		Total vitamin D	BVD
Age (years)	*r*	-0.21	-0.13
	p	0.020	0.385
Weight (kg)	*r*	-0.19	-0.18
	p	0.036	0.225
BMI (kg/m^2)	*r*	-0.24	-0.23
	p	0.007	0.118
BMI-SDS	*r*	-0.18	-0.29
	p	0.043	0.041
Waist Circumference (cm)	*r*	-0.07	-0.12
	p	0.557	0.557
WHtR	*r*	-0.04	0.06
	p	0.722	0.752
SBP (mmHg)	*r*	0.03	0.01
	p	0.786	0.972
DBP (mmHg)	*r*	-0.11	0.08
	p	0.269	0.637
Triglycerides (mg/dL)	*r*	0.06	-0.22
	p	0.564	0.704
Total cholesterol (mg/dL)	*r*	0.04	-0.04
	p	0.713	0.764
HDL-c (mg/dL)	*r*	0.17	0.19
	p	0.092	0.497
LDL-c (mg/dL)	*r*	-0.09	-0.10
	p	0.512	0.743
AST (UI/L)	*r*	-0.06	0.13
	p	0.653	0.825
ALT (UI/L)	*r*	-0.01	0.05
	p	0.917	0.743
PTH (pg/mL)	*r*	-0.31	-0.07
	p	0.001*	0.666
HOMA-IR	*r*	-0.24	-0.17
	p	0.008	0.140
HOMA-B	*r*	-0.31	-0.26
	p	0.001*	0.084
MATSUDA Index	*r*	0.28	0.25
	p	0.002*	0.026
IGI	*r*	-0.25	-0.38
	p	0.007	0.007
Derivative Control	*r*	-0.20	-0.29
	p	0.036	0.050
Proportional Control	*r*	-0.22	-0.31
	p	0.018	0.036
Fasting Plasma Glucose (mg/dL)	*r*	0.06	0.18
	p	0.512	0.558
Fasting Plasma Insulin (mU/L)	*r*	-0.25	-0.22
	p	0.004*	0.091
HbA1c (%)	*r*	0.12	0.13
	p	0.110	0.396
Total vitamin D (ng/mL)	*r*	-	0.68
	p	-	<0.001*
BVD (ng/mL)	*r*	0.68	-
	p	<0.001*	-
VDBP (µg/mL)	*r*	0.17	-0.51
	p	0.045	<0.001*

Nominal p-values were reported. *Significant p-values after further adjustment for multiplicity (by using the Benjamini–Hochberg step-up procedure). Abbreviations: BMI, Body Mass Index; WHtR, waist-to-height ratio; HOMA-IR, Homeostasis model assessment for insulin resistance; HOMA-B, Homeostasis model assessment for β-cell function; IGI, Insulinogenic index; HbA1c, glycated hemoglobin; VDBP, Vitamin D Binding Protein; BVD, Bioavailable Vitamin D in 47 subjects genotyped for VDBP variants; VDBP, Vitamin D Binding Protein.

Table 4 Multivariate linear regression analysis in the total sample for HOMA-IR, HOMA-B, Matsuda index, IGI, Derivative Control and Proportional Control using total vitamin D, age, gender, BMI SDS, pubertal stage e seasonality of vitamin D measurement as independent variables.

Dependent variables	Independent variables	β coefficient [95% CIs]	p-value	R^2
HOMA-IR	Total Vitamin D	-0.01 [-0.01 to 0.00]	0.038	0.239
	Age	0.01 [-0.03 to 0.04]	0.392	
	Gender	0.07 [-0.03 to 0.17]	0.076	
	BMI SDS	0.15 [0.09 to 0.21]	<0.001	
	Pubertal stage	0.01 [-0.09 to 0.10]	0.215	
	Seasonality	0.05 [-0.04 to 0.13]	0.556	
HOMA-B	Total Vitamin D	-0.02 [-0.03 to -0.004]	0.012	0.306
	Age	0.03 [-0.04 to 0.09]	0.471	
	Gender	0.24 [0.04 to 0.44]	0.018	
	BMI SDS	0.30 [0.17 to 0.43]	<0.001	
	Pubertal stage	0.08 [-0.12 to 0.28]	0.804	
	Seasonality	-0.10 [-0.31 to 0.11]	0.331	
Matsuda index	Total Vitamin D	0.01 [0.001 to 0.01]	0.020	0.238
	Age	0.004 [-0.03 to 0.03]	0.761	
	Gender	-0.04 [-0.14 to 0.05]	0.281	
	BMI SDS	-0.14 [-0.20 to -0.08]	<0.001	
	Pubertal stage	-0.01 [-0.01 to 0.08]	0.851	
	Seasonality	-0.04 [-0.13 to 0.06]	0.464	
IGI	Total Vitamin D	-0.02 [-0.03 to -0.001]	0.042	0.102
	Age	-0.01 [-0.09 to 0.07]	0.901	
	Gender	0.04 [-0.21 to 0.29]	0.691	
	BMI SDS	0.17 [0.02 to 0.33]	0.025	
	Pubertal stage	0.02 [-0.21 to 0.25]	0.902	
	Seasonality	0.06 [-0.18 to 0.30]	0.595	
Derivative Control	Total Vitamin D	-0.31 [-0.92 to 0.30]	0.315	0.046
	Age	0.27 [-2.73 to 3.27]	0.859	
	Gender	-0.51 [-9.87 to 8.85]	0.914	
	BMI SDS	2.94 [-2.94 to 8.81]	0.323	
	Pubertal stage	3.08 [-5.53 to 11.69]	0.480	
	Seasonality	-3.83 [-12.93 to 5.28]	0.406	
Proportional Control	Total Vitamin D	-0.01 [-0.01 to 0.00]	0.045	0.039
	Age	-0.02 [-0.05 to 0.02]	0.437	
	Gender	0.03 [-0.08 to 0.13]	0.351	
	BMI SDS	0.01 [-0.06 to 0.08]	0.677	
	Pubertal stage	0.033 [-0.06 to 0.13]	0.142	
	Seasonality	-0.04 [-0.15 to 0.06]	0.342	

Statistical test performed on log- or root-square transformed data. Abbreviations: HOMA-IR, Homeostasis model assessment for insulin resistance; HOMA-B, Homeostasis model assessment for β-cell function; IGI, Insulinogenic index; BMI, Body Mass Index.

Table 5 Multivariate linear regression analysis in the total sample for HOMA-IR, HOMA-B, Matsuda index, IGI, Derivative Control and Proportional Control using BVD, age, gender, BMI SDS, pubertal stage e seasonality of vitamin D measurement as independent variables.

Dependent variables	Independent variables	β coefficient [95% CI]	p-value	R^2
HOMA-IR	BVD	-0.05 [-0.23 to 0.13]	0.441	0.316
	Age	-0.02 [-0.08 to 0.03]	0.973	
	Gender	0.01 [-0.15 to 0.18]	0.480	
	BMI SDS	0.18 [0.09 to 0.27]	<0.001	
	Pubertal stage	0.07 [-0.08 to 0.23]	0.449	
	Seasonality	0.05 [-0.11 to 0.21]	0.864	
HOMA-B	BVD	-0.15 [-0.54 to 0.24]	0.440	0.348
	Age	-0.02 [-0.14 to 0.10]	0.709	
	Gender	0.12 [-0.24 to 0.48]	0.502	
	BMI SDS	0.40 [0.20 to 0.61]	<0.001	
	Pubertal stage	0.15 [-0.19 to 0.48]	0.387	
	Seasonality	-0.06 [-0.41 to 0.29]	0.732	
Matsuda index	BVD	0.13 [-0.05 to 0.30]	0.108	0.228
	Age	0.03 [-0.03 to 0.08]	0.614	
	Gender	0.004 [-0.16 to 0.17]	0.647	
	BMI SDS	-0.15 [-0.25 to -0.06]	0.001	
	Pubertal stage	-0.04 [-0.19 to 0.12]	0.971	
	Seasonality	-0.04 [-0.20 to 0.12]	0.902	
IGI	BVD	-0.43 [-0.83 to -0.02]	0.034	0.227
	Age	0.03 [-0.11 to 0.17]	0.485	
	Gender	-0.24 [-0.65 to 0.18]	0.108	
	BMI SDS	0.27 [0.02 to 0.52]	0.025	
	Pubertal stage	-0.05 [-0.17 to 0.63]	0.988	
	Seasonality	0.23 [-0.17 to 0.63]	0.392	
Derivative Control	BVD	-10.4 [-30.9 to 10.1]	0.504	0.140
	Age	1.76 [-4.54 to 8.16]	0.540	
	Gender	-9.72 [-28.7 to 9.2]	0.358	
	BMI SDS	4.83 [-6.9 to 16.6]	0.244	
	Pubertal stage	3.5 [-14.3 to 21.4]	0.735	
	Seasonality	-10.5 [-28.9 to 7.9]	0.761	
Proportional Control	BVD	-0.17 [-0.35 to 0.02]	0.082	0.132
	Age	-0.01 [-0.06 to 0.05]	0.657	
	Gender	-0.10 [-0.27 to 0.07]	0.171	
	BMI SDS	0.02 [-0.09 to 0.12]	0.716	
	Pubertal stage	-0.04 [-0.20 to 0.12]	0.752	
	Seasonality	-0.04 [-0.35 to 0.02]	0.426	

Statistical test performed on log- or root-square transformed data. Abbreviations: HOMA-IR, Homeostasis model assessment for insulin resistance; HOMA-B, Homeostasis model assessment for β-cell function; IGI, Insulinogenic index; BMI, Body Mass Index; BVD, Bioavailable Vitamin D in 47 subjects genotyped for VDBP variants.

2.3 Asprosin serum levels and glucose homeostasis in children with obesity.

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Introduction

Childhood obesity has become one of the major public health issues worldwide. Obese children and adolescents are more likely to become obese adults with an increased risk of cardio-metabolic complications [1, 2]. Adipose tissue plays an active role in the production of metabolically active molecules, including adipokines, involved in energy homeostasis, in several metabolic pathways, in inflammatory cascade and in pathophysiological processes that contribute to obesity-related cardio-metabolic complications [3].

Asprosin, firstly described in 2016 [4], is an adipokine involved in glucose homeostasis and in regulation of food intake and energy homeostasis. It is a 140-amino acid long C-terminal cleavage product encoded by exons 65 and 66 of Fibrillin 1 gene (FBN1) located in chromosome 15q21.1. This adipokine is mainly secreted by white adipose tissue, but adipocytes do not seem to be the only cells able to produce asprosin. In fact, FBN1 is expressed in several tissues, as skin, salivary glands and pancreatic β-cell, which would also appear to be involved in asprosin production, although the interaction between these different sites of asprosin secretion is not completely clear [4, 5, 6].

Asprosin serum concentration increases in fasting conditions and rapidly decreases with refeeding. Asprosin acts principally influencing hepatic glucose release and determining the activation of hypothalamic feeding center after crossing blood-brain barrier. Asprosin seems to increase activity of orexigenic agouti-related peptide (AgRP) neurons by G proteins-cAMP-protein-kinase-A (PKA) axis and, simultaneously, it seems to determine a GABA-dependent inhibition of anorexigenic pro-opiomelanocortin (POMC) neurons, finally, stimulating appetite [7]. The effect of asprosin also on hepatocyte glucose release was reported to be mediated by the G protein-cAMP-PKA axis [4]. This action leads to production and release of glucose and, indirectly, to an increased insulin secretion, which is able to suppress asprosin-mediated hepatic glucose release [4]. However, the role of asprosin in glucose homeostasis regulation remains still controversial [8, 9]. Few studies evaluated the relationships between asprosin concentration and obesity, insulin resistance (IR) or type 2 diabetes (T2D) in humans, and available results are conflicting, especially in pediatrics [10-12]. Evaluation of these aspects in obese children and adolescents not suffering from long-lasting obesity-related complications could broad knowledge about the mechanisms underlying asprosin pathophysiology.

Aims of the study were to compare fasting serum asprosin levels between children with obesity and controls, and to investigate the relationships of asprosin with body mass index (BMI) and biochemical

markers of IR, insulin sensitivity, β-cell function and cardio-metabolic risk in non-diabetic children with obesity.

Materials and methods

Study population

This is a single-center, cross-sectional, case-controlled study carried out from May to November 2019. Inclusion criteria were: BMI ≥ +2 standard deviation score (SDS) for obese children, in accordance with definition of obesity by World Health Organization (WHO) for children from the age of 5 years [13]; age ranged between 5 and 16 years; Caucasian ethnicity; born as healthy full-term infant adequate for gestational age. BMI SDS included in the normal range according to WHO criteria (BMI SDS > -2 SDS and < +2 SDS) [13] was considered for the recruitment of control group patients. Exclusion criteria were: genetic and/or endocrine causes of obesity; impaired glucose tolerance (IGT); diabetes; chronic diseases; chronic pharmacological therapies; smoking. All procedures were performed in accordance with the Declaration of Helsinki and were approved by Ethics Committee of Messina (N.552-17/04/2019). Written informed consent was obtained from all parents or legal tutors.

Clinical and biochemical evaluation

At recruitment, physical evaluation was performed according to standardized procedures, including evaluation of height, weight, BMI, BMI SDS, waist circumference (WC), WC-to-height ratio (WHtR), systolic and diastolic blood pressure [14]. Children underwent fasting biochemical assessments (lipid profile, oral glucose tolerance test (OGTT), thyroid, kidney, liver function tests), as previously described [15]. Homeostasis model assessment of insulin resistance (HOMA-IR), homeostasis model assessment of β-cell function (HOMA-B), Matsuda-index, insulinogenic-index (IGI) were calculated, as previously detailed [16]. Areas Under the Curves for glucose (AUCg) and insulin (AUCi) and their ratio were also evaluated. IR was defined as HOMA-IR > 2.5 in prepubertal children and > 4 in pubertal subjects [15]. Fasting asprosin serum levels were measured, after overnight fasting (at least 8 hours), using an enzyme-linked immunosorbent assay (ELISA) kit accordingly to the manufacturer's instructions (MyBioSource,USA; catalog number:MBS9716571). The detection threshold was 1 pg/mL and no significant cross-reactivity between human asprosin and analogues was reported; the intra-assay and inter-assay coefficient of variation (CV) values were <9% and <11%, respectively. Asprosin concentrations were expressed as pg/mL.

Statistical analysis

Numerical data were expressed as mean and SDS and categorical variables as number. The non-parametric approach was used since most of numerical variables were not normally distributed, as verified by Kolmogorov Smirnov test. To assess the existence of significant differences between patients and controls, male and female, or pubertal and prepubertal children, we applied the Mann Whitney test (for numerical parameters) and Chi Square test or exact Fisher test or Likelihood ratio test (for categorical variables), as appropriate. The interdependence between asprosin values and clinical, biochemical variables (Spearman correlation test) and between asprosin and dichotomous variables (point-biserial (pb) correlation) were evaluated. Partial correlation was calculated to evaluate abovementioned relations controlling for age, sex, pubertal stage and BMI. Moreover, asprosin variable was arbitrarily dichotomized, according to mean value, in order to create two ordered classes of values, because of lacking of definite range of asprosin serum levels in literature. Logistic regression models were estimated to assess the possible dependence of dichotomized asprosin (dependent variables) on some potential explicative variables as BMI SDS, age and sex. In particular, a crude model (model 1) with only BMI SDS, a second model in which age was added (model 2) and a final model (model 3) in which sex was added to model 2, were estimated. A receiver operating characteristic (ROC) curve analysis was performed in order to identify the best cut-off of BMI SDS into discriminate subjects belonging to asprosin classes in our cohort. Statistical analyses were performed using SPSS for Window package (version 22.0). A P-value <0.05 was considered to be statistically significant.

Results

Sixty-seven children were consecutively recruited, including 43 Caucasian children with obesity (group A) and 24 normal-weight controls (group B), matched for age (11.9±2.1 vs 10.7±2.9 years; p=0.07), sex (21 males/22 females vs 7/17; p=0.12) and pubertal stage (17 pre-pubertal/26 pubertal vs 10/14; p=0.86). Thyroid, liver and kidney function tests were normal in the entire population.

Fasting asprosin concentration was significantly lower in group A compared to group B (331.9±120.5 vs 358.1±74.1 pg/ml; p=0.013) (Figure 1). Comparing the entire cohort by sex, asprosin was lower in boys than in girls (313.7±59.5 vs 361.1±127.2 pg/ml; p=0.044) (Figure 2), while BMI SDS was higher in boys compared to girls (2.63±1.19 vs 1.71±1.51; p=0.024); however, in within-group comparison, these differences were not confirmed. Comparison analysis between pre-pubertal and pubertal subjects did not show significant differences in asprosin levels neither in the entire cohort nor within groups A and B (data not shown).

When children with obesity were categorized in IR and non-IR subjects according to HOMA-IR, no significant differences in asprosin levels were demonstrated between subgroups (Table 1).

Considering all study population, asprosin was negatively correlated with BMI (r=-0.275, p=0.024), BMI SDS (r=-0.239, p=0.044) and male sex (r_{pb}=-0.248, p=0.043) (Table 2).
No correlations were demonstrated between asprosin, clinical and biochemical variables in group A (data not shown). All these correlations remained statistically non-significant after adjustment by age, sex, pubertal stage and BMI (data not shown).
Moreover, the entire cohort was dichotomized in two classes based on the mean value of asprosin serum levels ≤ 341 and > 341 ng/ml, respectively. Accordingly, logistic regression models documented a significant negative association between BMI SDS and asprosin classes independently from age and sex (Figure 3 a, b, c). ROC curve analysis showed existence of the best cut-off for BMI SDS (+ 2.7 SDS) able to discriminate patients belonging to two asprosin classes in our cohort (AUC ROC= 0.7; p-value= 0.009) (Figure 3 d).

Discussion

In this study, we documented significantly lower asprosin serum levels in children with obesity compared to controls and a negative correlation between asprosin and BMI in the entire cohort. Only three others studies evaluated asprosin concentration in small cohorts of children with obesity [10-12]. Our findings are consistent with Long et al. results [10], but in contrast with other studies [11,12] which documented increased asprosin serum levels in children with obesity compared with normal-weight controls. Our results were confirmed by the finding of a significant association between higher BMI SDS values and lower asprosin serum levels class. Furthermore, ROC curve analysis suggested the potential diagnostic value of BMI +2.7 SDS, expression of a severe obesity, in predictability of asprosin serum levels in our cohort. However, these cut-offs cannot be generalized to discriminate between "low or high" serum asprosin in children, since univocal reference of serum asprosin levels has not yet been established. We also demonstrated lower asprosin serum levels in boys than in girls and a negative correlation between asprosin and male sex in the entire cohort. Similarly, Long et al. [10] documented that asprosin was lower in obese boys than in obese girls, suggesting an involvement of the well-known sex differences in obesity, related to thermogenesis, fat oxidation, lipid metabolism, to justify the difference in asprosin levels between sexes. However, this hypothesis should be confirmed by further studies and also evaluated in relation to the pubertal stage. In our cohort asprosin serum levels did not significantly differ between pubertal and pre-pubertal subjects.
Available studies, investigating on asprosin serum levels in obese human subjects, were not able to determine a cause-effect relationship between asprosin and obesity due to their cross-sectional design [4,11,17]. Moreover, in studies demonstrating a positive correlation between asprosin and BMI, asprosin concentration might be influenced by the fact that obese subjects with IR, IGT and T2D were

included [4,17,18]. In adults with IGT and T2D, higher circulating asprosin concentration in comparison with controls [18] and a positive correlation between asprosin serum levels and HOMA-IR [19] were documented. Furthermore, Zhang et al. demonstrated an alteration of meal-dependent circadian oscillation of asprosin in T2D adult patients compared to non-diabetic controls, consisting in a non-significant reduction of asprosin levels after OGTT [20]. In pediatric cohorts, Wang et al. [11] reported a positive correlation between asprosin and HOMA-IR, conversely Long et al. [10] documented a negative correlation between the two abovementioned variables. Inconsistently with these findings, we demonstrated no correlation neither between asprosin serum levels and HOMA-IR, nor between asprosin and other OGTT-derived variables expression of insulin sensitivity, β-cell function and insulin bioavailability, which were not evaluated in other pediatric cohorts, suggesting that asprosin serum levels regulation may be likely influenced not only by glucose homeostasis but also by other physiological pathways, including hormonal and neuronal pathways.

Further investigations, trying to define the role of asprosin in glucose homeostasis regulation, reported contrasting results. Asprosin has been implicated in impairment of skeletal muscle insulin sensitivity through PKC-δ-associated endoplasmatic reticulum stress/inflammation pathways [9] and in β-cells inflammation by toll-like receptor (TLR) 4 / c-Jun N-terminal kinase (JNK) phosphorylation pathway [5]. Moreover, a possible involvement of asprosin neutralization in β-cell function improvement has been suggested [4]. On the contrary, in murine models, it has been suggested that asprosin may prevent glucose-induced cardiomyocytes apoptosis through inhibition of malondialdehyde and reactive oxygen species production [21] and that it may protect myocardial mesenchymal cells from oxidative stress-induced apoptosis via activating ERK1/2-SOD2 pathway [22]. Considering these contrasting evidence, the possible association between asprosin concentration and T2D development needs to be further investigated. Particularly, it needs to be clarified if asprosin levels variation is the results of glucose homeostasis alteration, oxidative stress and inflammation or it is due to a protective mechanism consequential to metabolic disorders.

In relation to our findings and taking into account all available relevant knowledge in literature about asprosin, we suggested the hypothesis that decreased circulating levels of fasting asprosin in non-diabetic, normal glucose tolerant, children with obesity, could be expression of a self-regulatory mechanism able to prevent the asprosin-mediated increase of fasting glucose.

Cross-sectional design and small simple size represented two limitations of our study, which made us unable to verify the cause-effective relation between asprosin and metabolic variables. On the other hand, our study has significant strengths. First, our cohort consisted in a homogeneous sample of obese children without glucose metabolism impairment. Second, we investigated a several OGTT-derived variables expression of glucose metabolism. Finally, we utilized a high sensitive ELISA kit

to measure asprosin concentration. Indeed, in our cohort asprosin serum concentration ranged between 179.58 and 985.31 pg/ml that were markedly lower compared to values reported in other pediatric studies, in which asprosin ranges were in turn not homogenous among each other [10-12].
In conclusion, asprosin serum levels were significantly lower in obese children compared to control. Fasting asprosin decreased with increasing BMI, but it was not significantly affected by IR. Nevertheless, several aspects on asprosin function in physiological conditions as well as in obesity and glucose homeostasis alterations are still to be clarified.

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Tables and figures

Table 1 – Comparison analysis between insulin resistant and non-insulin resistant obese children.

	IR subjects (n=33)	**Non-IR subjects (n=10)**	**p-value**
Age (year)	12.05 ± 1.89	11.59 ± 2.68	0.585
Sex (M/F)	16/17	5/5	0.608
Pubertal stage (prepubertal/pubertal)	14/19	3/7	0.375
BMI SDS	3.05 ± 0.66	2.81 ± 0.59	0.147
WHtR	0.62 ± 0.061	0.57 ± 0.06	0.168
SBP (mmHg)	115.86± 12.16	110.50 ± 9.61	0.286
DBP (mmHg)	71.11 ± 8.49	67.63 ± 14.21	0.567
TSH (µUI/ml)	3.17 ± 1.68	2.73 ± 1.86	0.336
FT4 (µg/dl)	15.53 ± 1.50	15.92 ± 2.28	0.615
Uric acid (mg/dl)	4.74 ± 1.06	5.19 ± 0.99	0.300
AST (U/L)	20.03 ± 7.05	20.2 ± 3.8	0.739
ALT (U/L)	24.21 ± 15.31	19.6 ± 8.44	0.396
Total cholesterol (mg/dl)	174.15 ± 28.62	191.50 ± 31	0.256
LDL-cholesterol (mg/dl)	101.52 ± 28.28	112.1 ± 32.24	0.455
HDL-cholesterol (mg/dl)	49.55 ± 10.47	49.2 ± 10.78	0.977
Triglycerides (mg/dl)	81.24 ± 32.86	83.4 ± 40.4	0.863
Total cholesterol/HDL-ratio	3.63 ± .81	4.08 ± 1.1	0.244
Triglycerides /HDL-ratio	1.73 ± 0.77	1.87 ± 1.15	0.829
Fasting glucose (mg/dl)	93.73 ± 6.19	89.8 ± 7.52	0.274
Fasting insulin (µUI/ml)	23.55 ± 8.35	11.32 ± 3.13	**0.000**
HbA1c (%)	5.43 ± 0.33	5.33 ± 0.22	0.331
HOMA-IR	5.46 ± 1.98	2.50 ± 0.69	**0.000**
HOMA-B	285.35 ± 112.97	175.39 ± 105.93	**0.004**
Matsuda-index	2.2 ± 1.12	11.81 ± 24.48	**0.000**
IGI	2.78 ± 2.09	2.42 ± 1.69	0.438
AUC_g	248.18 ± 34.76	216 ± 72.64	0.111
AUC_i	230.05 ± 118.05	139.26 ± 102.93	**0.011**
AUC_i / AUC_g ratio	0.91 ± 0.4	0.58 ± 0.35	**0.013**
Asprosin (pg/ml)	329.43 ± 134.77	340.24 ± 56.15	0.238

Numerical data are express as mean ± SDS, categorical variables as number.

Insulin resistance was defined as a HOMA-IR > 2.5 in pre-pubertal and > 4 in pubertal subjects.

Insulin resistant (IR), body mass index (BMI), standard deviation score (SDS), waist circumference (WC), WC-to-height ratio (WHtR), systolic blood pressure (SBP), diastolic blood pressure (DBP), thyroid stimulating hormone (TSH), free thyroxine (FT4), alanine aminotransferase (ALT), aspartate aminotransferase (AST), glycated haemoglobin (HbA1c), homeostasis model assessment of insulin resistance (HOMA-IR), Homeostasis model assessment for β-cell function (HOMA-B), Insulinogenic index (IGI), Area under the curve for glucose (AUC_g) and insulin (AUC_i).

Table 2 – Correlation analysis between asprosin and BMI, BMI SDS and sex.

	Asprosin	
	r	p
BMI	- 0.275	0.024
BMI SDS	- 0.239	0.044
Sex (male)	- 0.248 [pb]	0.043

Body mass index (BMI), standard deviation score (SDS), point-biserial (pb)

Figure 1 – Box-plot of asprosin serum levels in obese children (Group A) and controls (Group B).

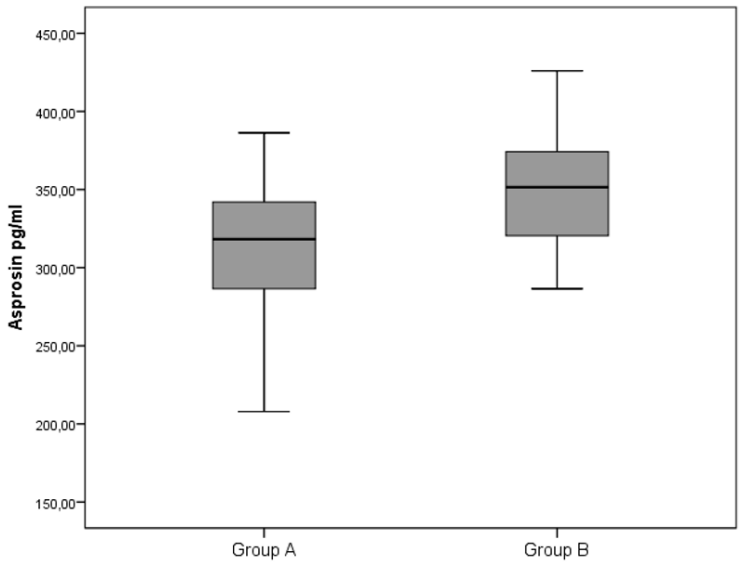


Figure 2 – Box-plot of asprosin serum levels in boys and girls in the entire cohort.

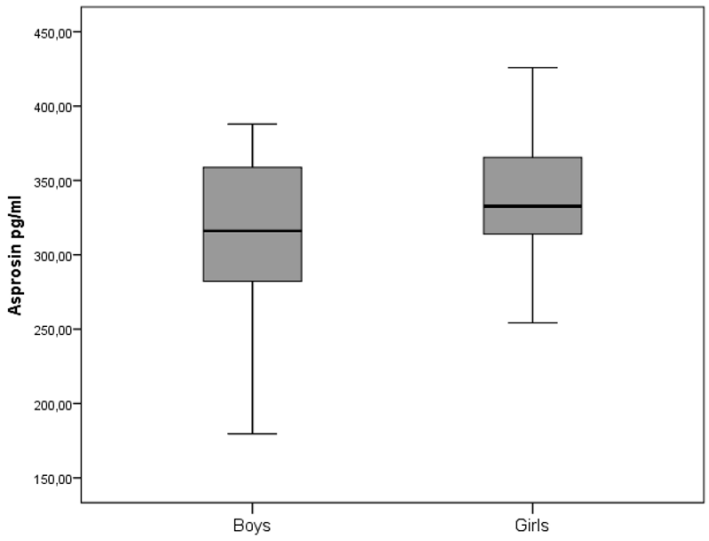


Figure 3 – Logistic regression models (a, b, c) and receiver operating characteristic (ROC) curve analysis (d).

a)

	B	Wald	P	OR	95%CI
BMI SDS	-0.519	6.879	0.009	0.595	0.404 – 0.877

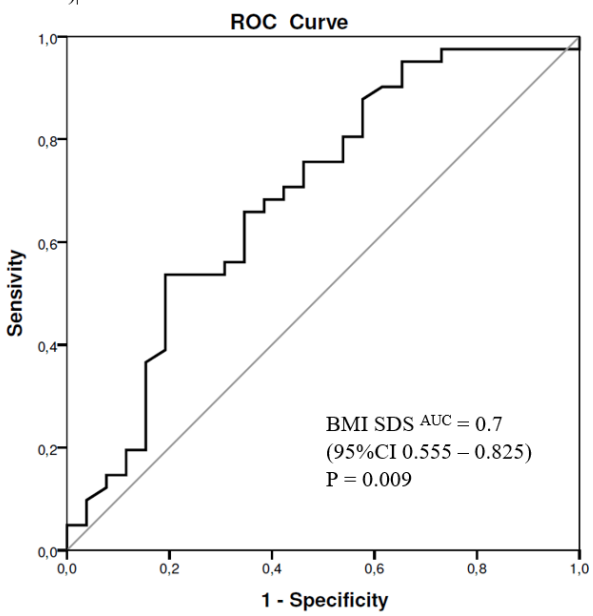
b)

	B	Wald	P	OR	95%CI
BMI SDS	-0.464	5.423	0.020	0.629	0.426 – 0.929
Age (years)	-0.207	3.429	0.064	0.813	0.653 – 1.012

c)

	B	Wald	P	OR	95%CI
BMI SDS	-0.419	4.013	0.042	0.658	0.437 – 0.991
Age (years)	-0.210	3.513	0.061	0.811	0.651 – 1.010
Sex (M/F)	0.434	0.548	0.459	1.544	0.489 – 4.874

d)



Logistic regression models were estimated to assess the possible dependence of dichotomized asprosin (<= 341 and > 341 ng/ml) on some potential explicative variables as body mass index standard deviation score (BMI SDS), age and sex (a, b, c). In particular, a crude model (a) with BMI SDS, a second model with BMI SDS and age (b), and a third model (c) including BMI SDS, age and sex, were estimated.
ROC curve analysis (d) was performed in order to identify the best cut-off of BMI SDS into discriminate subjects belonging to asprosin classes (<= 341 and > 341 ng/ml).
Regression coefficient (B), Wald test (Wald), p-value (P), Odds ratio (ORs), 95% confidence interval (95%CI), male (M), female (F), area under the curve (AUC).

2.4 Meal-related asprosin serum levels are affected by insulin resistance and impaired fasting glucose in children with obesity.

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Manuscript under review.

Introduction

Childhood obesity represents one of the most important health issues worldwide and it is associated with increased risk of metabolic complications, such as insulin resistance (IR) and impaired glucose regulation (IGR), including impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) (1, 2). IR has become a common feature of childhood obesity being directly related to adiposity (3). Adipose tissue is known to play an important role as endocrine organ secreting several adipokines involved in the pathogenesis of obesity-related IR (1). Asprosin is a recently identified adipokine, produced mainly by white adipose tissue (4), implicated in the pathophysiology of several conditions, such as obesity, IR, type 2 diabetes mellitus (T2DM), cardiovascular diseases, by preclinical and clinical studies (5, 6). Previous studies described asprosin involvement in glucose homeostasis consisting in appetite regulation through orexigenic AgRP+ neurons and promotion hepatic gluconeogenesis under fasting condition (4, 7). Accordingly, asprosin serum levels increases in fasting conditions and decreases with refeeding in physiological condition (4). Asprosin-related hepatic glucose release receptor occurs through OLFR734 receptor in mice (8), whereas the corresponding human receptor, OR4M1, has not yet been investigated as asprosin target (5). Several studies documented a positive correlation between asprosin levels and HOMA-IR (9-11) while others have not confirmed this correlation (6) or have found a negative correlation (12). Elevated serum asprosin concentration has been documented in subjects affected by T2DM compared to healthy control (11, 13, 14). In addition, an alteration of meal-related circadian oscillation of asprosin serum levels has been reported in T2DM patients compared to non-diabetic controls (15). Based on these findings, although partly contrasting, asprosin seems to play a role in glucose metabolism, but no data are available on meal-related asprosin levels changes in children and adolescents with obesity.

Aims of this single-center cross-sectional study were to evaluate variation between fasting and postprandial asprosin levels and to assess which metabolic variables condition this variation in non-diabetic children and adolescents with obesity.

Material and methods

This is a single-center, cross-sectional study carried out from October 2020 to May 2021. Inclusion criteria were: age ranged between 5 and 16 years; BMI ≥ +2 standard deviation score (SDS), in accordance with definition of obesity by World Health Organization (WHO) for children from the age of 5 years; Caucasian ethnicity; born as healthy full-term infant adequate for gestational age. Exclusion criteria were: genetic and/or endocrine causes of obesity; diabetes; chronic diseases; chronic pharmacological therapies; smoking. All procedures were performed in accordance with the Declaration of Helsinki and were approved by Ethics Committee of Messina (N.552-17/04/2019). Written informed consent was obtained from all parents or legal tutors.

Clinical and biochemical evaluation

At recruitment, physical evaluation was performed according to standardized procedures, including assessment of height, weight, BMI, BMI SDS, waist circumference (WC), WC-to-height ratio (WHtR), systolic and diastolic blood pressure (16). Pubertal stage is determined according to Tanner classification (16); patients were considered in a pubertal stage from Tanner stages B2 for females and G2 for males. Children underwent fasting biochemical assessment (lipid profile, thyroid, kidney, liver function tests, oral glucose tolerance test (OGTT)), as previously described (17). OGTT was performed according to a standard procedure (1.75 g/kg of body weight, up to a maximum of 75 g) according to the American Diabetes Association (ADA) guidelines (18), with sampling at 0, +30, +60, +90, +120 minutes for measurements of glucose and insulin. IFG was defined as fasting plasma glucose between 100 and 125 mg/dL and IGT as 2-h plasma glucose after 75-g OGTT levels between 140 and 199 mg/dL (18).

Blood samples for the serum asprosin assay were taken on a fasting state at the beginning of OGTT, after at least 8 hours of overnight fasting, and at the 120-minute OGTT timepoint (2h-postprandial asprosin). Asprosin serum levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit accordingly to the manufacturer's instructions (MyBioSource,USA; catalog number:MBS9716571). The detection threshold was 1 pg/mL and no significant cross-reactivity between human asprosin and analogues was reported; the intra-assay and inter-assay coefficient of variation (CV) values were <9% and <11%, respectively. Asprosin concentrations were expressed as pg/mL.

Homeostasis model assessment of insulin resistance (HOMA-IR), homeostasis model assessment of β-cell function (HOMA-B), Matsuda-index, insulinogenic-index (IGI) were calculated, as previously detailed (19). Areas Under the Curves for glucose (AUCg) and insulin (AUCi) and their ratio were also evaluated. IR was defined as HOMA-IR > 2.5 in prepubertal children and > 4 in pubertal subjects

(17). All patient underwent hepatic ultrasound assessment. Diagnosis of liver steatosis was made by conventional liver US according to the presence of at least two of the following abnormal findings: 1) diffusely increased echogenicity of the liver compared with kidney or spleen; 2) ultrasound beam attenuation; 3) poor visualization of intrahepatic structures (20, 21).

Statistical analysis

Numerical data were expressed as mean and SDS and categorical variables as absolute frequency and percentage. The non-parametric approach was used since most numerical variables were not normally distributed, as verified by the Kolmogorov-Smirnov test. The Wilcoxon test for dependent samples was applied in order to individuate possible significant differences between fasting and 2h-postprandial glycaemia, insulin and asprosin, both in the whole cohort and in subgroups defined according to sex, pubertal stage, BMI SDS (BMI SDS ≤ or > 2.5) and the presence of IR, IFG and hepatic steatosis. The Spearman correlation test was used to assess the existence of significant interdependence between asprosin levels (both fasting and 2h-postprandial levels) and clinical and biochemical parameters; in addition, partial correlation was also estimated in order to control for sex, pubertal stage and BMI SDS. A stepwise multiple logistic regression analysis was carried out in order to identify which metabolic predictors significantly affect the variation of asposin levels considering the dichotomous variable asprosin increase/non-increase between fasting and 2h-postprandial status, through the estimation of different models (Model 1: age, sex, BMI SDS, HOMA-IR, HOMA-B, IGI, Matsuda index, AUCi/AUCg-ratio. Model 2: age, sex, BMI SDS, fasting and 2h-postprandial glycaemia, fasting and 2h-postprandial insulin. Model 3: age, sex, BMI SDS, Triglycerides/HDL-ratio, Total Cholesterol/HDL-ratio. Model 4: age, sex, BMI SDS, Triglycerides, LDL, HDL, Total Cholesterol. Model 5: age, sex, BMI SDS, alanine aminotransferase (ALT), aspartate aminotransferase (AST), Gamma-Glutamyl Transferase (GGT), hepatic steatosis. In addition, further logistic regression models were estimated in order to assess the influence of IFG on the increase of asprosin levels, even after adjustment for HOMA-IR, BMI SDS, sex and pubertal stage (Model 1: crude model; Model 2: HOMA IR; Model 3: HOMA IR, BMI SDS; Model 4: HOMA IR, BMI SDS, sex; Model 5: HOMA IR, BMI SDS, sex, pubertal stage). Statistical analyses were performed using IBM SPSS for Windows, Version 22 (Armonk, NY, IBM Corp.). A p-value smaller than 0.05 was considered to be statistically significant.

Results

Seventy-nine children (aged 11.5 ± 2.6 years) were consecutively recruited, including 40 males and 39 females; 64.5% of them were in a pubertal stage. Clinical and biochemical characteristics of the

study population are shown in Table 1. Sixty-seven percent had IR, 33% had IFG and 8.8% IGT. No patient was diagnosed with T2DM. Thirty-nine percent of patients were diagnosed with hepatic steatosis. As expected, a significant increase in blood glucose and insulin levels was documented from fasting to 2h-timepoint of OGTT (Tab. 2). Conversely, fasting and 2h-postprandial asprosin serum levels did not significantly differ in the entire cohort (Tab. 2). The same trend of asprosin levels was confirmed considering sex and pubertal stage (Tab. 2). Fifty-five percent of patients had an increase in 2h-postprandial asprosin compared with fasting levels.

Categorizing patients according to the presence or not of IR, no significant change was documented between fasting and 2h-postprandial asprosin levels in either subgroup, as occurred by dividing the population in relation to BMI SDS or the presence of steatosis (Table 2). A trend for subjects with IFG to have higher 2h-postprandial asprosin levels was demonstrated, although this finding did not reach statistical significance (Tab. 2). Correlation analysis documented a significant positive relation of fasting and 2h-postprandial asprosin levels with lipid profile parameters (Tab. 3), also after adjustment for sex, pubertal stage and BMI SDS. A correlation between asprosin and BMI, BMI SDS, and glucose metabolism parameters was not documented.

Considering that more than half of patients had increased 2h-postprandial asprosin compared to fasting levels, to assess which metabolic variables significantly affected the condition of asprosin increase between fasting and 2-h postprandial levels a stepwise multiple logistic regression analysis was applied. The condition of asprosin increase was significantly influenced by fasting glycaemia, HOMA-IR and HOMA-B. Particularly, the increase of asprosin levels were significantly positively associated with HOMA-IR and fasting glycaemia and negatively associated with HOMA-B (Tab. 4). Conversely, no significant associations were found in the models that assessed the increase in asprosin with respect to parameters of lipid metabolism (model 3), transaminases and the presence of hepatic steatosis (model 4) (data not shown). Moreover, further logistic regression analysis documented a significant association between IFG and the increase in asprosin levels, even after adjustment for HOMA-IR, BMI SDS, sex, and pubertal stage (Tab. 5).

Discussion

Asprosin is a glucogenic adipokine that stimulate hepatic glucose release by cyclic-AMP (cAMP)-protein kinase A (PKA) pathway (4). Under physiological condition, asprosin levels decrease after meal intake in both murine models and in humans (4). For the first time, in our study we demonstrated, differently than expected, no significant variation between fasting and 2h-postprandial asprosin levels in obese, non-diabetic, children, suggesting an alteration in the meal-related regulation of asprosin secretion in these subjects. In addition, HOMA-IR, fasting glycaemia and IFG significantly

influenced the condition of asprosin increase in 2-h postprandial evaluation, suggesting that IR and IFG may affect the physiological meal-related variation in asprosin levels.

IR seems to play a central role in the relationship between obesity and the associated risk of IGR, T2DM, metabolic syndrome and cardiovascular disease (1, 17, 22). Obese children with a more significant alteration in insulin sensitivity are at greater risk of developing T2DM and cardiovascular diseases, compared with peers without IR, given the same BMI (1, 17, 22). Asprosin may be involved in the pathogenesis of IR and IGR. Romere et al first demonstrated elevated plasma asprosin levels in human and mice with IR, increased levels of glucose and 2h-insulin in fast mice undergoing continuous or pulsatile overexpression of asprosin, and a significant decrease in glucose and insulin levels secondary to reduced hepatic glucose release after immunologic o genetic asprosin action inhibition (4). Following several studies have shown higher serum asprosin levels in subjects with IR and /or T2DM and a correlation between these pathological conditions and asprosin serum levels (9-11, 13-15). Wang et al. documented higher plasma asprosin levels in IGR (including IFG and IGT subjects) and newly diagnosed T2DM patients compared to the subjects with normal glucose regulation (NGR), and they showed a significant positive correlation between asprosin and HOMA-IR and a negative correlation between asprosin and HOMA-B. Moreover, these authors reported higher asprosin levels, at fasting state and at all intravenous glucose tolerance test (IVGTT) samplings, in adults with IGR compared to NGR and newly diagnosed T2DM, suggesting a role of asprosin as biomarkers to predict prediabetes (11). Interestingly, an alteration of meal-dependent circadian oscillation of asprosin serum levels has been reported in T2DM adult patients compared to non-diabetic controls; in particular, Zhang et al documented a non-significant decrease of 2h-postprandial serum asprosin level during an OGTT (15). Our study found that asprosin levels did not significantly decrease at 2h-postprandial assessment in obese non-diabetic children and that this trend was significantly influenced by the presence of IR and IFG. We speculate that in obese children and adolescents with IR and IFG there may be an altered secretion of asprosin which in turn may promote IR worsening by stimulating hepatic glucose production and subsequent hyperinsulinemia. The condition of asprosin increase and, in particular, the alteration of its circadian secretion, might be an early biomarker of IGR in obese children with IR. Asprosin may also be implicated in the pathogenesis of the well-known association between IR and lipid profile alteration (23). Zhang et al demonstrated a strong correlation between asprosin and lipid metabolism (14). Similarly, in our study, fasting and 2h-postprandial asprosin levels significantly correlated with lipid profile parameters even after adjustment for sex, pubertal stage and BMI SDS.

Sample size represented a limitation of our study. Due to ethical reasons, it was not possible to undergo healthy children to an OGTT to perform the same assessments conducted on the cohort evaluated in the study.

Conclusions

This is the first study to demonstrate a non-significant variation between fasting and 2h-postprandial asprosin levels in obese, non-diabetic, children and to document an influence of IR, fasting glycaemia and IFG in the meal-related changes in asprosin serum levels. The alteration of asprosin circadian secretion might be an early biomarker of IGR in obese children with IR. Further studies on larger cohort are needed to confirm and verify these findings.

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Tables and figures

Table 1 – Clinical and biochemical features of study population.

	Mean	SDS
Age (years)	11.46	2.61
Weight SDS	2.25	0.58
Height SDS	0.48	1.18
BMI	29.49	4.33
BMI SDS	3.16	0.93
WC (cm)	89.50	10.29
WHtR	0.59	0.05
SBP (mmHg)	114.26	8.50
DBP (mmHg)	69.81	9.68
AST (U/L)	21.03	7.54
ALT (U/L)	23.82	16.28
GGT (U/L)	15.57	14.92
Total cholesterol (mg/dl)	171.92	28.08
LDL-cholesterol (mg/dl)	90.65	29.99
HDL-cholesterol (mg/dl)	53.15	16.95
Triglycerides (mg/dl)	88.24	38.97
Triglycerides /HDL-ratio	1.86	1.17
Total cholesterol/HDL-ratio	3.53	1.29
Uric acid (mg/dl)	5.04	1.19
CRP (mg/dl)	0.28	0.29
HbA1c (%)	5.28	0.33
Fasting glucose (mg/dl)	97.67	8.36
2h-postprandial glucose (mg/dl)	115.09	16.16
Fasting insulin (µUI/ml)	21.11	12.15
2h-postprandial insulin (µUI/ml)	119.98	90.82
HOMA-IR	5.17	3.15
HOMA-B	221.20	120.57
IGI	2.64	1.88
Matsuda-index	0.25	0.13
AUC_g	249.40	29.49
AUC_i	234.19	163.95
AUC_i / AUC_g ratio	0.93	0.62
FT4 (pmol/L)	15.48	2.32
TSH (uUI/ml)	2.68	2.94
Fasting Asprosin (pg/ml)	374.28	77.23
2h-postprandial Asprosin (pg/ml)	375.27	81.26

Body mass index (BMI), standard deviation score (SDS), waist circumference (WC), WC-to-height ratio (WHtR), systolic blood pressure (SBP), diastolic blood pressure (DBP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), Gamma-Glutamyl Transferase (GGT), glycated haemoglobin (HbA1c), 120-minutes OGTT glucose levels (2h-postprandial glucose), 120-minutes OGTT insulin levels (2h-postprandial insulin), model assessment of insulin resistance (HOMA-IR), homeostasis model assessment for β-cell function (HOMA-B), insulinogenic index (IGI), area under the

curve for glucose (AUC_g) and insulin (AUC_i), free triiodothyronine (FT3), free thyroxine (FT4), thyroid stimulating hormone (TSH), 120-minutes OGTT asprosin levels (2h-postprandial asprosin).

Table 2 – Variations in blood glucose, insulin and asprosin between fasting and 2-h postprandial levels

Subgroup (N° of patients)	Blood glucose			Insulin			Asprosin		
	T0	**T120**	**p**	**T0**	**T120**	**p**	**T0**	**T120**	**p**
Entire cohort (79)	97.67 ± 8.36	115.08 ± 16.158	0.000	21.11 ± 12.15	119.97 ± 90.82	0.000	374.28 ± 77.23	375.27 ± 81.26	0.837
Male (40)	97.35 ± 7.52	116.85 ± 16.02	0.000	19.26 ± 11.59	118.97 ± 98.32	0.000	375.03 ± 80.86	370.27 ± 70.11	0.840
Female (39)	98 ± 9.23	113.28 ± 16.3	0.000	23.00 ± 12.56	121.01 ± 83.72	0.000	373.52 ± 74.37	380.41± 91.96	0.548
Prepubertal (28)	96 ± 8.19	119.21± 16.25	0.000	15.2589 ±8.19972	119.51 ± 88.41	0.000	408.82 ± 111.14	395.04 ± 84.09	0.374
Pubertal (51)	98.59 ± 8.39	112.82 ± 15.81	0.000	24.32 ± 12.82	120.23 ± 92.99	0.000	355.33 ±40.0	364.42 ± 78.39	0.265
IR (53)	99.56 ± 7.63	117.39 ± 15.29	0.000	26.26 ± 11.47	138.77 ± 102.37	0.000	382.37 ± 89.01	383.07 ± 94.54	0.968
Non-IR (26)	93.81 ± 8.59	110.38 ± 17.13	0.000	10.58 ± 3.89	81.65 ± 40.75	0.000	357.79 ± 41.34	359.37 ± 40.23	0.568
Steatosis (31)	97.9 ± 7.97	115.9 ± 15.98	0.000	24.65 ± 13.43	131.06 ± 102.88	0.000	368.55 ± 80.95	366.09 ± 68.18	0.984
Non-steatosis (40)	97.5 ± 8.68	116.03 ± 16.88	0.000	17.95 ± 10.68	108.29 ± 75.84	0.000	377 ± 79.92	379.79 ± 90.34	0.819
BMI SDS > 2.5 (65)	97.05 ± 8.55	115.52 ± 16.83	0.000	21.16 ± 12.37	118.13 ± 89.86	0.000	378.93 ± 83.74	378.81 ± 86.12	0.959
BMI SDS ≤ 2.5 (14)	100.57 ± 6.93	113.07 ± 12.89	0.000	20.85 ± 11.48	128.52 ± 98.16	0.000	352.70 ± 25.56	358.81 ± 52.4	0.551
IFG (26)	107.15 ± 5.70	119.34 ± 15.3	0.000	27.38 ± 12.13	136.05 ± 84.23	0.000	361.12 ± 39.23	382.91 ± 101.32	0.078
Non-IFG (53)	93.02 ± 4.71	113 ± 16.29	0.000	18.03 ± 11.01	112.09 ± 93.64	0.000	380.74 ± 89.8	371.52 ± 70.19	0.383

T0: Fasting time point of OGTT; T120: two-hours time point of OGTT.
Body mass index (BMI), standard deviation score (SDS), insulin resistance (IR), impaired fasting glucose (IFG),

Table 3 – Correlations between asprosin and lipid profile parameters.

3a)

	Fasting asprosin			
	r	p	r*	p
LDL	0.353	0.002	0.123	0.294
TG	0.277	0.013	0.243	0.036
TG/HDL-ratio	0.352	0.002	0.244	0.035
TC/HDL-ratio	0.350	0.002	0.151	0.196
HDL	-0.207	0.069	-0.217	0.061
Total cholesterol	0.204	0.071	0.221	0.069

Spearman correlation after adjustment for sex, pubertal stage and BMI SDS (r).*
LDL-cholesterol (LDL), Triglycerides (TG), Triglycerides /HDL-ratio (TG/HDL-ratio), Total cholesterol/HDL-ratio (TC/HDL-ratio).

3b)

	2h-postprandial asprosin			
	r	p	r*	p
LDL	0.360	0.001	0.292	0.011
TG	0.222	0.049	0.250	0.031
TG/HDL-ratio	0.331	0.003	0.232	0.045
TC/HDL-ratio	0.388	0.000	0.185	0.112
HDL	-0.221	0.052	-0.149	0.202
Total cholesterol	0.217	0.054	0.122	0.297

Spearman correlation after adjustment for sex, pubertal stage and BMI SDS (r).*
LDL-cholesterol (LDL), Triglycerides (TG), Triglycerides /HDL-ratio (TG/HDL-ratio), Total cholesterol/HDL-ratio (TC/HDL-ratio).

Table 4 –Stepwise multiple logistic regression analysis of variables affecting serum asprosin levels.

Model 1: age, sex, BMI SDS, HOMA-IR, HOMA-B, IGI, Matsuda index, AUC_i/AUC_g-ratio

	B	**P**	**OR**	**95% CI**	
HOMA-IR	0.34	0.047	1.41	1.005	1.977
HOMA-B	-0.01	0.035	0.99	0.984	0.999

Dependent variable: asprosin increase/non-increase between 0 and 120 minutes OGTT time point evaluations.

Model 2: age, sex, BMI SDS, fasting and 2-hpostprandial glycaemia, fasting and 2-h postprandial insulin.

	B	**P**	**OR**	**95% CI**	
Fasting glycaemia	0.07	0.02	1.073	1.009	1.141

Dependent variable: asprosin increase/non-increase between 0 and 120 minutes OGTT time point evaluations.

Body mass index standard deviation score (BMI SDS), 120-minutes OGTT glucose levels (postprandial glucose), 120-minutes OGTT insulin levels homeostasis (postprandial insulin), model assessment of insulin resistance (HOMA-IR), homeostasis model assessment for β-cell function (HOMA-B), insulinogenic index (IGI), area under the curve for glucose (AUC_g) and insulin (AUC_i), Confidence interval (CI).

Table 5 – OR and 95% Confidence interval (CI) for impaired fasting glucose according to asprosin increase.

Models	**OR (95%CI)**	***P value***
1	3.040 (1.095 8.436)	0.033
2	3.325 (1.049 10.545)	0.041
3	3.287 (1.033 10.458)	0.044
4	3.286 (1.033 10.457)	0.044
5	3.211 (1.006 10.249)	0.048

Dependent variable: asprosin increase/non-increase between 0 and 120 minutes OGTT time point evaluations.

Model 1: crude model; Model 2: HOMA IR; Model 3: HOMA IR, BMI SDS; Model 4: HOMA IR, BMI SDS, sex; Model 5: HOMA IR, BMI SDS, sex, pubertal stage.

CHAPTER 3

Cardio-metabolic changes in childhood obesity

3.1 Precocious preclinical cardiovascular sonographic markers in metabolically healthy and unhealthy childhood obesity.

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Introduction

Obesity in childhood is related to a wide spectrum of cardiovascular and metabolic comorbidities. Obese children and adolescents are more likely to become obese adults, with an increased risk of premature morbidity and mortality due to cardiovascular diseases (CVD) (1–5). The presence of early signs of cardiovascular (CV) dysfunction have already been demonstrated in obese children and adolescents, even in absence of other obese-related comorbidities, as insulin resistance, dyslipidemia, and arterial hypertension (6). A categorization of obesity in two different phenotypes have been proposed. Accordingly, it is possible to distinguish the metabolically unhealthy obesity (MUO), characterized by "unfavorable" cardiometabolic profile, and the metabolically healthy obesity (MHO), with "favorable" lipid, glycemic, and blood pressure profiles (7). To date, health and clinical implications of this distinction remain controversial. Several prospective studies reported a lower risk of CVD in MHO subjects in comparison to MUO, without demonstrating an increased risk of CVD when compared with the general population (8, 9). On the contrary, other studies documented a long-term increased risk of CVD and early obesity-related complications also in MHO subjects (10, 11). Moreover, the absence of univocal diagnostic criteria to define MUO makes it difficult to compare both adulthood and childhood studies (7). Interestingly, a shift from MHO to MUO seems to occur more frequently during transition from adolescence to adulthood (12); however, less is known about the clinical implications of MUO phenotype in childhood. Structural and functional cardiovascular modifications, such as left ventricle hypertrophy, systolic/diastolic dysfunction, increased carotid intima–media thickness (CIMT), have been considered preclinical indices of CVD in obese adults (13), as well as in pediatric obese subjects (14, 15). In this context, two parameters in obese patients' echocardiographic assessment became increasingly important: the left ventricular (LV) global longitudinal strain (GLS) and the epicardial adipose tissue (EAT). GLS is considered a reliable and

reproducible parameter for the assessment of myocardial contractility (16). Strain imaging is able to early detect subclinical myocardial abnormalities in subjects affected by cardiovascular and metabolic diseases, showing a better diagnostic efficacy and prognostic value for predicting cardiovascular events compared to LV ejection fraction (EF) (17, 18). EAT is a metabolically active adipose tissue localized around the heart, between the myocardium and the visceral layers of the pericardium (19), that is strictly related to visceral and subcutaneous fat (19) and to the pathogenesis of the CVD associated with obesity (20). Relationships between precocious cardiovascular changes and metabolic alterations are not widely investigated in children. In light of these pieces of evidence, the aims of this study are (1) to identify precocious, preclinical, cardiovascular, structural, and functional sonographic modifications, in a cohort of overweight (OW) and obese (OB) children and adolescents; (2) to investigate the association between clinical and metabolic variables and cardiovascular sonographic parameters; (3) to evaluate their relation with two different phenotypes of obesity: MHO and MUO.

Materials and methods

Study Design and Population

This is a single-center, cross-sectional, case-control study carried out at the Pediatric Endocrinology Outpatient Clinic at the University of Messina, Italy, during a period of 6 months (from September 2017 to March 2018). Fifty-nine Caucasian OW and OB children and adolescents were brought to the Outpatient Clinic for first evaluation, and 20 age- and sex-matched, lean (BMI SD $\leq$ 1) controls, were consecutively recruited. The inclusion criteria were BMI > 1 SD according to the WHO definition (21), age range between 5 and 16 years, Caucasian ethnicity, and born as healthy full-term infant adequate for gestational age. The exclusion criteria were genetic and/or endocrine causes of obesity, diabetes, CVD, either pre-term or post-term birth, chronic diseases, chronic pharmacological therapies, and smoking.

Clinical Evaluation and Laboratory Assessment

Detailed history from the parents and from clinical records was obtained. At recruitment, physical evaluation was performed according to standardized procedures, as previously described (22). Body weight was measured to the nearest 0.1 kg on accurate and properly calibrated standard beam scales, in minimal underclothes and no shoes. Height was measured to the nearest 0.1 cm on standardized, wall-mounted height boards, according to standardized procedures. The children stood with the head aligned in the Frankfort plane, barefoot, with feet placed together and flat on the ground, heels, buttocks, and scapulae against the vertical backboard, arms loose and relaxed with the palms facing

medially. BMI was calculated using the equation: body weight (kg)/height (m)2. BMI values were standardized using age- and sex-specific standard deviation (SD) based on WHO growth references (21). Waist circumference (WC) was measured, to the nearest 0.5 cm while the subjects were standing, after gently exhaling, as the minimal circumference measurable on the horizontal plane between the lowest portion of the rib cage and the iliac crest (23). Waist-to-height ratio (WHtR), an index of body fat distribution, was calculated as previously described (24). Patients underwent a detailed physical examination and pubertal evaluation, assessed by five Tanner stages of breast development in girls and testicular volume in boys (25), performed by pediatric endocrinologists. Pubertal stage was defined from G2 or B2 to G5 or B5 Tanner's stages (25). Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were recorded at rest three times on the right arm in mmHg using a manual sphygmomanometer; for analysis, the average of three blood pressure values was used (26). A fasting blood sampling for plasma triglycerides, high-density lipoproteins (HDL), low-density lipoproteins (LDL), total cholesterol, glucose and insulin was performed at least 8 h after the last meal. These parameters were analyzed with standard techniques: triglycerides were measured enzymatically, the HDL-cholesterol fraction was obtained after precipitation using a phosphotungstic reagent, and glucose was measured using a glucose oxidase method; serum insulin was determined by a chemiluminescence immunoassay. Thyroid, liver, and kidney function tests were also performed. Oral glucose tolerance test (OGTT) was performed with the standard method (1.75 g/kg of body weight, up to a maximum of 75 g) in OW and OB patients only, measuring glucose and insulin serum levels at baseline and during OGTT (at 0, 30, 60, 90, and 120 min), and impaired glucose tolerance or type 2 diabetes were diagnosed according to the criteria of the American Diabetes Association (27). Insulin resistance was measured through homeostasis model assessment of insulin resistance (HOMA-IR). This index was calculated using the equation: Fasting insulin (μU/ml) × Fasting glucose (mg/dl)/405 (28). Insulin resistance was defined as a HOMA-IR > 2.5 in prepubertal children and >4 in pubertal subjects (29). Among OW and OB patients, MUO was defined as the presence of two or more of the following cardiometabolic risk factors (12): triglycerides ≥ 110 mg/dl or on cholesterol medication; HDL <40 mg/dl or on cholesterol medication; fasting glucose ≥ 100 mg/dl or on glucose/insulin medication; blood pressure ≥ 90th percentile for age, gender, and height or on blood pressure medication.

Echocardiographic Conventional Parameters

All participants underwent 2D transthoracic echocardiography (TTE) with Vivid E95 echocardiography equipment (GE Vingmed Ultrasound AS, Horten, Norway). Image acquisition was performed at a frame rate of 70–90 frames per second, and three cardiac cycles were stored in cine

loop format for subsequent off-line calculation using a specific software workstation (EchoPAC version 7.0.0; GE Vingmed Ultrasound AS). All the echocardiographic measurements were obtained according to the current guidelines (30, 31). Linear internal measurements of the LV and its walls were performed in the parasternal long-axis view. Specifically, interventricular septum (IVSD), diastolic left ventricle posterior wall (PWD), end-diastolic (LVEDD) and end-systolic (LVESD) LV diameters, were evaluated. Using these measurements, LV mass was automatically calculated; therefore, LV mass was divided to height (meters) to the power of 2.7 to obtain the LV mass index (LVM-index) (32). LV volumes and EF were calculated by the Simpson method in the apical three-, four-, and two-chamber views. Similarly, the left atrial (LA) volume was calculated in the apical four- and two-chamber views by the method of disks. EAT was measured as the echo-free space between the outer wall of the myocardium and the visceral layer of the pericardium perpendicularly to the free wall of the right ventricle at end systole in the parasternal long-axis view. Ascending aorta (AA) diameters were calculated from the parasternal long-axis view at the maximal diameter of the sinuses of Valsalva, in systole (AoS) and in diastole (AoD). AA stiffness was calculated by the following formula: (ln (SBP/DBP)/[(AoS – AoD)/AoD]) (33). Mitral peak early (E) and late (A) velocities, and E/A ratio, were obtained by pulsed-wave Doppler performed in the apical four-chamber view, placing the sample volume at the tip of mitral leaflets; septal and lateral early diastolic mitral annular velocities (E′) were evaluated by pulsed-wave tissue Doppler imaging (TDI), and the ratio between mitral peak early velocity and the averaged value of septal and lateral early diastolic mitral annular velocities (E/E′) was calculated; the maximum tricuspid regurgitation (TR) velocity was measured in the apical four-chamber view through continuous wave Doppler.

2D Speckle Tracking Echocardiography Analysis and Evaluation of Carotid Artery Stiffness

For the evaluation of LV 2D speckle tracking, GLS images were obtained from the apical four-, three- and two-chamber views. Using a customized commercial speckle-tracking software (EchoPAC version 7.0.0; GE Vingmed Ultrasound AS), LV GLS was calculated placing fiducial landmarks to define the base and apex of LV; the software automatically generated the region of interest and, after the processing, the bull's-eye maps that allowed the calculation of the averaged value of GLS (30). Using the same software, the LA endocardium surface was manually traced in the four- and two-chamber views by a point-and-click approach, excluding the appendage and pulmonary veins. An epicardial surface tracing was then automatically generated by the system, and the region of interest, divided into six segments, was identified. The software generates the longitudinal strain curves for each of these segments, together with a mean curve of all segments, whose maximal positive peak was used to calculate the LA reservoir strain value. The LA strain was determined as the average

value from all segments of the LA in the apical four-chamber and two-chamber views (30). The carotid ultrasound examinations were performed using a color Doppler echocardiography machine (Prosound Alpha 10, Aloka, Tokyo, Japan) equipped with a 7.5-MHz linear array probe high-resolution echo-tracking system that allows accurate measurements of carotid diameter changes. Pressure waveforms, calibrated on systolic and diastolic blood pressure values measured with a cuff-type manometer applied to the right upper arm, were non-invasively obtained using arterial diameter change (systolic–diastolic diameter). Validated parameters of arterial stiffness [β-index, pulse wave velocity (PWV) and augmentation index] were automatically calculated as a mean of five beats, as already reported (31, 34). Moreover, CIMT, defined as the distance between the lumen/intima and the media/adventitia interfaces, was evaluated. All measurements were taken manually at the far wall of the vessel from perfectly horizontal images of distal carotid common artery (about 1.5 cm proximal to the carotid bifurcation), in longitudinal planes, with a transducer depth of 4 cm and from a posterolateral approach.

Statistical Analysis

The numerical data were expressed as the mean and standard deviations (SD) while the categorical variables as number and percentage. The non-parametric approach was used due to sample size dimension and since not all numerical variables (prevalently metabolic variables) were normally distributed, as verified by the Kolmogorov–Smirnov test. The Mann–Whitney test was applied in order to compare cases and healthy controls with reference to anthropometric and cardiovascular parameters; the Chi Square test was applied in order to compare these groups with reference to categorical variables. Moreover, the Mann–Whitney test was performed, within cases, to evaluate possible differences between patients with or without insulin resistance and between MUO or MHO subjects. The Spearman correlation test was applied to assess the existence of any significant interdependence between numerical parameters. Two stepwise multivariable linear regression models were estimated in order to individuate the most significant predictors of each cardiovascular parameter (IVSD, PWD, LVEDD, LVSED, LVM index, LA volume, LA strain, E/A ratio, E′, E/E′ ratio, GLS, CIMT, PWV, β-index, Augmentation index, AA diameter, AA stiffness, EAT) according to the following models: Model 1 (age, gender, pubertal stage, BMI SD, WC, HOMA-IR, LDL, HDL, triglycerides, SBP, duration of obesity) and Model 2 (age, gender, pubertal stage, BMI SD, WHtR, HOMA-IR, LDL, HDL, triglycerides, SBP, duration of obesity). The normal distribution shown by the ultrasound parameters provides the methodological guarantee for the use of linear regression models. A $p < 0.050$ was considered to be statistically significant. Statistical analyses were performed using SPSS for Window package, version 22.

Results

Clinical Characteristics and Biochemical Evaluation

Seventy-nine children and adolescents were consecutively recruited: 59 OW and OB were included in group A, while 20 lean controls were included in group B. Groups A and B were comparable for age (9.8 ± 2.9 vs. 8.6 ± 2.9; p = 0.07), gender (33 males/26 females vs. 10/10; p = 0.65), and pubertal stage (30 pre-pubertal/29 pubertal vs. 12/8; p = 0.48). All subjects of group A presented with abdominal obesity (WHtR ≥ 0.5; 0.61 ± 0.05). Thyroid, liver, and kidney function tests were normal in the entire population (data not shown). Insulin resistance was observed in 47.5% of group A patients. OGTT documented a condition of impaired glucose tolerance in four patients of group A (6.8%) who had normal fasting glucose; diabetes was excluded in the entire cohort. OW and OB children showed significantly higher SBP compared to controls, although in seven patients of group A (11.8%), an above maximum of range SBP value was documented, according to the Flynn et al. criteria (26).

Cardiovascular Assessment

Comparison analysis documented a significantly higher IVSD (7.8 ± 1.1 vs. 7 ± 1.2 mm; p = 0.006), PWD (7.8 ± 1.3 vs. 5.9 ± 0.7 mm; p = 0.000), LVEDD (43.5 ± 4.6 vs. 36.9 ± 5.4 mm; p = 0.000), LVESD (27.3 ± 3.2 vs. 22.6 ± 3.8 mm; p = 0.000), LVM-index (37 ± 7.2 vs. 32.1 ± 9; p = 0.006), CIMT (4.9 ± 0.8 vs. 3.3 ± 0.3 mm; p = 0.000), β-index (3.2 ± 0.8 vs. 2.7 ± 0.4; p = 0.007), PWV (3.7 ± 0.5 vs. 3.3 ± 0.3 m/s; p = 0.004) in group A compared to group B (Table 1). Moreover, the E/A ratio (1.9 ± 0.4 vs. 2.2 ± 0.5; p = 0.003) and EF (67.3 ± 3.7 vs. 69.7 ± 4.0%; p = 0.041) were significantly lower, and GLS was significantly impaired (−18.7 ± 2.2 vs. −23.9 ± 2.4%; p = 0.000) in OW and OB subjects compared to controls. Comparison between group A patients with and without insulin resistance are reported in Table 2. Results of correlation analysis are reported in Table 3. In particular, BMI SD and HOMA-IR were positively significantly related to LV dimensions, LA volume, and EAT, and negatively to E/A ratio. WC was positively correlated to SBP, DBP, LV dimensions, LA volume, E/E′ ratio, CIMT, PWV, AA diameter, EAT, and negatively with LA strain. Moreover, EAT was positively significantly related to LV dimensions, LA volume, SBP, CIMT, AA diameter and negatively with LA strain. To investigate the independent effect of anthropometric and biochemical variables on cardiovascular parameters, two multivariate stepwise regression analyses were performed according to model 1 (Table 4) and model 2 (Table 5). WCs were very strong predictors of LV dimensions, LA volume and strain, AA stiffness and diameter (model 1). BMI SD

was significantly associated with EAT, LVM-index, and E/A ratio (models 1 and 2). HOMA-IR and triglycerides were significant predictors of GLS (models 1 and 2).

MUO vs. MHO: Clinical, Biochemical, Cardiovascular Evaluation

Thirteen OW and OB patients (22%) were classified as MUO, according to the Camhi et al. criteria (12). MUO patients showed significantly higher BMI SD, WC, WHtR, HOMA-IR, triglycerides, SBP, as well as LV dimensions, EAT, CIMT, AA diameter, carotid, and AA stiffness compared to MHO patients (Table 6). Moreover, GLS was significantly impaired in MUO (Table 6).

Discussion

The present study demonstrated a negative effect of childhood obesity on subclinical structural and functional cardiovascular modifications. Particularly, severity of overweight, abdominal obesity, and insulin resistance were the main predictors of cardiovascular remodeling, subclinical myocardial dysfunction, and amount of EAT. MUO patients seem to have a significant unfavorable cardiometabolic profile.

Systolic Myocardial Function and Myocardial Geometry

Evaluation of GLS has assumed increasing importance in LV systolic function assessment as a reliable and reproducible index of myocardial contractility. GLS is able to early identify subclinical myocardial abnormalities differently from EF that is characterized by intrinsic limitations, as late reduction only in an advanced stage of CVD, poor reliability in patients with LV hypertrophy and volume reduction, inter-observer and intra-observer variability due to apical foreshortening (16). Accordingly, in our study EF did not differ between groups. In our cohort, OW and OB children showed a significantly impaired GLS compared to controls, that is, expression of a decreased LV myocardial deformation. This result indicates an incipient, preclinical, systolic alteration, consistent with the results obtained in other obese pediatric cohorts (35, 36). Furthermore, GLS was significantly impaired among MUO patients compared to MHO, suggesting a negative effect of unfavorable metabolic profile on the early alteration of longitudinal myocardial deformation property. Moreover, GLS was significantly associated with HOMA-IR, and it was significantly impaired in OW and OB patients with insulin resistance. These findings let us speculate that insulin resistance is a strong independent predictor of subclinical LV dysfunction in obese, non-diabetic, children. Furthermore, insulin resistance has been demonstrated to have negative effects on myocardial function: a decreased myocardial glucose uptake, caused by a reduced availability of GLUT-4 sarcolemmal transporters, which results in a switch from aerobic glycolysis to a greater utilization of free fatty acids and in an increased oxidative stress and proinflammatory status (37); an activation of cardiomyocyte

authopaghy, which causes loss of contractile cells (38); an increased deposition of the extracellular matrix and collagen associated with reduction of the degradation mechanisms (39). GLS evaluation, in our cohort, assumes further importance and reliability in the assessment of the specific effect of obesity on myocardial contractility independent from diabetes and chronic arterial hypertension that could affect GLS (40, 41). As previously reported (15, 42, 43), we also demonstrated a significant increase in both LV dimensions and LVM-index in OW and OB children compared to controls. These parameters were significantly affected by the severity of overweight, abdominal obesity, and insulin resistance. Precocious modifications of cardiac geometry could be determined, at least in part, by preload/afterload increase related to obesity (42).

Diastolic Myocardial Function

Diastolic dysfunction has been associated with obesity both in adults as well as in children (44, 45). Accordingly, in our cohort, the E/A ratio was significantly lower in the OW and OB groups, and it was negatively significantly related to BMI SD, suggesting an incipient impaired myocardial relaxation of LV in these children. Moreover, the increased LVM-index and its significant association with BMI SD are expressions of the initial sign of obesity-related LV hypertrophy and early impaired myocardial relaxation (43). We also documented a significantly negative correlation between WC, LA strain, and E′, markers of diastolic dysfunction (46, 47). On the other hand, the E/E′ ratio as well as the LA volume, usually altered in the case of LV-elevated filling pressure in patients with chronic diastolic dysfunction, were included in the normal range, and they were not significantly different between groups. These findings are compatible with an early stage of LV dysfunction. Therefore, our results are consistent with the early signs of subclinical diastolic dysfunction significantly influenced by WC, BMI SD, and HOMA-IR.

EAT Evaluation

EAT is a metabolically active adipose tissue strictly related to visceral and subcutaneous fat (19). The echocardiographic assessment of EAT is a sensitive and reliable marker of visceral adiposity (48). EAT and visceral fat exhibit similar pro-inflammatory cytokine mRNAs likely involved in chronic inflammation and potentially contributing to CVD pathogenesis (49). Therefore, EAT pathological increase seems to be involved in obesity-related CVD pathogenesis, probably promoted by EAT direct interaction with coronary vessels and myocardium, and mediated by paracrine and vasocrine secretion of pro-inflammatory cytokines and free fatty acids (20). The amount of EAT in our patients was significantly higher among MUO patients, and it was strictly related to LV dimensions and mass, BMI SD, WC, and HOMA-IR; particularly, BMI SD was the stronger predictor of EAT amount. Data from the present series are consistent with the results of other pediatric studies, suggesting the

possibility of a routine ultrasound assessment of EAT to estimate the CVD risk in obese children (50–52). An interesting result of our study is the significant correlation between EAT and HOMA-IR, consistent with the reported data in adults (53, 54) and in contrast with the results of those few pediatric studies available (50, 55). In pediatric cohorts, despite not finding an association between EAT and HOMA-IR, EAT was able to identify patients with insulin resistance (19, 51). Linkage between EAT and insulin resistance may be explained by the findings of Fernadez-Trasancos et al. (56). These authors demonstrated, in EAT mesenchymal cells of patients with CVD, an association between a low adipogenic ability and insulin resistance, directly dependent from obesity, diabetes, and coronary artery disease. Insulin treatment rapidly improved adipogenic ability. Authors concluded that this association may contribute, at least partially, to the relation between EAT and CVD (56). Based on our findings, we speculate that the accumulation of EAT may be promoted by the severity of overweight, abdominal obesity, and insulin resistance even in children.

Arterial Evaluation

This study confirms a negative influence of childhood obesity on arterial structure and function. CIMT is considered a precocious non-invasive marker of atherosclerosis, strictly related to the severity of obesity and body fat distribution (57). In a large cross-sectional study, Hedblad et al. reported an increased CIMT in non-diabetic patients with insulin resistance (58). Arterial stiffness, able to reveal functional alteration, is a more sensitive parameter than intima–media thickness for the assessment of early vascular damage (59). An increased arterial stiffness, related to an increased CVD risk in adults (60), was documented also in obese pediatric cohorts, both in carotids (61, 62) as well as in aorta evaluation (63, 64), although, these findings were not univocally confirmed (65). Incipient signs of vascular remodeling, increased carotids and AA stiffness have been documented in our obese population, especially among MUO patients. Moreover, BMI SD and WC showed a significant association with structural and functional indices of carotid and aorta, although an influence of SBP needs to be highlighted. Importantly, further studies are required to clarify the role of obesity, body fat distribution, dyslipidemia, insulin resistance, and other CV risk factors in atherosclerotic damage in children. It might be argued that our study has some limitations. First, due to the cross-sectional design of the study, we are unable to verify the causal relationships between cardiometabolic risk variables and structural and functional myocardial modifications, which could be clarified in a longitudinal study involving a further enlarged cohort. Second, a 24-h blood pressure monitoring has not been performed in our patients.

In conclusion, severity of overweight, abdominal obesity, insulin resistance, and MUO phenotype negatively affect cardiovascular remodeling and subclinical myocardial dysfunction in OW and OB

children. GLS and EAT are non-invasive and reliable indices that might be considered in echocardiographic evaluation to stratify cardiovascular risk in obese children and adolescents. MUO phenotype, characterized by higher prevalence of metabolic alterations and early cardiovascular modifications, is likely to increase the risk of developing cardiometabolic complications since the pediatric age. Therefore, a distinction between MHO and MUO phenotypes might be useful in planning a personalized follow-up approach in obese children, although it is needed before to establish univocal diagnostic criteria.

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Tables and figures

Tab. 1 – Significant differences between OW and OB children (Group A) and controls (Group B).

	Group A (n = 59)	**Group B (n = 20)**	***p***
BMI SD	2.2 ± 0.5	- 0.3 ± 0.8	**0.000**
SBP (mmHg)	112.5 ± 13.4	103 ± 7.3	**0.006**
IVSD (mm)	7.8 ± 1.1	7 ± 1.2	**0.006**
PWD (mm)	7.8 ± 1.3	5.9 ± 0.7	**0.000**
LVEDD (mm)	43.5 ± 4.6	36.9 ± 5.4	**0.000**
LVESD (mm)	27.3 ± 3.2	22.6 ± 3.8	**0.000**
LVM-index	37 ± 7.2	32,1 ± 9	**0.006**
EF (%)	67.3 ± 3.7	69.7 ± 4	**0.041**
E/A ratio	1.9 ± 0.4	2.2 ± 0.5	**0.003**
GLS (%)	-18.7 ± 2.2	-23.9 ± 2.4	**0.000**
CIMT (mm)	4.9 ± 0.8	3.3 ± 0.3	**0.000**
β-index	3.2 ± 0.8	2.7 ± 0.4	**0.007**
PWV (m/s)	3.7 ± 0.5	3.3 ± 0.3	**0.004**

Numerical data are express as mean ± SD.
Body mass index standard deviation (BMI SD), systolic blood pressure (SBP), interventricular septum (IVSD), Diastolic left ventricle posterior wall (PWD), end-diastolic (LVEDD) and end-systolic (LVESD) left ventricular diameters, left ventricle mass index (LVM-index, early (E) and late (A) mitral peak velocities ratio (E/A ratio), global longitudinal strain (GLS), carotid intima-media thickness (CIMT), pulse wave velocity (PWV).

Tab. 2 – Comparison analysis between patients of group A with and without insulin resistance.

	Non-IR patients (n=31)	IR patients (n=28)	*p*
WC (cm)	87.6 ± 12.8	95.4 ± 13.5	**0.03**
WHtR	0.60 ± 0.05	0.64 ± 0.05	**0.03**
BMI SD	2.1 ± 0.4	2.4 ± 0.5	**0.02**
SBP (mmHg)	111.4 ± 14.1	113.8 ± 12.7	0.39
DBP (mmHg)	68.2 ± 11.3	67.3 ± 9.4	0.42
IVSD (mm)	7.7 ± 1.1	7.9 ± 1.2	0.48
PWD (mm)	7.7 ± 1.3	7.8 ± 1.2	0.69
LVEDD (mm)	42.7 ± 4.6	44.3 ± 4.5	0.34
LVESD (mm)	26.8 ± 3.2	27.9 ± 3	0.23
LVM-index	35.8 ± 5.8	38.5 ± 8.4	0.28
LA volume (ml)	40.8 ± 11.4	42.6 ± 11.7	0.61
LA strain (%)	40.5 ± 9.9	39.3 ± 10.8	0.54
EF (%)	67.5 ± 4.1	67.2 ± 3.2	0.74
E/A ratio	1.9 ± 0.5	1.8 ± 0.4	0.66
E' (cm/s)	15.8 ± 2.1	14.9 ± 2.6	0.25
E/E' ratio	6.3 ± 0.9	6.5 ± 1.1	0.28
GLS (%)	- 19.5 ± 1.4	- 17.8 ± 2.4	**0.03**
EAT (mm)	12.6 ± 2.1	13.3 ± 2.6	0.43
CIMT (mm)	4.8 ± 0.8	4.9 ± 0.7	0.59
β-index	3.1 ± 0.9	3.3 ± 0.7	0.38
PWV (m/s)	3.6 ± 0.6	3.8 ± 0.4	0.25
Augmentation index	4.4 ± 13.1	5.1 ± 15.4	0.93
AA diameter (mm)	23.4 ± 3.2	22.4 ± 2.8	0.33
AA stiffness	2.4 ± 0.4	2.7 ± 0.4	**0.003**

Numerical data are express as mean ± SD.
Insulin resistance was defined as a HOMA-IR > 2.5 in pre-puberal and > 4 in puberal subjects.
Insulin resistant (IR), homeostasis model assessment of insulin resistance (HOMA-IR), waist circumference (WC), waist-to-height ratio (WHtR), body mass index standard deviation (BMI SD), systolic blood pressure (SBP), diastolic blood pressure (DBP), interventricular septum (IVSD), Diastolic left ventricle posterior wall (PWD), end-diastolic (LVEDD) and end-systolic (LVESD) left ventricular diameters, left ventricle mass index (LVM-index), left atrial (LA), early (E) and late (A) mitral peak velocities ratio (E/A ratio), early diastolic mitral annular velocities (E'), septal and lateral early diastolic mitral annular velocities (E/E'), global longitudinal strain (GLS), carotid intima-media thickness (CIMT), pulse wave velocity (PWV), ascending aorta (AA), epicardial adipose tissue (EAT).

Tab. 3 – Bivariate correlation analysis.

		BMI SD	HOMA-IR	SBP	WC	WHtR	EAT
SBP	*r*	0.340	0.340	--	0.569	0.428	0.489
	p	**0.008**	**0.008**		**0.000**	**0.004**	**0.002**
DBP	*r*	0.099	0.225	--	0.375	0.282	-0.074
	p	0.456	0.087		**0.012**	0.064	0.665
IVSD	*r*	0.260	0.343	0.353	0.573	0.450	0.558
	p	**0.044**	**0.008**	**0.006**	**0.000**	**0.002**	**0.000**
PWD	*r*	0.323	0.222	0.282	0.517	0.423	0.706
	p	**0.012**	0.091	**0.030**	**0.000**	**0.004**	**0.000**
LVEDD	*r*	0.143	0.268	0.359	0.387	0.099	0.577
	p	0.280	**0.040**	**0.005**	**0.009**	0.524	**0.000**
LVESD	*r*	0.190	0.322	0.361	0.418	0.121	0.500
	p	0.150	**0.013**	**0.005**	**0.005**	0.432	**0.002**
EF	*r*	-0.022	-0.058	-0.021	-0.101	-0.044	0.020
	p	0.866	0.663	0.873	0.515	0.775	0.908
LVM-index	*r*	0.158	0.008	-0.07	-0.157	0.075	0,323
	p	0.244	0.954	0.61	0.326	0.640	0,055
LA volume	*r*	0.303	0.277	0.363	0.593	0.237	0.525
	p	**0.026**	**0.043**	**0.007**	**0.000**	0.136	**0.002**
LA strain	*r*	-0.101	-0.434	-0.352	-0.619	-0.235	-0.387
	p	0.571	**0.010**	**0.041**	**0.002**	0.291	**0.029**
E/A ratio	*r*	-0.407	-0.129	-0.001	-0.173	-0.361	-0.236
	p	**0.001**	0.331	0.994	0.262	**0.016**	0.159
E'	*r*	-0.140	-0.284	-0.257	-0.460	-0.297	-0.327
	p	0.342	***0.051***	0.078	**0.006**	0.088	0.067
E/E' ratio	*r*	-0.019	0.242	0.313	0.487	0.387	0.227
	p	0.900	0.098	**0.030**	**0.003**	**0.024**	0.211
GLS	*r*	-0.161	-0.314	-0.389	0.176	0.195	0.228
	p	0.355	0.066	**0.021**	0.422	0.372	0.210
CIMT	*r*	0.157	0.027	-0.006	0.242	0.330	0.441
	p	0.249	0.842	0.966	0.122	**0.033**	**0.008**
β-index	*r*	0.140	0.133	0.327	0.221	0.178	0.323
	p	0.298	0.323	**0.013**	0.155	0.253	0.055
PWV	*r*	0.252	0.283	0.644	0.472	0.383	0.294
	p	0.059	**0.033**	**0.0001**	**0.001**	**0.011**	0.081
Augmentation index	*r*	-0.137	-0.13	0.022	0.025	0.009	0.159
	p	0.315	0.923	0.874	0.873	0.956	0.362
AA diameter	*r*	0.087	0.238	0.267	0.554	0.275	0.465
	p	0.631	0.182	0.133	**0.011**	0.241	**0.006**
AA stiffness	*r*	-0.057	0.446	0.075	0.296	0.178	0.213
	p	0.753	**0.009**	0.680	0.205	0.453	0.233
EAT	*r*	0.431	0.349	0.489	0.519	0.396	--
	p	**0.008**	**0.034**	**0.002**	**0.011**	0.062	

Homeostasis model assessment of insulin resistance (HOMA-IR), waist circumference (WC), waist-to-height ratio (WHtR), body mass index standard deviation (BMI SD), systolic blood pressure (SBP), diastolic blood pressure (DBP), interventricular septum (IVSD), diastolic left ventricle posterior wall (PWD), end-diastolic (LVEDD) and end-systolic (LVESD) left ventricular diameters, left ventricle mass index (LVM-index), left atrial (LA), early (E) and late (A) mitral peak velocities ratio (E/A ratio), early diastolic mitral annular velocities (E'), septal and lateral early diastolic mitral annular velocities (E/E'), global longitudinal strain (GLS), carotid intima-media thickness (CIMT), pulse wave velocity (PWV), ascending aorta (AA), epicardial adipose tissue (EAT).

Tab. 4 - Stepwise multivariate linear regression analysis for WC, BMI SD, HOMA-IR, LDL, HDL, triglycerides, SBP, obesity duration, age, sex and pubertal stage (model 1).

IVSD			
Predictors	**B**	**SE**	***p*-Value**
WC	0.036	0.012	0.005
PWD			
WC	0.034	0.015	0.03
LVEDD			
WC	0.126	0.041	0.004
Triglycerides	0.042	0.016	0.013
LVESD			
WC	0.110	0.030	0.001
LVM-index			
BMI SD	7.016	2,968	0.023
LA volume			
WC	0.503	0.108	0.000
LA strain			
WC	-0.522	0.143	0.002
E/A ratio			
BMI SD	-0.391	0.139	0.008
E'			
WC	-0.095	0.025	0.001
E/E' ratio			
HOMA-IR	0.218	0.073	0.005
GLS			
HOMA-IR	0.780	0.285	0.013
Triglycerides	0.022	0.010	0.042
CIMT			
BMI SD	0.678	0.242	0.008
PWV			
SBP	0.020	0.004	0.000
HDL	-0.007	0.004	0.041
AA diameter			
WC	0.131	0.038	0.003
AA Stiffness			
WC	0.017	0.006	0.010
EAT			
BMI SD	3.744	0.976	0.001

Regression coefficient (B), standard error (SE), homeostasis model assessment of insulin resistance (HOMA-IR), waist circumference (WC), body mass index standard deviation (BMI SD), systolic blood pressure (SBP), interventricular septum (IVSD), Diastolic left ventricle posterior wall (PWD), end-diastolic (LVEDD) and end-systolic (LVESD) left ventricular diameters, left ventricle mass index (LVM-index), left atrial (LA), early (E) and late (A) mitral peak velocities ratio (E/A ratio), early diastolic mitral annular velocities (E'), septal and lateral early diastolic mitral annular velocities (E/E'), global longitudinal strain (GLS), carotid intima-media thickness (CIMT), pulse wave velocity (PWV), ascending aorta (AA), epicardial adipose tissue (EAT).

Tab. 5 - Stepwise multivariate linear regression analysis for WHtR, BMI SD, HOMA-IR, LDL, HDL, triglycerides, SBP, obesity duration, age, sex and pubertal stage (model 2).

IVSD			
Predictors	**B**	**SE**	***p*-Value**
BMI SD	0.749	0.322	0.02
PWD			
BMI SD	1.478	0.352	0.000
LVEDD			
HOMA-IR	0.459	0.218	0.042
Triglycerides	0.046	0.016	0.07
Obesity duration	0.333	0.147	0.03
LVESD			
HOMA-IR	0.343	0.166	0.043
Triglycerides	0.025	0.012	0.042
Obesity duration	0.230	0.112	0.041
LVM-index			
BMI SD	7.016	2,968	0.023
LA volume			
BMI SD	9.802	3.573	0.009
LA strain			
HOMA-IR	-3.247	1.234	0.016
E/A ratio			
BMI SD	-0.391	0.139	0.008
E'			
HOMA-IR	-0.670	0.188	0.001
E / E' ratio			
HOMA-IR	0.218	0.073	0.005
GLS			
HOMA-IR	0.780	0.285	0.013
Triglycerides	0.022	0.010	0.042
CIMT			
WHtR	6.436	1.997	0.003
PWV			
SBP	0.020	0.004	0.000
HDL	-0.007	0.004	0.041
AA diameter			
BMI SD	3.245	1.248	0.019
Obesity duration	0.319	0.130	0.026
AA Stiffness			
SBP	0.014	0.006	0.026
LDL	0.007	0.003	0.021
EAT			
BMI SD	3.744	0.976	0.001

Regression coefficient (B), standard error (SE), homeostasis model assessment of insulin resistance (HOMA-IR), waist-to-height ratio (WHtR), body mass index standard deviation (BMI SD), systolic blood pressure (SBP), diastolic blood pressure (DBP), interventricular septum (IVSD), Diastolic left ventricle posterior wall (PWD), end-diastolic (LVEDD) and end-systolic (LVESD) left ventricular diameters, left ventricle mass index (LVM-index), left atrial (LA), early (E) and late (A) mitral peak velocities ratio (E/A ratio), early diastolic mitral annular velocities (E'), septal and lateral early diastolic mitral annular velocities (E/E'), global longitudinal strain (GLS), carotid intima-media thickness (CIMT), pulse wave velocity (PWV), ascending aorta (AA), epicardial adipose tissue (EAT).

Tab. 6 – Comparison analysis between MUO and MHO phenotypes in group A.

	MUO (n=13)	**MHO (n=46)**	***p***
Age (years)	12.1 ± 2.8	10.7 ± 2.9	0.13
BMI SD	2.5 ± 0.6	2.1 ± 0.4	**0.02**
WC (cm)	103.3 ± 13.5	87.4 ± 11.2	**0.001**
WHtR	0.7 ± 0.1	0.6 ± 0.01	**0.001**
HOMA-IR	4.6 ± 2	3.1 ± 2.1	**0.004**
Total cholesterol (mg/dl)	166.8 ± 33.8	171.6 ± 26.5	0.79
HDL (mg/dl)	43.9 ± 7.4	53.5 ± 14	**0.002**
LDL (mg/dl)	98.2 ± 33.2	94.8 ± 27.9	0.87
Triglycerides (mg/dl)	105.9 ± 46.8	74 ± 27.7	**0.01**
GOT (U/l)	22.5 ± 5.4	21 ± 5.5	0.49
GPT (U/l)	31 ± 23	21.3 ± 7.1	0.19
PCR (mg/dl)	0.7 ± 1.5	0.3 ± 0.3	0.85
SBP (mmHg)	124.8 ± 14.7	109.1 ± 10.8	**0.001**
DBP (mmHg)	73.1 ± 12.7	66.3 ± 9.3	0.12
IVSD (mm)	8.4 ± 1.3	7.7 ± 1.1	**0.07**
PWD (mm)	8.4 ± 1.2	7.6 ± 1.3	**0.06**
LVEDD (mm)	46.3 ± 5.6	42.7 ± 4	**0.043**
LVESD (mm)	29.3 ± 3.7	26.8 ± 2.8	**0.02**
EF (%)	66.5 ± 3.3	67.6 ± 3.8	0.47
LVM-index	37.1 ± 7.2	36.5 ± 6.4	0.71
LA volume (ml)	45.4 ± 11.9	40.5 ± 11.3	0.26
LA strain (%)	37.6 ± 10.9	40.8 ± 10.1	0.48
E/A ratio	1.9 ± 0.4	1.9 ± 0.4	0.85
E' (cm/s)	13.5 ± 1.9	15.9 ± 2.3	**0.004**
E/E'ratio	7.2 ± 0.8	6.2 ± 1	**0.004**
GLS (%)	- 17.3 ± 2.1	- 19.1 ± 2	**0.042**
EAT (mm)	14.6 ± 1.7	12.6 ± 2.3	**0.03**
CIMT (mm)	5.5 ± 1.1	4.7 ± 0.8	**0.01**
β-index	3.6 ± 0.6	3.1 ± 0.9	**0.03**
PWV (m/s)	4.1 ± 0.3	3.6 ± 0.5	**0.002**
Augmentation index	0.1 ± 15.7	4.1 ± 19.1	0.33
AA diameter (mm)	25 ± 2	22.3 ± 3	**0.02**
AA stiffness	3 ± 0.6	2.4 ± 0.3	**0.006**

Numerical data are express as mean ± SD.

Metabolically healthy obesity (MHO), metabolically unhealthy childhood obesity (MUO); homeostasis model assessment of insulin resistance (HOMA-IR), waist circumference (WC), waist-to-height ratio (WHtR), body mass index standard deviation (BMI SD), glutamyl oxaloacetic transaminase (GOT), glutamyl pyruvic transaminase (GPT), systolic blood pressure (SBP), diastolic blood pressure (DBP), interventricular septum (IVSD), Diastolic left ventricle posterior wall (PWD), end-diastolic (LVEDD) and end-sistolic (LVESD) left ventricular diameters, left ventricle mass index (LVM-index), left atrial (LA), mitral peak early (E) and late (A) velocities ratio (E/A ratio), early diastolic mitral annular velocities (E'), septal and lateral early diastolic mitral annular velocities (E/E'), global longitudinal strain (GLS), carotid intima-media thickness (CIMT), pulse wave velocity (PWV), ascending aorta (AA), epicardial adipose tissue (EAT).

3.2 Prospective assessment of liver stiffness by shear wave elastography in childhood obesity: a pilot study.

Corica D, Bottari A, Aversa T, Morabito LA, Curatola S, Alibrandi A, Ascenti G, Wasniewska M. Endocrine. 2021 Jul 23. doi: 10.1007/s12020-021-02828-5.

Introduction

Childhood obesity has become one of the major health issues worldwide and it is associated with a wide spectrum of cardio-metabolic complications (1,2). The epidemic increase of obesity is driving an alarming increased prevalence of non-alcoholic fatty liver disease (NAFLD) which has become the most common liver disease in pediatric age. Among obese children NAFLD prevalence, evaluated by biochemical and ultrasound assessment, has been reported in a wide range from 22.5% to 80% (3-6). Accordingly, in a recent consensus has suggested a new acronym "MAFLD" (metabolic associated fatty liver disease) which should express more precisely the metabolic dysfunction associated with this condition (7). NAFLD is a multifactorial and progressive disease including a spectrum of liver abnormalities ranging from simple liver steatosis to non-alcoholic steatohepatitis (NASH), cirrhosis and hepatocellular carcinoma (8,9). Liver biopsy remains the gold standard method to diagnose NAFLD, to differentiate between simple steatosis and NASH and to exclude other causes of chronic liver diseases (10). The European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) recommends to consider liver biopsy in children with NAFLD when an advanced liver disease is suspected (11). However, biopsy is burdened by several limitations including invasiveness, relatively high risk of complications and elevated costs, that make it difficult to apply it as routine diagnostic procedure, especially in pediatrics. Magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS) could be used for identifying liver steatosis, while magnetic resonance elastography (MRE) could be used for assessing hepatic stiffness, but these methods are burdened by high costs and long execution time that limit their large application in clinical practice (12). Therefore, due to the increasing incidence of NAFLD alongside obesity and to the necessity to adequately manage obesity-related liver abnormalities, the need to identify a non-invasive, reliable and cost-effectiveness methodology to diagnose and monitor NAFLD has arisen. Identification and staging of liver fibrosis are crucial factors to establish prognosis of obesity-related liver abnormalities, since liver fibrosis has been strictly related to the development of long-term outcomes of NALFD (13,14). Up to date, ultrasound (US) is the most widely utilized non-invasive method to identify liver steatosis, although, it has low sensitivity to detect mild steatosis and to quantify hepatic fibrosis. In this context,

ultrasound elastrography (US-E) has encountered considerable interest for non-invasive liver evaluation, showing reliability and feasibility in quantitative assessment of liver tissue stiffness both in adults as in children (15-19). Transient elastography (TE) has proved to be accurate and reliable to identify significant hepatic fibrosis even in pediatric age (20,21). Recently, two-dimensional shear wave elastography (2D-SWE) has emerged as a reliable, non-invasive, tool to evaluate liver tissue elasticity in clinical practice. The main advantages of SWE, compared to TE, are that it is integrated with conventional gray-scale US probes allowing a practical use in several clinical settings and that it allows the operator to choose a homogeneous portion of liver parenchyma, avoiding focal lesions, large vessels and bile ducts (22). Particularly, 2D-SWE provides a shear-wave speed imaging, according to a color map, evaluating liver tissue elasticity/stiffness expressed in kilopascal (kPa) or m/s (22) and it is directly related to stiffness of liver parenchyma, which increases under pathologic conditions, such as fibrosis (23,24). However, SWE application in childhood obesity has not been largely evaluated yet.

Aims of this study were to longitudinally evaluate 2D-SWE changes in relation to loss of weight, metabolic profile and body composition modifications and to investigate the correlation between 2D-SWE variation and clinical and biochemical indices of cardio-metabolic risk in obese children.

Methods

Clinical, bioimpedenziometric and biochemical assessment

Eighty obese children have been consecutively evaluated from April 2018 to October 2018 according to the following inclusion criteria: BMI $\geq$ 2 SDS according to WHO definition (25), aged between 5 and 16 years, Caucasian ethnicity, born as healthy full-term infant adequate for gestational age. Patients were excluded if they were affected by liver diseases other than NAFLD, genetic and/or endocrine causes of obesity, diabetes, chronic diseases, if they undergone liver transplantation and in case of chronic pharmacological therapies, smoking, alcohol abuse.

Thirty-three children completed the 12-months follow-up evaluation and, therefore, they were included in the prospective study. All patients recruited undergone dietary consultation and a physical activity program both prescribed based on European Society of Endocrinology and the Pediatric Endocrine Society recommendations (26). Adherence control of prescribed diet and physical activity program was performed every 3 months (Online Resource 1). All subjects underwent anthropometric (height, weight, waist circumference, BMI), bioimpedenziometric (fat mass, FM; free fat mass, FFM; total body water, TBW) and fasting biochemical assessments at baseline (V0) and after 12 months (V12). At V0 and V12, venous blood samples were obtained from the 33 subjects, after at least 10-hours overnight fast, and glucose, insulin, total cholesterol (TC), high-density lipoproteins (HDL),

low-density lipoproteins (LDL), triglycerides (TG), glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), and gamma glutamyl transferase (GGT), thyroid stimulating hormone (TSH) and free thyroxine (FT4) were measured, as previously described (27). Oral glucose tolerance test (OGTT) were also performed in the entire cohort at V0 and V12. Homeostasis model assessment of insulin resistance (HOMA-IR), homeostasis model assessment of β-cell function (HOMA-β), Matsuda-index, TG/HDL-ratio, TC/HDL-ratio, Atherogenic-index of plasma (AIP), Areas Under the Curves for glucose (AUCg) and insulin (AUCi) and their ratio, were assessed as previously detailed (28). IR was defined as HOMA-IR > 2.5 in prepubertal children and > 4 in pubertal subjects (27).

Ultrasound and Ultrasound-elastography evaluation

In each patient, both conventional liver US and 2D-SWE were performed at V0 and V12 by two expert radiologists after at least 6-hours fast. US and 2D-SWE evaluation were performed with Mindray Resona 7 (Shenzen, China) US machine equipped with a broadband SC5-1U convex probe. B-mode and Doppler US imaging of the liver was performed before SWE measurements. Children were placed in the supine position with their right arm maximally abducted and evaluation of the liver right lobe was conducted through an intercostal space during mid-expiratory breath hold if possible, otherwise during free soft breathing. Diagnosis of liver steatosis was made by conventional liver US according to the presence of at least two of the following abnormal findings: 1) diffusely increased echogenicity of the liver compared with kidney or spleen; 2) ultrasound beam attenuation; 3) poor visualization of intrahepatic structures (29). The severity of steatosis was graded as follows: Grade 0 = no steatosis, defined as normal liver echotexture; Grade 1 = mild steatosis, as a slight and diffuse increase in fine parenchymal echoes with normal visualization of the diaphragm and portal vein borders; Grade 2 = moderate steatosis, as a moderate and diffuse increase in fine echoes with slightly impaired visualization of the portal vein borders and diaphragm; Grade 3 = severe steatosis, defined as fine echoes with poor or no visualization of the portal vein borders, diaphragm, and posterior portion of the right lobe (30).

2D-SWE stiffness was estimated in kPa, as the result of the average of 6 valid measurements, through an intercostal space, perpendicular to the capsule, in a homogeneous portion of parenchyma of the right liver lobe, avoiding large vessels and rib shadows, considering a region of interest (ROI) of 1 x 1.5 cm localized about 1-2 cm from liver capsule, at a maximum depth of 8 cm (31,32). The size and position of the ROI can be adjusted in a color-coded map by the examiner using the B-mode image for guidance. The best possible frame for measurement was indicated by two reliability indicators (M-STB Index and RLB Map) assuring better reproducibility and inter-/intra-observer agreement of

the measurements. An interquartile range/median ratio (IQR/M) < 30% of the measurements was the most important reliability criterion. Variation of clinical, bioimpedenziometric, biochemical and elastographic parameters was evaluated in intra- and inter-group comparison analysis in children who had not lost weight (Group A) and those who had lost weight (Group B) after 12-months follow-up. In addition, study population was also analyzed by dividing it into two groups with respect to liver elasticity value ≤ 10.6 kPa or > 10.6 kPa in accordance with Tutar et al findings (33).

Statistical analysis

The numerical data were expressed as mean and standard deviations score (SDS) and the categorical variables as absolute frequencies and percentages. The non-parametric approach was used since most numerical variables were not normally distributed, such as verified by Shapiro-Wilk test. In order to perform comparison between two time-points (V0 and V12) we applied the Wilcoxon test for numerical parameters and McNemar test for dichotomous variables. In order to compare Group A and Group B patients, Mann Whitney test was applied with reference to numerical parameters and Chi Square test with reference to categorical variables (as insulin resistance, IR), both at V0 and V12, separately. Mann Whitney test was also applied with reference to numerical parameters to evaluate any statistically significant differences between two patient groups defined on the basis of 2D-SWE cut-off value 10.6 kPa, both at V0 and V1. In order to perform within-groups comparison between two time-points (V0 and V12) we applied the Wilcoxon test for numerical parameters and McNemar test for dichotomous variables, both for Group A and Group B patients. The Spearman test was applied in order to assess the existence of any significant correlation between 2D-SWE variation and numerical parameters. Logistic regression models were estimated to assess the possible dependence of IR (dependent variables) on some potential explicative variables as 2D-SWE, age, sex, BMI SDS, waist circumference (WC)-to-height ratio (WHtR), GOT, GPT, TG and TC at V0 and V12. In particular, a crude model (model 1) with only 2D-SWE, a model in which age and sex ware added (model 2), a model including 2D-SWE, GOT, GPT (model 3), and a model with TG and TC (model 4), were estimated. Statistical analysis was performed using IBM SPSS Statistics for Windows, Version 22 (Armonk, NY, IBM Corp.). A p-value smaller than 0.05 was considered statistically significant.

Results

Baseline evaluation (V0)

Study population included 33 obese children (17 females, 16 male). Clinical, biochemical and bioimpedenziometric characteristic of patients at V0 are reported in Table 1.

At V0 evaluation, patients aged 11.5 ± 2.3 years and 11 of them were pre-pubertal (33.3%); mean BMI SDS was 2.9 ± 0.6. In 25 children IR was diagnosed based on HOMA-IR. In 7 children steatosis was documented by US (mild steatosis in 4 patients, moderate in 3 patients). 2D-SWE mean value was 10.5 ± 2.1 kPa (IQR/M mean value 15.5%). In particular, in 15 patients the mean 2D-SWE value was ≤ 10.6 kPa, but there were no significant differences with regard to clinical and biochemical variables compared with the 18 patients with a mean 2D-SWE value > 10.6 kPa. No significant correlation between 2D-SWE and anthropometric, bioimpedenziometric or biochemical variables were found.

Longitudinal evaluation (V12)

Clinical, bioimpedenziometric and biochemical assessment

At V12, BMI, BMI SDS and FM did not significantly change in the entire cohort. However, a significant increase of FFM (p=0.002) and TBW (p=0.004) was documented (Table 1a). With regard to biochemical variables, GOT, TG/HDL-ratio, HbA1c, OGTT 120-minutes glucose and insulin were significantly decreased (Table 1a), as well as the number of subjects with insulin resistance defined by HOMA-IR (Table 1b). Group A patients reported that they did not follow the dietary and physical activity recommendations given during the visits.

Inter-group comparison analysis of clinical and biochemical parameters between V0 and V12, documented significant differences for BMI, BMI SDS, fasting insulin, HOMA-IR, HOMA-β, TG, TG/HDL-ratio, AIP, TC/HDL-ratio, GOT, GPT, GGT, uric acid between Group A and Group B children after 12-months follow-up (Table 2). Bioimpedenziometric parameters did not significantly change between V0 and V12. Intra-group comparison analysis showed a significant decrease of BMI, BMI SDS and a significant improvement of metabolic profile, documented by the reduction of fasting insulin, OGTT 120-minutes glucose and insulin, HOMA-IR, HbA1c, TG, TG/HDL-ratio, AIP, GOT, GPT, GGT, uric acid, and by an increase of Matsuda-index and HDL-cholesterol, in children of Group B but not in those of Group A (Table 3). Furthermore, a significant decrease in number of children with IR was demonstrated in Group B children between V0 and V12 evaluation (p=0.016), but not in Group A (p=0.96). Bioimpedenziometric parameters did not significantly change between V0 and V12.

US and SWE evaluations

At V12, 2D-SWE mean value was 8.9 ± 2.3 kPa (IQR/M mean value 15.5%). US evaluation did not document significant difference regarding to steatosis between V0 and V12. 2D-SWE stiffness significantly decreased both in the entire population (p=0.002) (Table 1), as well as among Group B

children (p=0.004) evaluated between V0 and V12 in intra-group comparison analysis (Table 3). Variation of 2D-SWE (Δ-SWE) did not significant differ with regard to sex or pubertal stage, and did not significantly correlate with variation of clinical, biochemical and bioimpedenziometric parameters (Table 4). Moreover, 2D-SWE values did not significant correlate with anthropometric, bioimpedenziometric or biochemical variables. Logistic regression models did not document a significant association between IR, 2D-SWE and other analyzed variables. A decrease from 18 to 8 patients with mean 2D-SWE > 10.6 kPa and an increase from 15 to 25 patients with mean 2D-SWE ≤ 10.6 kPa were observed. Differences between clinical and biochemical variables of these two groups remained nonsignificant at V12.

Discussion

In this pilot study, for the first time, 2D-SWE has been longitudinally evaluated in obese children in relation to loss of weight, metabolic profile and body composition modifications. A significant reduction of mean 2D-SWE value, associated with an improvement of metabolic profile, was demonstrated both in the entire cohort and, particularly, in children who had lost weight.

NAFLD includes a wide spectrum of liver abnormalities, as asymptomatic steatosis with elevated or normal transaminases and NASH, characterized by the presence of macrovesicular steatosis, inflammation with hepatocyte alterations, as ballooning degeneration, apoptotic bodies and Mallory-Denk bodies, with a variable degree of liver fibrosis, although it is not necessary for NASH diagnosis (34,35). A NAFLD activity score has been used to classify the histologically determined liver damage in relation to steatosis, lobular inflammation and ballooning (35). IR and lipid metabolism alteration, well-known cardio-metabolic risk factors in childhood obesity, play a pivotal role in NAFLD development in children and adults with obesity. IR promotes triglycerides accumulation in liver, favoring inhibition of lipolysis in adipocytes, promoting the increased uptake and the reduced catabo¬lism of free fatty acid (FFA) in hepatocytes. Furthermore, inflammation injury due to oxidative stress, by activating hepatic stellate cells and promoting fibrosis, can significantly contribute to hepatic damage in obesity (36). In addition, a contribution to the pathogenesis of NAFLD could be determined by the increased levels of uric acid, which is considered a risk marker for NAFLD. Uric acid promotes an enhancement hepatic lipogenesis and an increase in pro-inflammatory and oxidative stress pathways (37). Liver biopsy is a highly invasive method that should be considered only for suspected advanced stage of NAFLD in children with obesity. Therefore, in this context, SWE could play a central role in non-invasive liver assessment along with US evaluation in order to provide a detailed assessment of liver parenchyma in children. The presence of steatosis is documented in different grades of NAFLD (34), but steatosis is a non-specific alteration, may have

variable distribution, may not be associated to elevated transaminases and could not be detected by US evaluation due to low sensitivity to detect mild steatosis. In particular, obesity-related hepatopathy in childhood is often characterized by normal transaminase values and by the absence of significant US alteration, at least in the early stages when subclinical inflammation may be present in absence of significant hepatic fibrosis. However, although ballooning degeneration and fibrosis are usually mild or absent in children with obesity, persistence of chronic inflammation could promote liver fibrosis and progression to NASH. In a rabbit model, it has been shown that, although liver fibrosis was the factor that most significantly affects liver elasticity, also simple steatosis, ballooning degeneration and inflammation were all independent factors affecting liver elasticity in rabbits without fibrosis, thus resulting in SWE measurement higher than normal liver group (38). These findings could be explained considering that liver elasticity can be affected by hepatocyte swelling due to the accumulation of lipid droplets and the increased tension of the liver tissue, which can be detected in both steatosis and ballooning degeneration. Therefore, these authors concluded that hepatic stiffness can be influenced not only by fibrosis but also by the presence of steatosis, ballooning degeneration and different degree of inflammation (38). These results were confirmed in studies involving adults with different stages of NAFLD; liver stiffness was greater in patients affected by NASH with inflammation but without fibrosis compared to those with simple steatosis, but it was lower than in patients with fibrosis (39). It has been shown that increased liver stiffness precedes the fibrosis onset; inflammatory cells infiltration, oedema and increased cross-linking of hepatic extracellular matrix could contribute to increase of hepatic stiffness and they seem to be directly related to subsequent fibrosis development (40,41).

The role of hepatic steatosis on liver stiffness degree, and thus on elastographic assessment, is still unclear and literature data are conflicting. Indeed, some studies documented higher elastographic values in subjects with hepatic steatosis (33,42), while other studies did not confirm this finding assuming that the presence of large fatty vacuoles in hepatic steatosis increases the space between cells, thus reducing the density of liver tissue and making it softer on elastographic evaluation (19,43-46). This variability in liver elastographic assessment could be explained, at least in part, by influence of other factors on liver stiffness, including the degree of inflammation. In our study, the presence of hepatic steatosis did not significantly influence the 2D-SWE values, although it must be considered that steatosis was proven in only 7 patients by US.

In a recent meta-analysis Kim et al. suggested that 2D-SWE would have better technical performance than TE given its methodological advantages, such as integration with conventional US and tissue elasticity measurement through a real-time color map of tissue elasticity in a specific ROI (47). Moreover, 2D-SWE demonstrated a better feasibility compared to TE (47,31). 2D-SWE has a high

specificity and sensitivity for distinguishing the presence of liver fibrosis; however, this technique does not seem to be able to accurately distinguish the different histological grades of liver fibrosis (according to Brunt classification) (33). Furthermore, in children with NASH, 2D-SWE values may not correlate with the presence of fibrosis (33). This may be explained by the influence of steatosis, ballooning degeneration and/or inflammation on increased liver stiffness even in the absence of fibrosis (48,49). In our population, we used the 2D-SWE cut-off 10.6 kPa proposed by Tutar et al (33) to identify patients who were likely or unlikely to have liver fibrosis, without, however, identifying any significant difference between the two groups with regard to clinical and metabolic variables. The significant decrease of 2D-SWE mean value in our entire cohort and in particular in children who had lost weight, might be attributed to the significant improvement in glucose and lipid profile documented in longitudinal evaluation. Furthermore, intra-group analysis documented a significant reduction of transaminases and uric acid among subjects who had lost weight during prospective observation. Our results suggest the presence of an association among overweight reduction, improvement of metabolic profile and transaminases levels and the decrease of stiffness degree documented by 2D-SWE, and, therefore, support the possible significant role of 2D-SWE in monitoring evolution of NAFLD in childhood obesity.

The limited period of follow-up and the small sample size, which is conditioned by a high dropout rate of young obese patients, are two limitations of our study. These factors might explain why a significant association between the reduction of 2D-SWE and the improvement of clinical and metabolic parameters was not documented. Liver biopsy was not performed to determine liver involvement, but it remain difficult to routinely apply because it is expensive and invasive. The assessment of alcohol consumption only at the time of anamnestic survey in presence of parents and the non-assessment of gene polymorphisms can be considered as study limitations. On the other hand, our study has significant strengths. First, 2D-SWE variation has been longitudinally evaluated according to clinical, biochemical and bioimpedenziometric variables. Second, our cohort consisted in a homogeneous sample of obese children. Moreover, we investigated several OGTT-derived variables expression of glucose metabolism.

In conclusion, SWE could play a significant role in the non-invasive assessment of NAFLD in children and adolescents with obesity. We suggested that weight loss and metabolic profile improvement might be associated with a decrease in 2D-SWE values. Further studies, carried out in larger cohorts of obese children, will be necessary to confirm diagnostic accuracy and reliability of 2D-SWE evaluation and its variation in relation to metabolic profile, also taking into account further development of elastographic method for NAFLD evaluation (50).

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Tables and figures

Table 1- Comparison analysis of clinical, bioimpedenziometric, biochemical and elastographic parameters between patients at baseline (V0) and after 12-months follow-up (V12).

1a) Wilcoxon test for numerical variable

	V0	**V12**	**p**
Age (years)	11.5 ± 2.3	12.6 ± 2.4	0.000
Pubertal stage (prepubertal/pubertal)	11/22	9/24	0.5
Height (cm)	151.2 ± 13.5	155.5 ± 14.2	0.000
Weight (kg)	67.6 ± 17.4	70.8 ± 19.1	0.005
BMI	29.0 ± 3.5	28.6 ± 4.1	0.231
BMI SDS	2.9 ± 0.6	2.9 ± 0.8	0.681
WC	87.8 ± 8.9	89.9 ± 13.1	0.334
WHtR	0.6 ± 0.1	0.6 ± 0.1	0.900
SBP (mmHg)	112.8 ± 11.5	113.0 ± 10.1	0.726
DBP (mmHg)	69.2 ± 9.6	67.6 ± 10.8	0.583
Fasting Glucose (mg/dl)	94.9 ± 7.4	95.2 ± 15.9	0.303
Fasting Insulin (µUI/ml)	22.0 ± 14.0	20.7 ± 12.1	0.207
Glucose OGTT-120’	127.6 ± 24.6	116.4 ± 20.8	0.012
Insulin OGTT-120’	143.8 ± 116.9	97.4 ± 67.1	0.025
HOMA-IR	5.3 ± 3.7	5.0 ± 3.4	0.266
HOMA-β	294.5 ± 287.6	253.9 ± 166.4	0.950
HbA1c	5.7 ± 0.4	5.3 ± 0.3	0.000
Matsuda index	2.47 ± 1.44	2.85 ± 1.6	0.022
AUCg	254.04 ± 39.89	237.93 ± 44.37	0.001
AUCi	251.99 ± 186.95	207.75 ± 131.98	0.026
AUCi / AUCg	0.96 ± 0.63	0.89 ± 0.57	0.201
TC (mg/dl)	164.1 ± 29.7	173.8 ± 24.1	0.006
HDL (mg/dl)	44.5 ± 10.0	50.1 ± 10.3	0.000
LDL (mg/dl)	95.2 ± 24.0	98.7 ± 25.6	0.274
TG (mg/dl)	87.8 ± 38.9	78.5 ± 28.9	0.092
TG/HDL	2.1 ± 1.1	1.7 ± 0.8	0.005
TC/HDL	3.9 ± 1.2	3.6 ± 0.9	0.086
AIP	0.3 ± 0.2	0.2 ± 0.2	0.009
GPT (U/L)	23.0 ± 13.6	21.5 ± 10.6	0.307
GOT (U/L)	32.5 ± 45.6	19.7 ± 5.4	0.003
GGT (U/L)	15.5 ± 6.2	13.9 ±6.5	0.520
Uric Acid (mg/dl)	5.2 ± 1.2	5.2 ± 1.3	0.324
PCR (mg/dl)	0.4 ± 1.1	0.2 ± 0.2	0.275
TSH (µUI/ml)	2.9 ± 1.4	2.8 ± 1.4	0.748
FT4 (ng/dl)	1.3 ± 0.1	1.8 ± 3.0	0.053
FM	28.3 ± 9.6	28.8 ± 10.6	0.487
FFM	39.5 ± 9.7	41.8 ± 10.7	0.002
TBW	28.8 ± 7.5	30.1 ± 8.1	0.004
2D-SWE (kPa)	10.5 ± 2.1	8.9 ± 2.3	0.002

Numerical data are express as mean ± SD.
Body mass index standard deviation score (BMI SDS), waist circumference (WC), waist-to-height ratio (WHtR), systolic blood pressure (SBP), diastolic blood pressure (DBP), oral glucose tolerance test (OGTT), homeostasis model assessment of insulin resistance (HOMA-IR), homeostasis model assessment of β-cell function (HOMA-β), Areas Under the Curves for glucose (AUCg) and insulin (AUCi), Total cholesterol (TC), Triglycerides (TG), atherogenic index of plasma (AIP), C protein reactive (PCR), glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), and gamma glutamyl transferase (GGT), thyroid stimulating hormone (TSH) and free thyroxine (FT4), fat mass (FM), free fat mass (FFM), total body water (TBW), two-dimensional shear wave elastography (2D-SWE).

1b) McNemar test for dichotomous variables

		INSULIN RESISTANCE V12 YES	INSULIN RESISTANCE V12 NO	p value
INSULIN RESISTANCE V0	YES	15	10	0.039
	NO	2	6	

Table 2- Inter-group analysis between Group A and Group B children at V0 and V12.

	Group A_{V0} (n=18)	**Group B_{V0} (n=15)**	***p***	**Group A_{V12} (n=18)**	**Group B_{V12} (n=15)**	***p***
BMI	29.23 ± 3.47	28.65 ± 3.60	0.664	30.46 ± 3.60	26.39 ± 3.69	0.011
BMI SDS	2.87 ± 0.32	2.93 ± 0.82	0.395	3.27 ± 0.61	2.46 ± 0.73	0.001
WC	89.64 ± 7.23	85.67 ± 10.37	0.175	95.38 ± 8.83	83.71 ± 14.96	0.132
WHtR	0.60 ± 0.06	0.57 ± 0.04	0.114	0.64 ± 0.05	0.56 ± 0.08	0.135
SBP (mmHg)	113.11 ± 10	112.5 ± 13.45	0.651	114.1 ± 8.98	111.80 ± 11.51	0.335
DBP (mmHg)	69.00 ± 8.22	69.40 ± 11.26	0.885	65.88 ± 9.64	69.53 ± 12.10	0.326
Fasting Glucose (mg/dl)	95 ± 7.25	94.86 ± 7.8	0.828	98.11 ± 20.07	91.6 ± 7.908	0.261
Fasting Insulin (μUI/ml)	23.3 ± 17.4	20.4 ± 8.58	0.800	26.1 ± 13.5	14.14 ± 5.39	0.003
Glucose OGTT-120’	129.5 ± 30.4	125.4 ± 15.6	0.731	121.3 ± 22.51	110.4 ± 17.4	0.153
Insulin OGTT-120’	136.1 ± 108.9	153.1 ± 129.1	0.800	120.6 ± 75.2	69.6 ± 43.5	0.071
HOMA-IR	5.5 ± 4.6	4.9 ± 2.35	0.914	6.5 ± 3.9	3.18 ± 1.14	0.003
HOMA-β	351.1 ± 380.5	226.6 ± 64.25	0.971	305.4 ± 196.9	192.02 ± 93.07	0.033
HbA1c (%)	5.7 ± 0.37	5.5 ± 0.4	0.365	5.4 ± 0.25	5.2 ± 0.3	0.108
Matsuda index	2.35 ± 1.48	2.6 ± 1.4	0.515	2.4 ± 1.67	3.3 ± 1.38	0.025
AUCg	262.5 ± 39.7	243.9 ± 39	0.386	238.3 ± 54.4	237.46 ± 30.05	0.664
AUCi	265.1 ± 194.7	236.2 ± 182.7	0.515	251.1 ± 159.8	155.7 ± 58.9	0.036
AUCi / AUCg	0.98 ± 0.65	0.92 ± 0.61	0.691	1.07 ± 0.69	0.66 ± 0.24	0.030
TC (mg/dl)	171 ± 24.8	155.8 ± 33.5	0.096	178.8 ± 21.1	167.8 ± 26.6	0.191
HDL (mg/dl)	42.7 ± 9.8	46.6 ± 10	0.365	47.4 ± 9.5	53.3 ± 10.6	0.115
LDL (mg/dl)	99.5 ± 23.15	90 ± 24.8	0.205	106.5 ± 21.38	89.2 ± 27.6	0.096
TG (mg/dl)	83.4 ± 33.3	93 ± 45.5	0.731	86.7 ± 31.5	68.5 ± 22.4	0.027
TG/HDL	2.07 ± 1.03	2.1 ± 1.2	0.786	1.9 ± 0.97	1.3 ± 0.5	0.019
TC/HDL	4.2 ± 1.34	3.4 ± 0.7	0.041	3.9 ± 0.9	3.3 ± 0.8	0.049
AIP	0.27 ± 0.19	0.26 ± 0.24	0.814	0.24 ± 0.19	0.09 ± 0.14	0.021
GPT (U/L)	26.27 ± 17.07	19 ± 6.01	0.285	26.8 ± 11.18	15 ± 4.8	0.000
GOT (U/L)	43.1 ± 60.25	19.8 ± 5.6	0.046	21.3 ± 5.06	17.6 ± 5.19	0.035
GGT (U/L)	15.6 ± 7.4	15.4 ± 4.5	0.599	16.2 ± 7.5	10.8 ± 3.2	0.023
Uric Acid (mg/dl)	5.5 ± 1.18	4.8 ± 1.2	0.124	5.8 ± 1.2	4.47 ± 0.9	0.007
PCR (mg/dl)	0.5 ± 1.5	0.2 ± 0.13	0.113	0.2 ± 0.2	0.2 ± 0.25	0.968
TSH (μUI/ml)	2.8 ± 1.39	2.96 ± 1.4	0.914	2.9 ± 1.4	2.7 ± 1.3	0.678
FT4 (ng/dl)	1.27 ± 0.15	1.3 ± 0.14	0.270	1.2 ± 0.17	2.38 ± 4.37	0.612
2D-SWE (kPa)	10.8 ± 2.2	10.05 ± 2.01	0.142	9.0 ± 2.18	8.65 ± 2.55	0.638
IR (yes/no)	14/4	11/4	0.54	13/5	4/11	0.01

Group A_{V0}= baseline assessment of subjects who will not have lost weight after 12 months of follow-up
Group B_{V0}= baseline assessment of subjects who will lose weight after 12 months of follow-up
Group A_{V12}= subjects who have not lost weight after 12 months of follow-up
Group B_{V12}= subjects who lost weight after 12 months of follow-up

Numerical data are express as mean ± SD. Chi Square test was applied for dichotomous variable (IR).
Body mass index standard deviation score (BMI SDS), waist circumference (WC), waist-to-height ratio (WHtR), systolic blood pressure (SBP), diastolic blood pressure (DBP), oral glucose tolerance test (OGTT), homeostasis model assessment of insulin resistance (HOMA-IR), homeostasis model assessment of β-cell function (HOMA-β), Areas Under the Curves for glucose (AUCg) and insulin (AUCi), Total cholesterol (TC), Triglycerides (TG), high-density lipoproteins (HDL), low-density lipoproteins (LDL), atherogenic index of plasma (AIP), C protein reactive (PCR), glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), and gamma glutamyl transferase (GGT), thyroid stimulating hormone (TSH) and free thyroxine (FT4), fat mass (FM) free fat mass (FFM), total body water (TBW), two-dimensional shear wave elastography (2D-SWE), insulin resistance (IR).

Table 3 - Intra-group analysis between Group A and Group B children at V0 and V12.

	Group A (n=18)			**Group B (n=15)**		
	V0	**V12**	**p**	**V0**	**V12**	**p**
BMI	29.23 ± 3.47	30.46 ± 3.60	0.071	28.65 ± 3.60	26.39 ±3.69	0.001
BMI SDS	2.87 ± 0.32	3.27 ± 0.61	0.001	2.93 ± 0.82	2.46 ± 0.73	0.001
WC	89.64 ± 7.23	95.38 ± 8.83	0.123	85.67 ± 10.37	83.71 ± 14.96	0.799
WHtR	0.60 ± 0.06	0.64 ± 0.05	0.362	0.57 ± 0.04	0.56 ± 0.08	0.400
SBP (mmHg)	113.11 ± 10	114.1 ± 8.98	0.609	112.47 ± 13.45	111.80 ± 11.51	0.955
DBP (mmHg)	69.00 ± 8.22	65.88 ± 9.64	0.288	69.40 ± 11.26	69.53 ± 12.10	0.842
Fasting Glucose (mg/dl)	95 ± 7.25	98.11 ± 0.07	0.777	94.86 ± 7.8	91.6 ± 7.91	0.123
Fasting Insulin (μUI/ml)	23.3 ± 17.4	26.1 ± 13.5	0.557	20.4 ± 8.58	14.14 ± 5.39	0.013
Glucose OGTT-120’	129.5 ± 30.4	121.3 ± 2.51	0.151	125.4 ± 15.6	110.4 ± 17.4	0.041
Insulin OGTT-120’	136.1 ± 108.9	120.6 ± 75.2	0.528	153.1 ± 129.1	69.6 ± 43.5	0.011
HOMA-IR	5.5 ± 4.6	6.5 ± 3.9	0.459	4.9 ± 2.35	3.18 ± 1.14	0.013
HOMA-β	351.1 ± 380.5	305.4 ± 96.9	0.679	226.6 ± 64.25	192.02 ± 93.07	0.191
HbA1c (%)	5.7 ± 0.37	5.4 ± 0.25	0.001	5.5 ± 0.4	5.2 ± 0.3	0.003
Matsuda index	2.35 ± 1.48	2.4 ± 1.67	0.500	2.6 ± 1.4	3.3 ± 1.38	0.009
AUCg	262.5 ± 39.7	238.3 ± 54.4	0.009	243.9 ± 39	237.46 ± 30.05	0.047
AUCi	265.1 ± 194.7	251.1 ± 59.8	0.215	236.2 ± 182.7	155.7 ± 58.9	0.061
AUCi / AUCg	0.98 ± 0.65	1.07 ± 0.69	0.879	0.92 ± 0.61	0.66 ± 0.24	0.1
TC (mg/dl)	171 ± 24.8	178.8 ± 21.1	0.162	155.8 ± 33.5	167.8 ± 26.6	0.014
HDL (mg/dl)	42.7 ± 9.8	47.4 ± 9.5	0.004	46.6 ± 10	53.3 ± 10.6	0.021
LDL (mg/dl)	99.5 ± 23.15	106.5 ± 1.38	0.098	90 ± 24.8	89.2 ± 27.6	0.826
TG (mg/dl)	83.4 ± 33.3	86.7 ± 31.5	0.705	93 ± 45.5	68.5 ± 22.4	0.008
TG/HDL	2.07 ± 1.03	1.9 ± 0.97	0.287	2.1 ± 1.2	1.3 ± 0.5	0.005
TC/HDL	4.2 ± 1.34	3.9 ± 0.9	0.163	3.4 ± 0.7	3.3 ± 0.8	0.307
AIP	0.27 ± 0.19	0.24 ± 0.19	0.368	0.26 ± 0.24	0.09 ± 0.14	0.011
GPT (U/L)	26.27 ± 17.07	26.8 ± 11.18	0.679	19 ± 6.01	15 ± 4.8	0.009
GOT (U/L)	43.1 ± 60.25	21.3 ± 5.06	0.061	19.8 ± 5.6	17.6 ± 5.19	0.003
GGT (U/L)	15.6 ± 7.4	16.2 ± 7.5	0.112	15.4 ± 4.5	10.8 ± 3.2	0.016
Uric Acid (mg/dl)	5.5 ± 1.18	5.8 ± 1.2	0.410	4.8 ± 1.2	4.47 ± 0.9	0.031
PCR (mg/dl)	0.5 ± 1.5	0.2 ± 0.2	0.193	0.2 ± 0.13	0.2 ± 0.25	0.675
TSH (μUI/ml)	2.8 ± 1.39	2.9 ± 1.4	0.586	2.96 ± 1.4	2.7 ± 1.3	0.268
FT4 (ng/dl)	1.27 ± 0.15	1.2 ± 0.17	0.142	1.3 ± 0.14	2.38 ± 4.37	0.233
2D-SWE (kPa)	10.8 ± 2.2	9.0 ± 2.18	0.068	10.05 ± 2.01	8.65 ± 2.55	0.004

Numerical data are express as mean ± SD. Wilcoxon test was applied for the statistical analysis.
Body mass index standard deviation score (BMI SDS), waist circumference (WC), waist-to-height ratio (WHtR), systolic blood pressure (SBP), diastolic blood pressure (DBP), oral glucose tolerance test (OGTT), homeostasis model assessment of insulin resistance (HOMA-IR), homeostasis model assessment of β-cell function (HOMA-β), Areas Under the Curves for glucose (AUCg) and insulin (AUCi), Total cholesterol (TC), Triglycerides (TG), high-density lipoproteins (HDL), low-density lipoproteins (LDL), atherogenic index of plasma (AIP), C protein reactive (PCR), glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), and gamma glutamyl transferase (GGT), thyroid stimulating hormone (TSH) and free thyroxine (FT4), fat mass (FM) free fat mass (FFM), total body water (TBW), two-dimensional shear wave elastography (2D-SWE), insulin resistance (IR).

Table 4 – Correlation analysis between 2D-SWE variation (Δ SWE) and variation (Δ) of clinical, biochemical and bioimpedenziometric parameters.

	Δ SWE	
	r_S	p
Δ BMI SDS	-0.028	0.883
Δ WHtR	-0.383	0.196
Δ HOMA-IR	-0.183	0.324
Δ Matsuda-index	0.045	0.811
Δ AUCi/AUCg	0.077	0.679
Δ GPT	-0.109	0.561
Δ GOT	-0.139	0.456
Δ TC	-0.141	0.450
Δ TG	0.273	0.137
Δ FM	-0.139	0.471
Δ FFM	0.033	0.867
Δ TBW	-0.100	0.606

Spearman's test was applied for the statistical analysis.
Body mass index standard deviation score (BMI SDS), waist-to-height ratio (WHtR), homeostasis model assessment of insulin resistance (HOMA-IR), Areas Under the Curves for glucose (AUCg) and insulin (AUCi), Total cholesterol (TC), Triglycerides (TG), glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), fat mass (FM) free fat mass (FFM), Spearman's correlation coefficient (r_S), total body water (TBW), two-dimensional shear wave elastography (2D-SWE).

CHAPTER 4

Conclusions

Overall results of the realized researches, concerning both pathophysiological pathways and innovative non-invasive methods for the early diagnosis of preclinical alterations, have shown that the severity of overweight and IR are the factors predominantly affecting the cardio-metabolic profile in children and adolescents with obesity.

New evidence provided by our results on the addressed topics can potentially have a significant impact in implementing management of children and adolescents with obesity, such as the role of asprosin and of the AGE/RAGE oxidative stress pathway in the pathophysiology of childhood obesity complications and the application of methods for non-invasive detection of precocious preclinical cardiovascular and metabolic alterations, such as 2D shear wave elastography and 2D speckle tracking echocardiography analysis.

Application of biochemical and instrumental markers, able to identify early obese subjects at higher risk of developing obesity-related cardio-metabolic complications and the personalized approach of obese patients represent the upcoming future in care of children and adolescents with obesity and for our scientific research.