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- XXXII CYCLE -

Ph.D. THESIS:

LINKING OBESITY, OBESITY-RELATED DISEASES, MEDITERRANEAN STYLE-DIET AND GUT MICROBIOTA IN PEDIATRICS

(MED/38 - MED/49)

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“Due cose riempiono l’animo di ammirazione e venerazione sempre nuova e crescente,
quanto più spesso e più a lungo la riflessione si occupa di esse:
il cielo stellato sopra di me, e la legge morale in me.”
Immanuel Kant (1724-1804)

Alla mia famiglia

SUMMARY

The worldwide prevalence of overweight and obesity among children and adolescents has risen dramatically. Obesity is a complex and multifactorial condition associated with an increased risk of multiple comorbidities, like diabetes, arterial hypertension, dyslipidemia, cardiovascular diseases and cancer. Most of them have been already described since pediatric age. However, precocious biomarkers for identifying “high risk” subjects to an unhealthy metabolic profile are lacking, especially in pediatrics. Thus, one of the aims of this Ph.D. project was to investigate the development of obesity-related diseases in obese children and adolescents, identifying precocious biomarkers that could allow early detection and promote prevention strategies. Besides, we reported that insulin resistance, metabolic syndrome, and hyperuricemia correlated with cardiovascular dysfunction in pediatric obesity. Furthermore, we identified novel metabolic risk factors, in particular high-normal estimated glomerular filtration rate and haptoglobin phenotypes.

The fundamental cause of overweight and obesity is an energy imbalance between calories consumed and calories expended. Certainly, the diet plays a key role and is a crucial variable for a healthy life. A rapidly growing number of studies, in recent years, support the hypothesis that the Mediterranean style-diet has beneficial effect. However, the urbanization of people living in the Mediterranean area modified lifestyle choices deviating to a “Western diet” richer in saturated fat, refined grains, simple carbohydrates and processed foods. Thus, we also aimed to describe the adherence to the Mediterranean style-diet in children and adolescents, assessing the potential effect of healthier behaviours. In nothern Italy, we described poor food quality which replaced the Mediterranean dietary pattern in children and adolescents, in particular among younger children. Moreover, in a systematic review, we supported skipping breakfast as an easy marker of the risk of overweight and obesity and metabolic-related diseases.

Besides, increasing evidence especially reported the involvement of the gut microbiota in the dysmetabolism associated with obesity. For this reason, exploring the role of the gut microbiota in the development of childhood obesity was another outcome of this Ph.D. project, for potentially revealing new strategies for obesity prevention and treatment. Our first baseline findings correlated gut microbiota to dietary pattern and adherence to the Mediterranean style-diet. This suggested that dietary intervention would have enormous potential in modulating the microbial composition and promoting

a more health-associated metabolic profile. However, few data are nowadays available concerning pediatrics, so this relationship awaits further studies.

Most of the above-mentioned results were published on international peer-reviewed scientific journals, hoping to contribute to the current knowledge on the crosstalk between obesity, obesity-related diseases, Mediterranean style-diet and gut microbiota.

Futhermore, during the Ph.D. project the G☺☺D-DAY Trial was designed and realized (ID NCT03154255). Although COVID-19 pandemic temporary stopped it, further results are awaited in the next months.

RIASSUNTO

La prevalenza mondiale di sovrappeso ed obesità in bambini ed adolescenti è drasticamente in aumento. Nell'obesità si riconosce una condizione complessa e multifattoriale associata ad un aumentato rischio di sviluppo di comorbidità, quali diabete, ipertensione arteriosa, dislipidemia, malattie cardiovascolari e neoplasie. Molte di queste complicanze correlate all'eccesso ponderale si sviluppano già a partire dall'età pediatrica. Tuttavia, ad oggi mancano marcatori precoci atti ad identificare soggetti "ad alto rischio" con un profilo metabolico sfavorevole, soprattutto in età pediatrica. Pertanto, uno degli obiettivi di questo progetto di Dottorato è stato studiare lo sviluppo di complicanze correlate all'obesità in bambini ed adolescenti obesi, identificandone gli eventuali marcatori precoci in grado di contribuire alla diagnosi precoce nonché alla promozione di strategie di prevenzione. A tal riguardo, l'insulino-resistenza, la sindrome metabolica e l'iperuricemia sono risultate positivamente correlate alla disfunzione cardiovascolare nell'obesità pediatrica. Inoltre sono stati identificati nuovi potenziali fattori di rischio metabolico nell'aumento del filtrato glomerulare e nei diversi fenotipi di aptoglobina.

La causa principale di sovrappeso ed obesità trova ragione in uno squilibrio energetico tra le calorie introdotte e quelle consumate. Certamente, la dieta svolge un ruolo chiave ed è risultata una variabile cruciale per uno stile di vita sano. Negli ultimi anni, un numero crescente di studi ha suggerito e dimostrato effetti benefici correlati all'aderenza alla dieta Mediterranea. Tuttavia, l'urbanizzazione crescente delle comunità che risiedono in particolare nell'area mediterranea, ne ha modificato le loro scelte ed il loro stile di vita promuovendo una "dieta occidentale" più ricca di grassi saturi, cereali raffinati, carboidrati semplici ed alimenti trasformati. Pertanto, tra i nostri obiettivi, si inserisce quello di descrivere l'aderenza alla dieta Mediterranea in età pediatrica, valutando il potenziale effetto di stili di vita più salutari. Nel Nord Italia è stata descritta una scarsa qualità nelle scelte alimentare che hanno sostituito il modello di dieta Mediterraneo soprattutto tra i bambini più piccoli. Inoltre, in un lavoro di revisione sistematica, è emerso come il "saltare" la colazione sia un più semplice indicatore del rischio di sovrappeso ed obesità nonché di malattie metaboliche correlate.

Crescenti evidenze hanno inoltre riconosciuto nel microbiota intestinale un suo coinvolgimento diretto nel dismetabolismo associato all'obesità. A tal proposito, si configura un ulteriore obiettivo di questo progetto di Dottorato ovvero esplorare il ruolo

del microbiota intestinale ed il suo impatto sullo sviluppo dell'obesità, al fine di identificare nuove strategie di prevenzione e trattamento. Dati preliminari hanno evidenziato una interessante correlazione tra microbiota intestinale ed aderenza alla dieta Mediterranea. Pertanto, l'intervento dietetico potrebbe avere un enorme potenziale nel modulare la composizione microbica e nel promuovere un profilo metabolico più salutare. Tuttavia, ad oggi, sono disponibili solo pochi dati in età pediatrica, di conseguenza ulteriori studi sono necessari per confermare tale ipotesi.

La maggior parte dei risultati sopra menzionati è stata pubblicata su riviste scientifiche internazionali peer-reviewed, nella speranza di poter contribuire alle attuali conoscenze sul crosstalk tra obesità e complicanze ad essa correlate, dieta Mediterranea e microbiota intestinale. Nel corso del progetto di Dottorato è stato inoltre condotto un Trial, registrato come G☺☺D-DAY (ID NCT03154255). Nonostante la temporanea interruzione correlata alla situazione contigente della pandemia da COVID-19, sono attesi ulteriori risultati nei prossimi mesi.

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1. GENERAL INTRODUCTION AND RATIONAL

1.1. THE EPIDEMIC OF OBESITY AND ITS HEALTHY IMPACT

Worldwide obesity has nearly tripled since 1975. Nowadays, most of the world's population live in countries where overweight and obesity kills more people than underweight. In 2016, more than 1.9 billion adults, 18 years and older, were overweight. Of these, over 650 million were obese. Therefore, over 340 million children and adolescents aged 5-19 years were overweight or obese. Latest data also reported in 2019 that 38 million children under the age of 5 were overweight or obese yet [1]. From 1985 to 2019, the unhealthiest changes like gaining too little height, too much weight for their height compared with children in other countries or both, occurred in many countries in sub-Saharan Africa, New Zealand and the USA for boys and girls, in Malaysia and some Pacific island nations for boys, and Mexico for girls [2]. All these data are alarming and underline how obesity is worldwilde a real threat, amplifying concerns over the health risks associated with this worsening problem. It is well known that obese children are more prone to become obese adults, with an enhanced risk of obesity-related complications [3]. A cluster of cardiovascular disease risk factors as arterial hypertension, dyslipidemia, chronic inflammation, increased blood clotting tendency, endothelial dysfunction, and hyperinsulinemia suggesting the early stages of metabolic decompensation, has been already identified in African-American children as young as 5 years of age affected by overweight and obesity [4]. Besides, recently, the collaborative groups of the Global Burden of Disease Study (GBD) reported that the number of global deaths and disability-adjusted life years (DALYs) attributable to high Body Mass Index (BMI) had more than doubled for both females and male between 1990 and 2017. Cardiovascular disease resulted in the leading cause of high-BMI-related DALYs, followed by diabetes and kidney diseases, and neoplasms, together accounting for 89.3% of all high-BMI-related DALYs [5]. Thus obesity represents an urgent issue that needs to be properly addressed. A successful response to the challenge of obesity requires an accurate understanding of its current burden.

1.2. AIMS OF THE Ph.D. PROJECT

The rising prevalence of obesity in the pediatric population represents a major public health concern. Pediatric obesity has forecasted to be an increased financial burden due to its comorbidities [6]. Since onset of the obesity-associated disease is generally subtle and asymptomatic, there is clearly an urgent need of biomarkers that could allow

early detection. Thus, the purpose of this Ph.D. project was, first, to investigate the development of obesity-related complications in obese children and adolescents and, second, to detect precocious biomarkers that could help the clinician identifying "high risk" subjects to an unhealthy metabolic profile (*Chapter 2 – Obesity-related diseases in pediatrics*). With appropriate changes in lifestyle, the progression of co-morbidities associated with obesity can be delayed or prevented, particularly in pediatrics [7]. Although Mediterranean style-diet is recognized as beneficial, with the urbanization of people living in the Mediterranean area, in particular children and adolescents are deviating to a "Western diet" richer in saturated fat, refined grains, simple carbohydrates and processed foods than traditional food habits [8]. This Ph.D. project also aimed to describe the adherence to the Mediterranean Diet and to assess the potential effect of healthy lifestyle choices versus unhealthy behaviours in pediatrics (*Chapter 3 – The Mediterranean style-Diet*). Alongside efforts in public health and policy to reverse the childhood obesity epidemic, medical providers seek to play effective roles in prevention and treatment. Limited interventional studies with effective long-term maintenance of weight loss in children are available in the literature [9]. Applying game design elements to traditionally non-game contexts, which is introducing the gamification concept in nutritional and educational intervention contrasting obesity in children and adolescents represents another aim of this thesis (*Chapter 4 – The Mediterranean style-Diet, microbiota, and obesity in pediatrics*). Therefore, the diet has been shown to modulate inflammation [10] and has a role in shaping gut microbiota composition and function, mainly in the first part of life [11]. Thus, understanding whether different nutritional components can modulate the metabolic responses in pediatric obesity also through microbiota shaping may define novel targets for the prevention of metabolic and cardiovascular diseases from the pediatric age.

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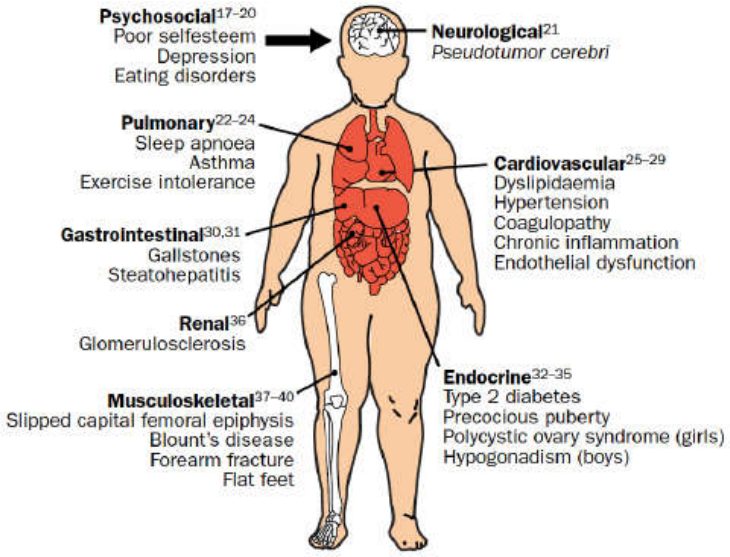
2. OBESITY-RELATED DISEASES IN PEDIATRICS

2.1. INTRODUCTION

Obesity is increasing worldwide in children and adolescents [1]. Moreover, there has been an alarming increase in the degree of obesity, resulting in extreme obesity in youth [2]. This increase in prevalence and degree of obesity has been linked to increased cardiovascular morbidity and mortality [3]. Childhood obesity is a multistystem disease [4] and it is well known that its consequences, such as glucose intolerance, arterial hypertension, and hypercholesterolemia in adulthood increase mortality rates. Also, childhood obesity seems to predict premature death because its early metabolic derangement [5]. If canonical cardiovascular and metabolic risk factors have been investigated in pediatrics, increasing evidence suggests that obesity may also damage the kidney being a risk factor for chronic renal injury in children [6]. Either insulin-resistance and other comorbidities, such as arterial hyperthension, dyslipidemia and dysglycemia, act in a vicious circle [7]. In addition, it has been recently reported that body mass index (BMI) at age 17 years was associated with an increased risk of mortality and morbidity for all cancers (except premenopausal breast and cervical cancer) at age 30 years [8]. However, mechanisms by which these effects are mediated have not been fully understood, suggesting that this issue requires further attention. This is of importance for the identification of precocious biomarkers and the detection of “high risk” profiles associated with obesity during childhood and adolescence.

Figure 2. Complications of pediatric obesity [4].



Aiming to explore the metabolic features, phenotypes, and complications of obesity in pediatric age, the Ph.D. activity was focused on the following topics: glucose metabolism and insulin resistance, metabolic syndrome, cardiovascular risk factors and kidney marker diseases.

The following papers have published in this regard:

- *Insulin resistance, serum uric acid and metabolic syndrome are linked to cardiovascular dysfunction in pediatric obesity*
- *High-normal estimated glomerular filtration rate and hyperuricemia positively correlate with metabolic impairment in pediatric obese patients*
- *Consensus document and recommendations for the prevention of cardiovascular disease in Italy*
- *Diagnosis, treatment and prevention of pediatric obesity: consensus position statement of the Italian Society for Pediatric Endocrinology and Diabetology and the Italian Society of Pediatrics*
- *Children Obesity, Glucose Tolerance, Ghrelin and Prader Willi Syndrome*
- *The relationship between cortisol and IGF-I influences metabolic alteration in pediatric overweight and obesity*
- *Haptoglobin phenotypes are associated with the post-load glucose and insulin levels in pediatric obesity*

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2.2.1. Insulin resistance, serum uric acid and metabolic syndrome are linked to cardiovascular dysfunction in pediatric obesity

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Abstract

Introduction. Childhood obesity is associated with cardiovascular abnormalities but little is known on the potential correlation between early cardiovascular and metabolic alterations.

Aim. Aims of this study were 1) to evaluate early cardiovascular abnormalities in a large population of obese children and adolescents compared with a normal weight counterpart, 2) to investigate their potential association with insulin resistance (IR), serum uric acid (sUA) and metabolic syndrome (MetS).

Methods. This was a single-center case-control study. Eighty obese (OB) subjects (6–16 years) and 20 normal weight (NW) matched controlswere consecutively recruited. In the whole population we performed an anthropometric and a cardiovascular assessment. OB patients also underwent an OGTT and biochemical evaluations.

Results. OB children showed greater left atrial (LA) and ventricular (LV) dimensions and mass and higher carotid artery intima-media thickness (CIMT), compared with NW controls. TheBMI z-score,waist circumference, IR and sUA were positively related with LA and LV dimensions and mass. OB subjects with MetS (46.3%) showed greater LA diameter ($p = 0.001$) and LV area ($p = 0.01$) and volume ($p = 0.04$) compared with OB children without MetS. LA diameter and LV dimensions and mass were significantly dependent on the number of criteria for MetS. Mets, sUA and IR were significant predictors of left heart dimensions and mass in obese children.

Conclusions. Obesity and MetS are associated with abnormal cardiovascular response during childhood. Hyperuricemia can be an earlymarker of cardiovascular dysfunction and the routine determination of circulating levels of sUA should be implemented during risk stratification among pediatric age.

Introduction

Childhood obesity causes a wide range of severe complications, increasing the risk of premature morbidity and mortality [1]. In addition, obese children are more prone to become obese adults, with higher risk of cardiovascular diseases (CVD) [2]. A cluster of CVD risk factors has been identified in children as young as 5 years of age [3] and, among adolescents, their presence correlates with asymptomatic coronary atherosclerosis [4]. Pediatric obesity has been related to an impaired cardiac structure and function as left atrial (LA) and left ventricular (LV) dimensions and mass significantly greater than lean controls [5–8] and impaired diastolic and systolic function [6,9]. Atherogenesis and arterial wall damage begin during childhood and, there is

evolving evidence that clinical indicators of atherosclerosis such as carotid artery intima-media thickness (CIMT), arterial stiffness, and endothelial function are altered in obese children [10,11]. Hyperuricemia has been recognized as a risk factor for CVD in adults being predictive of insulin resistance (IR), metabolic syndrome (MetS) and all its components [12]. However, little is known on the potential association between early cardiovascular alterations and hyperuricemia and MetS in obese children. The detection of a high cardiovascular risk profile associated with pediatric obesity and the identification of possible early markers of cardiovascular damage during childhood would let an early diagnosis of cardiovascular complications allowing prompt preventive therapeutic measures that might reduce cardiovascular morbidity and mortality at long term follow up. Therefore, aims of this study were 1) to evaluate early cardiovascular abnormalities in obese children and adolescents compared with a normal weight counterpart, 2) to investigate their potential association with IR, serum uric acid (sUA) and MetS.

Subjects and Methods

Study design and population

This was a single-center longitudinal case-control study conducted at Division of Pediatrics in Piedmont Region, Italy. We consecutively enrolled 80 Caucasian obese (OB) children and adolescents, aged 6–16 years, and 20 normal weight, age and sex matched controls (NW). OB patients were eligible if they were generally healthy, obese according to the International Obesity Task Force (IOTF) criteria [13], and not on a weight-loss diet. Exclusion criteria were specific causes of endocrine or genetic obesity, type 1 or type 2 diabetes, previous heart, respiratory, liver and kidney diseases, current or past use of hormonal or interfering therapies (lipid-lowering, hypoglycemic, or antihypertensive treatments). Control subjects were eligible if they were normal weight [13], with no history of organic or psychiatric diseases. The protocol was conducted in accordance with the declaration of Helsinki and was approved by the Local Ethic Committee (CE 95/12). Informed written consent was obtained from all subjects' parents.

Anthropometric and biochemical variables

Height, weight, waist circumference (WC), hip circumference (HC), systolic (SBP) and diastolic (DBP) blood pressure were measured as previously described [14] and bodymass index (BMI) was calculated. Pubertal stages were evaluated according to

Tanner criteria. In the OB group, after a 12-h overnight fast, blood samples were taken for measurement of: glucose (mg/dL), insulin (μUI/mL), total cholesterol (mg/dL), high density lipoproteincholesterol (HDL-c, mg/dL), triglycerides (mg/dL), sUA (mg/dL), using standardized methods in the Hospital's Laboratory [14]. Low density lipoprotein-cholesterol (LDL-c) was calculated by the Friedwald formula. sUA (mg/dL) was measured by Fossati method reaction using uricase with a Trinder-like endpoint. Obese subjects also underwent an OGTT (1.75 g of glucose solution per kg, maximum 75 g) and samples were drawn for the determination of glucose and insulin every 30 min. Insulin-resistance was calculated using the formula of homeostasis model assessment (HOMA)-IR. Insulin sensitivity at fasting and during OGTT was calculated as the formula of the Quantitative Insulin- Sensitivity Check Index (QUICKI) and Matsuda index (ISI) [28]. Glucose was expressed in mg/dL (1 mg/dL = 0.05551 mmol/L) and insulin in μUI/mL (1 μUI/mL = 7.175 pmol/L) in each formula [15]. Impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) were defined according to American Diabetes Association [16] and MetS by using the modified National Cholesterol Education Program/Adult Treatment Panel III (NCEP-ATP III) criteria of Cruz and Goran [17].

Echocardiographic assessment

Transthoracic echocardiogram using a Vivid 7 Pro ultrasound scanner (General Electric Healthcare, USA) was performed by a sonographer and the images were reviewed by an expert pediatric cardiologist, blinded to patients' clinical data. Measurements of left ventricle (LV end-diastolic diameter, LVEDD; LV end-systolic diameter, LVESD; interventricular septum at end diastole, IVSD; LV posterior wall at end diastole, LVPWD), relative wall thickness (RWT), left atrium diameter (LAD), the maximum LA volume, and LV ejection fraction were obtained according to established standards [18]. LV mass (LVM) was derived from the Devereux formula and indexed to body surface area (left ventricular mass index [LVMI]) [27]. Using pulsed wave Doppler, mitral inflow velocities, peak early diastolic velocity (E), peak late diastolic velocity (A), E/A ratio, were measured. Pulsed wave tissue Doppler of the lateralmitral annulus was used for themeasurement ofearly peak diastolic mitral annular velocity (E'). The E/E' ratio was calculated.

Vascular assessment

Vascular measurements were performed with a high-resolution ultrasonography (Esaote MyLab25™Gold, Esaote, Italy) using a 8 mHz linear transducer and a 5 mHz

convex transducer for the abdominal aorta, by an expert sonographer and images were then reviewed offline by an expert vascular surgeon blinded to patients' clinical status. CIMT, abdominal aortic diameter at maximum systolic expansion (Ds) and minimum diastolic expansion (Dd), brachial artery diameters, brachial artery peak systolic velocity (PSV) and end diastolic velocity (EDV) were measured as previously described and aortic strain (S), pressure strain elasticmodulus (Ep), pressure strain normalized by diastolic pressure (Ep*) and brachial artery flow-mediated dilation (FMD) were calculated. While S is the mean strain of the aortic wall, Ep and Ep* are the mean stiffness [19]. The brachial artery maximum diameter recorded following reactive hyperemia was reported as a percentage change of resting diameter (FMD = peak diameter – baseline diameter/baseline diameter) [20].

Statistical analysis

All data are expressed as mean ± standard deviation (SD), absolute values or percentages. A sample of 15 individuals has been estimated to be sufficient to demonstrate a difference of 10% in LV diameter with a SD of 0.44 cm with 90% power and a significance level of 95% in the Student t-test between obese and controls according to published data [6]. A cohort of 75 obese subjects has been estimated to be sufficient to demonstrate differences among numbers of MetS criteria (0–5 criteria according to NCEPATPIII classification) [17]. Distributions of continuous variables were examined for skewness and were logarithmically transformed as appropriate. ANCOVAwas used to determine the differences between obese and control subjects. Covariates were sex, age and pubertal stage. Correlation of cardiovascular parameters with continuous values of BMI zscore, WC, HOMA-IR, ISI, and sUA were examined using Pearson correlation coefficients. Partial correlation was used to correct for covariates. The stepwise regression model with two-tailed probability values and 95% confidence intervals was used to measure the strength of the association between cardiovascular variables and BMI zscore and each specific MetS criteria (abdominal obesity, hypertension, hypertrygliceridemia, low HDL-c, glucose intolerance: model 1) and other metabolic impairments (Model 2: model 1 + sUA; Model 3: model 2 + HOMA-IR). Statistical significance was assumed at $p<0.05$. The statistical analysis was performed with SPSS for Windows version 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Anthropometric and cardiovascular characteristics

Table 1 shows the auxological and cardiovascular data of the 80 OB (age 11.2 ± 2.7 yrs., 47.5% females, 36.3% prepubertal, BMI zscore 2.32 ± 0.51) and 20 age-, sex- and pubertal status-matched NW subjects (age 10.9 ± 2.5 yrs., 60.0% females, 45.0% prepubertal, BMI z-score−0.76±0.98). After adjusting for age, sex and puberty, OB children showed greater LV dimensions (LVEDD $p<0.02$, LVESD $p<0.02$, IVSD $p<0.0001$, LVPWD $p<0.001$), area ($p<0.0001$), volume ($p<0.002$) and LVM ($p<0.0001$), LAD ($p<0.005$) and lower E/A ratio ($p<0.005$) compared with NW subjects. No differences were found in the E/E' ratio, LV systolic function, LVMI and RWT. OB patients had larger abdominal aortic ($p<0.03$) and brachial artery ($p<0.006$) diameters compared with NW controls. CIMT was significantly higher in the OB group ($p<0.0001$) while no difference between the two groups was shown in aortic strain (S) and stiffness (Ep, Ep*) and in FMD of the brachial artery.

Table 1. Clinical and cardiovascular data of 80 obese (OB) and 20 normal weight (NW) subjects.

	OB	NW	p Value
Subjects	80	20	
Age (years)	11.2 ± 2.7	10.9 ± 2.5	ns
Female	38 (47.5%)	12 (60.0%)	ns
Prepubertal	29 (36.3%)	9 (45.0%)	ns
Height (cm)	149.6 ± 15.1	145.3 ± 15.3	**0.0001**
Height (SDS)	1.11 ± 1.21	0.65 ± 1.11	**0.03**
Weight (kg)	69.3 ± 22.6	36.5 ± 10.3	**0.0001**
BMI (kg/m^2)	30.0 ± 5.2	17.0 ± 2.4	**0.0001**
BMI z-score	2.32 ± 0.51	−0.76 ± 0.98	**0.0001**
Waist (cm)	90.2 ± 14.3	60.9 ± 6.6	**0.0001**
Hip (cm)	96.4 ± 15.0	70.4 ± 9.3	**0.0001**
N° WC ≥ 90° p	79 (98.7%)	4 (20.0%)	**0.001**
SBP (mm Hg)	123.7 ± 16.6	105.8 ± 16.1	**0.0001**
DBP (mm Hg)	78.2 ± 10.8	67.1 ± 8.8	**0.0001**
N° SBP or DBP ≥ 90° p	68 (78.7%)	6 (30%)	**0.001**
HR (b/min)	84.2 ± 12.1	78.1 ± 11.4	ns
EF (%)	69.5 ± 8.4	70.8 ± 6.1	ns
FS (%)	40.3 ± 6.7	40.2 ± 5.5	ns
LVEDD (mm)	44.5 ± 0.6	41.2 ± 0.5	**0.02**
LVESD (mm)	27.4 ± 0.5	24.6 ± 1.2	**0.02**
IVSD (mm)	7.6 ± 0.2	5.9 ± 0.4	**0.0001**
LVPWD (mm)	7.8 ± 0.2	6.1 ± 0.5	**0.001**
LAD (mm)	32.0 ± 0.6	27.8 ± 1.3	**0.005**
LV area (cm^2)	25.8 ± 0.6	19.9 ± 1.4	**0.0001**
LV volume (mL)	78.6 ± 3.0	56.3 ± 6.3	**0.002**
LA area (cm^2)	13.1 ± 3.7	12.1 ± 3.6	ns
LA volume (mL)	31.2 ± 12.4	29.4 ± 10.9	ns
LV mass (g)	113.3 ± 6.0	74.3 ± 13.4	**0.0001**
LV mass index (g/m^2)	66.9 ± 22.9	62.5 ± 24.2	ns
RWT	0.35 ± 0.08	0.31 ± 0.06	ns
Mitral E peak (cm/s)	102.0 ± 20.1	99.2 ± 10.3	ns
Mitral A peak (cm/s)	58.2 ± 14.1	50.1 ± 11.2	**0.03**
Mitral E/A ratio	1.8 ± 0.1	2.1 ± 0.1	**0.005**
Mitral lateral E' velocity (cm/s)	19.6 ± 3.2	19.2 ± 2.1	ns
Mitral lateral E/E' ratio	5.2 ± 0.9	5.1 ± 0.7	ns
CIMT (mm)	0.63 ± 0.02	0.46 ± 0.04	**0.0001**
AoDs (mm)	12.8 ± 0.2	11.4 ± 0.4	**0.006**
AoDd (mm)	10.3 ± 0.2	9.3 ± 0.4	**0.03**
S	0.23 ± 0.12	0.25 ± 0.14	ns
Ep (mm Hg)	459 ± 1170	302 ± 681	ns
Ep*	8.3 ± 24.1	4.0 ± 9.2	ns
BAD basal (mm)	3.5 ± 0.1	3.0 ± 0.1	**0.006**
BAD after (mm)	3.5 ± 0.1	3.2 ± 0.2	**0.04**
FMD (%)	3.5 ± 15.6	7.8 ± 13.6	ns
PSV basal (cm/s)	67.2 ± 26.0	71.3 ± 18.9	ns
EDV basal (cm/s)	11.3 ± 6.7	9.3 ± 7.6	ns
PSV immediately after (cm/s)	81.7 ± 27.6	89.2 ± 19.3	ns
EDV immediately after (cm/s)	25.1 ± 10.1	26.5 ± 8.7	ns
PSV after (cm/s)	72.4 ± 27.1	74.6 ± 17.1	ns
EDV afer (cm/s)	13.5 ± 8.0	11.3 ± 6.9	ns

Values are number (%) or means ± SD.

Legend: A, peak velocity of late diastolic transmitral wave; after, 2 min after the release of the pneumatic cuff; AoDd, abdominal aortic diastolic diameter; AoDs, abdominal aortic systolic diameter; BAD brachial artery diameter; BMI, body mass index; CIMT, carotid intima media thickness; DBP, diastolic blood pressure; E, peak velocity of early diastolic transmitral wave; E', peak early diastolic velocity on mitral annulus; EDV, brachial artery end-diastolic velocity; EF, LV ejection fraction; Ep, pressure strain elastic modulus; Ep*, pressure strain normalized for DBP; FMD, brachial artery flow-mediated dilation; FS, LV fractional shortening; HR, heart rate; immediately after, the release of the pneumatic cuff; IVSD, interventricular septum diastolic dimension; LA, left atrium; LAD, LA end-systolic diameter; LV, left ventricle; LVEDD, LV end-diastolic dimension, LVESD, LV end-systolic dimension; LVPW, LV posterior wall diastolic dimension; N°, number of subjects; ns, not significant; p, percentile; PSV, brachial artery peak systolic velocity; RWT, relative wall thickness; S, aortic strain; SBP, systolic blood pressure; SDS, standard deviation score; WC, waist circumference.

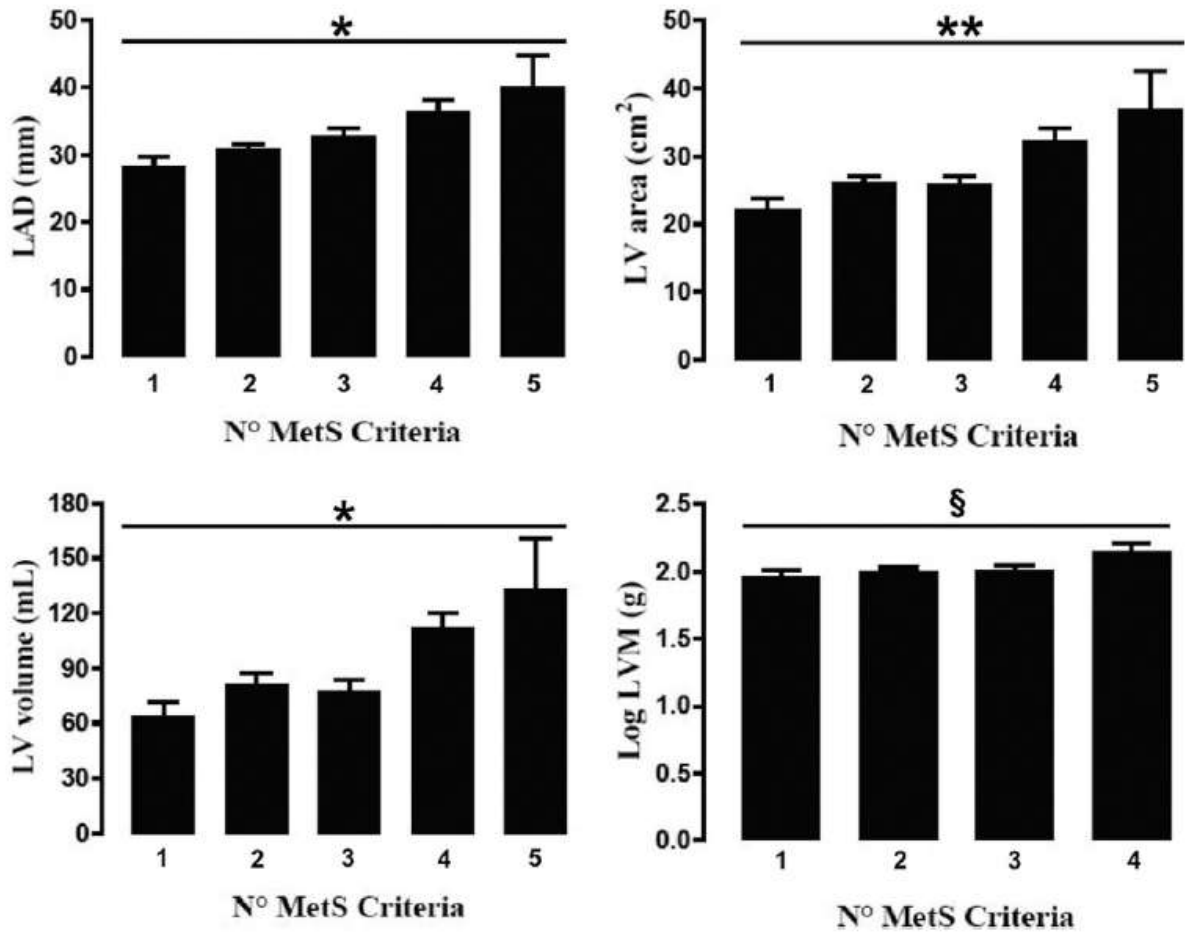
Associations between cardiovascular measurements and metabolic parameters

In the whole population, after adjusting for age, sex and pubertal status, both BMI z-score and WC were positively associated with LV dimensions (LVEDD, LVESD, IVSD, LVPWD), area and volume, LVM, LVM index, RWT, LAD, CIMT, abdominal aortic and brachial artery diameters and negatively relatedwith the E/A ratio. A negative relationship was found between BMI z-score and aortic stiffness. After adjusting for age, sex and puberty, insulin-resistance at fasting was positively correlated with LV dimensions (LVEDD, IVSD, LVPWD), area and volume, LVM and LVMI, RWT, LAD, LA area and volume, abdominal aortic diameter and negatively with the E/A ratio while insulin sensitivity during OGTT was negatively related with IVSD, LVPWD, LVM, RWT, LAD and abdominal aortic diameter. sUA was directly associated with LVEDD, LVESD, LVM, LV area and volume, brachial artery diameters and negatively with the E/A ratio, even after adjustment for confounding factors (Table S1). Associations between cardiovascular measurements and SBP and DBP are shown in Table S2 in the supplementary appendix. As regards lipid profile, after adjustment for confounding factors, total cholesterol was positively correlated with LVPWD and RWT; HDL-c was negatively related with LAD; LDL-c was directly associated with CIMT and triglycerides were positively correlated with LAD. Total cholesterol, LDL-c and triglycerides were negatively related with the E/A ratio.

Impact of MetS and sUA

Out of OB children, 37 (46.3%) matched the NCEP ATPIII criteria for MetS. Patients with MetS had greater LAD (p=0.001) and LV area (p=0.01) and volume (p=0.04) compared with OB subjects without MetS. LAD and LV area, volume and LVM were significantly dependent on the number of criteria of MetS. Increasing the number of matched criteria for MetS, LAD, LV area, volume and LVM significantly increased (Fig. 1). This correlation persisted after adjustment for HOMAIR for LAD, LV area and volume while was lost for LVM.

Figure 1. MetS criteria-dependent left atrium diameter (LAD) (mm), left ventricle (LV) area (cm2), LV volume (mL) and LV mass (LVM) (g) in 80 obese children and adolescents. Log transformation was used for skewed variables (LVM). *p<0.001; ** p<0.0001; §p<0.04. Significance was maintained when the model included homoeostasis model assessment of insulin resistance (HOMA-IR) as covariates for LAD, LV area and volume while was lost for LVM.



Stepwise multiple regression analysis showed that BMI z-score, HDL-c ≤10th percentile and triglycerides ≥90th percentile were independent predictors of LAD (model 1 and 2). In model 3, only BMI z-score and HDL-c in addition to HOMA-IR were confirmed as

significant predictors of increased LAD. LV area was predicted by BP ≥90th percentile and IGT (model 1). In model 2, only sUA and BP were significant predictors. In model 3, HOMA-IR and BP were found as significant predictors. LV volume was predicted by IGT and HDL-c ≤10th percentile (model 1). In model 2 and 3, sUA and HOMA-IR were the only significant predictors. Finally, LVM was predicted by BMI z-score and triglycerides ≥90th percentile. In model 2 and 3, BMI z-score, sUA, HOMA-IR and triglycerides were the only significant predictors (Table 2).

Table 2. Stepwise multiple regression analysis of BMI z-score, metabolic syndrome (MetS) components, serum uric acid (sUA) and insulin resistance (HOMA-IR) (as independent variables) on left atrium diameter (LAD), left ventricular (LV) area and volume and Log LV mass (dependent variables).

Dependent variable	Significant effects	B (95% CI)	β	p-Value
Model 1				
LAD (mm)	BMI z-score	2.286 (1.070;3.501)	0.375	<0.0001
	HDL-c ≤ 10th p	0.255 (0.036;0.473)	0.229	<0.02
	Triglycerides ≥ 90th p	0.278 (0.023;0.534)	0.218	<0.03
R:0.568				
Model 2 (sUA)				
LAD (mm)	BMI z-score	2.286 (1.070;3.501)	0.375	<0.0001
	HDL-c ≤ 10th p	0.255 (0.036;0.473)	0.229	<0.02
	Triglycerides ≥ 90th p	0.278 (0.023;0.534)	0.218	<0.03
R:0.568				
Model 3 (HOMA-IR)				
LAD (mm)	BMI z-score	2.081 (0.828;3.333)	0.341	<0.001
	HDL-c ≤ 10th p	0.275 (0.059;0.490)	0.247	<0.01
	HOMA-IR	0.042 (0.006;0.077)	0.240	<0.02
R:0.574				
Model 1				
LV area (cm^2)	BP ≥ 90th p	4.395 (1.137;7.653)	0.290	<0.009
	IGT	7.016 (1.548;12.484)	0.275	<0.01
R:0.428				
Model 2 (sUA)				
LV area (cm^2)	sUA	1.900 (0.928;2.873)	0.402	<0.0001
	BP ≥ 90th p	3.761 (0.636;6.886)	0.248	<0.02
R:0.513				
Model 3 (HOMA-IR)				
LV area (cm^2)	HOMA-IR	0.633 (0.207;1.060)	0.316	<0.004
	BP ≥ 90th p	4.150 (0.920;7.379)	0.273	<0.01
R:0.453				
Model 1				
LV volume (mL)	IGT	32.633 (5.271;59.995)	0.266	<0.02
	HDL-c ≤ 10th p	14.123 (0.140;28.105)	0.225	<0.05
R:0.365				
Model 2 (sUA)				
LV volume (mL)	sUA	10.284 (5.443;15.125)	0.449	<0.0001
R:0.449				
Model 3 (HOMA-IR)				
LV volume (mL)	sUA	8.177 (3.073;13.281)	0.357	<0.002
	HOMA-IR	2.370 (0.189;4.551)	0.242	<0.03
R:0.502				
Model 1				
Log LV mass (g)	BMI z-score	1.003 (0.615;1.390)	0.495	<0.0001
	Triglycerides ≥ 90th p	0.105 (0.023;0.187)	0.245	<0.01
R:0.598				
Model 2 (sUA)				
Log LV mass (g)	BMI z-score	0.765 (0.356;1.174)	0.378	<0.0001
	sUA	0.034 (0.009;0.058)	0.276	<0.007
	Triglycerides ≥ 90th p	0.100 (0.021;0.178)	0.233	<0.01
R:0.647				
Model 3 (HOMA-IR)				
Log LV mass (g)	BMI z-score	0.687 (0.279;1.095)	0.339	<0.001
	sUA	0.029 (0.004;0.053)	0.235	<0.02
	HOMA-IR	0.012 (0.001;0.023)	0.198	<0.04
	Triglycerides ≥ 90th p	0.080 (0.001;0.159)	0.186	<0.05
R:0.671				

Legend: BP, blood pressure; CI, confidence interval; HDL-c, HDL-cholesterol; IGT, impaired glucose tolerance; p, percentile. *Model 1*: independent variables were BMI z-score and MetS components, (waist circumference ≥90th percentile; BP ≥90th

percentile; triglycerides ≥90th percentile; HDL-c ≤10th percentile; impaired fasting glucose; impaired glucose tolerance).

Model 2: independent variables were those of Model 1 plus sUA.

Model 3: independent variables were those of Model 2 plus HOMA-IR.

Discussion

In this study, we found that pediatric obesity negatively affected cardiac remodelling and impaired vascular structure. Furthermore, IR and sUA and the presence of MetS might play an additional adverse role during heart remodelling among children and adolescents.

Cardiovascular alterations in pediatric obesity

Our study showed marked variations in the LA and LV dimensions in OB children compared to NW. These findings are in line with some previous studies reporting that LA and LV dimensions are significantly greater in OB children compared to NW [6,8,10,21]. Several investigations have also reported greater LVM in OB children [5,6,8,10,22], and this has been detected as early as 2 years of age [22]. Interestingly, we found no differences when normalizing LVM to body surface area between the two groups [9,21] possibly related to the overcorrection of measurements (normalized for parameters including weight) required for the obese group. Moreover, hypertrophy and dilatation of the LV might represent a physiological response to the enhanced preload and afterload dependent to the increased BMI. Regarding heart function, we found a significant reduction in the E/A ratio in the OB group (mainly related to the increased inflow velocities during atrial contraction/A-velocities) and no significant changes in E'-velocities or in the E/E'-ratio with a preserved global LV systolic function. Despite the well-known association between diastolic dysfunction and subsequent impaired systolic functional changes in the adulthood, this correlation is still conflicting when applied in pediatric subjects. The majority of studies, showed no difference between obese and normal weight children in the peak early mitral inflow velocity (E) and in the early diastolic mitral velocity assessed with PWTDI (E') as parameters for diastolic function [7,9,21]. According with our data, the lack of significant changes in early filling characteristics suggests an increased dependency of atrial filling related to a decreased LV early relaxation [9,21]. In line with this, we also found increased systolic and diastolic abdominal aortic and brachial artery diameters in the OB group, which might represent an early marker of vascular remodelling. In fact, the assessment of CIMT is a sensitive clinical marker of atherosclerosis, predictive of CV morbidity and

mortality in the adulthood and high-risk population. Interestingly, we found greater CIMT in obese patients, even in younger children, not related to the pubertal status and mainly due to the exposure to cardiovascular risk factors.

Potential mechanisms linking obesity to cardiovascular dysfunction

The excess of adipose tissue, enhanced metabolic activity and the subsequent increased preload, predispose to LA and LV dilatation, LV remodelling and hypertrophy as a compensatory mechanism for systolic and diastolicwall stress [23]. Obesity is associated with changes in the vascular system related to the development of early atherosclerosis, arterial hypertension and increase afterload. In this study the BMI z-score, waist circumference and systemic blood pressure are positively correlated with increasing CIMT. Surprisingly, we found an inverse relationship between the BMI z-score and arterial stiffness that might reflect an early compensatory mechanism depicted by the arteries that try to contrast the afterload via structural changes (increased diameter and thickness) before the rise in stiffness. According to previous data, in our obese population, the HOMA-IR correlates with increasing heart and aortic dimensions and decreasing diastolic function while no association was found between IR and CIMT and arterial stiffness [24]. IR can potentially induce to a decreased myocardial glucose uptake and increased fatty acid oxidation resulting in the accumulation of toxic intermediates of fatty acid metabolism that finally lead to myocardial dysfunction and arteries enlargement. Furthermore, compensatory hyperinsulinemia induces LV hypertrophy through binding of insulin to cardiac insulin-like growth factor 1 receptors [25]. In our OB group, MetS was associated with greater heart dimension and mass with straightforward linear raises when increasing the number of matched criteria for MetS. Subjects who met the MetS criteria, presented worst metabolic parameters (dyslipidemia, dysglycemia) with higher prevalence of hypertension and altered BMI with a significant impact on cardiac structural alterations. Our data highlight the influence ofMetS also in the pediatric subset, suggesting the usefulness of an early MetS investigation implemented with prompt cardiovascular imaging examination and aggressive therapeutic strategy in the obese children in order to prevent future cardiovascular dysfunction. Another interesting finding of the current study is the association between sUA and cardiovascular adverse remodelling. The association between sUA and levels of individual MetS components and their clustering has been previously reported [25–27]. The Bogalusa Heart Study showed that elevated sUA plays a crucial role in the pathogenesis of MetS with an influence that begins

during adolescence, suggesting that sUA may aid in the early identification and treatment of high risk individuals for MetS [27]. Indeed, in a group of 299 overweight/obese children aged 8–18 years from the STYJOBS/EDECTA cohort, sUA was the best predictor of unhealthy obesity [28]. Furthermore, recent investigations, has suggested that sUA level is an independent predictor of hypertension in adulthood [12]. In addition, Viazzi et al. showed that sUA was directly related to SBP and DBP independently of puberty, gender, BMI z-score, HOMA and renal function in a cohort of children and adolescents [29]. These data were confirmed by Feig et al. that showed a correlation between the reduction of sUA and normalization of blood pressure in 66% of hyperuricemic adolescents with hypertension, as compared to 3% in controls [30]. Finally, a reduction in sUA improved body weight and related CVD risk factors in young patients with hyperuricemia [31]. Moreover, sUA is an independent risk factor for heath failure and is associated with increased cardiovascular mortality in adults and children [12]. Out of our knowledge, this is the first study that demonstrates an association between sUA and LV volume and mass that is independent of BMI z-score, MetS and insulin resistance in a large cohort of obese pediatric patients. Studies conducted in adult populations suggest that hyperuricemia is associated with cardiac remodelling and LV hypertrophy and that sUA could be a marker of subclinical myocardial dysfunction [32], but this data were not fully confirmed during childhood. Reschke et al. showed, in a population of hypertensive children of which 21 overweight/obese and not evaluated for the presence of MetS, that 1 mg/dL increase in sUA over the baseline value was associated with an increase in LVM of 20.2 g. However, this association was no longer significant after adjustment for confounding factors [33]. Increased sUA levels may contribute to the echocardiographic abnormalities associated with obesity through effects on endothelial dysfunction and inflammation. Although uric acid seems to have antioxidant activity in the extracellular environment, once entered the cells, including vascular and heart muscle cells and adipocytes, might promote several detrimental effects. Injurious impacts of sUA include intracellular reactive oxygen species production, an inhibitory effect on nitric oxide formation, induction of platelet aggregation, and pro-inflammatory activity [12], also in children [34]. Furthermore, sUA activates the renin-angiotensin system causing hypertension. The renin-angiotensin system, in turn, has been proposed to cause LV hypertrophy and cardiac fibrosis though mechanisms including BP increase, direct action of angiotensin II on cardiac myocytes, and effects of aldosterone [32]. As suggested by Borghi and Cicero, sUA requires more attention in the evaluation of the metabolic risk profile of obese children

and adolescents [35] and, based on our findings, it could be an early marker of cardiovascular dysfunction in this population allowing a prompt and effective preventive measures.

Study limitations

Our study has several potential limitations. The major limit is the relatively small size of the population. Moreover, our study was not a randomized controlled trial. We perform a prospective data collection, which is certainly susceptible to selection bias. Finally a more extensive use of vascular imaging modalities including speckle tracking echocardiography and cardiac MRI would have certainly improved the results of the current study.

Conclusion

In conclusion, this study shows that obesity andMetS are associated with abnormal cardiovascular response during childhood. Moreover, hyperuricemia can be an early marker of cardiovascular dysfunction and the routine determination of circulating levels of sUA should be implemented during risk stratification among children and adolescents. Further larger studies and randomized trials are warranted to confirm our findings.

2.2.1.i. References

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2.2.2. Higher normal eGFR and hyperuricemia are related to metabolic impairment in pediatric obesity

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Abstract

Background. Childhood obesity represents a major health concern worldwide due to its well established detrimental effect on cardiovascular and its potential negative effect on kidney functions. However, biomarkers that can help diagnose early stages of kidney damage in obese children represent an unmet clinical need.

Objectives. In this study, we asked whether the prevalence of microalbuminuria, estimated glomerular filtration rate (eGFR) or hyperuricemia recorded in a wide cohort of obese children and adolescents would positively correlate with cardiometabolic dysfunction in these subjects.

Methods. We carried out a cross-sectional study on 360 obese children and adolescents between the ages of 3–18 years, enrolled in a tertiary care center. Clinical and biochemical evaluations including oral glucose tolerance tests (OGTTs) were performed on all patients. Microalbuminuria was defined as urinary albumin-to-creatinine ratio (u-ACR) of 30–300 mg/g. All data are expressed as mean ± standard deviation (SD), absolute values or percentages. Sex age-specific and eGFR SDs were used for statistical analyses. Serum uric acid ≥5.5 mg/dL was considered abnormal.

Results. The prevalence of microalbuminuria was 6.4%. Except for a lower insulinogenicindex, no correlations between microalbuminuria and cardiometabolic risk factors were detected. eGFR was <-1 SD and >1 SD in 1.4% and 60.8% of subjects, respectively. Subjects with an eGFR >1 SD had higher systolic blood pressure, liver enzymes, insulin resistance, glucose and insulin during OGTT, lower insulin sensitivity and a more prevalent microalbuminuria. Hyperuricemia (27.5%) increased the odds of hypertension, HDL ≤10th percentile and glucose ≥155.0 mg/dL after 60 minutes of OGTT.

Conclusions. A worse cardiometabolic profile was observed in subjects with an eGFR >1 SD compared to other subgroups. Therefore, pediatric obese patients with eGFR >1 SD or hyperuricemia should be closely monitored for microalbuminuria and post-challenge glucose and insulin secretion, all potential indicators of renal dysfunction in these young patients.

Introduction

The rising prevalence of pediatric obesity is one of the most challenging public health issues worldwide. Most of the major concerns derive from the fact that childhood obesity, besides increasing traditional cardiometabolic risk factors, can seriously hamper kidney function [1]. In this regard, the recent global obesity epidemic has

coincided with a dramatic rise in the prevalence of end-stage renal disease (ESRD) [1]. To make matters worse, emerging evidence suggests that renal dysfunction may start early during childhood, long before a diagnosis of hypertension with type 2 diabetes can be made, and it might be related to pediatric obesity [1]. Since onset of the obesity-associated renal disease is generally subtle and asymptomatic, there is clearly an urgent need of biomarkers that could allow early detection of kidney dysfunction in obese children. In this regard, mounting evidence indicates that in adults the prevalence of microalbuminuria, which is often associated with nephropathy and atherosclerosis, positively correlates with the degree of obesity [2,3]. Furthermore, a relationship between microalbuminuria and obesity has also been reported in children and adolescents [1], although long-term studies in these patients have yet to be conducted. Interestingly, the association between estimated glomerular filtration rate (eGFR) and some cardiometabolic risk factors appears non-linear as either low- or high-normal eGFR has been associated with increased risk of metabolic diseases and mortality [2]. However, whether eGFR represents a *bona fide* cardiometabolic risk indicator, especially in pediatric patients, still remains to be determined [2,4]. In addition to microalbuminuria, hyperuricemia is another well-established risk factor for chronic kidney disease (CDK) in adults [5]. This is probably due to the detrimental effects exerted by uric acid once it permeates a cell, which obviously counteracts its antioxidant activity in the extracellular environment [6]. Therefore, hyperuricemia has a negative impact on both metabolism and longevity independent of traditional cardiometabolic risk factors [5]. However, data concerning hyperuricemia in obese children and adolescents are still lacking. Here, we have conducted a cross-sectional study on a wide cohort of obese pediatric patients to determine 1) the prevalence of microalbuminuria; 2) the distribution of age- and sex-specific eGFRs; 3) the prevalence of hyperuricemia; and 4) any correlations between microalbuminuria, eGFR and uric acid and other known cardiometabolic markers.

Materials and Metods

Study design

This was a cross-sectional study. Study quality was assessed. We consecutively recruited Caucasian children and adolescents, aged 3–18 years, referred to our Pediatric Endocrine Service from January 2011 to June 2014 for simple obesity. Subjects were eligible if generally healthy, overweight or obese, according to the IOTF criteria [7], and naïve to a weight-loss diet. Among children that had been previously

subject to biochemical investigations due to any medical condition, only healthy children were included in the study. Subjects who refused to perform an oral glucose tolerance test (OGTT) were included only if they underwent fasting biochemical evaluations. Exclusion criteria were diagnosed or suspected endocrine or genetic obesity, type 1 diabetes and previous kidney diseases. Subjects referred to our Service for known comorbidities of obesity (e.g. glucose alterations, arterial hypertension, dyslipidemia, liver steatosis, hyperuricemia, etc.) were also excluded to avoid interferences due to previous lifestyle or pharmacological interventions. The protocol was conducted in accordance with the declaration of Helsinki and was approved by the Local Ethic Committee of AOU Maggiore della Carità of Novara (CE95/12). Informed consents was administered to all patients and parents of minors prior to the evaluations, and the study was carefully explained by the research team to all parents and children. Only those patients who provided a written informed consent were included in the study.

Anthropometric and biochemical measurements

Height was measured to the nearest 0.1 cm using a Harpenden stadiometer. Body weight was measured with light clothing to the nearest 0.1 kg using a mechanical column weighing scale (Wunder, weighing capacity 200 Kg). Body mass index (BMI) was calculated as body weight divided by squared height (kg/m2). The BMI standard deviation score (BMI-SDS) was calculated by the least median squares (LMS) method as previously described [8]. Waist circumference (WC) was measured at the high point of the iliac crest around the abdomen and was recorded to the nearest 0.1 cm. A non-elastic flexible tape was used, with the subjects being kept in a standing position with minimal respiration and no clothing covering the waist area or compressions on the skin. The waist-to-height ratio was calculated as well. Pubertal stages were determined by physical examination, using the criteria of Marshall and Tanner. Systolic BP (SBP) and diastolic BP (DBP) were measured three times at 2-minute intervals using a mercury sphygmomanometer with an appropriate cuff size after participants had been sitting quietly for at least 15 minutes, with their right arm being supported at the level of the heart, and feet resting flat on the floor, prior to other physical evaluations and at least 30 minutes after blood sampling. Mean values were used for all these analyses. Hypertension was determined only if BP values recorded at enrollment and testing day were both found elevated. After a 12-hour overnight fast, blood samples were taken for measurement of: glucose (mg/dL), insulin (µUI/mL), total cholesterol (mg/dL), high

density lipoprotein (HDL)-cholesterol (mg/dL), triglycerides (mg/dL), aspartate aminotransferase (AST, IU/L), alanine aminotransferase (ALT, IU/L), uric acid (mg/dL), creatinine (mg/dL), IGF1 (ng/mL), 25-hydroxy (OH) vitamin D (ng/mL), using standardized methods in the Hospital's Laboratory [9]. Low-density lipoprotein (LDL)-cholesterol was calculated by the Friedwald formula. AST-to-ALT ratio was calculated. Uric acid (mg/dL) was measured by the Fossati method using uricase with a Trinder-like endpoint. Serum creatinine concentration (mg/dL) was measured by the enzymatic method. Glucose was determined by the hexokinase method (Slein Method, Advia 1200/1800/2400 Autoanalyzer; Bayer Diagnostics, Leverkusen, Germany) with an intra-assay coefficient of variation of 0.7–2.3% (range 0.0 mg/dL-700.0 mg/dL). Insulin was determined by an immunoassay method (Advia Centaur1; Bayer Diagnostics, Leverkusen, Germany) with an intra- and inter-assay coefficient of variation of 3.2–4.6% and 2.6–5.9%, respectively (range 0.5 mU/L-300.0 mU/L). Urine albumin (mg/L) was determined by an advanced immunoturbidimetric assay, and urine creatinine (mg/dL) was measured using the enzymatic method. Subjects also underwent OGTT (1.75 g of glucose solution per kg, maximum 75 g), and samples were drawn for the determination of glucose and insulin every 30 minutes. The area under the curve (AUC) was calculated according to the trapezoidal rule. Insulin resistance was calculated using the formula for homeostasis model assessment (HOMA)-IR. Insulin sensitivity at fasting and during OGTT was calculated with the formula of the Quantitative Insulin-Sensitivity Check Index (QUICKI) and Matsuda index (ISI). Insulinogenic (InsI) and disposition (DI) indexes were also calculated as previously reported [10]. The stimulus for insulin secretion in the increment in plasma glucose as the insulinogenic index was calculated as the ratio of the changes in insulin and glucose concentration from 0 to 30 minutes (InsI). Beta-cell compensatory capacity was evaluated by the disposition index defined as the product of the ISI and InsI (DI) [11]. Glucose was expressed in mg/dL (1 mg/dL = 0.05551 mmol/L) and insulin in μUI/mL (1 μUI/mL = 7.175 pmol/L) in each formula.

Definitions

Subjects were classified as overweight or obese according to age- and sex-specific IOTF cut-offs [7]. WC percentiles were stratified according to sex and age, identifying abdominal obesity as the presence of WC ≥90th percentile or a waist-to-height ratio of 0.5 [10]. SBP and DBP values were evaluated according to percentiles for age, sex and height, and arterial hypertension was defined as SBP or DBP >95th percentile.

Triglycerides, LDL- and HDL-cholesterol percentiles for age and sex were classified according to the Lipid Research Clinic Pediatric Prevalence Study. Dyslipidemia was defined as the presence of triglycerides ≥90th percentile, HDL- cholesterol ≤10th percentile or LDL ≥90th percentile. Impaired fasting glucose and impaired glucose tolerance were defined as fasting plasma glucose ≥100–125 mg/dL (5.6 to 6.9 mmol/ L) and 2-hour post-OGTT, glucose ≥140–199 mg/dL (7.8 to 11.0 mmol/L), respectively. Uric acid ≥5.5 mg/dL was considered abnormal [12]. According to the NKF-K/DOQI Guidelines for chronic kidney disease (CKD) in children and adolescents [13], the eGFR was calculated using the modified Schwartz's formula [14]: eGFR (mL/min/1.73 m2) = [0.413 x patient's height (cm)] / serum creatinine (mg/dL). The normal renal function of patients [mean eGFR ± stan- dard deviation (SD) in mL/min/1.73 m2] was calculated based on age and gender according to NKF-K/DOQI Guidelines [13]. 2–12 year-old males and females: 133±27 mL/min/1.73 m2; 13– 21 yearold males: 140±30 mL/min/1.73m2; and 13–21 year-old females: 126±22 mL/min/1.73 m2. Because only 6 subjects had eGFR lower or higher than ±2SD, the population was divided into four categories according to age and gender ±1SD (range: <-1 SD; -1-0 SD; 0–1 SD; >1 SD). All subjects collected first-morning urine samples at rest. Urine albumin-to creatinine- ratio (u-ACR; mg/g) was calculated as follows: [urine albumin (mg/dL)/urine creatinine (g/dL)]. Microalbuminuria was defined as u-ACR ranging from 30 to 300 mg/g [15]. We collected two more samples from the subjects found positive for microalbuminuria to confirm the measurement. Microalbuminuria was diagnosed if all the three samples were found positive.

Statistical analysis

All data are expressed as mean ± SD, absolute values or percentages. In the case of microalbuminuria, the u-ACR mean values of the three first-morning samples were used as continuous variables. With an expected prevalence of 14% of microalbuminuria [6], a confidence level of 99.0% and a margin of error of 5.0%, a population size of 320 individuals was estimated sufficient to reflect our target population. Skewed variables were logarithmically transformed. ANOVA was used to determine the differences among sex, the presence of microalbuminuria, hyperuricemia, and the eGFR subgroups with a Bonferroni post-hoc test for multiple comparisons in the latter. Analysis of covariance (ANCOVA) was also used for hyperuricemia and eGFR and covariates were age, sex, puberty and BMI (Model 1) or WC (Model 2), according to the significant relationship with dependent variables.

Logistic regression was used to determine the association of microalbuminuria, eGFR and uric acid with the odds ratio (OR, 95% CI) of each cardiometabolic risk factor. Covariates of model 1 and 2 were also used in logistic regression for hyperuricemia and eGFR. Correlations as well as partial correlations were performed. Significance was assumed at p <0.05. The analysis was carried out with SPSS for Windows version 17.0 (SPSS Inc., Chicago, IL, USA). The database of the study is available upon request for validation or collaboration purposes as it includes other data (e.g. family history and other biochemical variables) not yet analyzed.

Results

Anthropometric and metabolic characteristics of patients

Nineteen out of 379 subjects selected were excluded because they did not satisfy inclusion criteria (fifteen subjects did not have adequate blood sampling, and 4 were without at least 3 urine collections). The final dataset included 360 participants (180 males and 180 females), aged 3 to 18 years, with a mean age of 10.9±3.0 years. Of the 360 participants, 18 subjects did not undergo OGTT (fifteen refused, 3 had analyses missing for technical problems), but had a complete fasting biochemical evaluation. Among patients 88% of them were obese, and 12% overweight. Almost all subjects had a WC ≥90th percentile (97.8%) with an overall mean of the waist-to-height ratio of 0.63±0.11, without differences between sexes. The clinical and biochemical characteristics of subjects are reported in Table 1. Hypertension was diagnosed in 216 (60.0%) subjects. Eighty-eight subjects (24.4%) had triglycerides ≥90th percentile; 148 (41.1%) had HDL-cholesterol ≤10th percentile; and 29 (8.0%) had LDL-cholesterol ≥90th percentile. Twenty subjects (5.5%) had impaired fasting glucose, 19 (5.2%) impaired glucose tolerance and 4 (1.1%) both metabolic dysfunctions. One patient had type 2 diabetes.

Table 1. Clinical and biochemical features of the study population according to sex.

		Overall	M	F
Subjects		360	180	180
Age (years)		10.9±3.0	10.7±2.8	11.1±3.3
Puberty	PP	155 (43.1%)	98 (54.4%)	57 (31.7%) †
	P	205 (56.9%)	82 (45.6%)	123 (68.3%) †
BMI (kg/m^2)		28.12±4.52	27.83±3.85	28.40±5.09
Obesity IOTF		317 (88.0%)	159 (88.3%)	158 (87.8%)
BMI SDS (kg/m^2)		2.09±0.46	2.06±0.41	2.13±0.50
Waist circumference (cm)		90.8±13.4	90.6±12.0	91.1±14.6
Waist/Height ratio		0.63±0.11	0.62±0.10	0.64±0.11
SBP (mmHg)		126±16.1	126±16.8	126±15.3
SBP percentile		90±14.9	89±15.3	90±14.5
DBP (mmHg)		79±10.8	79±10.8	79±10.7
DBP percentile		87±15.3	87±15.1	87±15.3
Total cholesterol (mg/dL)		145.6±27.7	144.6±26.9	146.8±28.5
HDL-c (mg/dL)		42.8±8.7	43.1±8.6	42.5±8.7
LDL-c (mg/dL)		87.3±23.5	86.9±23.4	87.7±23.7
Triglycerides (mg/dL)		77.8±43.5	72.4±35.8	83.1±9.6 *
AST (IU/L)		23.8±7.0	25.2±7.0	22.5±6.8 †
ALT (IU/L)		24.3±13.0	26.4±15.8	22.2±8.9 *
AST/ALT ratio		1.10±0.39	1.11±0.38	1.10±0.39
Uric acid (mg/dL)		4.87±1.20	4.97±1.30	4.76±1.07
IGF-1 (ng/mL)		286.0±129.7	259.2±129.7	313.1±124.2 †
eGFR (mL/min/1.73m^2)		119.78±19.70	120.6±19.4	118.91±19.95
GlcT0' (mg/dL)		87.9±7.3	88.3±7.1	87.5±7.5
GlcT30' (mg/dL)		134.8±22.4	136.9±23.0	132.7±22.5 *
GlcT60' (mg/dL)		115.1±25.9	116.1±26.5	114.0±25.3
GlcT90' (mg/dL)		108.9±20.0	108.7±19.5	107.5±21.3
GlcT120' (mg/dL)		108.0±21.6	109.9±18.3	106.0±18.3 *
AUC Glc (mg/dL*h/dL)		13816.4±2866.7	14035.7±3513.6	13587.8±1965.3
Mean Glc (mg/dL)		111.7±20.5	113.4±24.6	109.9±14.9
InsT0' (mUI/L)		16.4±11.4	14.7±10.0	18.1±12.4 *
Mean Ins (mUI/L)		75.6±62.3	72.9±67.5	78.3±56.6
HOMA-IR		3.66±2.80	3.37±2.72	3.96±2.85 *
ISI		4.71±4.70	4.77±3.78	4.66±5.50 *
QUICKI		0.33±0.05	0.33±0.04	0.33±0.05 *
InsI		2.14±4.04	2.05±4.88	2.22±2.96
DI		6.79±16.84	6.38±11.96	7.22±20.73
u-ACR (mg/g)		11.30±26.99	9.11±20.64	13.48±32.01 *

Data are expressed as mean±SD. p value <0.01*; < 0.0001†.

OGTT data are available for 342 or 345 subjects.

Legend: ALT: alanine aminotransferase; AST: aspartate aminotransferase; AUC: area under the curve; BMI: body mass index; DI: disposition index; DBP: diastolic blood

pressure; eGFR: estimated glomerular filtration rate; F: female; GlcT0': fasting glucose; GlcT30',T60',T90',T120': post-challenge glucose; HDL-c: high density lipoprotein cholesterol; HOMA-IR: homeostatic model assessment of insulin resistance; InsI: insulinogenic index; InsT0': fasting insulin; IOTF: International Obesity Task Force; ISI: insulin sensitivity index; LDL-c: low density lipoprotein cholesterol; M: male; P: pubertal; PP: prepubertal; QUICKI: quantitative insulin-sensitivity check index; SBP: systolic blood pressure; u-ACR: urinary albumin-to-creatinine ratio.

Microalbuminuria and associations between eGFR, uric acid, and other cardiometabolic variables

Microalbuminuria was detected in 6.4% (23/360) of subjects. In patients with or without microalbuminuria, uric acid was ≥5.5 mg/dL in 34.7% (8/23) and 27.0% (91/337) of them, respectively (p=0.278). All patients with microalbuminuria had eGFR >0 SD. In particular, 69.6% (16/23) of them had eGFR >1 SD. In the entire cohort without microalbuminuria, 91.7% (309/337) had eGFR >0 SD, and in 60.2% (203/337) of them the eGFR was >1SD. Subjects with microalbuminuria had lower insulin levels at 30-minute post-OGTT (81.8± 72.8 mUI/L *vs* 122.3±163.5; p<0.02) and lower insulinogenic index (InsI) (0.74±5.08 *vs* 2.24± 4.04; p<0.05) than those without it. No correlations between u-ACR as a continuous variable and cardiometabolic alterations were found.

eGFR evaluation and its association with microalbuminuria, uric acid, and other cardiometabolic variables

In 1.4% (5/360) of patients, eGFR was <-1 SD, while in 4 of them it was <-2 SD. Furthermore, eGFR was >1SD in 60.8% (219/360) of subjects, with 2 of them displaying an eGFR >2SD. Anthropometric and metabolic characteristics of subjects according to eGFR categories are reported in Table 2.

Table 2. Anthropometric and metabolic characteristics of the study population according to eGFR stratified for percentiles, age and sex dependent, based on NKF-K/DOQI Guidelines.

		Categories of eGFR				*P* value
		< - 1 SD	- 1–0 SD	0–1 SD	> 1 SD	
Subjects		5 (1.4%)	23 (6.4%)	113 (31.4%)	219 (60.8%)	
Age (years)		11.1±4.0	11.7±3.0	10.7±3.1	10.9±2.9	*ns*
Sex	**M**	3 (60%)	5 (21.7%) **	44 (38.9%) ††	128 (58.5%)	*< 0.0001*
	F	2 (40%)	18 (78.3%) **	69 (61.1%) ††	91 (41.5%)	
Puberty	**PP**	2 (40%)	5 (21.7%)	57 (50.4%)	91 (41.5%)	*ns*
	P	3 (60%)	18 (78.3%)	56 (49.6%)	128 (58.5%)	
BMI (kg/m^2)		30.13±6.74	29.50±5.43	27.50±4.98	28.25±4.06	*ns*
BMI SDS (kg/m^2)		2.35±0.63	2.21±0.55	2.03±0.48	2.11±0.43	*ns*
Waist circumference (cm)		97.1±20.3	93.8±14.1 §	87.5±13.0 ††	92.1±13.0	*< 0.009*
Waist/Height ratio		0.64±0.08	0.63±0.09	0.61±0.08	0.641±0.11	*ns*
SBP (mmHg)		135±10	128±15.5	123±16.0 ††	128±16.2	*< 0.02*
SBP percentile		98±1.3	92±9.23	86±17.5 ††	91±13.7	*< 0.01*
DBP (mmHg)		86±4.1	83±10.9	79±11.1	79±10.6	*ns*
DBP percentile		98±1.9	92±8.3	87±14.5	86±16.1	*ns*
Total cholesterol (mg/dL)		141.8±21.3	138.9±27.5	141.6±25.6	148.5±28.6	*ns*
HDL-c (mg/dL)		38.8±8.9	42.3±9.2	43.5±9.4	42.6±8.2	*ns*
LDL-c (mg/dL)		86.4±12.5	83.9±26.9	83.4±22.7	89.7±23.6	*ns*
Triglycerides (mg/dL)		83.6±54.3	70.5±22.6	74.6±39.6	80.0±46.8	*ns*
AST (IU/L)		23.6±10.7	19.5±6.0 §,**	22.9±5.9 ††	24.8±7.4	*< 0.002*
ALT (IU/L)		31.8±25.3	23.7±10.2	20.8±8.3 ††	25.9±14.5	*< 0.001*
AST/ALT ratio		0.86±0.29 ‡	0.89±0.31 §	1.20±0.37 ††	1.08±0.39	*< 0.001*
Uric acid (mg/dL)		4.70±0.43	5.30±0.89	4.72±1.16	4.90±1.24	*ns*
IGF-1 (ng/mL)		255.5±102.6	324.4±113.7	280.8±133.1	286.6±130.3	*ns*
GlcT0' (mg/dL)		86.6±4.9	87.04±5.75	86.9±7.2	88.5±7.5	*ns*
GlcT30' (mg/dL)		152.5±28.0 †	123.5±18.5 §, #	134.2±23.7	135.8±22.3	*< 0.0001*
GlcT60' (mg/dL)		122.2±36.4	106.7±19.5	111.0±27.2	117.6±25.3	*ns*
GlcT90' (mg/dL)		121.0±24.7 †	96.0±16.4 **	104.1±19.17 ††	110.8±20.6	*< 0.001*
GlcT120' (mg/dL)		115.6±21.1	100.0±13.6 **	104.4±17.4 ††	110.3±20.6	*< 0.009*
AUC Glc (mg/dL*h/dL)		22287.0±17283. 9 †	12614.2±1278.7 **	13393.9.±2008.8 ††	13930.7.±1868.0	*< 0.004*
Mean Glc (mg/dL)		168.8±117.3 *	102.9±9.5 **	108.5±15.0 ††	112.6±14.3	*< 0.005*
InsT0' (mUI/L)		21.7±8.8	13.7±7.1	13.7±9.7 ††	17.9±12.3	*< 0.006*
Mean Ins (mUI/L)		106.0±39.8	60.2±29.7	65.8±47.5	80.9±69.9	*ns*
HOMA-IR		4.62±1.92	2.97±1.50	2.95±2.13 ††	4.08±3.12	*< 0.02*
ISI		2.18±1.01	4.24±1.16	6.05±6.85 ††	4.18±3.40	*< 0.005*
QUICKI		0.31±0.01	0.33±0.02	0.35±0.05 §§	0.32±0.04	*< 0.001*
InsI		2.14±1.27	2.81±3.75	2.20±1.91	2.04±4.79	*ns*
DI		5.56±4.48	9.09±9.04	10.30±13.70 ††	4.92±18.61	*< 0.05*
u-ACR (mg/g)		7.35±3.81	4.83±5.18	9.91±18.83	12.78±31.72	*ns*

Data are expressed as mean±SD. p value -1SD vs -1-0 SD: <0.05*; <0.01†. -1SD vs 0-1SD: <0.05‡. -1-0 SD vs 0±1 SD: <0. 05§; <0.0001; -1-0 SD vs >1SD: <0.05#; <0.01**. 0±1 SD vs >1SD: < 0.05††; < 0.01‡‡; < 0.0001§§. ns: not significant. ANOVA analysis with a Bonferroni post-hoc test was used.

OGTT data are available for 342 or 345 subjects.

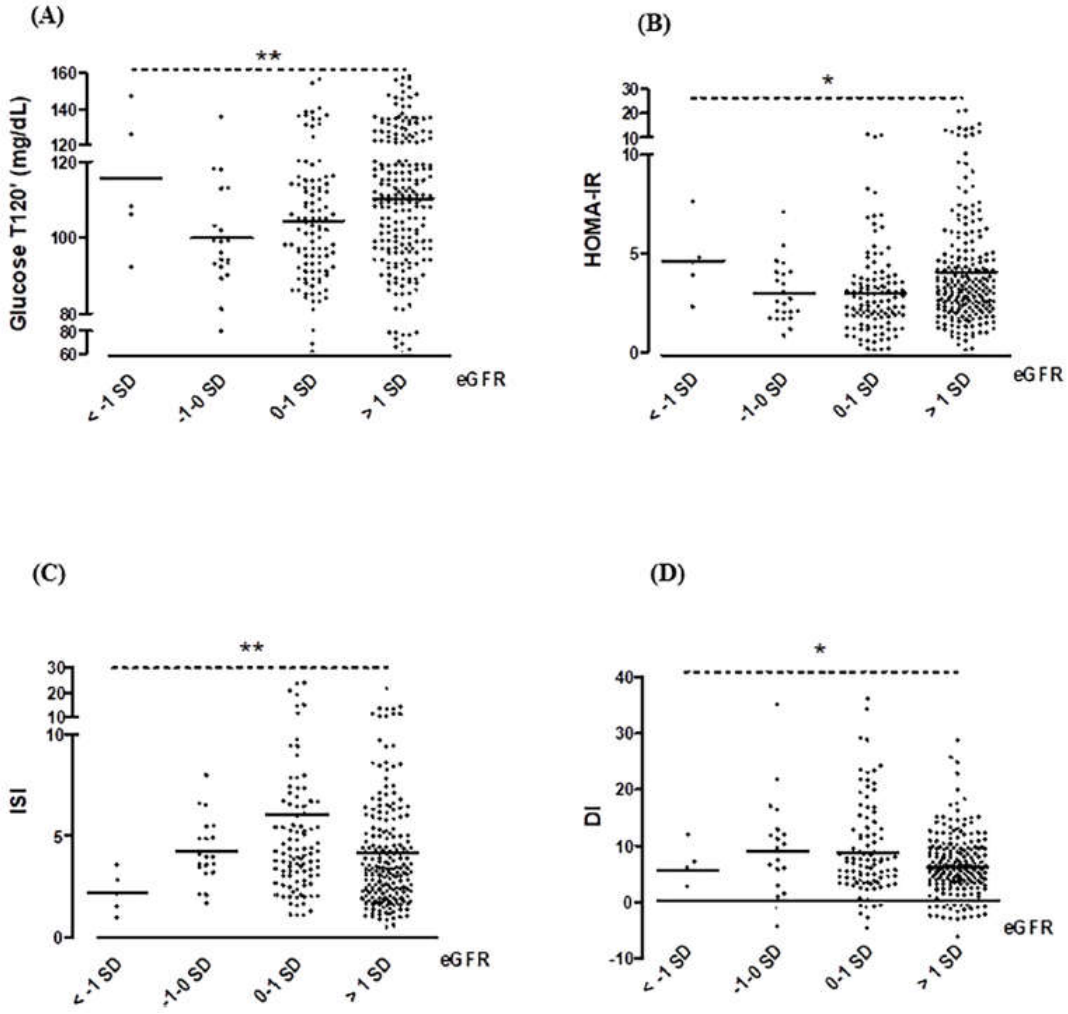
Legend: ALT: alanine aminotransferase; AST: aspartate aminotransferase;

AUC: area under the curve; BMI: body mass index; DI: disposition index; DBP: diastolic blood pressure; eGFR: estimated glomerular filtration rate; F: female; GlcT0': fasting glucose; GlcT30',T60',T90',T120': postchallenge glucose; HDL-c: high density

lipoprotein cholesterol; HOMA-IR: homeostatic model assessment of insulin resistance; InsI: insulinogenic index; InsT0': fasting insulin; IOTF: International Obesity Task Force; ISI: insulin sensitivity index; LDL-c: low density lipoprotein cholesterol; M: male; P: pubertal; PP: prepubertal; QUICKI: quantitative insulin-sensitivity check index; SBP: systolic blood pressure; u-ACR: urinary albumin to-creatinine ratio.

Compared with subjects with eGFR <-1SD, patients with eGFR >1SD showed higher SBP, AST, ALT, glucose and insulin during OGTT, insulin resistance (Fig 1 , Panel A and B); they also had lower sensitivity indexes after both crude analysis and ANCOVA (Fig 1 , Panel C and D).

Figure 1. Relationship between glucometabolic parameters and stratified estimated glomerular filtration rate (eGFR) levels. (Panel A) Plasma glucose levels (mg/dL) after 2-hour post-glucose tolerance test (OGTT) (T120'); (Panel B) homeostatic model assessment of insulin resistance (HOMA-IR); (Panel C) insulin sensitivity index (ISI); (Panel D) disposition index (DI); p value <0.05*; <0.01** after ANOVA corrected for confounding factors (i.e. sex, puberty, BMI).



Subjects with an eGFR within 0 and 1 SD had higher odds to have triglycerides <90th percentile in both crude and controlled models (model 2; OR 1.750; CI 1.002±3.056; p < 0.04). eGFR was correlated with many variables in the crude model. After adjusting for age, sex, puberty, and BMI, eGFR was positively associated with WC, fasting insulin, glucose levels at 90 and 120 minutes, AUC and mean glucose, insulin at 120 minutes, mean insulin and u-ACR, and negatively associated with DPB, uric acid, ISI, and QUICKI. After adjusting for age, sex, puberty, and WC, eGFR was positively associated with fasting insulin, glucose levels at 90 and 120 minutes, AUC and mean

glucose, HOMA-IR and u-ACR, and negatively associated with DPB, uric acid, ISI, and QUICKI (Table 3).

Table 3. Partial correlations between eGFR and microalbuminuria, uric acid and other cardiometabolic variables.

eGFR	Model 1		Model 2	
	r	*P* value	r	*P* value
Age (years)				
BMI (kg/m^2)			-0.062	*ns*
BMI SDS (kg/m^2)				
Waist circumference (cm)	**0.150**	*< 0.005*		
Waist/Height ratio				
SBP (mmHg)	0.034	*Ns*	0.016	*ns*
DBP (mmHg)	**- 0.122**	*< 0.002*	**-0.131**	*< 0.01*
Total cholesterol (mg/dL)	0.092	*Ns*	0.084	*ns*
HDL-c (mg/dL)	0.065	*Ns*	0.071	*ns*
LDL-c (mg/dL)	0.067	*Ns*	0.066	*ns*
Triglycerides (mg/dL)	0.026	*Ns*	-0.002	*ns*
AST (IU/L)	0.094	*Ns*	0.085	*ns*
ALT (IU/L)	0.037	*Ns*	0.012	*ns*
AST/ALT ratio	-0.11	*Ns*	-0.004	*ns*
Uric acid (mg/dL)	**-0.172**	*< 0.0001*	**-0.217**	*< 0.0001*
IGF-1 (ng/mL)	0.021	*Ns*	0.042	*ns*
GlcT0' (mg/dL)	0.078	*Ns*	0.090	*ns*
GlcT30' (mg/dL)	0.032	*Ns*	0.039	*ns*
GlcT60' (mg/dL)	0.077	*Ns*	0.083	*ns*
GlcT90' (mg/dL)	**0.174**	*< 0.001*	**0.176**	*< 0.001*
GlcT120' (mg/dL)	**0.164**	*< 0.002*	**0.168**	*< 0.002*
AUC Glc (mg/dL*h/dL)	**0.132**	*< 0.01*	**0.128**	*< 0.001*
Mean Glc (mg/dL)	**0.131**	*< 0.01*	**0.140**	*< 0.01*
Mean Ins (mUI/L)	**0.109**	*< 0.04*	0.080	*ns*
HOMA-IR	**0.164**	*< 0.02*	**0.141**	*< 0.009*
ISI	**-0.131**	*< 0.01*	**-0.108**	*< 0.05*
QUICKI	**-0.176**	*< 0.01*	**-0.140**	*< 0.009*
InsI	0.009	*Ns*	0.002	*ns*
DI	-0.047	*Ns*	-0.041	*ns*
u-ACR (mg/g)	**0.124**	*< 0.02*	**0.128**	*< 0.01*

ns: not significant. OGTT data are available for 342 or 345 subjects.

Legend: ALT: alanine aminotransferase; AST: aspartate aminotransferase; AUC: area under the curve; BMI: body mass index; DI: disposition index; DBP: diastolic blood pressure; eGFR: estimated glomerular filtration rate; GlcT0': fasting glucose; GlcT30',T60',T90',T120': post-challenge glucose; HDL-c: high density lipoprotein cholesterol; HOMA-IR: homeostatic model assessment of insulin resistance; InsI: insulinogenic index; InsT0': fasting insulin; ISI: insulin sensitivity index; LDL-c: low density lipoprotein cholesterol; QUICKI: quantitative insulin-sensitivity check index; SBP: systolic blood pressure; u-ACR: urinary-albumin-to-creatinine ratio.

Model 1: controlled for sex, age, puberty and BMI.

Model 2: controlled for sex, age, puberty and waist circumference.

Hyperuricemia evaluation and its association with microalbuminuria, eGFR and other cardiometabolic variables

Hyperuricemia was present in 27.5% (99/360) of subjects, of whom 8.1% (8/99) had microalbuminuria, whereas microalbuminuria was found in 5.7% (15/261) of subjects without hyperuricemia. Interestingly, 64.6% (64/99) of subjects with hyperuricemia had eGFR >1SD, and 26.3% (26/99) were between 0 and 1SD. In contrast, no subject with eGFR <-1 SD had hyperuricemia. Conversely, 59.4% (155/261) of subjects without hyperuricemia had eGFR >1 SD, and 33.3% (87/261) were between 0 and 1 SD. Subjects with hyperuricemia were older, had higher BMI, BMI SDS, waist circumference, SBP, DBP, triglycerides, ALT and IGF-1 levels and lower HDL-cholesterol, AST, AST to ALT ratio, eGFR and u-ACR compared with those with normal acid uric levels. Moreover, subjects with hyperuricemia showed higher glucose and insulin, either at fasting or as responses to OGTT, associated with higher insulin resistance and lower insulin sensitivity than those without hyperuricemia. After controlling for confounding factors, subjects with hyperuricemia maintained higher levels of IGF-1 and lower eGFR compared to those with normal acid uric levels (Table 4).

Table 4. Anthropometric and metabolic characteristics of the study population according to uric acid.

	URIC ACID *P* value			
	Normal	High	Model 1	Model 2
Age (years)	10.2±3.0	12.7±2.5		
BMI (kg/m^2)	27.1±4.5	30.8±4.7		*ns*
BMI SDS (kg/m^2)	2.03±0.46	2.27±0.54		*ns*
Waist circumference (cm)	87.5±13.4	100.3±12.4	*< 0.0001*	
Waist/Height ratio	0.62±0.10	0.64±0.12	*ns*	
SBP (mmHg)	123.2±16.1	134.5±17.7	*< 0.01*	*ns*
SBP percentile	88.7±15.0	92.1±13.7	*< 0.01*	*ns*
DBP (mmHg)	77.8±10.7	84.2±10.8	*< 0.05*	*ns*
DBP percentile	85.6±16.2	90.2±12.4	*< 0.05*	*ns*
Total cholesterol (mg/dL)	146.9±28.1	142.1±26.4	*ns*	*ns*
HDL-c (mg/dL)	43.8±8.7	40.1±7.9	*ns*	*ns*
LDL-c (mg/dL)	88.2±23.8	84.8±22.8	*ns*	*ns*
Triglycerides (mg/dL)	74.6±43.5	86.1±49.7	*ns*	*ns*
AST (IU/L)	24.2±7.0	22.9±6.6	*ns*	*ns*
ALT (IU/L)	23.1±13.1	27.5±16.6	*ns*	*ns*
AST/ALT ratio	1.16±0.39	0.95±0.30	*ns*	*ns*
Uric acid (mg/dL)	4.3±0.8	6.3±0.7	*ns*	*ns*
IGF-1 (ng/mL)	258.6±115.5	353.8±132.4	*< 0.04*	*< 0.03*
eGFR (mL/min/1.73m^2)	121.8±19.4	113.6±18.5	*ns*	*< 0.001*
25-OH VitD (ng/mL)	20.3±9.2	20.3±9.6	*ns*	*ns*
GlcT0' (mg/dL)	87.3±8.9	90.4±12.3	*ns*	*ns*
GlcT30' (mg/dL)	135.3±23.5	133.2±20.6	*ns*	*ns*
GlcT60' (mg/dL)	111.7±25.8	123.2±26.4	*ns*	*ns*
GlcT90' (mg/dL)	106.4±20.7	112.6±22.0	*ns*	*ns*
GlcT120' (mg/dL)	106.6±18.7	111.8±19.7	*ns*	*ns*
AUC Glc (mg/dL*h/dL)	1370.2±2870.7	14109.1±1943.1	*ns*	*ns*
Mean Glc (mg/dL)	110.7±20.6	114.3±14.9	*ns*	*ns*
InsT0' (mUI/L)	146.1±13.1	22.3±18.9	*< 0.01*	*ns*
InsT30' (mUI/L)	123.6±186.2	108.4±80.8	*ns*	*ns*
InsT60' (mUI/L)	80.0±86.7	109.7±104.8	*ns*	*ns*
InsT90' (mUI/L)	68.9±65.2	86.6±75.2	*ns*	*ns*
InsT120' (mUI/L)	71.2±92.7	100.5±126.3	*ns*	*ns*
AUC Ins (mUI*h/dL)	9508.7±8260.3	10988.4±7915.6	*ns*	*ns*
Mean Ins (mUI/L)	72.9±62.6	85.6±64.2	*ns*	*ns*
HOMA-IR	3.27±4.25	5.33±6.84	*< 0.05*	*ns*
ISI	5.13±4.79	3.66±2.91	*ns*	*ns*
QUICKI	0.34±0.04	0.32±0.05	*ns*	*ns*
InsI	2.17±4.50	2.06 ±2.50	*ns*	*ns*
DI	7.25±6.84	5.62±6.18	*ns*	*ns*
u-ACR (mg/g)	11.59±26.93	10.53±24.50	*ns*	*ns*

Data are expressed as mean±SD. ns: not significant.

OGTT data are available for 342 or 345 subjects.

Legend: ALT: alanine aminotransferase; AST: aspartate aminotransferase; AUC: area under the curve; BMI: body mass index; DI: disposition index; DBP: diastolic blood pressure; eGFR: estimated glomerular filtration rate; F: female; GlcT0': fasting glucose; GlcT30',T60',T90',T120': post-challenge glucose; HDL-c: high density lipoprotein cholesterol; HOMA-IR: homeostatic model assessment of insulin resistance; InsI: insulinogenic index; InsT0': fasting insulin; InsT30',T60',T90',T120': post-challenge glucose; ISI: insulin sensitivity index; LDL-c: low density lipoprotein cholesterol; M: male; QUICKI: quantitative insulin-sensitivity check index; SBP: systolic blood pressure;

u-ACR: urinary albumin-to-creatinine ratio; uric acid: high (≥5.5 mg/dL); 25OH VitD: 25-0H vitamin D.

Model 1: controlled for sex, age, puberty and BMI.

Model 2: controlled for sex, age, puberty and waist circumference.

Uric acid levels were positively associated with ALT, IGF-1, HOMA-IR, fasting insulin, glucose levels at 60, 90 and 120 minutes, AUC and mean glucose, insulin at 60 minutes, and negatively associated with HDL-cholesterol, AST to ALT ratio, eGFR, and QUICKI, also when corrected for covariates (Table 5).

Table 5. Partial correlations between uric acid and microalbuminuria, eGFR and other cardiometabolic variables.

URIC ACID	Model 1		Model 2	
	r	*P* value	r	*P* value
Age (years)				
BMI (kg/m^2)			0.015	*ns*
BMI SDS (kg/m^2)				
Waist circumference (cm)	**0.169**	*< 0.002*		
Waist/Height ratio				
SBP (mmHg)	**0.105**	*< 0.05*	0.078	*ns*
DBP (mmHg)	0.076	*ns*	0.088	*ns*
Total cholesterol (mg/dL)	0.052	*ns*	0.044	*ns*
HDL-c (mg/dL)	**-0.153**	*< 0.004*	**-0.150**	*< 0.005*
LDL-c (mg/dL)	0.081	*ns*	0.079	*ns*
Triglycerides (mg/dL)	**0.105**	*< 0.04*	0.087	*ns*
AST (IU/L)	0.018	*ns*	0.009	*ns*
ALT (IU/L)	**0.115**	*< 0.03*	**0.104**	*< 0.05*
AST/ALT ratio	-0.118	*< 0.02*	**-0.122**	*< 0.02*
IGF-1 (ng/mL)	**0.187**	*< 0.0001*	**0.217**	*< 0.0001*
eGFR (mL/min/1.73m^2)	**-0.172**	*< 0.001*	**-0.217**	*< 0.0001*
GlcT0' (mg/dL)	0.070	*ns*	0.074	*ns*
GlcT30' (mg/dL)	0.096	*ns*	0.089	*ns*
GlcT60' (mg/dL)	**0.209**	*< 0.0001*	**0.206**	*< 0.0001*
GlcT90' (mg/dL)	**0.118**	*< 0.03*	**0.111**	*< 0.04*
GlcT120' (mg/dL)	**0.137**	*< 0.01*	**0.136**	*< 0.01*
AUC Glc (mg/dL*h/dL)	**0.176**	*< 0.001*	**0.168**	*< 0.002*
Mean Glc (mg/dL)	**0.179**	*< 0.001*	**0.178**	*< 0.001*
InsT0' (mUI/L)	**0.181**	*< 0.001*	**0.014**	*< 0.009*
Mean Ins (mUI/L)	**0.114**	*< 0.003*	0.072	*ns*
HOMA-IR	**0.140**	*< 0.008*	**0.117**	*< 0.03*
ISI	**-0.120**	*< 0.02*	-0.096	*ns*
QUICKI	**-0.150**	*< 0.0001*	**-0.113**	*< 0.03*
InsI	-0.037	*ns*	-0.049	*ns*
DI	-0.090	*ns*	-0.091	*ns*
u-ACR (mg/g)	-0.085	*ns*	-0.080	*ns*

ns: not significant.

Legend: ALT: alanine aminotransferase; AST: aspartate aminotransferase; AUC: area under the curve; BMI: body mass index; DI: disposition index; DBP: diastolic blood pressure; eGFR: estimated glomerular filtration rate; GlcT0': fasting glucose; GlcT30',T60',T90',T120': post-challenge glucose; HDL-c: high density lipoprotein

cholesterol; HOMA-IR: homeostatic model assessment of insulin resistance; InsI: insulinogenic index; InsT0': fasting insulin; ISI: insulin sensitivity index; LDL-c: high density lipoprotein cholesterol; QUICKI: quantitative insulin-sensitivity check index; SBP: systolic blood pressure; u-ACR: urinary albumin-tocreatinine ratio.
Model 1: controlled for sex, age, puberty and BMI.
Model 2: controlled for sex, age, puberty and waist circumference.

Lastly, hyperuricemia was associated with hypertension (OR: 2.086, CI 1.257±3.460, p <0.003), HDL-cholesterol ≤10th percentile (OR: 2.001, CI 1.252±3.198; p <0.003) and glucose ≥155.0 mg/dL at 60 minutes after OGTT (OR: 2.350, CI 1.045±5.282; p <0.03) in both crude and controlled models.

Discussion

This study shows a worsened metabolic profile in obese children with normal eGFR higher than 1SD or with abnormally high serum uric acid levels. In particular, post-OGTT glucose levels were found to be higher, albeit within standard cutoffs, in those individuals with eGFR >1 SD. Furthermore, subjects with microalbuminuria did not display a major impairment in their cardiometabolic alterations, although all of them had eGFR >0 SD. In recent years, a positive correlation between microalbuminuria and obesity in adult patients has been clearly established [3]. However, in pediatric patients such correlation appears to be less obvious and only partially understood probably due to the lack of consistent data on large-sized cohorts. In this regard, here we show a prevalence of 6.4% of microalbuminuria in a large cohort of obese children and adolescents, which is in good agreement with previous data reporting prevalence of microalbuminuria ranging between 0.3% and 10.1% in similar patient groups [16-21]. These studies, including ours, however appears to underestimate the prevalence of microalbuminuria when compared to other reports showing a much higher prevalence of microalbuminuria in obese children, which could go as high as 37.0% [22,23]. This discrepancy could be explained by different modalities of urine sample collection [22] or by the measurement of urinary albumin excretion rate per minute time rather than u-ACR [23]. Moreover, other variables such as postural changes and exercise before the testing session, as well as ethnicity, might account for data variability [24], even though no evidence indicating that this is indeed the case in obese children has been found in previous studies [18,19]. Associations between microalbuminuria and other cardiometabolic markers in obese children and adolescents have been reported in some [16,18,20-22,25] but not all studies [17,19,26], including ours. Although this lack

of correlation could be explained by the young mean age of our patients or the low prevalence of microalbuminuria registered in these latter, our findings do not support a routine assessment of microalbuminuria in all obese children and adolescents. Interestingly, we find a lower insulinogenic index in subjects with microalbuminuria, which is in good agreement with a recent study demonstrating a positive correlation between microalbuminuria and HbA1c in obese Korean adolescents [21]. In this regard, it has been hypothesized that the prevalence of microalbuminuria progressively increased as plasma glucose values climbed through the `normal range' into the impaired range of glucose tolerance, suggesting that the effect of glucose may be continuous. Furthermore, frequent daily postprandial states of relatively higher glucose levels could increase oxidative stress on the vessels leading to increased urinary albumin excretion secondary to endothelial dysfunction [16]. Moreover, because HbA1c and insulinogenic-index are both related to insulin-resistance, also the latter could be a responsible for this alteration. This is in agreement with the evidence that insulin-resistance contributes to micro and macrovascular disease [1,16]. Since our results appear to indicate that subjects with eGFR >0SD have microalbuminuria and a suboptimal metabolic phenotype, further longitudinal studies on larger populations are clearly needed to fully establish whether eGFR could be used as a useful marker to stratify high-risk obese youths. According to NKF-K/DOQI guidelines [13], we also report eGFR <-1SD in 1.4% of our population, who was free of known CKDs. Based on a normal population distribution, a much greater percentage would be expected to be below -1SD and again below normal -2SD. The fact that the majority of our subjects were stratified above 0 and, to a greater extent, 1SD indicates a quite alarming skewed distribution of eGFR in obesity, in agreement with some [27], but not all authors [20,28]. In fact, differently by us, the distribution observed in the National Health and Nutrition Examination Survey (NHANES) resulted toward lower eGFR beyond what is expected. However, the NHANES cohort included only adolescents aged 12±19 years of general U.S. population [28], while ou population was much younger. These contrasting findings could be due also to different obesity trajectories or different formulas used to determine eGFR from serum creatinine concentration (i.e Jaffè technique vs Schwartz's formula) [14]. Therefore, previous reports have clearly shown that adulthood obesity is associated with glomerular hyperperfusion and hyper-filtration as an early sign of physiologic maladaptation leading, in part, to afferent arteriolar vasodilatation [1]. Of note, we show that microalbuminuria was present only in subjects with eGFR >0SD in good agreement with a previous report [29]. Thus, our findings, together with the

observation that childhood obesity positively correlates with a fast decline of eGFR over time, with a 2- to -3 fold higher risk of developing ESRD [30], should prompt physicians to evaluate the possibility of renal dysfunction in obese children. We also show that subjects with eGFR >1SD presented with an increased burden of cardiometabolic alterations as recent studies in adults seem to suggest [2]. In particular, in our young patients with eGFR >1SD, systolic blood pressure, glucose, and insulin levels in response to OGTT and insulin resistance were higher, whereas insulin sensitivity was lower compared to other subgroups, suggesting a glucose dysregulation mainly after OGTT. The same condition was also present in patients with eGFR <-1SD, suggesting a U-shaped relationship, even though the low number of subjects in the left part of the curve does not allow us to draw any firm conclusions about its generalizability. A similar Ushaped effect between eGFR, blood pressure and microalbuminuria has been recently reported by Di Bonito P. et al. [29], although these authors could not find a significant relationship between eGFR and the glyco-insulinemic profile at fasting. This discrepancy might be due to the different eGFR cutoff in adults used in this study. Overall, our findings raise the possibility that eGFR >1SD may be an early predictor of dysglycemia and pre-diabetes, a possibility that could be further explored by investigating the relationships among post-challenge glucose, insulin levels and kidney function in youths. Our data are also consistent with findings by Matsushita et al., who have recently shown that the inclusion of eGFR and u-ACR among traditional risk predictors greatly improved the discrimination of cardiometabolic outcomes in adults [4]. However, additional longitudinal studies are clearly needed to establish the evolution and distribution of eGFR in obese children and explain its pathophysiological significance over time. In our study, we show an inverse correlation between eGFR and uric acid, which is in line with previous reports on several adult populations [6] and adolescents with type 1 diabetes [31]. While in the past hyperuricemia was thought to result from a decreased uric acid clearance due to kidney damage, it now seems that uric acid per se might play a role in the natural history of GFR decline [6]. In this regard, here we show that hyperuricemic patients are at increased risk of having a 1-hour post-OGTT glycemia ≥155.0 mg/dL. This cut-off seems to be associated with an increased metabolic risk in subjects with a post-challenge normal glucose tolerance as well as with the development of an overt type 2 diabetes rather than fasting glucose [32]. Moreover, serum uric acid levels are closely related to both earlyphase insulin secretion and 2-hour post-challenge glucose levels in adults with apparently normal glucose regulation [33]. To the best of our knowledge, this is the first study in a pediatric obese

population where a positive association between uric acid and glucose response after OGTT has been found. In line with previous evidence [5], we confirm a worse cardiometabolic profile in subjects with hyperuricemia, which increased the odds of hypertension and HDL-cholesterol ≤10th percentile. In agreement with recent studies in adults [34], we also observed an association between uric acid and ALT, which suggests that uric acid may be an independent risk factor for liver diseases. Although serum uric acid seems a good predictor of renal and cardiometabolic diseases, its normal values in children and adolescents are still undefined. In this regard, we report an age-dependent effect on serum uric acid levels. Thus, the fixed cut-off is probably improper in the pediatric population, while a distribution according to age and sex may be more appropriate. All in all, our results are limited to a Caucasian population. We included only Caucasian children and adolescents because ethnic influences on microalbuminuria and serum uric acid have been reported [18,19,24,35]. Therefore, further studies on more heterogeneous populations are needed. Our study has some limitations. First of all, the cross-sectional design does not allow us to conclude that there is a causal relationship between variables; longitudinal studies might clarify this aspect. Moreover, a normal-weight control group is lacking, because the study was performed in a tertiary referral center. Another limit is that microalbuminuria was measured on spot morning urine samples; however, spot u-ACR correlates very well with the urine collection at the 24-hour time point [19]. Furthermore, we failed to observe a normal distribution of eGFR in obesity, and a very low percentage of subjects could be stratified in the extreme tails (±2SD). Thus, studies on larger populations are needed to confirm our data and investigate the metabolic phenotype of those with an eGFR above or below ±2SD. On the other hand, our study includes a large sample of subjects as well as the availability of OGTT for the majority of patients. Moreover, microalbuminuria was confirmed on three samples, and eGFR was stratified according to the pediatric cut-off, unlike most publications on pediatric obesity. In conclusion, our study suggests that eGFR may be helpful in clinical practice to identify an unhealthy metabolic profile in pediatric obesity. Thus, more attention should be paid to this relatively inexpensive parameter. Therefore, in subjects with an eGFR >1SD or hyperuricemia, we encourage to investigate the early-phase insulin secretion and 2-hour post-challenge glucose levels. Serum uric acid seems to be another useful tool to diagnose subjects at high risk of metabolic impairment. However, studies based on larger population are needed to establish normal references values according to age and sex. Finally, based on our data, we strongly recommend the inclusion of

microalbuminuria only in routine screenings of pediatric obese patients with eGFR greater than 1 SD. Further studies on large-sized pediatric cohorts are needed to confirm our finding also in obese children with an eGFR less than -2SD or greater than 2SD.

2.2.2.i. References

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2.2.3. Documento di consenso e raccomandazioni per la prevenzione cardiovascolare in Italia 2018:
Interventi su popolazioni specifiche: bambini e adolescenti*

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Introduzione

Da decenni, un numero sempre crescente di evidenze ha dimostrato come il rischio cardiovascolare si instauri precocemente, già in giovane età. Il processo di aterosclerosi ha infatti inizio durante l'infanzia e la sua progressione si manifestera clinicamente in presenza di un numero crescente di fattori di rischio cardiovascolare persistenti [1]. Addirittura, l'esposizione a fattori di rischio pre-natali sembra influire a lungo termine sul rischio cardiovascolare. A partire già dall'epoca gravidica e peri-natale si riconoscono come fattori determinanti la nutrizione e il peso materno, il fumo di sigaretta, il consumo di caffeina, il parto da taglio cesareo e un basso peso alla nascita [2]. Inoltre, già nell'infanzia e possibile riconoscere la presenza di fattori di rischio cardiovascolare che persistono poi nell'età adulta [3, 4]. In particolare, è stato riconosciuto alla condizione di obesità infantile un aumentato rischio di morte prematura per tutte le cause, soprattutto, di natura cardiovascolare in età adulta [5]. Negli ultimi anni, in età pediatrica, si è assistito ad un drammatico incremento nella prevalenza di casi di obesità severa. Dal 1980 al 2013, infatti, la prevalenza combinata di sovrappeso ed obesità in bambini e adolescenti è aumentata, su scala mondiale, del 47.1% [6]. Dato ancora più allarmante consiste nell'insorgenza precoce, già in età pediatrica, di comorbilità cardio-metaboliche associate all'eccesso ponderale, riconosciute nella sindrome metabolica, quali ipertensione arteriosa, alterazioni del profilo lipidico, prediabete e diabete mellito di tipo 2, con un peggioramento della prognosi in termini di morbilità e mortalità [7]. Come dichiarato dall'Endocrine Society, risulta pertanto fondamentale sensibilizzare la popolazione a questo problema ed aiutare i genitori a prenderne coscienza, nonchè a sottoporre questa popolazione ad un'attenta valutazione clinico-metabolica, in quanto la diagnosi precoce della condizione di sovrappeso/obesita permettera di instaurare un adeguato trattamento, che puo arrestare la progressione delle sue sequele [8]. Si raccomandano quindi ripetute rilevazioni ambulatoriali della pressione arteriosa in bambini e adolescenti in sovrappeso/obesi, interpretandone i valori attraverso la consultazione di specifici nomogrammi suddivisi per sesso, eta e statura [9]. Trova inoltre raccomandazione, in bambini obesi a partire dai 6 anni di età, la valutazione del profilo lipidico e della glicemia a digiuno e l'esecuzione della curva da carico orale di glucosio in particolari condizioni [10]. Accanto all'eccesso ponderale, quale causa più frequente di rischio cardiovascolare, si riconoscono anche altre categorie a rischio in età pediatrica [11] (Tabella 1). La malattia cardiovascolare, infatti, è in molti casi prevenibile attraverso una riduzione dei fattori che causano direttamente o indirettamente la sua insorgenza.

In particolare, con lo scopo di ridurre la probabilità che un bambino possa sviluppare sovrappeso nei primi 6 anni di vita ed incorrere nelle complicanze metaboliche, la Società Italiana di Pediatria Preventiva e Sociale ha promosso la divulgazione di 10 semplici azioni [12] (Tabella 2).

Tabella 1. Catergorie di rischio in età pediatrica

Livello	Categoria di rischio	Razionale	Processo/condizione patologica
Tier 1	Rischio alto	Malattia coronarica manifesta prima dei 30 anni di età: evidenze cliniche	FH omozigote Diabete mellito di tipo 1 Insufficienza renale cronica/ESRD Post-trapianto cardiaco ortotopico Malattia di Kawasaki in presenza di aneurismi coronarici
Tier 2	Rischio moderato	Aterosclerosi accelerata: evidenze fisiopatologiche	FH eterozigote Malattia di Kawasaki con regressione degli aneurismi coronarici Diabete mellito di tipo 2 Malattia infiammatoria cronica
Tier 3	A rischio	Contesti di alto rischio per aterosclerosi accelerata: evidenze epidemiologiche	Pazienti oncologi post-trattamento anti-tumorale Cardiopatia congenita Malattia di Kawasaki senza evidenza di coinvolgimento coronarico

ESRD, malattia renale in stadio terminale; FH, ipercolesterolemia familiare.

Tabella 2. Le 10 azioni per contrastare lo sviluppo di sovrappeso nei primi 6 anni di vita

	Azione	Indicatore
1.	Allattare al seno	Almeno 6 mesi.
2.	Svezzamento	Introdurre cibi complementari dopo i 6 mesi.
3.	Apporto proteico	Controllato (in particolare nei primi 2 anni).
4.	Bevande caloriche	Evitare succhi, tisane, soft drink, tè, ecc.
5.	Biberon	Da sospendere entro i 24 mesi.
6.	Mezzi di trasporto	Evitare l'uso del passeggino dopo i 3 anni e l'uso di moto e auto elettriche, favorire il raggiungimento a piedi della scuola (Pedibus).
7.	Controllo dell'IMC	Identificare se si verifica l'"adiposity rebound" precoce (prima dei 6 anni).
8.	TV, giochi sedentari	Solo dopo i 2 anni, massimo 8 ore/settimana.
9.	Gioco e stile di vita	Regalare e incentivare i giochi di movimento, adatti alle varie età del bambino.
10.	Porzioni corrette per l'età pre-scolare	Usare l'Atlante Fotografico delle porzioni degli alimenti (www.scottibassani.it).

IMC, indice di massa corporea.

Obiettivi terapeutici nel bambino e nell'adolescente

L'obiettivo cardine prevede in primo luogo atti di prevenzione primaria con lo scopo di favorire in tutti i bambini un corretto stile di vita, che si traduce in raccomandazioni dietetiche ed indicazioni all'attivita fisica quotidiana. Si tratta in primis di affrontare un intervento educazionale, considerando la minima aderenza alle raccomandazioni per un corretto stile di vita, come è emerso dallo studio IDEFICS (Identification and Prevention of Dietary- and Lifestyle-Induced Health Effects in Children and Infants) condotto in Europa su una casistica di bambini di eta pre-scolare [13]. Le attuali evidenze tradotte dalla linee guida ESPGHAN sottolineano l'importanza di un introito calorico adatto alle esigenze metaboliche proprie dell'eta evolutiva, indipendentemente

da una specifica distribuzione dei macronutrienti principali (carboidrati, proteine e grassi), nell'arco di 5 pasti nel corso della giornata, con incoraggiamento quotidiano al consumo della colazione [14]. E' stato inoltre confermato come il pattern dietetico mediterraneo sia inversamente associato allo sviluppo di sovrappeso/obesità e malattie cardiovascolari, configurandosi pertanto come fattore protettivo in età evolutiva [15]. In età infantile, inoltre, l'attività fisica e motoria dovrebbe essere incorporata nella programmazione curricolare quotidiana e promossa attraverso il gioco con proposte divertenti, appropriate per sesso ed età e il più possibile diversificate [16]. Tali indicazioni trovano altresì supporto negli Standard Italiani per la Cura dell'Obesità (Tabella 3) [17]. Indagando altre potenziali strategie a disposizione, da un lato, per contrastare il dilagare dell'eccesso ponderale e, dall'altro, per gestire efficacemente bambini ed adolescenti obesi, l'American Heart Association ha suggerito alcuni interventi a promozione della salute basati anche sull'utilizzo di social network e media [18]. D'altra parte, si ritiene da scoraggiare il tempo sedentario totale a scuola e a casa, dedicato in particolare a televisione, computer, tablet e smartphone. L'American Academy of Pediatrics, infatti, vieta il ricorso ai media fino a 18 mesi di vita, consentendone un uso comunque estremamente limitato nelle fasce di età successive [19].

Tabella 3. Standard Italiani per la Cura dell'Obesità [17]; raccomandazioni per la pratica dell'attività fisica in età pediatrica

• Motivare i genitori a uno stile di vita più attivo *(livello di prova I, forza della raccomandazione A).*
• Programmare la riduzione del tempo dedicato ad attività sedentarie, in particolare il tempo di video-esposizione *(livello di prova I, forza della raccomandazione A).*
• Promuovere il gioco attivo, possibilmente all'aria aperta e in gruppo *(livello di prova VI, forza della raccomandazione A).*
• Promuovere la pratica di un'attività motoria regolare organizzata (sport) gradita al bambino, divertente e in cui l'obiettivo principale non è la competizione ma l'attività fisica *(livello di prova VI, forza della raccomandazione A).*
• L'intensità dell'esercizio programmato dovrebbe inizialmente essere moderata (non >65% della frequenza cardiaca massimale o 55% del massimo consumo di ossigeno) *(livello di prova VI, forza della raccomandazione A).*
• È consigliato un tipo di esercizio aerobico (nuoto, bicicletta, camminata, ecc.) da praticare quotidianamente. Si possono anche associare esercizi che stimolano la flessibilità e la forza soprattutto di braccia e tronco, adeguati all'età e allo stadio dello sviluppo del bambino, con frequenza di 2-3 volte/ settimana *(livello di prova I, forza della raccomandazione A).*
• La durata dell'esercizio dovrebbe essere inizialmente di 30 minuti, da aumentare gradualmente nelle sedute successive *(livello di prova VI, forza della raccomandazione A).*

Conclusioni

Sulla base di queste considerazioni, risulta essenziale identificare precocemente i bambini a rischio cardiovascolare, riconoscerne il grado sulla base della coesistenza di diversi fattori di rischio, ed avviarli precocemente ad un programma di correzione dello stile di vita, affiancato da follow-up ed eventuale terapia, cosi da contrastare lo sviluppo di malattie cardiovascolari in età giovane o adulta.

Take home messages

- Il rischio cardiovascolare si può instaurare già in giovane età, al punto che anche l'esposizione a fattori di rischio pre-natali sembra influire a lungo termine sul rischio cardiovascolare.
- È fondamentale favorire nei bambini un corretto stile di vita mediante raccomandazioni dietetiche e indicazioni all'attività fisica quotidiana.
- Negli ultimi anni si è assistito ad un netto aumento della prevalenza di obesità in età pediatrica, che suggerisce un'accurata valutazione clinico-metabolica e del profilo lipidico e glucidico ai fini di una diagnosi precoce, oltre che interventi di politica sanitaria volti a migliorare lo stile di vita nei bambini e negli adolescenti.

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2.2.4. Diagnosis, treatment and prevention of pediatric obesity: consensus position statement of the Italian Society for Pediatric Endocrinology and Diabetology and the Italian Society of Pediatrics

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Abstract

The Italian Consensus Position Statement on Diagnosis, Treatment and Prevention of Obesity in Children and Adolescents integrates and updates the previous guidelines to deliver an evidence based approach to the disease. The following areas were reviewed: (1) obesity definition and causes of secondary obesity; (2) physical and psychosocial comorbidities; (3) treatment and care settings; (4) prevention. The main novelties deriving from the Italian experience lie in the definition, screening of the cardiometabolic and hepatic risk factors and the endorsement of a staged approach to treatment. The evidence based efficacy of behavioral intervention versus pharmacological or surgical treatments is reported. Lastly, the prevention by promoting healthful diet, physical activity, sleep pattern, and environment is strongly recommended since the intrauterine phase.

Background

Contrasting pediatric obesity is among the priority goals in the healthcare agenda of the Italian National Healthcare System. Beyond the high prevalence and persistence of pediatric obesity [1], robust evidence demonstrates that physical and psychosocial complications are already present in obese children [2] and worsen in adulthood. Therefore, prevention and treatment of pediatric obesity and complications are key strategic goals, in order to reduce morbidity, mortality, and expected costs for the care of obese adults. The very fruitful scientific research on pediatric obesity of the last decade justified to update the guidelines, in order to provide the best evidence-based reccomendations. Therefore, the Italian Society for Pediatric Endocrinology and Diabetology and the Italian Society of Pediatrics, with other Pediatric Societies joined in the common objective of contrasting pediatric obesity, made this Consensus on “Diagnosis, therapy and prevention of obesity in children and adolescents”, updating the document published in 2006 [3].

Methods

Four main topics were defined: 1) diagnostic criteria, secondary obesity; 2) comorbidities; 3) treatment and care settings; 4) prevention. Coordinators were identified for each topic and specific questions listed. Twenty experts’ groups were set up, embracing all the skills needed for document processing. Each group systematically revised the literature on the assigned themes limited to the time frame 1 January 2006 to 31 May 2016 and patients’age range 0–18 years. The article search

was done through PubMed using MeSH terms or descriptors. Scientific articles, systematic reviews, meta-analysis, consensus, recommendations, international and national guidelines published on pediatric obesity even prior to 2005 were considered and deemed useful to the Consensus. The level of evidence (LOE) and the grade of raccomendation were established in accordance with the National Manual of Guidelines [4] (Additional file 1). Each working group prepared a preliminary draft reporting LOE for each specific recommendation, followed by a brief description of the scientific evidence in support, epidemiological data, and any notes deemed as useful. A Consensus Conference was held in Verona, on June 9th, 2016 in the presence of the document extensors and delegates of the Scientific Societies to discuss and approve the preliminary draft. The final document was sent on October 10th, 2016 to all the extensors and members of the Pediatric Obesity Study Group of the Italian Society for Pediatric Endocrinology and Diabetology and approved on 28th February 2017 in its definitive form. Literature search was updated before preparing the final draft; no additional relevant publication was identified which might have required a change in the statements.

Diagnosis

Diagnostic criteria for defining overweight, obesity and severe obesity

The definition of overweight and obesity is based on the use of percentiles of the weight-to-length ratio or body mass index, depending on sex and age. LOE V-A In children up to 24 months, the diagnosis of overweight and obesity is based on the weight-to-length ratio, using the World Health Organization (WHO) 2006 reference curves [5]. After the age of 2 years it is based on the Body Mass Index (BMI), using the WHO 2006 reference system [5] up to 5 years and the WHO 2007 reference system [6] thereafter (Table 1). The recommendation of using the WHO standard is based on the need to propose a reference system which, although is not an ideal model to assess adiposity in single children or groups, it has a greater sensitivity in identifying children and adolescents with overweight and obesity, in a period of particular seriousness of the pediatric obesity epidemic in Italy. On the contrary, the Italian BMI thresholds [7] underestimate the prevalence of obesity compared to WHO, probably because they were based on measurements taken during the epidemic increase of obesity [8].

The cut-off to define severe obesity is represented by the BMI > 99th percentile. LOE VI-B

It has been demonstrated that the 99th percentile of BMI identifies subjects with higher prevalence of cardiometabolic risk factors and persistence of severe obesity in adulthood with respect to the lower percentiles [9]. The WHO system provides the values of the 99th percentile of BMI which approximate + 3 SDS from 2 years upwards. However, as for overweight and obesity classification, the WHO terminology for severe obesity differs between younger (0–5 years) and older children/adolescents (5–18 years): the 99th percentile identifies "obesity" in the former group, and "severe obesity"in the latter. This cautious approach is motivated by the fact that the growth process differs between younger and older children; moreover few data are available on the functional significance of the cut-offs for the upper end of the BMI-for-age distribution in pre-school age [10, 11]. A scientific statement from the American Hearth Association proposed the 120% above the age and sex 95th percentile of BMI or an absolute BMI ≥ 35 kg/m^2 (equivalent to class 2 obesity in adults) as an alternative to the 99th percentile [12]. The impact of this system using the WHO thresholds has yet to be assessed in clinical practice.

Table 1. Diagnostic criteria to classify overweight and obesity

Age	0–2 years	2–5 years	5–18 years
Index	Weight-to-lenght ratio	BMI	BMI
Reference	WHO 2006	WHO 2006	WHO 2007
>85th percentile[a]	At risk of overweight	At risk of overweight	Overweight
>97th percentile[a]	Overweight	Overweight	Obesity
>99th percentile[a]	Obesity	Obesity	Severe obesity

[a]the 85th, 97th and 99th percentiles approximate z-scores of + 1, + 2 and + 3, respectively.

Secondary obesity

The clinical suspicion of secondary obesity arises after careful anamnestic, anthropometric and clinical evaluations. LOE III-A

Obesity may be ascribed to a specific cause (endocrine, hypothalamic, genetic, iatrogenic). Therefore, clinical history, peculiar signs and symptoms must be accurately assessed such as: 1) onset of obesity before 5 years and/ or rapid progression, especially in association with clues suggesting secondary causes (i.e. genetic forms); 2) continuous and/or rapid weight gain associated with reduced height velocity or short stature; 3) delayed cognitive development; 4) dismorphic features; and 5) use of drugs inducing hyperphagia (i.e. corticosteroids, sodium valproate, risperidone, phenothiazines, ciproeptadine) [13]. Early-onset obesity occurring in a child with

delayed psychomotor development, cognitive deficiency, short stature, cryptorchidism or hypogonadism, dysmorphisms and characteristic facial features, ocular and/or auditory alterations, is suggestive of a syndromic form [14]. Prader-Willi syndrome is the most common one, whereas Bardet-Biedl, Alström, Cohen, Borjeson-Forssman and Carpenter are more rarely observed [15–20]. Obesity occurs frequently in children with trisomy 21, Klinefelter and Turner syndromes [21–23]. The monogenic forms, albeit uncommon, are nevertheless the most frequent causes of obesity with early onset compared to endocrine and syndromic forms [24] and are due to dysregulated hunger satiety circuits [25]. Certain monogenic forms are characterized by tall or normal stature [14]. Suspicion of syndromic or monogenic forms is confirmed by genetic investigations.

Comorbidities

Hypertension

Blood pressure measurement is recommended in all children with overweight or obesity from the age of 3 years. LOE I-A

Obesity is the main risk factor for hypertension in children and adolescents [26, 27]. The risk increases with obesity severity [28]. As blood pressure (BP) levels change according to sex, age, ethnicity and obesity, the prevalence of high BP levels and especially hypertension is heterogeneous (7–30%) in obese children [29, 30]. White coat hypertension may cause overestimation of the high BP prevalence, but the effect tends to disappear if BP is measured on at least 2–3 occasions [29]. Screening can be anticipated in children < 3 years if there is a history of neonatal complications, cardiac malformations, genetic diseases, acquired or congenital kidney diseases, neoplasms, drug use, illnesses which induce increased intra-cranial pressure [31] (LOE III-B).

The definition of high BP levels requires a precise methodology and the use of tables expressing by sex and age the percentile of systolic and diastolic blood pressure as a function of the height percentile. LOE III-A

The method of measuring BP and the definition of high systolic (SBP) and diastolic BP (DBP) values are based on the guidelines of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Children and Adolescents and the European Society for Hypertension (Table 2) [32, 33]. Primary forms of hypertension are mainly associated with obesity and more frequent in children > 6 years. Secondary forms are predominant in younger children. Nephropathy, nephrovascular pathologies and coarctation of the aorta account for 70–90% of the

causes of secondary hypertension in pediatric age, while hypertension by endocrine causes is rare [34]. Various drugs (steroids, erythropoietin, theophylline, beta-stimulants, cyclosporin, tacrolimus, tricyclic antidepressants, antipsychotics, monoamino oxidase inhibitors, nasal decongestants, oral contraceptives, and androgens) can increase BP. If stage I hypertension is confirmed on 3 different visits, the following diagnostic work-up is recommended: 1) assessment of blood urea nitrogen, creatinine, glycemia, electrolytes, lipids, urine examination, microalbuminuria (may be influenced by physical activity) (LOE II-A); 2) measurement of glomerular filtration by formulas for renal function monitoring (LOE III-B); 3) echocardiography to assess organ damage (left ventricular hypertrophy, altered cardiac structure) (LOE III-A) [35]. Left ventricular remodeling or concentric hypertrophy are associated with high BP levels and other comorbidities such as visceral obesity and atherogenic dyslipidemia [36, 37]. Weight loss and reduced sodium intake are recommended. If stage II hypertension or secondary causes are present, the patient must be referred to a specialist for further investigations and treatment [31, 34, 35].

Table 2. Definition of the blood pressure values

Normal BP	SBP and DBP < 90th percentile by gender, age and height
High normal BP	SBP and/or DBP ≥90th but <95th percentile by gender, age and height (BP > 120/80 mmHg even <90th percentile are considered as high normal BP).
Hypertension (Stage I)	SBP and/or DBP ≥95th <99th percentile + 5 mmHg by gender, age and height.
Hypertension (Stage II)	SBP and/or DBP ≥99th percentile + 5 mmHg by gender, age and height.

BP Blood pressure, SBP Systolic blood pressure, DBP Diastolic blood pressure

Prediabetes and type 2 diabetes mellitus

Fasting blood glucose measurement is recommended in all children and adolescents with overweight and obesity since the age of 6, as the first step for screening prediabetes and type 2 diabetes. LOE V-A

The diagnosis of prediabetes, i.e. high fasting blood glucose and impaired glucose tolerance (IGT) or overt type 2 diabetes (T2D) is based on fasting plasma glucose or oral glucose tolerance test (OGTT) [38]. The use of hemoglobin glycosilated A1c (HbA1c) is still controversial in pediatric age [38–42]. The criteria for defining prediabetes and T2D are summarized in Table 3. The screening must be repeated after

3 years, unless rapid weight increase or the development of other cardiometabolic comorbidities occur. Since evidences provided from national studies suggest that prediabetes is already present in about 5% obese children < 10 years [43], it is recommended to start the screening by testing fasting glucose in all overweight or obese children after the age of 6 years. The OGTT is indicated after the age of 10 years or at onset of puberty in agreement with the criteria of the American Diabetes Association [38] (Table 4). Certain conditions, such as non-alcoholic fatty liver disease (NAFLD), fasting blood glucose ≥86 mg/dL, or a combination of triglycerides (TG) > 100 mg/dL plus fasting blood glucose > 80 mg/dL, or TG to HDL-cholesterol ratio (TG/HDL-C) ≥2.2, have been associated with increased risk of IGT [44–47] and therefore, an OGTT may be considered in latter cases (LOE VI-B) (Table 4).

Table 3. Criteria for the diagnosis of prediabetes and diabetes mellitus

Prediabetes Impaired fasting glucose: plasma glucose (after 8 h of fasting) between 100 (5.6 mmol/l) and 125 mg/dl (6.9 mmol/l) Impaired glucose tolerance: plasma glucose after 2 h of the OGTT between 140 and 199 mg/dl (7.8 mmol/l) HbA1c between 5.7–6.4% (39–47 mmol/mol)
Type 2 diabetes Random glycemia ≥200 mg/dl (11.1 mmol/l) and symptoms suggestive of diabetes (glycosuria without ketonuria, polydipsia, weight loss). Confirmation with a second test is not necessary. If symptoms are lacking, diagnosis is made whether one of the following criteria is fullfilled: 1. Fasting glycemia ≥126 mg/dl after 8 h of fasting. 2. Glycemia ≥200 mg/dl after 2 h of the OGTT. 3. HbA1c ≥6.5% or ≥ 48 mmol/l (IFCC reference method using high-performance liquid chromatography (caution in pediatric age). If one test is positive, the diagnosis must be confirmed by a second test. Whenever the two tests are discordant, the patient should be strictly monitored and the positive test repeated within 3–6 months. If the diagnosis of diabetes is made, the assessment of the autoimmune markers (ICA, GAD, IA2, IAA o ZnT8) is needed to exclude type 1 diabetes. Genetic screening for monogenic diabetes is recommended in the rare cases presenting with obesity, diabetes, negative autoimmunity tests and family history for T2D.

Table 4. Indication for the oral glucose tolerance test in children and adolescents with overweight or obesity

Children with fasting plasma glucose ≥100 mg/dl or HbA1c ≥5.7–6.4% (39–46 mmol/mol)
Adolescents (> 10 years of age) or at onset of puberty with overweight (BMI > 85th percentile) and at least one of the following risk factors:
- Family history of T2DM in first- or second-degree relatives;
- Race/ethnicity (African American, Latino, Native American, Asian American, or Pacific Islander);
- Signs or conditions associated with insulin resistance (hypertension, dyslipidaemia, polycystic ovary syndrome, acanthosis nigricans, or small for gestational age at birth)
- Maternal history of diabetes or gestational diabetes during the child's gestation
- Non alcholic liver disease
- TG/HDL-Cholesterol ≥2.2
- Fasting plasma glucose ≥86 mg/dl
- TG > 100 mg/dl and fasting plasma glucose > 80 mg/dl

Dyslipidemia

The measurement of cholesterol, HDL-cholesterol and triglycerides is recommended in all children and adolescents with obesity since the age of 6. LOE I-A

The dyslipidemic pattern associated with childhood obesity consists of a combination of elevated TG, decreased HDL-C, and low density lipoprotein cholesterol. The prevalence of dyslipidemia among obese children was 46–50.4% [48, 49]. Because the association of obesity/ hyperlipidemia (expecially hypertriglyceridemia) is predictive of fatal and non fatal cardiovascular events in adult life [50], the screening of dyslipidemia is recommended, and should be repeated after 3 years, if negative, or more frequently if rapid increase in weight or development of other cardiometabolic comorbidities occurs [51, 52].

In the absence of national reference values, the diagnosis of dyslipidemia is based on the criteria proposed by the expert panel on integrated guidelines for cardiovascular health and risk reduction in children and adolescents. LOE III-B

The cut-offs for the definition of abnormal lipid levels as proposed by the Expert Panel [51] are summarized in Table 5. Recent studies have shown that the TG/HDL-C ratio is associated with insulin resistance and early organ damage (heart, liver, and carotid) [53–55]. The Tg/ HDL-C > 2.2 can be considered as a marker of atherogenic dyslipidemia and an altered cardiometabolic risk profile in obese children in Italy [55,

56] (LOE V-A). Children with TG ≥ 500 mg/dL or LDL-Cholesterol persistently ≥ 160 mg/dL need lipid specialist consultation [51].

Table 5. References values to define dyslipidemia according to the Expert Panel on Integrated Guidelines for Cardiovascular Health and Risk Reduction in Children and Adolescents [60]

Cathegory	Acceptable	Borderline-high	High
Total cholesterol (mg/dl)	<170	170–199	≥200
LDL-cholesterol (mg/dl)	<110	110–129	≥130
Non HDL-cholesterol (mg/dl)	<120	120–144	≥145
Triglycerides (mg/dl)			
0–9 years	<75	75–99	≥100
10–19 years	<90	90–129	≥130
	Acceptable	Borderline-low	Low
HDL-cholesterol (mg/dl)	>45	40–45	<40

Lipids are determined after at least 12 h of fasting LDL Cholesterol is calculated by the Friedewald's formula as total Cholesterol minus HDL cholesterol minus (Triglycerides/5) (provided that triglycerides are < 400 mg/dL). Non HDL cholesterol is calculated as total Cholesterol minus HDL Cholesterol.

Gastroenterological complications

Non-alcoholic fatty liver disease

The assessment of transaminases and liver ultrasound is suggested in all children and adolescents with obesity starting at age of 6 years. LOE V-B

The prevalence of NAFLD in obese children is 38–46% [57, 58]. Bright liver on ultrasound examination, with or without elevation of alanine aminotransferase (> 26 U/L in boys and > 22 U/L in girls) suggests NAFLD [59]. Weight reduction and re-testing after 6 months are initially recommended [60] (LOE III-A). If liver hyperechogenicity and/or elevated alanine aminotransferase persist despite weight loss, other causes of hepatic disease (i.e. viral hepatitis, Wilson's disease, autoimmune hepatitis, alpha 1 anti-tripsin deficiency, etc.) should be investigated. If ALT persistently exceeds twice the normal limit, the patient must be referred to a pediatric hepatologist [61]. Liver biopsy is the gold standard for diagnosis, but its invasiveness and the possible complications limit its use only to selected cases [61] (LOE VI-A). Assessment of

biochemical markers (i.e. retinol-binding protein 4, cytokeratine 18, hyaluronic acid) [62, 63] as indicators of hepatic histological damage, or clinical-laboratory scores as indicators of prognostic risk is not recommended in the clinical practice [64, 65] (LOEV-D). Non-invasive investigations (magnetic resonance, computed tomography, elastography, ultrasound elastography) [66] are promising but again their use is not recommended. (LOE V-D). NAFLD may be screened also in overweight children presenting with waist-to-height ratio > 0.5 and the assessment yearly repeated [67].

Gallstones

There is no evidence to recommend the screening for colelithiasis. LOE IV-C

Gallstone disease occurs in approximately 2% obese children and adolescents [68, 69]. The rate increases up to 5.9% in obese patients with rapid weight loss [70]. The disease is rarely diagnosed, since it is symptomatic only in 20% cases [69, 71] In the presence of pain, primarily in the right upper quadrant, nausea and vomiting, assessment of serum transaminases, gamma glutamil transpherase, alkaline phosphatase, bilirubin and liver ultrasonography are diagnostic [71–73].

Gastroesophageal reflux

Gastroesophageal reflux is suspected in the presence of evocative symptoms (such as pyrosis, heartburn, regurgitation). LOE VI-B

The prevalence of gastroesophageal reflux in obese children and adolescents is 13–25% (diagnosis made through questionnaires) [74–78]. Suggestive symptoms are pyrosis, epigastralgia, regurgitation. Weight loss may improve these symptoms. However, if symptoms persist or more severe symptoms occur (dysphagia, vomit) despite weight loss, referral for specialist investigations (gastrointestinal contrast study, endoscopy and oesophageal pH or impedance monitoring) and treatment is required [79].

Polycystic ovary syndrome

The components of the polycystic ovary syndrome should be considered in all female adolescents with obesity. LOE VI-A

Polycystic ovary syndrome (PCOS) is characterized by hyperandrogenism (acne, hirsutism and alopecia) and ovary dysfunction (oligo-amenorrhea). It is associated with increased risk of infertility, T2D, metabolic syndrome and cardiovascular disease in adulthood [80, 81]. In adult women, the diagnosis is based on at least two of the

following criteria: a) oligo-ovulation and/or anovulation; b) clinical and/or biochemical signs of hyperandrogenism; c) polycystic ovary [82]. Since there is no widely accepted definition for PCOS in the teenage, it is suggested to identify and treat the single components of the syndrome [83]. Referral for specialist investigations is required to exclude other hyperandrogenic causes (congenital adrenal hyperplasia, androgen-secreting tumors, Cushing syndrome/disease) [80–84].

Respiratory complications

Respiratory symptoms and signs should be sought in children and adolescents with obesity. LOE V-A

The prevalence of respiratory problems, such as asthma, obstructive sleep apnea syndrome (OSAS), and obesity hypoventilation syndrome (OHS) is higher in obese children and adolescents compared to the general population [85, 86]. OSAS affects 13–59% of obese children [85, 87–89]. The severity is strongly associated with excess weight, while adeno-tonsillar hypertrophy, skull-facial abnormalities, Afro-American and Asian ethnicities are modulation factors [85, 90]. The OHS is less frequent, affecting 3.9% obese patients [89]. Children and adolescents may present with increased breath rate, dyspnea after moderate efforts, wheezing, chest pain. OSAS is associated with intermittent hypoxemia, hypercapnia, and disrupted sleep. Specific symptoms and signs are: snoring/noisy breathing (> 3 nights/week), pauses in breathing, mouth breathing, awakening headache that may persist during the day, daytime sleepiness, inability to concentrate, poor academic performance, hyperactivity, cognitive deficits. Rarely, growth delay, systemic hypertension pulmonary and artery hypertension have been reported in severe obesity [91, 92]. OHS is characterized by severe obesity, chronic daytime alveolar hypoventilation (defined as PaCO2 levels > 45 mmHg and PaO2 < 70 mmHg), a pattern of combined obstruction and restriction, in absence of other pulmonary, neuromuscular, metabolic, or chest diseases that may justify daytime hypercapnia [89]. In the presence of respiratory symptoms/signs, transcutaneous saturation of O2 should be determined; for values < 95%, arterial blood emogasalysis should be performed. If asthma and/or any other ventilatory dysfunction are suspected, respiratory function (spirometry, pletismography, six minute walking test) should be measured. Allergological evaluation is not necessary, unless a history of atopia is reported [86], neither is necessary measuring the exhaled nitric oxide [93, 94]. Night polysomnography is the gold standard for diagnosis of sleep disorders. The apnea/hypopnea index (ratio between total number of apnea/hypopnea episodes and

duration of sleep in hours) indicates the severity (1–5 very mild; 5–10 mild; 10–20 moderate;> 20 severe). Alternatively, overnight pulse oximetry can be used, which is very specific but less sensitive. Otorhinolaryngoiatric or odontoiatric evaluations complete the diagnostic work-up. Cardiology referral should be considered in severe and long-lasting OSAS for assessing lung or systemic hypertension, and left ventricular hypertrophy [91]. Cognitive assessement may be required to assess neurocognitive damage and behavioral disorders [95].

Orthopaedic complications

Orthopaedic complications should be sought in the presence of musculoskeletal pain and joint limit ation at the lower extremity. LOE V-A

Severity of obesity and sedentary lifestyle influence the morphology of osteo-cartilaginous structures and growth plate, leading to serious orthopedic consequences [96, 97]. The main orthopaedic complications are: slipped capital femoral epiphysis, Blount's disease or tibia vara, valgus knee, flat foot [98–103]. Slipped capital femoral epiphysis may affect one or both hips; it usually occurs during the pubertal growth spurt. Hip pain and/or knee pain, an acute or insidious onset of a limp and decreased range of motion in the affected hip are the main symptoms/signs [104]. Blount disease is characterized by the varus deformity of the leg. Clinical manifestation is the instability of the knee in walking and lateral movements, simulating lameness [100]. Valgum knee is characterized by the deformity of the femoro-tibial angle in valgism; other deformities are associated, such as deviations in rotation of the tibia [101, 102]. Flat foot is characterized by flattening of the medial arch and heel valgus. Pain may be reported along the medial part of the foot, with more specific complaints after exercises or long walks [105]. Although obesity may exhibit higher risk of fracture, the measurement of bone density is not recommended. LOE V-D The risk of fracture is increased in obese children, even for low energy injuries [106–108]. Inactivity, abnormalities in biomechanics of locomotion, inadequate balance may expose the obese child to fall and consequently to fracture, especially of the forearm [109]. There is no evidence that obesity results in a reduction of bone density [110]: while some studies have described an increased or normal bone mineral content, others reported a reduced bone mass in relation to bone size and weight [107].

Renal complications

There is insufficient evidence to recommend screening of kidney complications in non-diabetic and non-hypertensive children and adolescents with obesity. LOE IV-D

In adults, obesity is an independent risk factor for chronic kidney disease [111]. Obesity complication, (i.e. hypertension, dyslipidemia, insulin resistance, T2D, inflammatory state, autonomous system dysfunction) indeed, can alter the kidney function [112]. Peculiar to obesity, the obesity-related glomerulopathy is a secondary form of segmental focal glomerulosclerosis occurring tipically in obese patients and that improves after weight loss [112]. Obesity is likely to be a risk factor for chronic renal disease in children too. Indeed, children with renal disease have BMI higher than healthy population [113] and kidneys transplanted from obese donors have reduced glomerular filtration and higher rate of dysfunction than the kidneys obtained from normal weight donors [114]. In the light of current evidence [115–119], the assessment of microalbuminuria is not recommended in non-diabetic and non-hypertensive obese children (LOE IV-D). Individual cases of severe obesity (BMI > 40) that may be associated with proteinuria in the nephrotic range remain to be evaluated individually (LOE VI-C).

Idiopathic endocranic hypertension

Headache, vomiting, photophobia, transiently blurred vision, diplopia should be sought in subjects with overweight/obesity, especially if adolescents. LOE V-A

Idiopathic endocranial hypertension is rare but potentially serious condition that can cause permanent loss of vision [120–122]. Prevalence and risk of recurrence increase with the severity of BMI [123–125]. Some symptoms occur frequently in adolescents as in adults (headache, vomiting, photophobia, transiently blurred vision, diplopia), while irritability, apathy, drowsiness, dizziness, cervical and dorsal pain are less frequent [123, 126]. The diagnosis is based on the presence of increased intracranial pressure documented with a lumbar puncture, papilledema, normal neurologic examination results (except for cranial nerves), normal cerebrospinal fluid composition, normal appearance of neuroimaging studies, and no other identifiable cause of increased intracranial pressure [127].

Migraine and chronic headache

Promoting healthy lifestyle habits and weight control can be a protective factor of migraine and chronic headache. LOE V-B

Recent studies have reported greater risk of episodic or recurrent migraine or daily chronic headache or tension headache in obese children and adolescents than the normal population [128, 129]. Some drugs used for headache and migraine have weight gain as side effect [129]. Negative lifestyle factors, which may influence the prevalence of recurrent headache, are possible targets for preventive measures [130]. An intervention study reported improvement in migraine symptoms after weight loss [131].

Psychosocial correlates

Psychosocial discomfort may affect therapeutic success, therefore it should be identified as part of the multidisciplinary assessment. LOE V-A

Recognition of psycho-social correlates (unsatisfactory body image, depressive and anxiety symptoms, loss of eating control, weight concern, dysfunctional social relationships, inactivity due to problematic body image, obesity-related stigma, low self-esteem, academic failure) is crucial to promoting specific strategies that improve the results in weight loss programs [132–134]. Although obesity is not a psychopathological and behavioral disorder, referral for specialist consult is needed in the suspicious of depressive and/or anxious symptoms, dysmorphophobic traits, suicidal risk, and eating disorders [135, 136].

Binge eating disorder

The presence of binge eating disorder should be considered in the multi-professional assessment of an obese child or adolescent. LOE V-B

Binge Eating Disorder (BED) is the most common Nutrition and Eating Disorder found in pediatric obesity. It is indicative of psychopathology and is a serious risk factor for the development of obesity, especially in the presence of family history of obesity and marked negative experiences coupled with factors predisposing to psychiatric disorders [137]. BED is often preceeded by uncontrolled eating since childhood, occasional bulimia, obesity, but also by an attention deficit and hyperactivity disorder [136–138]. Upon referral to appropriate medical subspecialists and/or mental health personnel, the diagnosis of BED is critical to the therapeutic success. It may be necessary associating psychological and pharmacological therapy (only in selected cases) within the weight-loss treatment program [136, 137, 139].

Treatment

Changes in diet and lifestyle leading to a negative caloric balance is recommended to gradually reduce the BMI. LOE I-A

The main objective is a permanent change in the child's eating habits and lifestyle, rather than attaining rapid weight loss through low-calorie diets. It is indispensable involving the whole family and setting realistic goals. Further goals: - maintaining an appropriate growth rate and achieving an healthier weight-to-height ratio; - reducing weight excess (without necessarily achieving the ideal weight), in particular the fat mass, while preserving the lean mass; - maintaining or promoting good mental health (self-esteem, correct attitudes toward food and body image, health related quality of life); - treatment and improvement/resolution of complications, if present, in the shortest time possible; - achieving and mantaining a healthier weight-to-height ratio and preventing relapses.

Diet

A balanced and varied diet is recommended (LOE I-A)

The classic diet-therapy based on the prescription of a low calorie diet is not effective in the medium/long term with relapses and failures, increased risk of dropout and progression into more complicated forms [140] (LOE III-B). The educational process starts from the assessment of the child's and family's dietary habits, by means of the assessment of meal composition, portions, frequency of food intake, food preferences or aversions, use of condiments, cooking methods and food presentation [141–145] (LOE I-A). Food diary is an excellent tool for assessing eating behavior; it should be compiled by the child together with the parents or by the adolescent and evaluated by the operator [146, 147] (LOE I-B).

Dietary advice

1. Eat 5 meals a day (three meals and no more than two snacks) [148] (LOE V-B).
2. Have an adequate breakfast [149] (LOE II-B).
3. Avoid eating between meals [150] (LOE III-B).
4. Avoid high-energy and low nutrient density foods (eg. sweetened or energizing drinks, fruit juices, fast food, high-energy snack) [151, 152] (LOE III-B).
5. Increase intake of fruit, vegetables and fiber rich cereals [153, 154] (LOE VI-A).
6. Limit portions [155, 156] (LOE I-A).

If a hypocaloric diet is needed, it should fulfill the National Recommended Energy and Nutrient Intake Levels, based on sex, age and ideal weight for stature (proteins 1

g/kg/day; carbohydrates 45–60% of total calories; simple sugars < 15% of total calories, lipids 20–35% of total calories starting from 4 years of age, saturated fatty acids < 10% of total calories) [157] (LOE VI-A).

Efficacy of dietary regimens

There are currently no randomized controlled trials (RCTs) examining the effects of different diets on child's weight and body composition, regardless of potential confounders such as treatment intensity, behavioral or physical activity strategies [158, 159].

Very low caloric diet

It is the most effective regimen in terms of weight loss [160]. One example is the protein-sparing modified fast (600–800 kcal/day, protein 1.5–2 g/kg ideal weight, carbohydrates 20–25 g/day, multivitamins + minerals, water > 2000 mL/day), which can be prescribed in selected patients with severe obesity, under close medical surveillance and in specialized pediatric centers. The aim is to induce rapid weight loss (duration of this restrictive diet no longer than 10 weeks) followed by a less restrictive diet regimen balanced in macronutrients. RCTs are not available to evaluate medium to long-term efficacy compared to other diet-therapies and possible adverse effects on growth (LOE III-C). Traffic light and modified traffic light diets Reduced caloric intake (1000–1500 kcal/day) is achieved trough categories of foods grouped by nutrient density [161]. They were found to produce a significant improvement of BMI in 8–12 year old children even in the long term [162] (LOE III-C).

Non-restrictive approach

It does not consider a given caloric intake or nutrient composition, rather it focuses on the consumption of low-fat and high-nutrient density foods (LOE III-C).

Replacement meals

They are not recommended, since efficacy and safety have not been tested in children/adolescents. No significant effect has been demonstrated for diets with specific macronutrient composition and medium caloric content in children. In particular:

Hypocaloric diets with low glycemic index and low glycemic load

Although an effect on satiety is suggested, their superiority compared with other dietary approaches has not been proved over the medium term [163–165] (LOE I-C).

Exercise

It is recommended to associate physical exercise to diet. LOE I-A

Physical exercise ameliorates body composition and reduces cardio-metabolic risk factors. [166–171]. Change in body composition (in particular fat reduction) rather than reduced BMI is sensitive to evaluating the effectiveness of exercise [166, 172]. It has not yet been proven which is the ideal exercise for obese children [170]. Low evidence demonstrates that combining aerobic and resistance exercises results in fat mass reduction, especially with programs of at least 2 weekly sessions and duration > 60 min [173] (LOE I-B).

The evidence is limited that exercising at higher intensity is more effective in modifying the body composition (LOE I-B).

Owing to difficulty of obese subjects to practice exercise at high intensity, there is no evidence that vigorus efforts result in greater body fat reduction [166]. Children and adolescents should practice 60 min or more of physical activity every day, which should be mainly represented by aerobic exercises at least of moderate intensity; resistance exercises are suggested for at least 3 times a week, adjusted to the physical abilities of the obese child [174, 175]. Examples of aerobic and resistance exercises for obese children and adolescents are synthetized in Table 6. The practice of recreational activities and sports that involve a large amount of body mass such as swimming, soccer, basketball, volleyball, handball, rugby or require anaerobic and neuromuscular power, such as gymnastics or judo is encouraged. In severe obesity exercises that put constant weight or repeated impact on the child's legs, feet and hips should be avoided.

Sedentary behaviors

It is suggested to reduce the time spent in sedentary behaviours (television viewing, videogaming, internet surfing). LOE II-B

Weight gain may be only partially due to sedentary behaviors [176–179]; in the case of television viewing, it may be associated with overfeeding [180]. Interventions targeting sedentary behaviour were more effective in children aged 5–12 years [181].

Use of active video games may be suggested to increase daily energy expenditure in obese and sedentary children. LOE I-B

Active video games represent an additional strategy to reduce sedentary behaviors. They do not replace 'real' sports activities, but can contribute to increase energy

expenditure beyond the sedentary activity threshold, provided they are supervised by adults [182–187].

The systematic use of active video games for weight loss and improvement of body composition is not discouraged. LOE III-C

While studies are not consistent with the recommendation to use active video games to obtain weight loss or improve body composition, their use is not recommended but neither discouraged to obtain other effects (improvement in vascular response, heart rate and VO2max or obesity-related comorbidities; positive psycho-behavioral and psycho-social effects) [182–184].

Table 6. Examples of aerobic and resistance exercises suggested for obese children and adolescents

Aerobic exercises[a]	exercises on treadmill, cycle ergometer, elliptical trainer
	water activities (swimming or water aerobics)
Resistance exercises[a]	body weight exercise (push-ups, sit-ups, abdominal crunches), lifting free weights, using weight training machines and elastic resistance bands, circuit training

[a]under qualified supervision

Cognitive and family-based behavioral therapy

Cognitive behavioral treatment or family-based behavioural treatment are both recommended to favor better adhesion to diet and physical activity. Cognitive behavioral treatment LOE III- B; family-based behavioral treatment LOE I- A

Cognitive behavioral techniques are effective. Neverthless, they are not easily applicable requiring specific training of the multidisciplinary team [188– 190]. The most effective techniques are goal setting, self-monitoring (through food and physical activity diaries), contingency training, stimulus control, positive reinforcement, cognitive restructuring, problem solving [191]. Family-based behavioral treatments involve multicomponent interventions aimed at changing the lifestyle of the whole family, with goals shared between parents and children [191–194]. Interventions in which parents are active participants are more effective than interventions in which they are not encouraged to make their own behavioral changes. On the other hand, family-based therapies require greater investment of resources in terms of time and staff involved [188, 190, 192–198]. In children, they are more effective than treatments not involving

parents. There is no robust evidence demontsrating their superiority in adolescents [189, 190, 194] (LOE I-A). Therapeutic education has been proposed in the recent years, using tools of cognitive-behavioral approach and motivational interview, such as reflective listening, therapeutic alliance, family approach, modeling, motivational counseling, narrative approach, positive reinforcement, goal setting, negotiating treatment objectives. It requires professional skills of all the team members with ongoing training [199–201] (LOE VI-B). Child Appetite Awareness Training and Cue Exposure Treatment are still considered experimental and require further studies [202, 203] (LOE V-C).

Indicators of successful treatment

The BMI standard deviation score is recommended to estimate weight loss. LOE V-B

The reduction of the BMI Standard Deviation Score (BMI-SDS) is the best indicator of the weight loss amount taking into account the patient's age and gender [204]. A reduction > 0.5, but even > 0.25 (consistent with a 1 kg/m^2 BMI reduction or stable weight for more than 1 year in a growing child) was associated with improved body composition and decreased cardio-metabolic risk [205]. Waist circumference and waist/height ratio can be used to monitor abdominal fat variations but are subject to error and offer no benefit over BMI [204, 206–208]. The same is true for the skinfold thicknesses [204, 209].

Other behavioral indicators (related to diet, lifestyle, physical fitness or quality of life) can be considered if no substantial reduction in the BMI-SDS occurs. LOE VI-B

Since the percentage of weight loss is generally low, evaluation based solely on the BMI-SDS may induce a sense of failure in the family and healthcare workers. In order to maintain the adherence to treatment, a stable modification of diet, physical activity and sedentary behavior, the increase of physical fitness and improvement of the quality of life should be considered as index of good compliance [210, 211].

The scarce effect of treatment in the long term demands the development of long-lasting care models and their validation. LOE VI-B

The effectiveness of treatment programs based on diet and lifestyle on BMI-SDS reduction was shown only in the short term (6–12 months) [167, 212]. In a European multicentre study, the success rate (BMI SDS reduction > 0.25) was 7% at 2 years; it reached 50% in a few number of centers, which differed for the greater intensity of intervention and training of the multi-disciplinary team [213, 214]. Only two national

studies based on diet education, cognitive or cognitive-behavioral strategies and family involvement reported BMI-SDS reduction of 0.44 after three years [199] and 1.49 after 5 years of follow up [188], respectively.

It is necessary to monitor the possible onset of eating disorders, especially when the weight loss is rapid. LOE IV-A

Dissatisfaction with body image may be related to the onset of eating disorders, especially bulimia nervosa and binge eating, but also of anorexia nervosa [215–218]. Diet education undertakings can accentuate the perceived stigma in subjects with obesity, causing drastic strategies of weight control [219, 220]. In some cases, the onset is triggered by an initially desired restriction of food, which then becomes uncontrollable. Careful evaluation of excessive weight variations and related bodily experience, especially when hypocaloric diets are prescribed, is recommended [217–219, 221].

Pharmacological intervention

Pharmacological therapy can only be applied after the failure of the multidisciplinary lifestyle intervention. LOE VI-B

When clinically significant weight loss cannot be achieved through lifestyle-based interventions, use of drugs is considered, especially in severe obesity with cardiometabolic, hepatic or respiratory disorders [222–226]. Management of drugs should be done in specialist centers [225].

Orlistat is the only drug available for the treatment of children and adolescents with severe obesity age. LOE II-B

Few studies, with small sample size and short duration, are available on the effects of anti-obesity medications in pediatric age [227–230]. Orlistat (tetra-hydro-lipstinate) is the only drug approved for the treatment of obesity in pediatric age. It seems producing significant weight loss and favoring behavioral changes [231–233]. It does not affect the mineral balance, if the low-calorie diet is associated with normal mineral content; on the contrary, attention must be paid to prevent liposoluble vitamins deficiency [234].

Bariatric surgery

Bariatric surgery is the ultimate solution in adolescents with severe obesity and resistant to all other treatments, especially when serious complications are present. LOE VI-B

The indications for surgery in the adolescent are (LOE III-B) [235, 236]: - BMI ≥35 kg/m^2 with at least one severe comorbidity, such as T2D, moderate to severe obstructive sleep apnea (AHI > 15), idiopathic endocranial hypertension, NAFLD with significant fibrosis (Ishak score > 1). - BMI ≥40 kg/m2 with less serious comorbidities, such as mild sleep apnea (apnea/hypopnea index > 5), hypertension, dyslipidemia, carbohydrate intolerance. More prudently other guidelines suggest a BMI > 40 kg/m^2 with one severe comorbidity or > 50 kg/m^2 with less serious comorbidities [223, 237]. Eligibility criteria are: adolescents with long lasting severe obesity; a. previous failure of any dietetic, behavioral or pharmacological intervention (after at least 12 months of intensive treatment); b. family and social support in managing the multidisciplinary care programs; c. decisional capacity for surgical management and the post-surgery follow-up; d. able to express the informed assent.

Surgery should be performed in a highly specialized center that guarantees the presence of an experienced multidisciplinary team. LOE III-A

The multidisciplinary team carefully evaluates the case and poses the indication for the surgey taking care of the pre-surgical assessment and post-surgical follow-up [238, 239]. The preoperative phase includes a comprehensive assessment of the patient and the family, with particular regard to physical and psychological maturation of the adolescent and his/her adherence to treatment [235, 237, 240]. Neuropsychiatric counseling should be undertaken to identify cases at risk of psychotic disorders, severe major depression, personality or eating disorders, alcoholism and drug dependence [235–237]. In the postoperative follow-up anthropometric, clinical and nutritional assessment, and counseling are performed and early or late complications are monitored. For the adverse effect on height velocity, the adolescent should have reached adequate skeletal maturation or a pubertal stage IV according to Tanner [223, 236, 237, 241] (LOE III-A). Contraindications to surgery are documented substance abuse problem and/or drug dependencies; patient inability to care for him/herself or to participate in life-long medical follow-up, no long-term family or social support that will warrant such care and follow-up; acute or chronic diseases even not directly associated with obesity threatening life in the short term; high anesthetic risk; pregnancy or planned pregnancy within the first two years after surgery, current breast-feeding [237] (LOE VI-A).

Indication for surgery must be given on a case-by-case basis by the multidisciplinary team (LOE VI-A)

Surgical procedures performed mostly by laparoscopy in adolescents and supported by at least 3 years of follow-up, are: a. restrictive interventions, including adjustable gastric bandage and sleeve gastrectomy; b. restrictive/malabsorbitive interventions, such as Roux-en Y gastric by-pass (RYGB) (LOE III-B).

Although the RYGB is the gold standard, there is no enough evidence to support this specific surgical technique compared to the others in terms of effectiveness, side effects, long-term complications and benefits [239]. Although studies in adolescents have increased, lack of RCTs makes it difficult to establish the effective efficacy at this age. There is no evidence or expert opinion supporting the efficacy of anticipating bariatric surgery to the teenage with respect to adults. A recent Cochrane review identified four RCTs in progress with expected results in the near future [242]. Several non-randomized and non-controlled trials were published with at least three years follow-up on the use of bariatric surgery in adolescents [243–247]. The published studies showed an average BMI decrease of 16.6 kg/m2 after RYGB, 11.6 kg/m^2 after gastric bandage and 14.1 kg/m^2 after sleeve gastrectomy [248]. All interventions have been associated with improvement or complete restoration of comorbidities. Most studies are consistent in demonstrating improvement of the quality of life [244, 248–250].

Care settings

For the multifactorial nature of obesity, variability in its severity, and the health implications, treatment should be conducted in multiple settings with different levels of treatment. LOE III-A

Health services should be organized in a network of services [150, 251–254]. Fundamental is the periodic training of all network operators on motivational counseling, parenting and teamwork [251]. A child- and family-centered approach is based on sharing simple and realistic objectives about eating habits, sedentary behaviours, physical activity, and verification of results related to improving nutritional status, quality of life and complications if present [255–258].

Primary care pediatricians represent the first level treatment. LOE III-A

Primary care pediatricians' responsibilities are summarized in Table 7 [259, 260]. They are the reference point for obese children/adolescents and their family, participating in the various proposals for action and decisions, when a more aggressive approach is proposed (e.g., hospitalization or surgery). The efficacy of obesity treatment in the primary care setting is still modest [261, 262], but it might improve if paediatricians are

assisted by other professionals experienced in pediatric obesity (dieticians/nutritionist, psychologist) and trained in family education and interdisciplinary work [258, 259, 263, 264] (LOE VI-B).

District or hospital outpatient services represent the second level of care. LOE VI-A

In the second level centers, the multidisciplinary team (pediatrician, dietician and psychologist) experienced in pediatric obesity defines the clinical condition of children referred by the primary care pediatricians, and runs the multidisciplinary intervention that is centered on diet education and lifestyle modification [150, 260, 265, 266]. The patient is referred to the third level health care center in case of no response to the treatment, severe comorbidities, compromised psychological balance or significantly impaired quality of life.

Specialized centers for pediatric obesity represent the third level of care. LOE VI-A

Third level centers are organized on a multidisciplinary and multiprofessional basis for comorbidity management or bariatric surgery. They admit patients who are suspected of secondary obesity or require more specialistic diagnostic assessment and/or intensive care programs, including bariatric surgery. They coordinate the networking activities as well as the training of operators and promote research activities and intervention trials in the context of specific protocols [267–271].

Transition

Pediatric obesity care should include a transition path from pediatric to adult care. LOE VI-B

It is necessary to test a transition model for adolescents with severe obesity and/or complications, particularly with metabolic syndrome, NAFLD, hypertension [272–274]. Unfortunately, the experience is extremely limited for the high drop-out, poor consideration about obesity as chronic illness, absence of pre-established pathways, possible transition to structures that follow the specific complications (eg.

hypertension), no availability of cost-effective models [275].

Table 7. Primary care pediatricians' responsibilities

Conditions	Responsabilities
Risk factors: Prenatal life: first-degree familiarity for obesity, low socioeconomic status; Neonatal life: small for gestational age, or macrosomic infant; Postnatal life: no breastfeeding, early complementary feeding, excessive weight gain in the first two years of life, early adiposity rebound	Monitoring the child's weight and length linear growth Educating to a balanced diet and healthy lifestyle since the earliest years of life Assuring appropriate timing of complementary feeding
Children and adolescents with overweight or moderate, uncomplicated obesity	Early identification of children's excess weight Promoting parental awareness of children's excess weight Motivating and supporting the family to change, possibly involving other professionals trained in childhood obesity
Severe obesity or psychological co-morbidity, or additional risk factors, or biochemical alterations, or treatment failure within 4–6 months	Identification of severe obesity Promoting parental awareness of children's excess weight Motivating and supporting the family to more intensive levels of care
Suspicion of secondary obesity	Referral to specialized centers

Prevention

Given the multifactorial nature of obesity, preventive interventions should be designed to modify the environmental and social determinants. Health and non-health professionals should be involved in implementing healthy food education and promoting physical activity. Promotion of balanced nutrition and healthy lifestyle implies the need to remodel economic, agricultural, industrial, environmental, socio-educational, recreational and health policies, including those aimed at contrasting socio-economic and ethnic minorities' inequalities [276]. To be effective, actions must be multicomponent and multilevel, building agreements and alliances among many stakeholders, including families, community organizations such as schools and sport institutions, health care providers [277–279]. Primary prevention actions begin from the prenatal age, involving the "Birth Pathway" within the family counselling services, spanning to the adolescence with actions spread at individual, family and community levels [260].

Prevention is based on behavioral modification starting from the prenatal age. LOE I-A

Lifestyle-based interventions are able to achieve mild but significant effects on dysfunctional behaviors (diet, physical activity, sedentary behaviours) and BMI [280]. Maintaining the BMI in a growing child is an important health objective. The best results have been obtained in school settings and in children 6–12 years [263]. Further studies are needed to determine the effectiveness of preventive interventions in children under 3 years and adolescents [281].

The family involvement is strongly recommended. LOE III-A

Similarly to treatment, preventive interventions involving the whole family are recommended as more successful and long lasting compared to child-centered interventions, though they were more effective in children than adolescents [263, 282–

284]. Interventions targeting at specific behaviors, such as taking fruits and vegetables and reducing sedentary behaviours have been found effective as well [283].

Prenatal age

Women should start pregnancy with appropriate weight and control their weight gain following an healthy lifestyle. LOE III-A

An excessive weight gain during pregnancy is associated with fetal macrosomy and increased risk of obesity [285–290]. This effect is independent of maternal hyperglycemia, which is also a well-known risk factor for future obesity [291]. Recommended gestational weigh gain is between 11.5 and 16 Kg in normal weight women, 7 to 11.5 Kg, in overweight and 5 to 9 Kg in those who with prepregnancy obesity [292].

Tobacco smoke in pregnancy is banned. LOE III-A

Maternal smoking in the perinatal age increased the risk of overweight at age 7 regardless of birth weight; the risk increased for maternal smoking not only in pregnancy but also in the post-natal period. There was a dose-dependent effect. Hence, smoking exposure must be banned in preand post-natal life [293, 294].

Diet

First two years of life

Avoid excessive weight gain and/or increased weight-tolength ratio from the very first months of life. LOE III-A

Early rapid weight gain increases the risk of overweight and obesity in childhood [295]. Prevention in infants is focused on quality, quantity and timing of food intake. In particular: Exclusive breastfeeding is recommended up to 6 months [296–299]. LOE III-A. Solid foods and beverages other than breast milk or infant formulas should be introduced no earlier than 4 months and no later than 6 months [300–305]. LOE III-B. Protein intake should be limited to less than 15% of the daily energy intake [302, 306–309]. LOE I-B. Reduction of lipid intake to percentages indicated for adults is not recommended [310]. LOE II-D. Sweetened drinks should be avoided [311]. LOE III-A. There is insufficient evidence that complementary responsive feeding practices, such as baby-led weaning (which is associated with early satiety-responsiveness acquisition), are protective against obesity respect to usual complementary feeding mode [312–314]. LOE V-C.

From preschool age to adolescence

Low energy density diet is recommended, based on the principles of the Mediterranean diet, promoting at least 5 servings of fruit and vegetables and plant based proteins [315]. Food should be distributed in no more than 5 daily meals and household consumption of meals should be promoted [316, 317]. LOE V-A. The use of fast food and fast food-based venues should be limited [318, 319].LOE V-A. Avoid sweetened drinks, including sports drinks and juice additives; alcoholic and energy drinks should also be avoided in adolescents [320–322]. LOE I-A.

Physical activity

It is recommended that children/adolescents spend on average 60 min a day on moderate/vigorous physical activity. LOE III-A

Prospective studies have shown a negative association between levels of physical activity and overweight/obesity [323, 324]. Even moderate physical activity is sufficient to improve aerobic fitness, an important marker of metabolic health which is independent of adiposity [325, 326]. 210, 211 Moderate physical activity is more effective and easier to implement in children who are sedentary or overweight. The increase of physical activity levels can be achieved starting from the age of 2–3 years by active play, walking, using the tricycle, and after 5–6 years, promoting also sports participation 2/3 times a week. Exercise should primarily stimulate aerobic capacity, but also strength and flexibility, be adequate to the child's ability and stage of physical and psychomotor development [174, 175, 327].

Sedentary behaviours

The use of television and electronic games is discouraged in children < 2 years of age. LOE VI-B

Although there are no specific studies on the effects of video exposure on overweight/obesity in this age group, video exposure should be discouraged since it may disturb sleep regularity [328, 329].

Sedentary behavior, especially the time spent in front of a screen (TV, video games, computers, mobile phones, etc.) should be reduced to less than 2 h a day in children > 2 years of age. LOE III-B

The association between sedentary behaviour, obesity and cardiometabolic risk factors is weak, and it is reduced when corrected for physical activity levels [330]. On the

contrary, the evidence based on prospective studies and RCTs show a strong relationship between television hours, obesity and cardio-metabolic risk factors, presumably because overfeeding frequently occurs [331, 332]. Several studies demonstrated a greater amount of television hours in children who have a television in their bedroom, but there is no clear evidence that its removal reduces the duration of the video exposure [333]; on the contrary the installation of an electronic television time manager seems effective [334]. Decreasing sedentary behaviour was more successful in reducing BMI in children 5–12 years [181]. Prospective studies showed that interrupting prolonged sedentary periods with mild physical activity had beneficial effects on metabolic outcomes in adults [335]. Although evidence is lacking in pediatric age, it is suggested breaking up prolonged sitting time at home and school.

Sleep duration and quality

Adequate sleep duration and quality should be promoted in infants, children and adolescents. LOE III-B

A short sleep duration is a potential risk factor for overweight/obesity through neuroendocrine and metabolic influences [336, 337]. One meta-analysis of longitudinal studies indicated a risk of obesity more than doubled in children with a sleep duration lower than recommended [338]. Three intervention studies aimed at changing sleeping hours within a multicomponent obesity treatment were not effective in reducing the BMI [339]. Waiting for stronger evidence, we endorse the recommendation for optimal amount of sleep in children and adolescents released by the American Academy of Sleep Medicine [340] syntethized in Table 8. Turning off all “screens” 30 min before bedtime is also suggested to ensuring adequate sleep.

Table 8. Recommended amount of sleep in children and adolescents

4–12 months	12–16 h/day (including afternoon naps)
1–2 years	11–14 h/day (including afternoon naps)
3–5 years	10–13 h/day (including afternoon naps)
6–12 years	9–12 h/day
13–18 years	8–10 h/day

Involvement of school settings for implementing preventive actions

It is recommended to include the school settings in obesity prevention programs. LOE I-A

The school is institutionally devoted to the education of children and is certainly a privileged area for the implementation of preventive actions. Studies support with moderate/high evidence that promoting healthy nutrition and physical activity at school prevent excessive weight gain and reduce the prevalence of overweight/ obesity [341, 342]. The most effective and promising changes are summarized in Table 9 [334].

Table 9. Effective environmental strategies to prevent pediatric obesity at school

Support school personnel's strategies for implementing health promotion programs.
Improvement of overall school food environment:
Removal of vending machines selling sugar sweetened beverages or snacks high in fat, sugar or salt; banning sales of this kind of food; reformulation of school lunches to reduce high calorie unhealthy food.
Provision of a healthy breakfast
Provision of free or low-cost fruit
Provision of free/low cost water
Improvement of overall school physical activity environment:
Increase of the daily formal PA session organized during and after school hours.
Availability of school playgrounds for structured/unstructured PA during and after regular school hours

Conclusions

This paper is a Consensus position document on the care of pediatric obesity in children and adolescents produced by experts belonging to the Italian Society for Pediatric Endocrinology and Diabetology and the Italian Society of Pediatrics, and endorsed by the main Italian scientific societies involved in tackling obesity and its complications. Consistent evidences suggest that the disease-burden of obesity on the overall health starts very early in life and is particularly serious for the development of cardiometabolic disease risk factors during childhood and adolescence and the association with premature mortality in adults. Furthermore, the mechanical and psychosocial comorbidities undermine physical functioning and the health-related quality of life. Several systematic reviews and meta-analyses on treatment and prevention indicate that weight control may be obtained by multicomponent intervention focused on a life-long change in the child's eating habits and lifestyle, involving the whole family and the surrounding social environment (school, communities). The effectiveness of treatment programs based on diet and lifestyle on excess weight reduction was shown only in the short term. Further study is needed to evaluate the effectiveness and safety of the different modalities of treatment, including pharmacotherapy and/or bariatric surgery, in the long term.

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2.2.5. Children Obesity, Glucose Tolerance, Ghrelin and Prader Willi Syndrome

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Abstract

Prader Willi syndrome (PWS) is caused by a genomic imprinting disorder. Its major manifestations include childhood-onset hyperphagia and morbid obesity with specific fat distribution, relative hypoinsulinemia, preserved insulin sensitivity, growth hormone (GH) deficiency, hypogonadism, and mild mental retardation. Hyperghrelinemia is a common feature of the syndrome preceding obesity in infancy, with a different pattern of acylated (AG) and unacylated ghrelin (UAG) secretion across PWS nutritional phases. Alterations in the ghrelin system have been investigated in the context of PWS, such as uncontrolled eating, fat accrual, and specific glucose metabolism, probably as compensatory mechanisms. This chapter discusses findings on the role of ghrelin and obestatin in these PWS clinical features, paying particular attention to the regulation of insulin secretion and sensitivity from childhood to adulthood. The regulation of the ghrelin system by new treatments for hyperphagia and obesity in PWS has been also addressed.

Introduction

Prader Willi syndrome (PWS) is a complex multisystemic genetic disorder caused by the lack of expression of paternally inherited genes known to be imprinted and located in the chromosome 15q11-q13 region, named the PWS region [1, 2]. Genomic imprinting is an epigenetic phenomenon whereby phenotype is modified by gender of the parent contributing that allele, and PWS was the first example described in humans [3]. It is known that 70% of PWS subjects present a noninherited deletion in the paternal PWS region. However, 25% of cases are due to a maternal disomy 15 (UPD: maternal uniparental disomy), and the remaining cases are due to genomic imprinting defects such as microdeletion, epimutation, or balanced chromosome translocations [4, 5]. The PWS region includes a few protein-coding genes and multiple paternally expressed noncoding RNAs, several of which were previously suggested to regulate alternative splicing [6]. It has been estimated that PWS occurs at a birth incidence of 1 in 30,000 individuals, and population prevalence is around 1 in 50,000 in Caucasians [7, 8]. Although genotype-phenotype correlations have been widely described, it can be summarized that PWS is characterized by typical phenotypes including neonatal hypotonia, uncontrolled and precocious hyperphagia, morbid obesity, short stature, hypogonadism, and other somatic, endocrine, and psychological problems [2, 9]. Indeed, PWS is considered the most common genetic syndrome leading to life-threatening obesity. Notably, the loss of several paternally expressed genes in the

PWS region is thought to contribute to the abnormalities of this syndrome; MKRN3, MAGEL2, NDN, SNURF-SNRPN, and several clusters of small nucleolar RNAs (snoRNAs) like Snord116, HBII-85, and NPAP1 are all expressed in the brain, including hypothalamus, which suggests that many features depend on hypothalamus-pituitary and brain-signaling derangements [4, 9–11]. This genetic condition appears to be a contiguous gene syndrome caused by the loss of at least two of a number of genes expressed exclusively from the paternal allele, but it is not yet well known which specific genes in this region are associated with this syndrome. In fact, the functions of the vast majority of genes residing in the PWS region remain to be determined [12]. The syndrome also recognizes a clinical overlap with other diseases, which makes it difficult to accurately diagnose. Prader Willi-like Syndrome (PWLS) shares features of the PWS phenotype, however, the genetic basis of these rare disorders differs. The implication is that the gene functions disrupted in PWLS are likely to lie in genetic pathways involved in the development of PWS phenotypes and require further research [12]. Recent surveys have highlighted the high rates and varied causes of morbidity and mortality throughout the natural history of the disease, due mostly to disorders related to severe obesity, and endocrine and metabolic impairment [13]. It has to be underlined that the PWS neuroendocrine and metabolic phenotype are partly different as occurs in simple obesity, which would be an interesting model to investigate the complex and redundant regulation of fat distribution, energy balance, feeding, and central and peripheral signaling. As the outcome of the studies on the investigation of PWS using a model of morbid obesity with special characteristics are partly different from those of primary obesity, the aim of this chapter is to review knowledge about obesity in PWS children and adults, paying particular attention to glucose metabolism and the ghrelin system, both of which seem to have typical regulation in this syndrome.

Clinical Features of PWS: Childhood-Onset Obesity, Body Composition, and Hyperphagia

PWS is the most commonly recognized genetic cause of early-onset obesity. The most prominent feature of PWS is childhood onset hyperphagia that can lead into morbid obesity. However, PWS can be divided into two distinct clinical phases. The second phase has been recently divided into two subphases to describe childhood, adolescence, and adulthood [2]. Stage 1 occurs from birth to early infancy at around 2 years old. It is not characterized by obesity; on the contrary, PWS neonates and infants present with mild prenatal growth retardation, and severe hypotonia with poor suck and

feeding, with or without failure to thrive [2, 4, 9, 14, 15]. Stage 2 starts between 18 and 36 months of life and is characterized by a progressive weight increase. First, weight presents a hyperbolic trend to increase without significant higher caloric intake or interest in food. Afterward, children increase their weight with the onset of a higher interest in food and mild hyperphagia [2, 4]. This stage is also characterized by low growth velocity and, as a consequence, short stature. Growth features seem to be mostly due to GH deficiency (GHD) [2, 9, 16–18]. Stage 3 ranges from 4 to 15 years of age. This is the stage whose features are classically associated with PWS with uncontrollable hyperphagia and aggressive behavior in food-seeking. PWS subjects present not only with further weight gain but also delayed meal termination and gastric emptying, and early meal initiation after previous food ingestion [19–21]. Consequences of unattended hyperphagia lead to maintenance of more than 200% ideal body weight in at least 33% of the PWS population and occasionally death due to choking or stomach rapture [22–24]. All these aspects suggest a derangement of hormonal and metabolic satiety responses to food intake, and most of the recent studies have been focused on endocrine and functional neuroimaging abnormalities. Stage 4 occurs in a subset of individuals after 30 years of age. It is still characterized by increased appetite but without uncontrolled hyperphagia [2] and does not seem to present abnormal gastric emptying [25]. Childhood-onset PWS obesity shows a specific body composition. In fact, PWS subjects present with hypotonia in both the prenatal and postnatal first months of life due to persistent poor muscle tone. This feature proceeds with age, and reduced lean muscle mass, bone mineral content, and density are associated with increased body fat from infancy to adulthood characterized by increased fat to lean mass ratios [26–28]. Total lean mass is significantly lower for arms, trunk, and especially legs, and therefore PWS differs from simple obesity in which an increase in lean tissue is observed along with an increase in adipose tissue [29]. Notably, subjects with PWS have a reduced energy expenditure not only from reduced physical activity but also from lower energy utilization due to reduced lean body mass, which consists primarily of muscle in all ages [30, 31]. Accordingly, the low resting metabolic rate is normal when corrected for reduced lower free fat mass and higher fat mass [32–34]. During the entire lifespan, the percentage of body fat is significantly greater in the PWS population than in control individuals with a comparable degree of obesity [26–28, 35], and most of the fat accumulation tended to be in extra-abdominal areas [29, 36]. In fact, PWS subjects present with less trunk and, as a consequence, fat mass with respect to controls matched for obesity [29, 35]. This is

evident as lower absolute visceral adipose tissue volume, the percentage of total adipose tissue or body mass. In contrast, no significant decrease in the amount of total or abdominal subcutaneous and nonvisceral internal adipose tissue is detected with respect to simple obesity [33, 37]. Fat distribution is rather similar between sexes, with a mean percentage body fat of 53% and 44% in adult PWS females and males, respectively, with similar results in children and adolescents [26, 32, 38]. Magnetic resonance imaging (MRI) has been used to characterize body composition, demonstrating that PWS subjects have greater subcutaneous and intermuscular abdominal adipose tissue and lower volumes of skeletal muscle, suggesting a sarcopenic obesity-like phenotype [39]. Moreover, subcutaneous adipose tissue in PWS is characterized by increased adipocyte size and changes in inflammation and extracellular matrix genes, features that could be related to the increased capacity of adipocyte cell expansion [40]. Hence, it becomes clear that morbid obesity in the PWS population more closely resembles body composition of sedentary elderly and patients suffering from GHD than that of essential obesity. Consistent with this hypothesis, GH/IGF-I axis has been widely explored in PWS individuals. GHD may be one of the candidate factors contributing to increased fat mass and, especially, to decreased muscle mass. Multiple studies on PWS children and adults described the status of reduced GH response to various GH secretory stimuli, as well as inadequate spontaneous 24-h GH secretion and decreased circulating IGF-I levels [2, 41–43]. However, the etiology of impaired GH secretion is still controversial because it could be a functional effect due to obesity alone, and references cut off of normal responses to pharmacological stimuli are unavailable in such severe obesity [2, 9, 16, 17]. In addition, because of different body composition with respect to simple obesity, cut-offs simply stratified for body mass index (BMI) are not really useful in PWS individuals. However, it has been shown that PWS adults present with a lower GH response to GHRH + arginine stimulus with respect to controls matched not only for BMI but also for the percentage of fat mass. A severely impaired GH response is also associated with low IGF-I levels. In this light, a higher prevalence of GHD has been also found in obese PWS children in comparison to nonobese PWS subjects [44]. Moreover, genetic subtypes also seem to influence the response to the stimulation tests [43]. These data support the hypothesis that GHD may be present in a significant subset of PWS patients. However, biochemical GHD has been reported to be less prevalent in ages up to 18 months (27%) compared with older children (81%), suggesting that pituitary GH reserve gradually declines in pediatric cohorts with increasing age and BMI [44].

Probably, GHD in PWS may reflect an evolving process, in which very young PWS children seem to have impaired hypothalamic GHRH secretion with a normal GH pituitary reserve [45]. This hypothesis is in line with recent evidence that the pituitary gland is smaller and often has a flat shape in PWS individuals. Moreover, its volume did not increase with age in contrast with healthy controls [46]. However, further studies are needed to confirm these data. Finally, reduced GH secretory pattern in individuals with PWS may reflect excessive fat body mass. Both children and adults with PWS share increased body fat, decreased lean body mass, reduced muscle strength, impaired bone mineral density, cardiovascular risk, osteoporosis, and psychological impairment with subjects affected by classical GHD [42, 43]. Nowadays, replacement with rhGH in PWS children is approved worldwide for some well-documented benefits, in particular, the achievement of final adult height and improvement in body composition, although without complete normalization [18]. In fact, early replacement with rhGH during PWS childhood can significantly increase lean mass toward the normal range and delay fat tissue accumulation [28]. In particular, replacement GH dosing exerts a crucial role to effectively sustain the improvement of body composition in children with PWS [43]. Data documenting real GHD status in adults also supports the replacement in adulthood, which also demonstrated an improvement of body composition, muscle size and strength, and exercise tolerance in several trials [41, 42, 47, 48]. Data on GH therapy and bone mass density in PWS, however, are not univocal in terms of beneficial effects on bone mineralization and bone geometry in PWS patients. These contrasting results suggest that bone impairment is not exclusively driven by GHD in PWS, but it may be related to several involved factors including body weight status, lifestyle factors, duration of the rhGH treatment, sex hormone replacement, and other hormonal disturbances such as hyperghrelinemia [43]. GHD and other dysfunctions such as hypogenitalism, central hypothyroidism (in particular, up to 2 years of age), temperature, and sleep disorders described in PWS subjects suggest complex hypothalamus-pituitary abnormalities. Accordingly, defects in the hypothalamus have been reported in studies using functional MRI, including altered resting-state functional connectivity between hypothalamus and right and left lateral occipital complex, or in postmortem quantification of hypothalamic neuropeptides [33, 46, 49–51]. Pituitary MRI abnormalities including empty sella, anterior and posterior pituitary hypoplasia, and longer pituitary stalk have been also reported [46, 52]. Neuroanatomical and functional hypothalamic abnormalities may contribute to hyperphagia and behavioral PWS problems. Interestingly, PWS hypothalamus presents

low oxytocin cell numbers, otherwise orexin A, GHRH, NPY, and AgRP neurons are dysfunctional [33, 46, 49, 51, 52]. A complex deregulation in reward and satiety circuitry is also present, including hypothalamus functional connectivity [46]. Given the effect of hyperphagia on health and quality of life in the PWS population, delineation of gene dysregulation and brain development and function is a challenge. It has already been shown that glucose administration is followed by a delayed signal reduction in the hypothalamus, ventromedial prefrontal cortex, insula, and nucleus accumbens in these patients [50]. This effect is associated with a hyperresponsive neural network related to food process and disruption of reward circuitry relating to food motivation in MRI-functional studies [53–55]. Divergent neural mechanisms seem to be associated with behavioral phenotypes in genetic subtypes of PWS suggesting that hyperphagia is a complex phenomenon, and mechanisms linked to it are differently regulated by maternally imprinted or paternally expressed genes [21]. PET protocols also demonstrated higher metabolism in prefrontal and temporal lobe during fasting and reduced tracer binding to GABA-A receptors in the same regions [56, 57]. The study of hormonal abnormalities associated with neural derangement and hyperphagia is in its infancy. Fasting and postprandial ghrelin levels are also elevated in PWS subjects before the onset of obesity [58], although peptide levels fall to a normal extent after meal ingestion [59]. Acute somatostatin infusion also inhibits ghrelin secretion in PWS subjects but without modulating appetite [60]. Peptide YY (PYY) secretion is also modified in PWS with fasting lower levels and a blunted postprandial response, in particular after a hedonic meal, a mechanism at least theoretically able to reactivate the central rewarding system and block early meal termination [61–63]. A similar altered profile has been observed for the pancreatic polypeptide [61, 62]. Furthermore, plasma orexin A levels were also higher in PWS children than controls [64], as well as orexin A gene expression in lymphoblastoid cells derived from adult males with PWS than nonsyndromic obese subjects [65]. It appears clear that morbid obesity and hyperphagia are the major problems of PWS, and obesity management requires environmental control with a low-caloric balanced diet and physical activity until pathogenetic mechanisms would not be clearly explained.Morbid obesity and hyperphagia are considered the major problems of PWS, thus obesity management requires environmental control with a low-caloric balanced diet and physical activity until pathogenetic mechanisms would not be clearly explained and identified specific pharmacological therapies. As far as this remains a challenge, supervision, restriction to access to food, and money are needed.

Glucose Metabolism and Insulin Sensitivity in PWS

Type 2 diabetes has been reported in about 25% of adults with PWS with a mean age of 20 years [66]. Obesity is a common component of type 2 diabetes, which plays an important role in the development of hyperinsulinemia and insulin resistance. The increase of visceral fat plays an integral role in the development of insulin resistance, glucose intolerance, and hyperlipidemia in obesity [67]. Thus the development of metabolic syndrome and complications of obesity in PWS children and adults is largely dependent on obesity status. The frequency of insulin resistance, hyperlipidemia, and hypertension in obese PWS children is similar to obese controls, with these conditions being quite rare in nonobese PWS children [68]. However, glucoregulatory mechanisms are different in obese PWS with respect to obese non-PWS subjects according to the unusual fat patterning of the syndrome [38, 69, 70]. In fact, subsequent studies have shown obese PWS children have greater insulin sensitivity and less fatty liver disease compared to obese controls; these findings have also been reported to persist into adulthood [71]. Euglycemic PWS patients have a reduced beta-cell response to glucose stimulation associated with an increased hepatic insulin extraction and a dissociation between obesity and insulin-resistance with respect to healthy obese subjects in both pediatric and adult ages after intravenous and oral glucose tolerance tests [69]. In particular, the first- and the second-phase insulin secretion responses are both lower in PWS with respect to matched obese subjects [69]. A state of relative hypoinsulinemia also occurs in PWS individuals after mixed meals [69, 72]. Therefore nondiabetic PWS patients present normal or increased insulin sensitivity suggesting that they may be protected from obesity-associated insulin resistance and metabolic syndrome [73–75]. Abnormal fat composition seen in PWS with increased subcutaneous fat and decreased visceral fat deposition is one of the components hypothesized to have a role [71]. Accordingly, insulin and insulin resistance indexes in PWS are not associated with adipokines widely shown in simple obesity. Adipokines, like adiponectin and resistin, are differently modulated in PWS [76, 77]. In particular, high molecular weight (HMW) isoform of adiponectin and HMW/total adiponectin ratios are increased in PWS children; these data are of particular interest because this isoform and the ratios correlate better with insulin sensitivity [75]. However, high levels of adiponectin may be a consequence, rather than a cause, of increased insulin sensitivity and hypoinsulinemia in PWS. Recently, the increased insulin sensitivity of PWS children was found to be associated not only with higher levels of adiponectin but also lower levels of high-sensitivity C-reactive protein and IL-6

[78]. Further long-term studies are needed to confirm and explain these findings. Many other mechanisms could be linked to these metabolic PWS features, in particular, GH and IGF/I deficiency. Glucose abnormalities and insulin resistance have been reported by some [69] but not all studies [41, 79, 80]. However, it has to be pointed out that GH treatment is followed by a modest increase in insulin, glucose, HbA1c, HOMA index, and prevalence of metabolic syndrome in both pediatric and adult populations [41, 81], suggesting that GH/IGF-I axis and fat distribution, which is influenced by hormone replacement, could have a role. Although there is a state of better insulin sensitivity in PWS, some individuals develop type 2 diabetes. Following the visceral fat area reported by Despres, PWS subjects with visceral fat area higher than 130 cm2 were found to have insulin sensitivity and resistance similar to obese-matched controls suggesting that this population could be at higher risk of obesity-related disorders [70]. Moreover, diabetic PWS patients continue to have higher glucose levels with decreased precocious insulin levels during oral glucose tolerance test (OGTT), which are secondary to the reduced beta-cell response to glucose stimulation [37, 69, 74]. More recently a multicenter Italian Cohort Study has been published evaluating a large cohort of children, adolescents, and young adult PWS. They demonstrated a high prevalence of altered glucose metabolism, which was more frequent in obese and adult subjects [82]. Similar results have been shown in Korean PWS patients, either children and young adults; BMI and insulin resistance resulted in strong predictive factors for the development of diabetes [83]. Although the etiology of the development of type 2 diabetes in PWS has not yet been clarified, surely the improvement in weight control remains the most important goal of any PWS treatment program.

Ghrelin and Obestatin Regulation

PWS subjects typically present with higher ghrelin levels [9, 84, 85]. Ghrelin is a 28-amino acid peptide, isolated from the stomach, but also shown to be expressed in other tissues such as the pancreas, testes, placenta, pituitary, and hypothalamus [86]. Ghrelin has been identified as an endogenous ligand of the orphan GH secretagogue (GHS)-receptor type 1a [86, 87]. It circulates in the blood in two forms, acylated (AG) and unacylated ghrelin (UAG). The acyl group, which binds to ghrelin at the serine-3 residue, seems to be essential for binding to GHS-receptor type 1a and the resulting neuroendocrine functions, namely GH secretion [86, 87]. UAG, which is devoid of the acyl group, represents the most abundant circulating form [88, 89]. UAG is biologically active, although it does not have direct neuroendocrine actions, suggesting the

existence of some GHS-receptor subtypes [86, 87, 89–91]. More recently, the acyltransferase that octanoylates ghrelin has been identified and named ghrelin O-acyl-transferase (GOAT). It is widely expressed, in particular in the pancreas, stomach, and gut [92, 93]. Interestingly, the lipids used for the acylation process are, at least in part, directly derived from the pool of ingested dietary lipids, in particular, the medium chain-triglycerides, which can be directly absorbed into the circulation without being broken down by lipases and bile acids [93–95]. In line with this evidence, recent studies with genetically modified mice demonstrated that the GOAT-ghrelin system is a nutrient sensor informing the presence of nutrients, rather than the absence. In particular, transgenic mice overexpressing ghrelin and GOAT have higher body weight and fat mass, and decreased energy expenditure than wild-type mice. This is shown when medium-chain fatty acids are available in the diet, whereas AG levels decrease when mice were fed a low-fat carbohydrate-rich chow diet [93–95]. Moreover, since its discovery, ghrelin emerged as a player in the regulation of food intake and energy expenditure, with AG the most potent peripheral orexigenic hormone known to date [86, 89, 91, 93]. In animals and humans, AG both after central and peripheral administration has been shown to induce appetite and food intake [89, 93]. Despite these clear orexigenic effects of AG, the actual physiological role of UAG in the regulation of appetite is still a matter of debate [89, 96]. It has also been clearly shown that both forms of ghrelin also influence energy metabolism at the peripheral level, most likely influencing fat oxidation [97, 98]. Reduced cellular fat oxidation and promotion of adipogenesis each contribute to an increase in fat mass induced by AG. These effects are mediated by central and peripheral mechanisms, including the sympathetic nervous system [89, 93, 97, 99, 100]. Diversely, data on UAG are conflicting. Some authors showed that high pharmacological amounts of UAG administered centrally to mice increases adiposity through mechanisms similar to AG [101], whereas others showed decreased activation of gene programs regulating lipogenesis [100]. Furthermore, mice overexpressing UAG have lower body weight and fat mass than wild-type controls [93]. Consistent with its effects on food intake and its involvement in energy balance, circulating ghrelin levels are negatively associated with BMI in humans [89, 102, 103]. Several studies indicate that ghrelin hyposecretion in essential obesity is a functional impairment in response to body weight alterations [104]. Furthermore, the majority of the data come from studies that have exclusively analyzed total ghrelin levels. Recent findings have also shown that UAG is decreased in obesity, yet there are no concordant results for AG [89, 105, 106]. Ghrelin is implicated in the regulation of

glucose homeostasis by modulating insulin secretion and action. It has also been demonstrated that insulin modulates ghrelin levels through inhibition [86, 89, 91, 107]. Ghrelin secretion is also modulated by nutrients. Lipids decrease more weakly total ghrelin and AG levels in several models than carbohydrates and proteins. Moreover, carbohydrates induce a biphasic pattern on AG secretion with a rebound 2–3 h after the meal [93, 108]. The system has recently been complicated by the discovery that ghrelin is a lipid- and a chemosensor. In line with that previously introduced describing GOAT, lipids, specifically medium-chain fatty acids, do not blunt but rather AG levels, whereas a mixed cocktail of lipids and amino acids decrease its secretion [93, 95, 108–110]. Furthermore, the regulation of ghrelin secretion after meals is age-dependent with refractoriness in neonates and lean children, and an inhibition that starts with puberty. On the other hand, total ghrelin levels are decreased after meals, irrespective of pubertal stages in obese children and adolescents [108]. In 2005, Zhang and coworkers, using bioinformatics from a conserved region of preproghrelin sequence, identified a 23-amino acid peptide named obestatin based on previously reported activities in animal models. It was initially characterized as the active ligand of the orphan receptor GPR39, but this result is strongly discussed because zinc ions appeared to be the endogenous ligand for this receptor [99, 111–113]. Furthermore, due to several similarities between the emerging metabolic actions of obestatin and those of GLP-1, obestatin was shown to bind to and upregulate the GLP-1 receptor [114]. Furthermore, a recent study has shown that obestatin's effect on insulin secretion is mediated by GHS-R in pancreatic β-cells [115]. Other authors have suggested that obestatin may activate an adenylate cyclase-linked G-protein-coupled receptor (GPCR), at least in the cardiovascular system [116]. In all, it is possible that the hormone binds to and activates several tissue-specific receptors. Obestatin, like ghrelin, is expressed in several tissues, which include among others the stomach, duodenum, pancreas, and brain [99]. On the other hand, the obestatin/ghrelin coexpression in tissues is debated [117]. Obestatin initially appeared to be a new regulator of appetite and body weight [111]. Following studies, however, failed to reproduce obestatin's anorectic and antiobesity effects, and these actions are still questioned [111, 117]. Diversely, data on the pancreas' role of physiology are more detailed. Although the precise pancreatic actions of obestatin remain unclear, actual evidence highlights beneficial effects on beta-cell metabolism and survival coupled with a modulation of insulin levels and inflammation [114, 117, 118]. Its role seems more complex in adipose tissue. Recently, it has been demonstrated to promote

preadipocyte differentiation, lipid accumulation, and leptin secretion. These effects are coupled with decreased and increased lipolysis during differentiation and adipogenesis, respectively [119]. These results are debated, in particular, due to in vitro and in vivo models used by the authors [117]. On the other hand, the majority of the studies in humans observed that obestatin levels are reduced in subjects with type 2 diabetes and impaired glucose tolerance, as well as in obesity, yet are increased in anorexia and type 1 diabetes [117, 120, 121].

Ghrelin and Obestatin Regulation in PWS

As previously discussed, PWS individuals present higher ghrelin levels (from 3.0- to 3.6-fold) despite morbid obesity [84, 85, 122, 123]. This feature is typical of the syndrome, unlike those with simple obesity, hypothalamic obesity due to craniopharyngioma, or other forms of genetic obesity [122, 123]. Hyperghrelinemia is also present in PWS infants, precedes morbid obesity, and maintains the age-dependent decrease [85, 124]. Kweh et al. [125] studied total ghrelin levels in PWS categorized by nutritional phase and showed that total ghrelin levels are elevated at the beginning in the earliest nutritional phase characterized by poor appetite and feeding. These results are in agreement with observations in a transgenic mouse model for the region equivalent to the human PWS region in which high ghrelin levels were observed from the third day after birth [126]. Some studies do not confirm higher ghrelin concentration in very young children, in particular, aged 17–60 months [58, 127, 128], but these results could be influenced by rhGH therapy in the population [129]. Accordingly, high fasting total ghrelin levels in GH-untreated PWS children were found to be decreased in the GH-treated group, although AG levels were not modified [85, 129, 130]. Indeed, total ghrelin could not be considered a surrogate for AG and the GH replacement, at least in PWS population, modulating ghrelin secretion in terms of AG/total ghrelin ratios [129]. Notably, gastric ghrelin-expressing cell density and quantity are both increased in the fundus and body of the stomach of PWS patients. This increase is not related to IGF-I levels, whereas these parameters are similar in not-PWS GHD patients, and lean and obese normal controls [20]. The increased density and quantity of ghrelin-expressing cells could also explain higher AG levels in PWS, according to the GOAT expression in stomach and its mainly intracellular activity [92, 129–131]. Few data are available about the UAG form. One study evaluated UAG secretion in PWS without differences with respect to obese-matched controls [131]. More recently, Tauber and colleagues demonstrated in early infancy a hormonal

pattern different from children and adults, with higher UAG levels. In particular, AG is normal and stable from 1 to 48 months of age, diversely from elevated UAG [132]. After this period, AG levels seem increased with UAG levels as healthy lean controls [133]. The different AG/UAG ratio across lifespan could explain the failure to thrive in infancy driving in later infancy the phenotype toward hyperphagia and obesity [132, 133]. Because AG has also positive effects on brain plasticity, memory, and cognition [93, 134], it has been hypothesized that relatively low AG levels in the first months of life may contribute to PWS patients' intellectual disability later in life. Preliminary conflicting data show obestatin is not increased and elevated in PWS children [31, 135]. Although Park and coworkers failed to find a different regulation of obestatin secretion, this peptide is positively correlated with BMI standard deviation score in this population, similar to what was observed in some, but not all studies, performed in simple obesity [135–138]. Indeed, hyperghrelinemia in PWS seems to be a typical feature that involves the specific process of acylation without modulating the other forms derived from the prepropeptide after the first 2 years of age. Besides these results, many hypotheses on increased AG and total ghrelin secretion have been widely discussed. According to very early hyperghrelinemia in PWS infants, this hormonal derangement could play a role in early-onset obesity [85]. Moreover, it could explain at least two other major endocrine dysfunctions of the syndrome, such as obesity with reduced and delayed satiety and GHD [2, 84, 85, 123]. Interestingly, very recently Leibel and coworkers demonstrated in an elegant study that the downregulation of Nhlh2 and Pcsk1 in Snord116p–/m + mice suggests that paternal deletion of Snord116 is at least sufficient for the downregulation of Nhlh2 and Pcsk1 in vivo. Humans and mice deficient in the prohormone convertase PC1 (encoded by PCSK1) display hyperphagic obesity, hypogonadism, decreased GH, and hypoinsulinemic diabetes due to impaired prohormone processing. Moreover, Snord116p–/m + mice displayed in vivo functional defects in prohormone processing of proinsulin, pro-GH–releasing hormone, and proghrelin [139]. This seems to be one of the first mechanisms linked to hyperghrelinemia (both AG and UAG levels) in PWS. Data regarding age-dependent regulation of GOAT are still lacking. However, the role of elevated AG levels in PWS hyperphagia is questioned. Long-acting octreotide treatment successfully blunted fasting and postprandial AG and UAG levels without being followed by changes in body weight and composition, appetite, or behavior toward food in PWS children and adolescents [124, 140]. Similar data were obtained in PWS adults after an acute somatostatin infusion [60]. It has been suggested that the lack of reduction of appetite

could be due by consensual reduction of PYY, but this effect has been obtained in the acute study on adults rather than in that with chronic administration in children [59, 60, 140]. Alternatively, the plasmatic inhibition of the peptide could not be associated with a consensual inhibition of its expression or activity. Indeed, increased appetite may be secondary to abnormal expression of nonghrelin genes, in particular, serotoninergic pathways [141, 142]. Notably, similar falls in ghrelin levels after somatostatin infusion or octreotide therapy in PWS as in other studies of non-PWS individuals suggest that the cause of hyperghrelinemia probably is not an intrinsic primary abnormality of ghrelin-expressing cells, but more likely a loss of inhibitory, or excess of stimulatory, neural or hormonal inputs [59, 60, 124, 143, 144]. This hypothesis is strongly supported by the data of Leibel and coworkers as discussed earlier [139]. As vagotomy increases plasma ghrelin in rodents, the elevated AG and UAG concentration in PWS subjects might also result from the reduced parasympathetic vagal efferent tone in PWS in agreement with similar data on disturbed parasympathetic cardiac autonomic function tests [123, 145]. Other mechanisms could not be excluded, in particular an imbalance between AG and other anorectic peptides besides UAG, including polypeptide P, which was shown to be reduced in PWS infants in the age of transition to hyperphagia, and PYY, which secretion was blunted both in the anticipatory cephalic phase and in the postprandial period [63, 128]. Furthermore, a role in the development of GHD in PWS is also questioned. Goldstone and coworkers failed to demonstrate an association between plasma ghrelin and IGF-I levels even when adjusting for adiposity and insulin levels in PWS adults [123]. Similarly, GHRH neurons do not present abnormalities in postmortem PWS hypothalamus [146]. However, as previously discussed, GH replacement blunts ghrelin secretion, suggesting at least a functional role between these systems [129, 130, 133]. It is clear that hyperghrelinemia is a new hormonal feature of PWS, but its pathophysiological role is still widely unclear at least with respect to hyperphagia, obesity, and GH status. The different AG/UAG ratio in PWS during life, and the lack of modulation of obestatin, remain to be investigated and could offer new hypotheses. An increase in ghrelin gene expression, a decline in transcription inhibitory factors, or a decrease in ghrelin clearance are all mechanisms to further explore.

PWS, Glucose Metabolism, and Ghrelin System: What Is the Link?

Insulin is a physiological modulator of glucose and ghrelin levels, as previously introduced. Insulin possesses an inhibitory effect on ghrelin secretion independent of

plasma glucose concentration, although an additional effect cannot be excluded [89, 93, 108, 147]. Insulin levels and resistance have been shown to be major predictors of reduction of UAG and total ghrelin levels in obesity and metabolic syndrome [89, 93, 105, 106, 108, 147]. PWS individuals present morbid obesity with typical fat distribution, relatively hypoinsulinemia, higher insulin sensitivity, and grossly elevated ghrelin levels, in particular AG. As a consequence, the contribution of ghrelin system to the PWS metabolic phenotype has been widely explored with the contribution of studies in animal models. It has been reported that deficit of insulin and glucagon during fetal and postnatal life are prominent features in the transgenic PWS deletion mouse models, which also present hyperghrelinemia in the postnatal period at the onset of severe hypoglycemia [148]. These findings suggest that higher ghrelin levels could be an adaptive response to restore normal glucose levels or glucose sensing. The same mechanism could also increase eating in this condition [126, 149]. Furthermore, insulin alone, which is reduced in PWS, is insufficient to lower basal ghrelin levels [149, 150]. Accordingly, Goldstone and coworkers have shown how higher ghrelin concentration in PWS adults may be partially contributed, but are not solely explained, by their abnormal fat distribution, relative hypoinsulinemia, or reduced insulin resistance in both fasting and fed conditions [59, 123]. These data are in agreement with similar results in type 1 diabetes in which has been shown that basal insulin is sufficient to suppress postprandial ghrelin in this, whereas the lack of meal-induced ghrelin suppression could be caused by a severe insulin deficiency and might explain hyperphagia in uncontrolled type 1 diabetes individuals [151]. On the other hand, the relative hyperadiponectinemia is advocated as one of the causes of relative hypoinsulinemia in PWS, but its role on the regulation of ghrelin secretion is still unexplored [78, 152, 153]. However, regulatory influences in PWS secretion seem to be intact. Ghrelin levels maintain a normal postprandial fall by 32% in euglycemic PWS adults [59, 135, 154] and are inhibited by somatostatin or its long-acting analogs in the same extent of non-PWS individuals [124, 140]. Moreover, the insulin-induced ghrelin suppression is more pronounced in PWS children during an euglycemic hyperinsulinemic clamp [149, 150]. Also, AG levels are inhibited by carbohydrate administration in euglycemic PWS children with more sensitivity suppression by insulin [130, 131, 135, 149] but, unlike total ghrelin, are not modulated by GH replacement in a previous paper [130]. More recent data showed lower fasting and postglucose challenge AG levels in GH-treated PWS patients than in those who stopped GH therapy [154]. This variation of AG levels after a glucose challenge showed a significant

correlation with whole-body insulin sensitivity index, and the decrease promptly starts with the delayed insulin surge [135]. A preliminary study evaluated fasting and postprandial AG and total ghrelin levels in PWS individuals with respect to their glucose tolerance. Notably, total ghrelin levels were similar whereas AG levels and the AG:/total ghrelin ratios were decreased in dysglycemic with respect to euglycemic PWS adults. Fasting AG and ratio decreases have been hypothesized to be linked to higher insulin resistance in dysglycemic PWS despite similar adiposity. Moreover, AG levels increased after meals only in glucose-intolerant individuals and were predicted by glucose levels during the oral glucose load, in particular in the first 60 min, suggesting that a fast increase of AG could modulate the relative hypoinsulinemia and hyperglycemic response to meals in PWS [155]. Alternatively, more pronounced low insulin levels are insufficient to inhibit postprandial AG levels with an inversion of the reciprocal pattern between insulin and ghrelin with respect to fasting and feeding conditions [151, 155, 156]. As previously introduced, UAG levels were similar between PWS and healthy obese controls after the first 2 years of life. Furthermore, AG and UAG secretion seem to be differently modulated by carbohydrates ingestion. Both peptides at fasting are correlated with a whole-body insulin sensitivity index, but this relationship is lost after glucose challenge for UAG unlike AG [131]. Moreover, as previously discussed, AG promptly decreases with insulin surge whereas UAG response is delayed for up to 90 min after glucose administration in one study [131], yet another one showed a concomitant, although less pronounced, inhibition with AG after 30 min [154]. In a study with an efficacious inhibition of UAG, lower UAG levels at 30 and 120 min after glucose load were associated with a higher insulin/glucose ratio at 120 min [154]. Because of different patterns of response, it has been hypothesized that insulin better inhibits AG in PWS or, conversely, a larger decrease in AG should induce greater insulin responses in PWS to preserve glucose tolerance [131, 155]. Obestatin have been shown elevated as well as not increased in PWS children [31, 135]. The authors also failed to find an association between obestatin and insulin levels in the syndrome, suggesting a different regulation among the two circulating forms of ghrelin and obestatin, at least in glucose metabolism in PWS [135]. All these data suggest that the ghrelin system is deeply involved in regulation of glucose metabolism in PWS. It is also clear that AG, UAG, and obestatin are differently modulated. More data are needed to better clarify the pathophysiology of hyperghrelinemia in PWS, which has been based on the gross evaluation of total ghrelin until now. Relative hypoinsulinemia, and likely, hyperadiponetinemia, has a key role in fasting and postprandial regulation of

these peptides probably acting on many molecular pathways. The evaluation of PWS individuals with respect to their metabolic assessment, in terms of glucose tolerance, despite only total fat mass and fat distribution could offer new findings and potential therapeutic options.

Are the New Therapies Effective on Ghrelin in PWS?

Treatment is imperative, as demonstrated by the international PWS association's slogan "Still hungry for a cure" [157]. However, the optimal treatment for PWS patients is still controversial. In the following sections, emerging therapies aiming to improve body weight and food cues in PWS are described in their relationship with the ghrelin system. GH. As previously introduced, the replacement with rhGH in PWS children is approved worldwide for some well-documented benefits, in particular, the achievement of final adult height and improvement in body composition, although without complete normalization. Many studies revealed that rhGH reduces body fat and weight gain in PWS children [18, 43, 158–163]. High fasting total ghrelin levels in GH-untreated PWS children were found to be decreased in the GH-treated group, although AG levels were not modified [85, 129, 130]. Indeed, total ghrelin could not be considered a surrogate for AG and the GH replacement, at least in the PWS population, which modulates ghrelin secretion in terms of AG/total ghrelin ratios [129]. Notably, gastric ghrelin-expressing cell density and quantity are both increased in the fundus and body of the stomach of PWS patients. This increase is not related to IGF-I levels, whereas these parameters are similar in not-PWS GHD patients, and lean and obese normal controls [20]. The increased density and quantity of ghrelin-expressing cells could also explain higher AG levels in PWS, according to the GOAT expression in the stomach and its mainly intracellular activity [92, 129–131]. On the other hand, the effects of rhGH on eating behavior in PWS children were poorly investigated. No studies in children exist, meanwhile in a small group of 12 PWS adults, rhGH was neutral on eating behaviors evaluated using Visual Analogue Rating Scales and individual eating curves [25]. Interestingly, Balikcioglu et al. reported that GH therapy in PWS children resulted associated with increased insulin sensitivity and attenuated postprandial suppression of total ghrelin after a high carbohydrate meal. The authors hypothesized that after feeding the GH treatment causes a relative decrease of insulin, which in turn incompletely inhibits ghrelin secretion, then explains the lesser decrease in ghrelin levels in response to carbohydrate in GH-treated PWS patients [164]. Other authors failed to observe any differences in ghrelin suppression after a mixed liquid meal in GH-

untreated or -treated PWS subjects [165]. Somatostatin analogs. Somatostatin and its analogs (octreotide and lanreotide) bind to somatostatin subtype 5 receptors on the beta-cell membrane, which limits insulin release and, consequently, may decrease adipogenesis. Somatostatin also suppresses ghrelin secretion in normal subjects. These two mechanisms suggest a role in the treatment of PWS [143, 166]. However, a short-term somatostatin infusion in adults [60], a subcutaneous octreotide administration for 1 week [167], and a monthly treatment with octreotide for 4 months were all able to suppress about half of the fasting ghrelin levels (both AG and UAG), and also postprandial ghrelin levels, but not appetite or BMI, discouraging their use in the clinical practice [168]. The short-term IV infusion with somatostatin, but not the long-term intramuscular treatment with octreotide, also inhibited PYY secretion. GLP-1 receptor agonists. GLP-1 receptor agonists (exenatide, liraglutide albiglutide, dulaglutide, lixisenatide, semaglutide, and taspoglutide) are used in the treatment of type 2 diabetes [169]. Their use is coupled with a significant BMI decrease due to their pleiotropic actions. GLP-1 directly stimulates POMC/CART neurons and indirectly inhibits neurotransmission in neurons expressing NPY and AgRP via GABA-dependent signaling acting on GLP-1 receptors. Exenatide is the first studied and commercialized, but liraglutide seems to be the more effective molecule because of its ability to cross the blood-brain barrier and to have central actions, and recently it has been also approved for the treatment of obesity at a dose of 3.0 mg/day [170]. Focusing on GLP-1 pleiotropic action, the use of its receptor agonist is intriguing in PWS, also considering the GLP-1 role in the central processing and regulation of palatable food intake [171]. Moreover, GLP-1 receptor agonists reduced ghrelin secretion in several animal models [172]. Positive effects on appetite behavioral modifications and HbA1c resulted after a 6-month treatment of exenatide administered 10 mcg twice daily to children and adolescents with PWS. This was the first longitudinal investigation of the effects of a GLP-1 receptor agonist. However, no significant changes in weight, BMI, and plasma AG and PP levels were detected [173]. Diversely, a case report in a young PWS female with type 2 diabetes reported efficacy in appetite and weight decrease coupled with a reduction in the daily insulin requirement [174]. The partial effects could be explained by the need of a higher and more frequent dose of exenatide during a chronic treatment, as demonstrated in Snord116 deletion animal models of PWS [175]. On the other hand, an acute dose of 10 mcg of exenatide is able to increase satiety and lower glucose and insulin levels with a higher insulin secretion rate after a meal in PWS adults. These benefits are coupled with a decrease in PYY and GLP-1, but not in

ghrelin levels [176]. Although liraglutide is more effective, only two case reports on PWS young females with diabetes have been published. Liraglutide at lower doses than those used for the treatment of obesity (0.9 and 1.8 mg/day) were both efficacious in improving type 2 diabetes and reducing weight, visceral fat, and appetite. Decreased AG levels were also reported in one of them [177, 178]. However, the role of GLP-1 in PWS, if any, is yet to be established. Thus larger, controlled, longer-term trials are needed to confirm these results and to evaluate whether its use might induce weight loss in PWS patients. Oxytocin. The pathogenesis of the various behavioral concerns in PWS is currently unclear, but a role of oxytocin has been hypothesized [179]. Oxytocin is an anorexigenic neuropeptide, secreted by the hypothalamic paraventricular nucleus, that also modulates social interactions and mother-infant bonding [180, 181]. Recent studies showed decreased expression of both oxytocin and its receptor expression within the hypothalamic paraventricular neurons, a condition coupled with increased oxytocin levels in PWS subjects compared to healthy controls. Altogether, the oxytocin system in patients with PWS appears to be dysfunctional. The elevated oxytocin levels may reflect a loss of the regulatory feedback due to decreased oxytocin receptor expression resulting in an oxytocin resistance. This should lead to the hyperphagia and obsessive-compulsive tendencies in PWS [182]. Thus given the possible dysfunction of the oxytocin system in PWS with consequences on socials skills, food intake, and body weight, it has been hypothesized that the oxytocin supplementation in subjects with PWS would improve social behavior and hyperphagia. Animal data demonstrated that an early treatment with oxytocin restores sucking after birth and decreases aggressive behavior [183]. Recently, a short course of repeated intranasal oxytocin administrations was well tolerated and improved oral feeding and social skills in infants and children with PWS, although these effects are still not confirmed in older PWS subjects [113, 184–186]. These effects are coupled with an increase in circulating AG, which might counterbalance the excess of UAG and possibly drives anorexia [185]. The stimulating effect of oxyciton on ghrelin secretion results via the oxytocin receptor in a cellular model [187]. Conversely, ghrelin could have a compensatory positive feedback on oxytocin, as suggested by neurohypophyseal in vitro models [188]. The regulation of ghrelin-oxytocin axis is still in its infancy. Beloranib. Beloranib is an irreversible inhibitor of methionine aminopeptidase 2 (MetAP2), an enzyme that removes N-terminal methionine residues from newly synthesized proteins. MetAP2 inhibitors were previously used in cancer therapy and were found to reduce food intake, body weight, and adipose tissue mass at doses lower than those needed to inhibit angiogenesis and

tumor growth. In the two-dose regimens (1.8 and 2.4 mg/day), beloranib produced improvement in the hyperphagia-related behaviors and weight loss, but the trial was precociously ceased due to serious venous thrombotic adverse events. No data on ghrelin were reported, but the secretory profile of adiponectin and leptin was improved [189]. Bariatric surgery. Recently, bariatric surgery was also considered in PWS. Several procedures have been proposed including biliopancreatic diversion, gastric or jejunoileal bypass, intragastric balloon, sleeve gastrectomy, gastroplasty, and truncal vagotomy in many case series. The majority of the studies used biliopancreatic diversion. Although there was initial enthusiasm, PWS patients have a significantly increased incidence of complications following bariatric surgery without the benefits of sustained weight loss in the long-term. Thus the risk-benefit ratio of bariatric surgery in PWS does not still appear to be a favorable choice [190]. On the other hand, several authors recently used sleeve gastrectomy with a significant reduction in body weight and minimal morbidities [191, 192]. Similar to what was shown in simple obesity, serum ghrelin levels were reduced after the bariatric surgery procedure [192]. Future intervention studies are necessary to clarify the role of ghrelin in PWS. The administration of specific inhibitors of ghrelin secretion, ghrelin acylation, or ghrelin receptor activity is needed with aiming to impact on the regulation of appetite in PWS.

Conclusions

PWS presents complex genetic and multiple phenotypes that show a need for multidisciplinary and environmental approaches to reduce morbidity and mortality. Clinical features evolve from the neonatal period to adulthood with hyperphagia, typical fat distribution, and preserved insulin sensitivity. Alterations in the ghrelin system have been advocated to have a key role in this phenotype. Growing data suggest that hyperghrelinemia in PWS could more likely be a compensatory mechanism to other biochemical and hormonal alterations, in particular, neonatal hypoglycemia and relative hypoinsulinemia. However, clear results are still scant due to major investigations on total ghrelin levels with only recent data on AG, UAG, and obestatin. Studies better focused on the complex evolution of the syndrome, paying particular attention to who develops glucose intolerance, could offer new hypotheses on the role of the ghrelin system in PWS phenotypes as well as in features of simple obesity.

2.2.5.i. References

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2.2.6. The relationship between cortisol and IGF-I influences metabolic alteration in pediatric overweight and obesity

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Abstract

Background/Objective. Data on metabolic impairments in Cushing's syndrome and GH deficiency all suggest that the relationship between cortisol and GH/IGF-I axis in obesity may have a role in the related diseases. However, studies focusing only on one of these hormones are often controversial in paediatrics. We aimed to explore the simultaneous relationship between cortisol and IGF-I with the metabolic alterations in pediatric obesity.

Subjects/Methods. Retrospective cross-sectional study in a tertiary care center. We recruited 876 (441 males, 435 females) overweight and obese children and adolescents. A complete clinical and biochemical evaluations including OGTT was performed. Cortisol and IGF-I SDS were divided in quartiles and then crossed to explore the reciprocal influence of high/high, low/low, and high/low levels of each one on the metabolic alterations of obesity.

Results. Subjects in the higher quartiles of IGF-I-SDS and cortisol had an increased risk of hypertension, hypercholesterolemia, high levels of triglycerides, and reduced HDL cholesterol. Diversely, lower IGF-I-SDS quartiles were associated with higher blood glucose, insulin, insulin resistance and reduced insulin sensitivity levels with the rise of cortisol quartiles.

Conclusions. We observed that, apart glucose metabolism that is associated with low IGF-I and high cortisol levels, the other parameters known to be associated with increased cardiovascular risk were related to high levels of both IGF-I and cortisol, even if within normal range. Cortisol and IGF-I play a complex role in the comorbidities of obesity, and the evaluation of both variables could clarify some of the discordant results.

Introduction

Obesity is associated with a complex derangement of the endocrine regulation due to compensatory mechanisms. Many of them can have a role in the development of persistent metabolic alterations, resulting in the metabolic unhealthy obese phenotype.

Several studies have shown that abdominal obesity, hypertension, high triglycerides, low HDL-cholesterol levels are associated with functional hypercortisolism [1, 2, 3], suggesting that a pharmacological inhibition of cortisol could be a valid option to avoid the kind of complications [4, 5]. We also recently demonstrated in children that ACTH and cortisol are differently associated with the risk of comorbidities in children and adolescents [6]. Moreover, obesity is associated with functional alterations of GH/IGF-I

axis: in obese subjects, a reduction of GH levels has been detected both at baseline and in dynamic tests and weight loss rescues normal values [7]. Although GH secretion is blunted, the dysregulation of the IGF-I system in obesity is uncertain, since IGF-I is detected as low, normal or elevated, also in the pediatric age [8, 9, 10, 11]. Population studies in adults show that IGF-I secretion is dependent on BMI with an inverse U-shaped curve and higher levels between a BMI of 30–35 Kg/m2, representing a critical metabolic and nutrient-sensing regulator [12]. Furthermore, the interaction with several comorbidities is complex with direct or curvilinear associations [13], also influencing mortality [14]. The regulation of IGF-I in obesity is complex, and many factors have a role, as hypothalamic neuropeptides, ghrelin, insulin, circulating free fatty acids, and cortisol [11]. Among the several players involved, both chronically raised cortisol levels and relative hypoadrenalism are associated with an impaired GH secretion, suggesting a dual dose-dependent effect [15]. On the other hand, GH and IGF-I influence 1β-hydroxysteroid dehydrogenase 1 activity in many organs, including adipose tissue and pancreas [5]. This evidence and data on metabolic impairments present in Cushing's syndrome and GH deficiency suggest that the relationship between cortisol and GH/IGF-I axis in obesity may have a role in the associated health diseases. Based on this hypothesis, the aim of this study is to explore the relationship between IGF-I and cortisol by crossing their quartiles with the metabolic alterations in pediatric obesity.

Methods

Study design and population

This was a cross sectional study. Data of 900 subjects, referred to the Paediatric Endocrine Service of our hospital for overweight and obesity, were collected from January 2005 to December 2016. Eligibility criteria were a general healthy status and overweight or obesity. Subjects with prior engagement in diet programs were excluded from the study. Other exclusion criteria were: diagnosis of diabetes, high blood pressure (BP), use of drugs influencing glucose or lipid metabolism, endocrine or genetic obesity, prematurity, distress during blood sampling and problematic phlebotomy (more than 5 minutes). The protocol was conducted in accordance with the declaration of Helsinki and approved by the Local Inter-Hospital Ethic Committee (Maggiore Hospital Ethical Committee, n. 95/12). All parents gave prior informed consent and careful explanations were provided to each patient.

Clinical measurements and biochemical analysis

Waist circumference, blood pressure levels, fasting and post-oral glucose tolerance test, glucose levels, insulin resistance, lipid profile, and liver enzymes were all considered as cardiometabolic risk factors. Pubertal stages were determined by a physical examination, using the criteria of Marshall and Tanner (1-5) [16]. Height was measured three times to the nearest 0.1 cm using the Harpenden stadiometer and body weight with light clothing was assessed to the nearest 0.1 kg using a manual weighing scale. Body mass index (BMI) was calculated as body weight divided by squared height (kg/m2). BMI deviation score (BMI z-score) was calculated by L, M, S method [17]. All data were classified according to IOTF criteria [17]. Waist circumference was measured in the area between the ribs and the iliac crest at the lowest horizontal circumference while hip circumference at the level of the imaginary horizontal circumference passing through the femoral trochanters, in standing position, at the end of a normal breath. Both were recorded to the nearest 0.1 cm. Waist/height ratio was calculated as well. Systolic BP (SBP) and diastolic BP (DBP), classified according to the American Paediatric Society's Guidelines [18], were measured three times at the right arm with participants seated quietly for at least 5 minutes using a standard digital oscillometric sphygmomanometer. Blood samples were collected after 12 hours of fasting in order to assess cortisol, Insulin like Growth Factor-I (IGF-I), alanine- (ALT), aspartate-aminotransferase (AST), glucose, insulin, total cholesterol, HDL-cholesterol and triglycerides. Low density lipoprotein (LDL)-cholesterol was calculated using the Friedwald formula. Obese subjects also underwent an oral glucose tolerance test (OGTT; 1.75 g of glucose solution per kg, maximum 75 g). Plasma samples were immediately processed and stored at -80°C. All participants were evaluated for signs suggestive of Cushing's syndrome, according to the Endocrine Society's Guidelines (19). In case of positive screening, a 1 mg overnight dexamethasone suppression test and urinary free cortisol measurement were performed. Cortisol levels (μg/dL) were measured by LIAISON Analyzer with the SPALT principle (Solid Phase Antigen Linked Technique). Inter- and intra-assay coefficients of variation were respectively 3.5% and 3.2% with analytical sensitivity of 0.16 μg/dL.Serum IGF-I values were measured by LIAISON Analyzer with a chemo-luminescence (CLIA) methods and an analytical sensitivity less than 3 ng/mL. IGF-I standard deviation score (IGF-I SDS) was calculated. Other analytical methods on ALT, AST, glucose, insulin, total cholesterol, HDL-cholesterol and triglycerides were previously published [20, 21].

Definitions

Hypertension was defined ≥ 95th percentile for sex and age, as suggested by National High Blood Pressure Education Program Working Group of American Academy of Pediatrics [18]. Impaired fasting glucose (IFG), impaired glucose tolerance (IGT), and type 2 diabetes were defined according to American Diabetes Association [22]. Using OGTT parameters, HOMA-IR, the Quantitative Insulin-sensitivity Check Index (QUICKI), and insulin sensitivity obtained from the Matsuda Index (ISI) were calculated. Formulas have been previously reported [23].

Statistical analysis

All data are expressed as mean ± standard deviation (SD), absolute values or percentages. Skewed variables were logarithmically transformed. Student T test was used to investigate sex differences. IGF-I SDS and cortisol levels were categorised into quartiles. The ANOVA was used to evaluate the association among continuous clinical and metabolic variables (BMI, BMI Z-score, weight and waist circumference, SBP, DBP, total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides, fasting glucose levels, glucose levels at 120 min after the OGTT, fasting insulin, HOMA-IR, QUICKI, ISI index, AST, ALT), cortisol, and IGF-I SDS quartiles. The ANOVA was also adjusted by confounding factors (Model 2: sex, age, Tanner stage, BMI, and waist circumference; Model 3: Model 2 + HOMA-IR). Among each IGF1-SDS quartile, logistic regression has been performed in order to calculate the association between cortisol quartiles and the odd ratio (OR, 95% IC) of each cardiovascular risk factor (hypertension, IFG, IGT, LDL-cholesterol > 75 percentile, HDL cholesterol ≤ 10th percentile; triglycerides ≥ 90th percentile). Cortisol quartiles were analysed as independent variables with the first quartile as reference. The analysis was adjusted for the same covariates used in the previous Model 3. Statistical significance was set up for $p < 0.05$. Samples ranging from of 20 to 202 individuals have been estimated enough to demonstrate a difference of 20% in predicted prevalence of metabolic alterations across quartiles with 90% power and a significance level of 95% basing on our previous data. Because the prevalence of glucose alterations is relatively low in paediatric obesity, a reduction of 20% in HOMA-IR with a SD 1.8 has been considered (6). Statistical analysis was performed with SPSS for Windows version 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Population

The final dataset included 876 patients (441 males and 435 females). Twenty-four children were excluded because they did not satisfy inclusion criteria (18 with a difficult

blood sampling, 3 with hypothyroidism, and 3 with signs of infections at blood count cells). Clinical, biochemical and hormonal characteristics of the population, stratified for sex, are reported in Table 1. Among them, 405 (46.1%) subjects were prepubertal and 471 (53.8%) were pubertal. Moreover, 215 (24.5%) subjects were overweight, and 661 (75.5%) obese (271 of them were severely obese), according to the IOTF criteria, without a sex imbalance. Most of the population presented visceral obesity, in particular 841 (96%) subjects had a waist circumference ≥ 90th percentile. Of the total cohort, 522 (59,6 %) subjects had hypertension and 77 (8,8%) IFG or IGT. Nobody had type 2 diabetes. Levels of triglycerides ≥ 90th percentile and HDL ≤ 10th percentile were more frequent in boys. Cortisol levels were lower than 5 µg/dL in 65 subjects, 40 of them repeated the measurement with normal values, whereas the other 25 subjects underwent the dynamic test with a normal response (24). Nobody had IGF-I levels and a phenotype suggestive for GH deficiency.

Associations among cortisol, IGF-I SDS quartiles and cardio-metabolic parameters

Patients have been divided into IGF-I SDS and cortisol quartiles. Each quartile included 219 subjects. Then, we crossed quartiles in order to evaluate the influence of both quartiles on the cardiovascular and metabolic risk factors. Subjects have been classified into 16 groups based on the IGF-1 SDS and cortisol quartiles, as shown in Supplementary Table 1. BMI, BMI Z-score, weight and waist circumference were similar in all cortisol and IGF-I SDS quartiles. In Model 1 and 2, age was positively correlated with the rise of cortisol quartiles of subjects in I and II quartiles of IGF-I SDS (I quartile $p < 0.05$; II quartile $p < 0.01$). SBP increased with cortisol quartiles in all the IGF-I SDS quartiles in all the models (Model 3: I IGF-I SDS quartile: $p < 0.02$; II IGF-I SDS quartile: $p < 0.02$; III quartile: $p < 0.01$; IV quartile: $p < 0.02$). Diversely, DBP did not vary at the increase of cortisol quartiles, independently from IGF-I SDS quartiles and model. Total cholesterol was positively associated with cortisol into high levels of IGF-I SDS in all the models (IV quartile, Model 3: $p < 0.01$). The same trend was observed in the II quartile of IGF-I SDS only in model 2 ($p = 0.056$). HDL cholesterol level did not change with the increase of IGF-I SDS and cortisol quartiles in the crude model, while assuming significance and increasing with the rise of cortisol quartiles in IV IGF-1 SDS quartile in Model 2 ($p < 0.02$) and 3 ($p < 0.02$). LDL cholesterol was positively correlated with cortisol quartiles in IV IGF-I SDS quartile in model 1 and 2 ($p < 0.02$), diversely by Model 3. Triglycerides increased with cortisol quartiles in I and IV IGF-I SDS quartile ($p < 0.01$) in both model 1 and 2. On the other hand, by using model 3, the significance

was maintained only in IV IGF-1 SDS quartile (p <0.01) (Figure 1). Fasting glucose levels increased according to the increase of cortisol qua tiles in I IGF-I SDS quartile (p <0.05) and in IV IGF-I SDS quartile (p <0.02) in model 1. In models 2 and 3, this significance was lost, while that in II IGF-I SDS quartile was obtained (p <0.03). Regarding glucose levels at 120 min after the OGTT, there was no significance in any IGF-I SDS quartile. Fasting insulin was positively correlated with cortisol in I IGF-I SDS quartile (p <0.03) in the crude model but this was lost when covariates were inserted. Diversely, insulin resistance at fasting, expressed as HOMA-IR, and insulin sensitivity at fasting, expressed as QUICKI, increased and decreased, respectively, with the increase of cortisol quartiles in I IGF1-SDS quartile in all models (HOMA-IR: p <0.04; QUICKI: p <0.05) (Figure 2). Similarly, ISI index decreased with the increase of cortisol quartiles in I quartile of IGF1-SDS (Model 1: p <0.04). AST values increased with the increase of cortisol in I (p <0.05) and III IGF-I SDS quartile in all the models (Model 1 and 2: p<0.008; Model 3: p <0.01), meanwhile ALT values increased with the rise of cortisol quartiles only in IV IGF-1 SDS (Model 1 and 2: p<0.03; Model 3: p <0.04).

Associations among cortisol, IGF-I SDS quartiles and metabolic alterations

Subjects included in the III IGF-I SDS quartile had a high risk of hypertension in III (OR: 2.665, CI95%1.029-6.900, p <0.04) and IV cortisol quartile (OR: 2.700, CI95%1.069-6.819, p <0.03). The risk of high levels of total cholesterol increased within III and IV cortisol quartiles in subjects in II, III and IV IGF-I SDS quartiles (Table. 2). Subjects included in the IV IGF-I SDS quartile had a higher risk of having HDL cholesterol ≤ 10^{th} percentile when cortisol levels were in III (OR: 4.048, CI95%1.560-10.505, p <0.004) and IV quartile (OR: 1.238, CI95%1.005-3.031, p <0.05). The risk of having triglycerides ≥ 90^{th} percentile increased in the IV cortisol quartile (OR: 3.291, CI95%1.117-9.698, p <0.03) in the I IGF-I SDS quartile. No significance was detected for IFG or IGT presence.

Table 1. Biochemical and clinical characteristics of subjects.

	All (876)	M (441)	F (435)
Age	10.6 ± 3.2	10.6 ± 2.9	10.7 ± 3.4
Prepubertal	405 (46.1%)	246* (55.8%)	159* (36.6%)
Pubertal	471 (53.8%)	195* (44.2%)	276* (63.4%)
BMI Z-score (kg/m^2)	2.0 ± 0.5	2.0 ± 0.5	2.0 ± 0.6
BMI (kg/m^2)	27.0 ± 4.7	27.1 ± 4.4	26.9 ± 5.0
Waist circumference (cm)	88.1 ± 13.6	88.8 ± 13.0	87.4 ± 14.2
SBP (mmHg)	124.8 ± 16.2	125.9 ± 16.4	123.7 ± 15.91
DBP (mmHg)	79.7 ± 11.1	80.6 ± 10.9	78.9 ± 11.2
SBP/DBP > 95°	522 (59.6%)	270 (61.2%)	252 (57.9%)
Total cholesterol (mg/dL)	145.1 ± 26.8	144.0 ± 26.5	146.2 ± 27.1
HDL (mg/dL)	43.2 ± 9.8	42.9 ± 9.3	43.5 ± 10.2
TG (mg/dL)	75.3 ± 42.7	72.8 ± 39.5	77.8 ± 45.7
LDL (mg/dL)	86.7 ± 22.6	86.4 ± 22.5	87.0 ± 22.8
Glc0' (mg/dL)	86.5 ± 8.8	87.5 ± 9.0	85.4 ± 8.3
Glc120' (mg/dL)	108.9 ± 18.9	110.2 ± 18.4	107.6 ± 19.3
Insulin0' (uUI/mL)	14.3 ± 9.8	13.4 ± 8.5	15.3 ± 10.9
HOMA-IR	3.2 ± 2.4	3.0 ± 2.2	3.3 ± 2.5
ISI	4.33 ± 3.92	4.40 ± 3.41	4.27 ± 5.34
QUICKI	0.34 ± 0.06	0.34 ± 0.04	0.34 ± 0.05
AST (IU/L)	24.2 ± 7.8	25.4 ± 8.3	22.9 ± 7.2
ALT (IU/L)	23.8 ± 13.3	25.9 ± 16.0	21.6 ± 9.2
Cortisol (µg/dL)	11.3 ± 5.2	11.3 ± 5.0	11.3 ± 5.5
IGF-1 (ng/mL)	268.5± 121.4	237.8 ± 113.3	299.6 ± 121.5
IGF-1 SDS	0.15 ± 0.61	0.01 ± 0.58	0.30 ± 0.61

Significant differences between males and females: * = p <0.001, ** = p <0,003.

All data are expressed as mean ± standard deviation (SD), absolute values or percentages. Abbreviations: SBP = systolic blood pressure; DBP = diastolic blood pressure; HDL = High density lipoprotein; LDL = low density lipoprotein; TG = triglycerides; glc0' = fasting plasma glucose, glc120' = blood glucose after 120 minutes of oral glucose tolerance test; Ins0' = fasting insulin; HOMA-IR = homeostatic model assessment insulin resistance; ISI = insulin sensitivity index; QUICKI = quantitative insulin sensitivity index; AST = aspartate amino transferase; ALT = alanine amino transferase; IGF-1 SDS = insulin-like growth factor-1 standard deviation score.

Table 2. Logistic regression of LDL cholesterol ≥ 75°per centile in quartiles of cortisol (µg/dL) and IGF-1 SDS quartiles.

		LDL ≥ 75° Centile		
		$\chi2 = 2.915$ p = ns		
	Cortisol quartiles	OR	95% CI	*P value*
I IGF1-SDS	**I**	1,000		
	II	0.642	0.563-2.534	ns
	III	1.041	0.277-3.913	ns
	IV	1.823	0.503-6.609	ns
		$\chi2 = 9.633$ $p < 0.02$		
	Cortisol quartiles	OR	95% CI	*P value*
II IGF1-SDS	**I**	1,000		
	II	1,347	0.371-4.887	ns
	III	**4.042**	**1.280-12.771**	**< 0.01**
	IV	**4.177**	**1.38-15.336**	**< 0.03**
		$\chi2 = 5.432$ p = ns		
	Cortisol quartiles	OR	95% CI	*P value*
III IGF1-SDS	**I**	1,000		
	II	2.597	0.690-9.781	ns
	III	1.781	0.410-7.738	ns
	IV	**4.096**	**1.167-14.382**	**0.02**
		$\chi2 = 8.127$ p = ns		
	Cortisol quartiles	OR	95% CI	*P value*
IV IGF1-SDS	**I**	1,000		
	II	1.431	0.454-4.507	ns
	III	**2.911**	**1.164-8.791**	**< 0.05**
	IV	1.879	0.562-6.285	Ns

Marked values represent significant results. Cortisol values were subdivided into quartiles as follows: I≤7.3 µg/dL, II: 7.4-10.2 µg/dL, III: 10.3-14.4 µg/dL, IV:> 14.4 µg/dL. The values of IGF-1 SDS were divided into quartiles as follows: I: ≤ -0.242, II: 0.243-0.078, III: 0.0079-0.045, IV:> 0.045. The variables used in Model 3 were: gender, age, Tanner stage, BMI Log, HOMA and waist circumference.

Discussion

Obesity is strongly associated with several comorbidities, including modification of hypothalamic-pituitary-adrenal (HPA) and GH/IGF-I axis. The development of cardiovascular diseases begins in paediatric age; therefore, the aim of the present study was to verify the presence of a possible association between cortisol, IGF-I values and obesity-related complications in overweight and obese patients. The data herein presented showed an association between different metabolic parameters, cortisol and IGF-I SDS. Although our results establish the usual cardiovascular and metabolic cluster, they differ from previous results in some variables between the lowest and the highest level of IGF-I. These differences can be due to a different evaluation of the complex metabolic relationship between HPA and GH/IGF-1 axis. First, despite an expected association based on literature [25, 26, 27], in our study, weight, BMI, BMI Z-score and waist circumference did not vary in correspondence of an increase in cortisol and IGF-I SDS. However, discordant findings could partly derive from different inclusion criteria. Studies that reported associations of cortisol or IGF-I values with BMI and waist circumference included a normal-weight group of subjects, although often limited in dimension [25], or obese subjects belonging to a specific ethnic background [28]. Other studies found an association only when daily urinary cortisol, urinary steroid metabolites or IGF-I binding proteins [27, 29] or more precise measures of fat mass were considered (3). Furthermore, during the paediatric age, HPA and GH/IGF-I axis regulation can be influenced by other factors, including obesity comorbidities. We introduced several factors to explain, at least partially, how metabolic parameters, cortisol and IGF-I interact. We demonstrated that SBP increases significantly, when cortisol increases in all the IGF-I SDS quartiles as well as the risk of hypertension exists in the III IGF-I SDS quartile for the highest levels of cortisol. IGF-I levels have been associated to the risk of hypertension both in condition of GH or IGF-I deficiency and acromegaly [30, 31]. Three mechanisms could have a major role: i) the lack of the stimulation of the production of nitric oxide, increasing vasodilation and reducing platelet activation for the low levels of IGF-I [32], ii) the stimulation of stiffening and vasoconstriction of vessel muscle cells and progression of atherosclerotic disease [33, 34], iii) and the augmented sodium retention with the increase of circulating volume (35) for the high levels. In literature, some discordant results are present. Similar [31], higher [36, 37], or lower [38] IGF-I levels have been shown in hypertensive patients compared to normotensive ones. Recently, a Dutch study reported a U-shaped association between IGF-I levels and cardiovascular risk factors [39], partly in

accordance with our results. Apart from IGF-I levels, we clearly showed that high SBP levels and risk of hypertension is present when cortisol levels are in the highest range of the normal values, suggesting that cortisol plays a decisive role in hypertension. Various mechanisms contribute to blood pressure increase: among the main ones, there is mineralocorticoid activity of cortisol. In hypercortisolism, renin-angiotensin-aldosterone system is altered, secondary to both central and peripheral over-expression of angiotensin II receptors [40]. Moreover, cortisol induces a reduction in nitric oxide synthesis, inhibiting activity of nitric oxide synthase [41], against what IGF-I usually causes. Moreover, other studies in obese children, identified correlation between increased SBP and cortisol [28, 42, 6]. As a result, some contrasting data in literature on IGF-I could be relative to the cortisol balance. As regarding lipid profile, total cholesterol, LDL-, HDL-cholesterol, and triglycerides increased with the rise of cortisol levels with high IGF-1. This has been confirmed also in the logistic regression analysis, where an increased risk of hypercholesterolemia and hypertriglyceridemia with cortisol values ≥10.2 μg/dL (III and IV quartile) was highlighted. This result agrees with literature data: activation of HPA axis can promote alteration of lipid profile in both adults [43] and children [6] due to complex mechanisms, involving the recycling of lipids, acting on the liver and adipose tissue [44, 45]. Triglycerides level is the only variable that increased also in patients with low levels of IGF-I with the rise of cortisol. Low levels of IGF-I have a burden on cardiac and metabolic risk factors, increasing over production of triglycerides also in paediatric obesity [46]. An intriguing association is that on HDL-cholesterol. We showed that HDL-cholesterol increased with the rise of cortisol levels with high IGF-I, but subjects in the IV IGF-I SDS quartile had however an increased risk of HDL-cholesterol ≤ 10^{th} percentile when cortisol levels were high. Two different physiological mechanisms could be hypothesized. Considering the role of IGF-I, a positive association between IGF-I and HDL-cholesterol values was found in several studies, including one in obese adolescents [47]. The exact mechanism underlying the relationship between IGF-I and HDL cholesterol in obesity is not fully clarified: the main reason may be that IGF-I inhibits the hepatic expression of the class B1 scavenger receptor on the surface of the hepatocytes, causing a reduction in the hepatic intake of HDL cholesterol and an increase in its circulating levels [48]. Furthermore, by considering cortisol, it has been highlighted that the adrenal gland uses cholesterol contained in HDL and not the one contained in LDL to synthesize steroid hormones [49, 50]. Indeed, patients with low levels of HDL had a low response to the ACTH test, suggesting that a condition of partial adrenal insufficiency should be

related to a reduction of HDL cholesterol [51]. On the other hand, subjects in the IV IGF-I SDS quartile with highest cortisol levels had an increased risk of HDL cholesterol ≤ 10th percentile when cortisol levels were high. We cannot exclude that, in the context of obesity, the state of low-grade inflammation associated with cortisol as well as hyper-nutrition, is able to stimulate a pro-atherogenic lipid profile even in the paediatric age [52, 53]. However, also a statistical bias could be hypothesized, linked to the sample size, although it resulted enough for all the cardiometabolic alterations considered, or to the lack of a control group of normal-weight children. Regarding glucose metabolism, diversely by other factors discussed until now, we showed that fasting glucose and insulin resistance augmented and in correspondence of that, QUICKI and ISI decreased with high cortisol levels in the I IGF-I SDS quartile. This result could be linked to the failure of insulin-like activity of IGF-I when its levels are low, resulting in an altered glycaemic and insulin status. Indeed, IGF-I can stimulate the use of glucose by activating GLUT4 and inhibiting gluconeogenesis, all insulin-like effects that improve insulin sensitivity [54]. The absence of an association between fasting glucose and insulin resistance, with the increase of cortisol quartiles in IV of IGF-I SDS quartile, could be an attempt to overcome a condition of subtle hyperglycaemia and hyperinsulinemia resulting from obesity. This would be through by stimulating IGF-I secretion and exploiting its hypoglycaemic effects [27]. Diversely, the lack of an association with altered glucose levels as IFG or IGT might be secondary to underpowered sample size, being these conditions a rare event in Caucasian paediatric obese children [6, 55]. Lastly, AST and ALT showed a direct association both with cortisol and IGF-I SDS, especially in III and IV quartiles. ALT represents the most specific hepatocyte damage marker, even in paediatric age [56], and liver steatosis is the most common cause for overweight and obese children and adolescents ranging up to 34% [56, 57] and being associated with a lipid profile characterized by hypertriglyceridemia and increased non-HDL cholesterol [58]. This has been confirmed by our results. However, it has not been possible to perform abdominal ultrasound or liver biopsy to all enrolled children. Due to this absence of data, the increase of AST and ALT with the liver steatosis remains a hypothesis in this population. Although obesity is strongly associated with hepatic steatosis, an excess of adipose tissue is not the only cause for liver steatosis development. Indeed, patients with lipodystrophy show a marked insulin resistance and easily develop hepatic steatosis and type 2 diabetes, suggesting that it is not the obesity that causes the pathology, making the lipolytic activity dysfunction the main factor [59]. In our study, this could be justified by

the lipolytic effect linked to functional hypercortisolemia associated with higher IGF-I levels suggestive of hyper-nutrition. This study has several limitations. First, we did not recruit healthy normal-weight children. We tried to overcome this point by investigating a huge cohort composed of overweight, obese and severely obese children and adolescents in the hypothesis that trends of comorbidities, cortisol and IGF-I levels were associated with the progressive increase of weight scores. However, we cannot exclude that a control group with and without subtle metabolic alterations could modify some of our findings. Further studies including also healthy children are mandatory to confirm our suggestions. Second, we limited used quartile-based categories to evaluate the relationship between cortisol/IGF-I and metabolic alterations. While this approach is largely used and generally produces valid results [60-62], other options (e.g. flexible modelling of the interaction of continuous variables) are available as well and could produce different results. Third, the role of hyper-nutrition or unbalanced nutrition on the relationship among IGF-I, cortisol and cardiometabolic parameters is a hypothesis in our study but also an exciting issue. Recent findings suggest that the adherence to a Mediterranean diet, mainly protein and milk intake are associated with the functionality of the GH/IGF-I axis and the cardiometabolic profile in adult obesity [63, 64]. Moreover, skipping breakfast, and then reducing milk intake, has been associated with a worse metabolic profile also in paediatric obesity [65]. Other foods have been associated with inflammation and may modulate the HPA axis [66]. In the view of the role of nutrients as metabolic sensors, a complete picture of food habits is required in further studies.

In conclusion, this study aimed to establish the association between cortisol, GH/IGF-I axis, and metabolic complications in overweight and obese children and adolescents. We observed that some of the parameters known to be associated with increased cardiovascular risk were related to high levels of IGF-I and cortisol, even if within normal range. We showed that subjects with high IGF-I and cortisol had an increased risk of hypertension, hypercholesterolemia, high levels of triglycerides, and reduced HDL cholesterol. Diversely, lower IGF-I levels were associated with higher blood glucose, insulin, insulin resistance and reduced insulin sensitivity levels with the rise of cortisol. These data suggest that cortisol and IGF-I play a complex role in the comorbidities of obesity. The evaluation of both variables could clarify some of the discordant results shown in literature on the role of IGF-I.

Figure 1. Triglycerides (mean±SD) in the I (a: p <0.01 in Model 2; b: p <0.01, c: p <0.02 and d: p <0.01 in Model 1), II, III, and IV IGF-1 SDS quartile (a: p <0.01 in Model 3; b: p <0.05 and c: p <0.02 in Model 1).

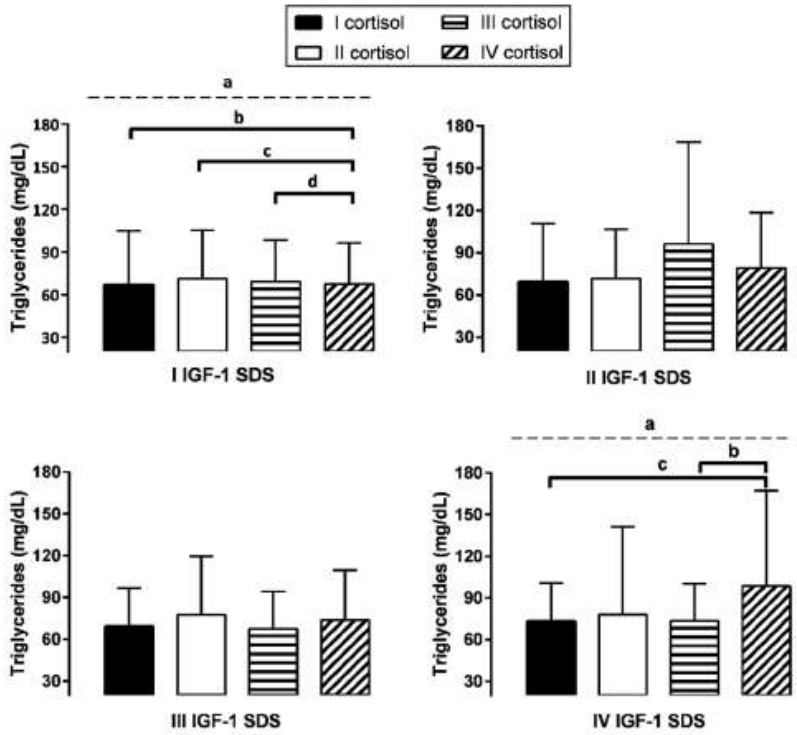
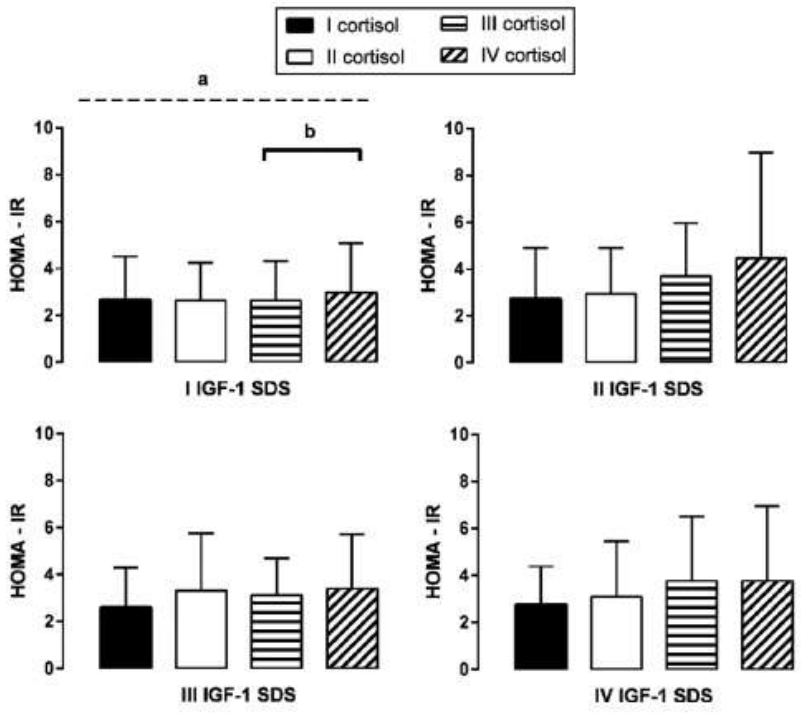


Figure 2. HOMA Index (mean±SD) in the I (a: p <0.05 in the Model 2; b: p <0.02 in Model 1), II, III and IV IGF-1 SDS quartile.



2.2.6.i. References

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2.2.7. Haptoglobin phenotypes are associated with the post-load glucose and insulin levels in pediatric obesity

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Abstract

Purpose: Haptoglobin (Hp) is a protein involved in the acute-phase reaction of inflammation. Humans have three major phenotypes (Hp1-1, Hp1-2, and Hp2-2). Several studies have shown altered Hp regulation in adults with obesity and metabolic alterations. Hp2-2 phenotype is associated with a high cardiovascular risk. Our aim was to investigate if Hp levels and Hp2-2 phenotype are associated with glucose metabolism in pediatric obesity.

Methods: We retrospectively studied 192 participants (92 males and 100 females), aged 4- 18 years. Clinical and biochemical data were collected. Hp phenotype (Hp1-1, Hp1-2, and Hp2-2) was identified through Western immunoblot.

Results: Subjects carrying Hp1-1, Hp1-2 and Hp2-2 phenotype were 13.6, 50.8, and 35.6%, respectively. Hp serum, fasting glucose, and insulin levels, as well as HOMA-IR, were similar among groups. Post-load glucose and insulin levels (as insulin AUC were progressively higher from Hp1-1 to Hp2-2 phenotype.

Conclusion: To our knowledge, this is the first study on Hp phenotypes conducted in a pediatric population with obesity. We showed that the presence of Hp2 allele is associated with a worse response of glucose load in term of both glucose and insulin levels. Thus, Hp2-2 phenotype could predispose in pediatrics, at the same degree of obesity, to a worse glycemic and -insulinemic compensation.

Introduction

Haptoglobin (Hp) is a tetrameric protein constituted of two α and two β-chains connected by a disulphide bridge. There are two main alleles at the Hp locus, Hp1 and Hp2, that encode for the same β chain but for two α-chains that differ in length. The two alleles are inherited in a co-dominant manner and may combine to originate the three major human phenotypes (Hp1-1, Hp2-2 and Hp1-2) [1]. Hp is involved in the acute-phase reaction of inflammation and its main activity is to bind to cell-free hemoglobin, preventing oxidative stress and tissue damage [2-4]. Hp is mainly produced by the liver, but also at quite lower concentration in immune cells including monocytes and white adipose tissue [4]. Several findings have shown a direct correlation between Hp levels and obesity in humans [4-7], suggesting that it may represent a new chemokine involved in the complex phenomenon of obesity, chronic inflammation, and cardiovascular risk. Furthermore, animal models of Hp deficiency at high-fat diet develop blunted obesity-associated comorbidities [4, 8]. Interestingly, previous studies suggested that clinical data on total Hp levels should be interpreted with caution

because although correlated to obesity and cardiometabolic risk, different metabolic outcomes may be associated to the different Hp phenotypes rather than the total levels. In type 2 diabetes the Hp2-2 phenotype has been associated with increased disease complications, mainly high myocardial infarction, and mortality risk [9-11]. Very recently, a post-hoc analysis of the ACCORD study revealed that intensive glucose lowering therapy was efficacious in preventing incident coronary heart disease and cardiovascular events only in individuals showing the Hp2-2 phenotype. This is because hyperglycemia through several mechanisms lead to an increased amounts of circulating Hp2:Hb complexes with an increased oxidative activity and paradoxically turn the HDL into a proatherogenic and prothrombotic lipoprotein [12]. To date, only a few studies have analyzed Hp levels in obese children [13-15] or young patients with diabetes [16] but none of them considered the Hp phenotype. The aim of our study was to investigate if the Hp levels and Hp2-2 phenotype were associated with obesity comorbidities and glucose metabolism even in childhood.

Methods

Study design and population

We studied 196 obese children and adolescents, aged 4 to 18 years, referred to the Pediatric Endocrine Service of our Hospital (January 2005 - December 2016) and included in an observational protocol on childhood obesity approved by the Local Ethics Committee (CE 95/12) and was conformed to the guidelines of the European Convention of Human Rights and Biomedicine for Research in Children. Subjects included should satisfy all the following inclusion criteria: 1) obese, according to the International Obesity Task Force (IOTF) body mass index criteria [17]; 2) not on a weight-loss diet; 3) biochemical evaluations including also a 2-hour oral glucose tolerance test (OGTT) had been performed; 4) plasma samples to evaluate the Hp phenotype were available. Exclusion criteria were specific causes of endocrine or genetic obesity, type 1 diabetes, and type 2 diabetes treated pharmacologically, previous kidney or hepatic diseases, and use of specific drugs (oral hypoglycemic agents or antihypertensives). Informed written consent was obtained from all parents before the evaluations after careful explanations were given to each patient. Subjects with pre-diabetes in medical nutrition therapy were included, while subjects with type 2 diabetes treated with oral hypoglycemic were excluded. No diagnosis of type 2 diabetes was done.

Anthropometric and biochemical measurements

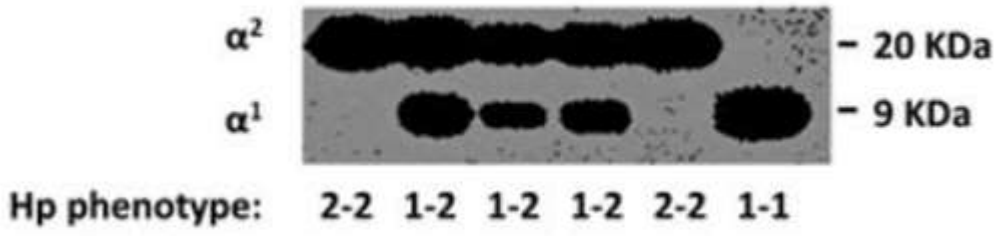
Height was measured to the nearest 0.1 cm using a Harpenden stadiometer, and body weight with light clothing to the nearest 0.1 kg using a manual weighing scale. BMI was calculated as body weight divided by squared height (kg/m2). The BMI standard deviation score (BMISDS) was calculated by the least median squares (LMS) method. Waist circumference (WC) was measured at the high point of the iliac crest around the abdomen and was recorded to the nearest 0.1 cm according to standard methods [18]. Waist-to-height ratio was calculated. Pubertal stages were determined by physical examination, using the criteria of Marshall and Tanner [19]. Systolic (SBP) and diastolic blood pressure (DBP) were measured three times at 2-minute intervals using a standard sphygmomanometer with an appropriate cuff size after participants were seated quietly for at least 15 minutes according to standards [20] and at least 30 minutes after blood sampling. Mean values were used for the analyses. After a 12-h overnight fast, blood samples were taken for measurement of: glucose (mg/dL), insulin (μUI/mL), total cholesterol (mg/dL), HDL-cholesterol (mg/dL), triglycerides (mg/dL), using standardized methods in the Hospital's Laboratory previously described [21]. LDL-cholesterol was calculated by the Friedwald formula. Uric acid (mg/dL) was measured by Fossati method reaction using uricase with a Trinder-like endpoint. Serum Hp levels (mg/ml) were measured by immunonephelometry, using as reagent the N antisera anti human haptoglobin (Siemens, Healthcare Diagnostics, Deerfield, IL, USA), with sensibility of 0.26 – 8.3 g/L, coefficient of variation inter-cycle 2.3%, intra-cycle 3.5% and total 3.9%. Subjects also underwent an OGTT (1.75 g of glucose solution per kg, maximum 75 g) and samples were drawn for the determination of glucose and insulin every 30 min. The area under the curve (AUC) was calculated according to the trapezoidal rule. Insulin-resistance was calculated using the formula of homeostasis model assessment (HOMA)-IR. Insulin sensitivity at fasting and during OGTT was calculated as the formula of the Quantitative Insulin-Sensitivity Check Index (QUICKI) and Matsuda index (ISI) as previously reported [22]. The stimulus for insulin secretion in the increment in plasma glucose as insulinogenic index was calculated as the ratio of the changes in insulin and glucose concentration from 0 to 30 min (InsI). Glucose was expressed in mg/dL (1 mg/dL = 0.05551 mmol/L) and insulin in μUI/mL (1 μUI/mL = 7.175 pmol/L) in each formula.

HP phenotype identification by Western Immunoblot

Plasma samples of patients collected by two consequential centrifugations (1300 rpm for 10', 2400 rcf for 25') were diluted 1:10 in Dulbecco's Phosphate buffered saline

(Sigma-Aldrich, St. Louis, MO) and then loaded in equal amounts in 15% SDS-PAGE gels under reduced conditions. After electrophoretic transfer, the immuno-blot polyvinylidene difluoride (PVDF) membranes (BioRad, Hercules, CA) were blocked in Tris-buffered saline 0.1% Tween-20 containing 5% non-fat milk for 1h, then incubated with a primary antibody for Hp α (Sigma-Aldrich) diluted 1:5000 and detected with horseradish peroxidase-conjugated secondary anti-mouse IgG diluted 1:5000 (Merck Millipore, Darmstadt, Germany). Immunoreactive proteins were detected using enhanced chemiluminescence (Pierce Biotechnology Inc, Rockford, IL, USA) with image capture performed using CCD-camera linked to ChemiDoc (Bio-Rad). In the current study, Hp phenotype were defined by the presence of α-chain bands at either ~9 kDa (α1: Hp 1-1), ~20 kDa (α2: Hp 2-2) or both (Hp1-2). Figure 1 is representative of a western immunoblot.

Figure 1. Hp-phenotypization by Western Immunoblot in plasma samples of obese pediatric subjects.



Diluted plasma samples from six representative obese pediatric subjects were analysed by WIB under reduced condition to identify the Hp phenotype using a specific antibody against α chain. The band ~ 9 kDa correspond to the α1 chain, whereas the band at ~ 20 kDa correspond to the α2 chain. In the presence of only one band, 20 kDa or 9 kDa, the phenotype was respectively 2-2 and 1-1, when both bands were detectable the phenotype was heterozygous 1-2.

Definitions

Subjects were classified as obese according to age- and sex-specific IOTF cut-offs [17]. According to the criteria of Marshall and Tanner [19], the pubertal stage was defined in the presence of a testicular volume of 4 mL for males and breast at stage 2 for females. SBP and DBP values were evaluated according to percentiles for age, sex, and height, of National High Blood Pressure Education Program (NHBPEP) Working Group [20]. Arterial hypertension was defined according to the following criteria: (1) >

95th percentile as suggested by the (NHBPEP) Working Group of American Academy of Pediatrics (AAP) [20]. Hypertension was determined if BP values recorded on both the enrollment day and blood sample day were elevated. Dyslipidemia was considered if hypertriglyceridemia as triglycerides ≥ 150 mg/dL or reduced HDL-cholesterol levels as HDL-cholesterol ≤ 40 mg/dL as suggested by IDF (International Diabetes Federation) criteria for metabolic syndrome classification in children [23]. Impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) were defined by a fasting plasma glucose of ≥ 5.6 to 6.9 mmol/L (100-125 mg/dL), and, as 2-h post-OGTT, glucose of ≥ 7.8 to 11.0 mmol/L (140-199 mg/dL), respectively, according to American Diabetes Association [24].

Statistical analysis

All data are expressed as mean ± SD, absolute values or percentages. A sample of 24 individuals for each of three Hp phenotypes been estimated to be enough to demonstrate a difference of 20 mg/dl in fasting or stimulated glucose levels with a 90.0% of power and a precision of 5.0% according to recently published data on obese children [25, 26]. Due to the lower prevalence of Hp 1 genotype in Caucasian and American subjects, the frequency of Hp 1-1 phenotype was considered and the population size was increased at 63 individuals in each group [27]. Distributions of continuous variables were examined for skewness and were logarithmically transformed as appropriate. The differences between genders and pubertal stages were analyzed with the Student T-Test. ANOVA was used to determine the differences between Hp phenotypes. The ANOVA analysis was also carried out with the following covariates: age, sex, Tanner's stage (Model 1) and BMI in addition to Model 1 (Model 2). Statistical significance was assumed at $P < 0.05$. The statistical analysis was performed with IBM SPSS Statistics for Windows version 22.0 (Chicago, IL, USA). Linkage disequilibrium calculation and haplotype frequencies determination was performed with the Haploview software (Center for Human Genetic Research, Massachusetts General Hospital, and the Broad Institute of Harvard & MIT).

Results

Of the 196 included subjects, 4 were excluded, 2 because the plasma samples were incorrectly conserved, and 2 because both Hp levels and phenotypes were not detected, and the number was not enough to be compared to the other subjects. The final dataset included 192 participants (92 males and 100 females), aged 4-18 years, with an age of 11.5±2.8 years. Of those subjects, 60 (31.4%) were prepubertal and 132

(68.6%) pubertal. The distribution of the three Hp phenotypes was 13.6, 50.8, and 35.6% for Hp1-1, 1-2, and 2-2, respectively, which was in Hardy-Weinberg equilibrium. Clinical and biochemical characteristics of the sample were summarized in Table 1 and HP phenotypes comparison in Table 2 considering each phenotype.

Table 1. Clinical and biochemical characteristics of the sample.

	All	Prepubertal	Pubertal	P value
Subjects	192	60	132	ns
Sex (M/F)	92/100	33/27	59/73	ns
Age (years)	11.5 ± 2.8	8.6 ± 1.8	12.8 ± 2.2	<0.0001
Height (cm)	150.2 ± 14.7	135.0 ± 10.8	157.2 ± 10.12	ns
Weight (Kg)	65.1 ± 21.5	49.5 ± 13.4	76.9 ± 16.6	<0.0001
BMI (Kg/m^2)	29.6 ± 4.8	26.8 ± 3.5	30.9 ± 4.8	<0.0001
BMI z-score (Kg/m^2)	2.2 ± 0.5	2.1 ± 0.4	2.3 ± 0.6	ns
Waist circumference (cm)	92.4 ±13.1	82.9 ± 10.6	96.8 ± 11.8	<0.0001
SBP (mmHg)	126.9 ± 18.4	119.3 ± 14.3	130.4 ± 19.0	<0.0001
DBP (mmHg)	82.0 ±11.3	77.5 ± 10.5	84.1 ± 11.1	<0.0001
Total cholesterol (mg/dL)	142.2 ± 25.1	145.2 ± 26.7	140.8 ± 24.3	ns
LDL-c (mg/dL)	84.2 ± 21.6	86.0 ± 22.8	83.5 ± 21.0	ns
HDL-c (mg/dL)	41.2 ± 9.7	43.2 ± 9.6	40.3 ± 9.6	<0.01
HDL-cholesterol ≤ 40 (mg/dL)	92 (47.9%)	25 (13.0%)	67 (34.9%)	ns
Triglycerides (mg/dL)	83.2 ± 44.8	77.5 ± 39.4	85.8 ± 47.0	ns
Triglycerides ≥ 150 (mg/dL)	14 (7.3%)	4 (2.1%)	10 (5.2%)	ns
GlcT0' (mg/dL)	88.8 ± 7.2	87.2 ± 7.5	89.6 ± 7.0	<0.02
GlcT120' (mg/dL)	111.8 ± 18.9	108.4 ± 14.1	113.2 ± 20.5	ns
InsT0' (mUI/L)	19.2 ± 12.5	15.2 ± 10.0	21.0 ± 13.1	<0.0001
InsT120' (mUI/L)	96.4 ± 110.9	80.6 ± 114.5	103.0 ± 109.7	<0.01
IFG	14 (7.3%)	3 (1.5%)	11 (5.8%)	ns
IGT	16 (8.3%)	1 (0.5%)	15 (7.8%)	<0.02
HOMA-IR	4.3 ± 2.9	3.3 ± 2.2	4.7 ± 3.1	<0.0001
ISI	3.72 ± 2.99	4.35 ± 3.00	3.47 ± 2.98	<0.01
QUICKI	0.323 ± 0.043	0.332 ± 0.033	0.318 ± 0.046	<0.0001
Haptoglobin (mg/dL)	104.8 ± 41.5	106.4 ± 33.4	104.1 ± 44.4	ns
Hp-phenotype 1-1	26 (13.6%)	10 (38.5%)	16 (61.5%)	
Hp-phenotype 1-2	97 (50.8%)	31 (32.0%)	66 (68.0%)	ns
Hp-phenotype 2-2	68 (35.6%)	19 (27.9%)	49 (72.1%)	

All data are expressed as mean ± SD (Standard Deviation) or percentage.

Abbreviations: BMI: body mass index; DBP: diastolic blood pressure; F: female; GlcT0': fasting glucose; HDL-c: high-density lipoprotein; HOMA-IR: homeostatic model assessment of insulin resistance; IFG: impaired fasting glucose; IGT: impaired glucose tolerance; InsT0': fasting insulin ISI: insulin sensitivity index; Hp: haptoglobin; LDL-c: low-density lipoprotein; M: male; ns: not significant; QUICKI: quantitative insulin-sensitivity check index; SBP: systolic blood pressure.

Table 2. Clinical and biochemical characteristics according to each HP phenotype.

	HP 1-1	HP 1-2	HP 2-2	P value
Subjects	26	97	68	ns
Sex (M/F)	11/15	50/47	30/38	ns
Age (years)	10.7 ± 2.7	11.4 ± 2.8	11.8 ± 2.8	ns
P/PP	16/10	66/31	49/19	ns
Height (cm)	151.0 ± 16.2	150.0 ± 14.8	150.9 ± 14.0	ns
Weight (Kg)	70.3 ± 24.6	66.7 ± 16.6	69.9 ± 19.4	ns
BMI (Kg/m^2)	29.9 ± 5.4	28.9 ± 4.3	30.1 ± 4.6	ns
BMI z-score (Kg/m^2)	2.3 ± 0.5	2.3 ± 0.4	2.2 ± 0.5	ns
Waist circumference (cm)	87.4 ± 16.6	90.5 ± 12.9	94.1 ± 13.0	ns
SBP (mmHg)	128.1 ± 15.2	125.3 ± 18.3	128.2 ± 20.1	ns
DBP (mmHg)	82.2 ±10.7	82.2 ± 10.4	80.6 ± 12.5	ns
Total cholesterol (mg/dL)	141.5 ± 27.6	143.1 ± 25.4	143.4 ± 25.3	ns
LDL-c (mg/dL)	82.7 ± 22.9	84.4 ± 23.8	85.9 ± 21.6	ns
HDL-c (mg/dL)	43.1 ± 7.1	42.2 ± 10.1	40.7 ± 7.8	ns
Triglycerides (mg/dL)	78.3 ± 36.5	77.2 ± 43.0	82.7 ± 40.7	ns
GlcT0' (mg/dL)	89.4 ± 1.5	88.4 ± 0.7	89.2 ± 0.1	ns
GlcT30' (mg/dL)	133.4 ± 4.2	134.2 ± 2.3	136.9 ± 2.6	ns
GlcT60' (mg/dL)	115.4 ± 4.9	115.8 ± 2.7	127.4 ± 3.0	p<0.009
GlcT90' (mg/dL)	110.3 ± 4.4	110.4 ± 2.4	120.8 ± 2.7	p<0.01
GlcT120' (mg/dL)	109.3 ± 3.7	108.7 ± 2.1	116.5 ± 2.3	p<0.03
InsT0' (mUI/L)	20.2 ± 2.5	17.8 ± 1.3	20.7 ± 1.5	ns
InsT30' (mUI/L)	94.0 ± 17.1	130.5 ± 8.8	151.1 ± 10.1	p<.0.04
InsT60' (mUI/L)	79.1 ± 20.8	99.1 ± 11.7	128.9 ± 13.0	p<0.05
InsT90' (mUI/L)	76.1 ± 55.4	76.7 ± 10.5	128.7 ± 11.8	p<0.02
InsT120' (mUI/L)	64.4 ± 19.5	75.4 ± 12.0	128.7 ± 13.5	P<0.05
HOMA-IR	4.5 ± 3.0	4.3 ± 2.9	4.6 ± 3.3	ns
ISI	2.80 ± 1.22	2.53 ± 0.80	2.91 ± 2.60	ns
QUICKI	0.321 ± 0.021	0.312 ± 0.030	0.311 ± 0.052	ns

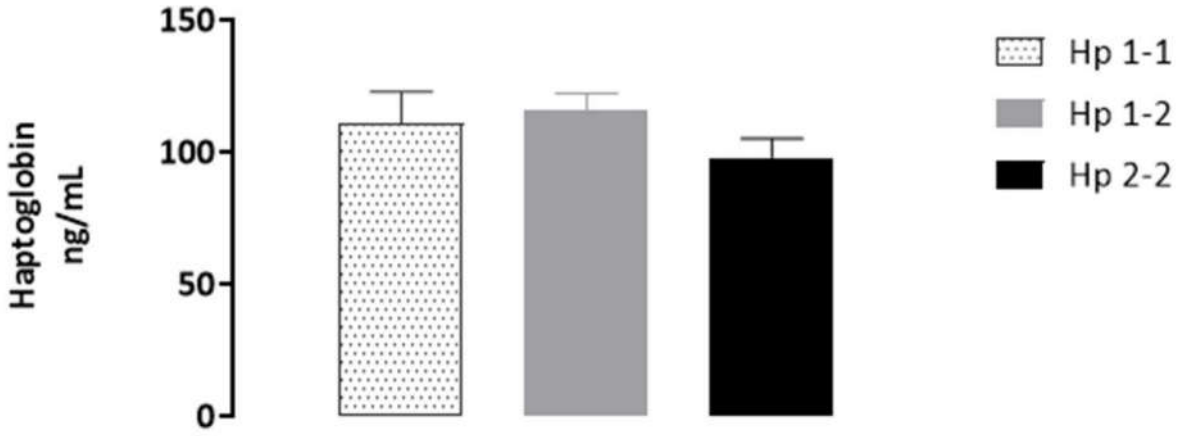
All data are expressed as mean ± SD (Standard Deviation). Significance is relative to the trend.

Abbreviations: BMI: body mass index; DBP: diastolic blood pressure; F: female; GlcT0': fasting glucose; HDL-c: high-density lipoprotein; HOMA-IR: homeostatic model assessment of insulin resistance; IFG: impaired fasting glucose; IGT: impaired glucose tolerance; InsT0': fasting insulin ISI: insulin sensitivity index; Hp: haptoglobin; LDL-c: low-density lipoprotein; M: male; ns: not significant; P: pubertal; PP: prepubertal; QUICKI: quantitative insulin-sensitivity check index; SBP: systolic blood pressure.

Of subjects with hypertriglyceridemia, 1 subject (7.0%) had Hp1-1, 10 subjects (72.0%) Hp1-2 and 3 (21.0%) Hp2-2 phenotype. Of subjects with reduced HDL cholesterol levels, 10 subjects (11.0%) had Hp1-1, 47 subjects (50.0%) Hp1-2, and 37 (39.0%) Hp2-2 phenotype. Of subjects with IFG, 2 subjects (12.0%) had Hp1-1, 6 subjects (38.0%) Hp1-2, and 8 (50.0%) Hp2-2 phenotype. Of those with IGT, 2 subjects (12.0%) had Hp1-1, 6 subjects (38.0%) Hp1-2, and 8 (50%) Hp2-2 phenotype. Nobody had type 2 diabetes. The prevalence of each alteration was not different between prepubertal and pubertal subjects. Of those subjects with Hp1-1 phenotype, 1 subject (4.0%) had hypertriglyceridemia, 10 subjects (38%) reduced HDL cholesterol levels, 8 subjects

(16.0%) had either IFG or IGT. Of carriers of Hp1-2 phenotype, 10 subjects (10.0%) had hypertriglyceridemia, 47 subjects (48.0%) reduced HDL cholesterol levels, 12 subjects (12%) had either IFG or IGT. Of subjects with Hp2-2 phenotype, 3 subjects (4.0%) had hypertriglyceridemia, 37 subjects (54.0%) reduced HDL cholesterol levels, 16 subjects (24%) had either IFG or IGT (Table 2). HDL cholesterol levels ($p < 0.007$), ISI index ($p < 0.008$) and QUICKI ($p < 0.0001$) were higher, while fasting glucose levels ($p < 0.02$), fasting ($p < 0.0001$) and post-OGTT insulin levels (120 minutes; $p < 0.01$), and HOMA-IR ($p < 0.0001$) were lower in prepubertal than pubertal subjects. All subjects were evaluated depending on the Hp phenotype. Hp blood concentrations were similar among the three phenotypes (Figure 2). Fasting blood glucose levels overlapped among them; meanwhile post-load glucose levels progressively increased from Hp1-1 to Hp2-2 phenotype (time points: 60' $p < 0.009$; 90' $p < 0.01$; 120' $p < 0.03$). Significances were not modified by covariates (Model 2, time points: 60' $p < 0.03$; 90' $p < 0.01$; 120' $p<0.05$). Consensually, the blood glucose AUC had the same trend ($p < 0.02$) (Figure 3). Similarly to blood glucose levels, post-load insulin levels were progressively higher from Hp1-1 to Hp2-2 (time points: 30' $p < 0.04$; 60' $p<0.05$; 90' $p < 0.02$; 120' $p < 0.05$), and significances were not modified by covariates (Model 2, time points: 60' $p < 0.01$; 90' $p < 0.002$; 120' $p <0.05$). The insulin AUC (330.2 ± 70.5 vs 400.4 ± 38.3 vs 550.9 ± 42.6 µUI/mL; $p < 0.009$) and the sum of insulin levels ($p < 0.02$) were progressively higher from Hp1-1 to Hp 2-2 (Figure 3). The HOMA-IR, QUICKI, ISI, and insulinogenic index were similar among Hp phenotypes.

Figure 2. Levels of Haptoglobin in the Hp1-1, Hp1-2, Hp2-2 phenotypes.



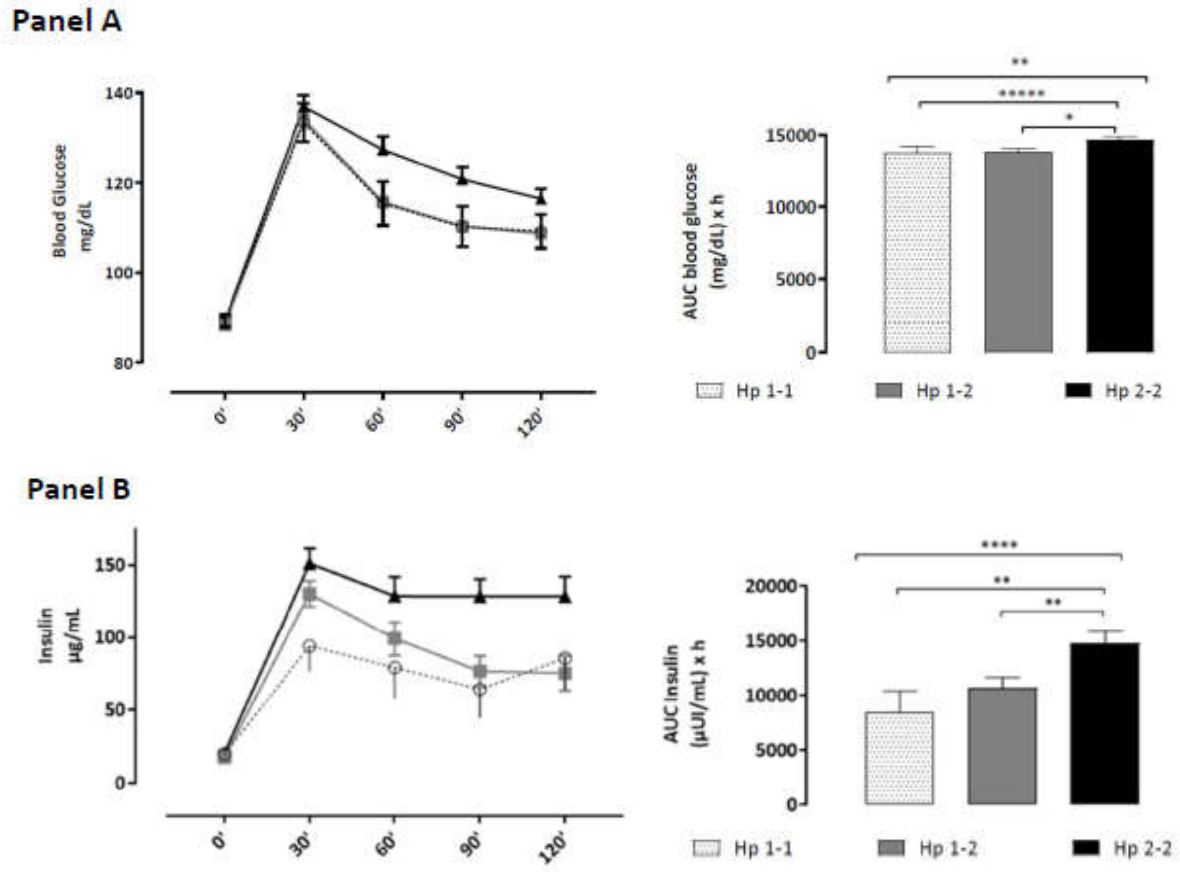
Data are expressed as mean ± SD. Phenotype 1-1: speckled line and bar; phenotype 1-2: grey line and bar; phenotype 2-2: black line and bar.

Figure 3. Post-load glucose and insulin levels among Hp phenotypes.

PANEL A: Glucose levels (mg/dL) at each time point and as AUC (mg/dL x h) at OGTT among Hp phenotypes.

PANEL B: Insulin levels (mg/dL) at each time point and as AUC (mg/dL x h) at OGTT among Hp phenotypes.

Data are expressed as mean ±SD. Legend: AUC = Area Under the Curve. Phenotype 1-1: speckled line and bar; phenotype 1-2: grey line and bar; phenotype 2-2: black line and bar. * p<0.01; ** p<0.02; **** p<0.04; *****p<0.05.



Discussion

A series of studies have shown higher Hp levels in adult subjects with obesity and metabolic alterations carrying the Hp2-2 phenotype. We demonstrated higher post-load glucose and insulin levels in obese children and adolescents carrying the Hp2-2 than Hp1-1 and Hp1-2 phenotypes. To our knowledge, this is the first study on Hp phenotypes conducted in a pediatric population with obesity. Hp is an acute-phase protein mainly produced by the liver, as previously introduced, and is also a player in oxidative stress [28, 29]. However, our interest in the Hp role in glucose metabolism derives by several reports on other functions of this molecule, including its involvement in vascular complication in type 2 diabetes [27]. Mainly, it is involved in the

development of insulin-resistance and accumulation of visceral adipose tissue [8, 13, 28-30]. Hp-null mice had better insulin sensitivity due to modifications of the insulin signal cascade in visceral adipocytes, hepatocytes, and muscle cells. Moreover, Hp null mice also had adipocytes of reduced size and number, highlighting the role of Hp in the regulation of adipose tissue depots [28]. First of all, the distribution of Hp phenotypes in our study was similar to other published reports on wide cohorts [12, 31-33], suggesting that the domination in some populations of Hp2 had provided some selective advantage [27]. Although some Authors reported low Hp serum levels in subjects carrying the Hp2-2 phenotype, we failed to show any difference among the three phenotypes in agreement with other studies [7, 26]. Thus, although we cannot exclude that the population size was not enough to demonstrate subtle different concentrations, the metabolic responses of the subjects seem to be more correlated to the Hp phenotype than to the protein blood concentration. The discrepancy among the studies on the role of Hp circulating levels probably depends on the fact that the phenotype of Hp has not often been previously considered [13, 15, 34, 35]. We observed higher stimulated insulin levels from Hp1-1 to Hp2-2 phenotype. Some data in literature show that Hp levels are directly correlated with obesity [4, 6, 36] and insulin levels more than insulin resistance [6, 37]. Authors suggested that Hp is a marker of hyperinsulinemia and that insulin could modulate Hp production by liver and white adipose tissue or both [4, 6]. It has been noted that several Authors failed to observe an association between insulin and Hp concentrations in African but not West Americans, and this could derive by the lack of the investigation of Hp phenotype [36, 37]. In mice with Hp deficiency insulin signaling is affected in those cells that overexpress Hp if obesity is present, in particular, visceral adipose tissue, and to a much lesser extent liver and muscle. These animals exhibit a higher response to insulin stimulus [4, 8]. Besides this, insulin signaling could be influenced not only by Hp deficiency but also by the phenotype. Since exon 3 contains the Hp-multimerization domain, the valences of two alleles are different: Hp1 is monovalent while Hp2 is bivalent. This difference influences the protein's structure (dimers in Hp1-1 subjects, linear polymers in Hp1-2, cyclic polymers in Hp2-2) and affects the oxidation capability [2, 12, 38]. Because Hp1-1 has more anti-inflammatory properties, Hp1-1 could promote a reduction in macrophage infiltrates and pro-inflammatory cytokines in white adipose tissue, resulting in improving the insulin pathway. This hypothesis is in line with findings in obese Hp-deficient models [28]. Moreover, we have also shown that the Hp phenotype is important for the metabolic function of the protein and is associated with a better post-load glucose response in

subjects carrying Hp1-1, while Hp2 is associated with a worse trend in glucose metabolism in regards of the genetic impact. In humans, several observational and longitudinal studies have established that the Hp2-2 phenotype is an independent risk factor for cardiovascular diseases in adults with type 2 diabetes, mainly in those with higher glucose levels [9, 11, 12]. This phenomenon seems dependent on its lower antioxidant efficacy, more pronounced by glycosylation of the hemoglobin [33, 39, 40]. Because we showed both higher glucose and insulin-stimulated levels in subjects carrying the Hp2-2 phenotype, we can hypothesize that hyperinsulinemia is secondary to subtle peripheral insulin resistance in feeding conditions. This hypothesis is supported by recent findings. Free hemoglobin and HbA1c are bound by Hp forming a complex that is cleared by CD163, a scavenger receptor that is also a novel biomarker of adipose tissue macrophage activation. Expression of CD163 is reduced in Hp2-2 carriers and is also further impaired in condition of hyperglycemia [12, 41]. A recent study observed that CD163 is inversely associated with insulin sensitivity and beta-cell function at OGTT and the risk of dysglycemia in adults at risk for type 2 diabetes [42]. Indeed, higher inflammation and lower oxidative capacity could have a role in altered glucose metabolism, mainly if obesity is present. Our findings could have a role in clinical practice. Obesity in the pediatric age increases the risk of the incidence of type 2 diabetes in early adulthood in both sexes [43]. Moreover, some children and adults have metabolic healthy obesity and several hypotheses have been suggested, none of these conclusive [44, 45]. The Hp2-2 phenotype could be one of the mechanisms linking inflammation, insulin resistance, and adipocyte biology with a role in the deterioration of glucose tolerance. Since it does not change with time and specific conditions together with the easy and relatively inexpensive determination, the Hp phenotype could be a promising marker to select young obese patients for a tight follow-up. Studies are also needed to understand if treatments with insulin-sensitizing agents, as metformin, as a different response in insulin resistance based upon Hp phenotypes. In conclusion, for the first time, we demonstrated higher post-load glucose and insulin levels in obese children and adolescents carrying the Hp2-2 phenotype than those carrying the other two Hp-phenotypes. Thus, it seems that the Hp2-2 phenotype predisposes in pediatrics, at the same degree of obesity, to a worse glycemic compensation. Further studies are needed to understand if the worst stimulated glucose and insulin levels in Hp2-2 obese children are suggestive of earlier development of altered glucose metabolism. Moreover, studies on long-term metabolic

complications of pediatric obesity are required to evaluate if Hp-phenotype is a good biomarker to stratify those at high cardiovascular risk.

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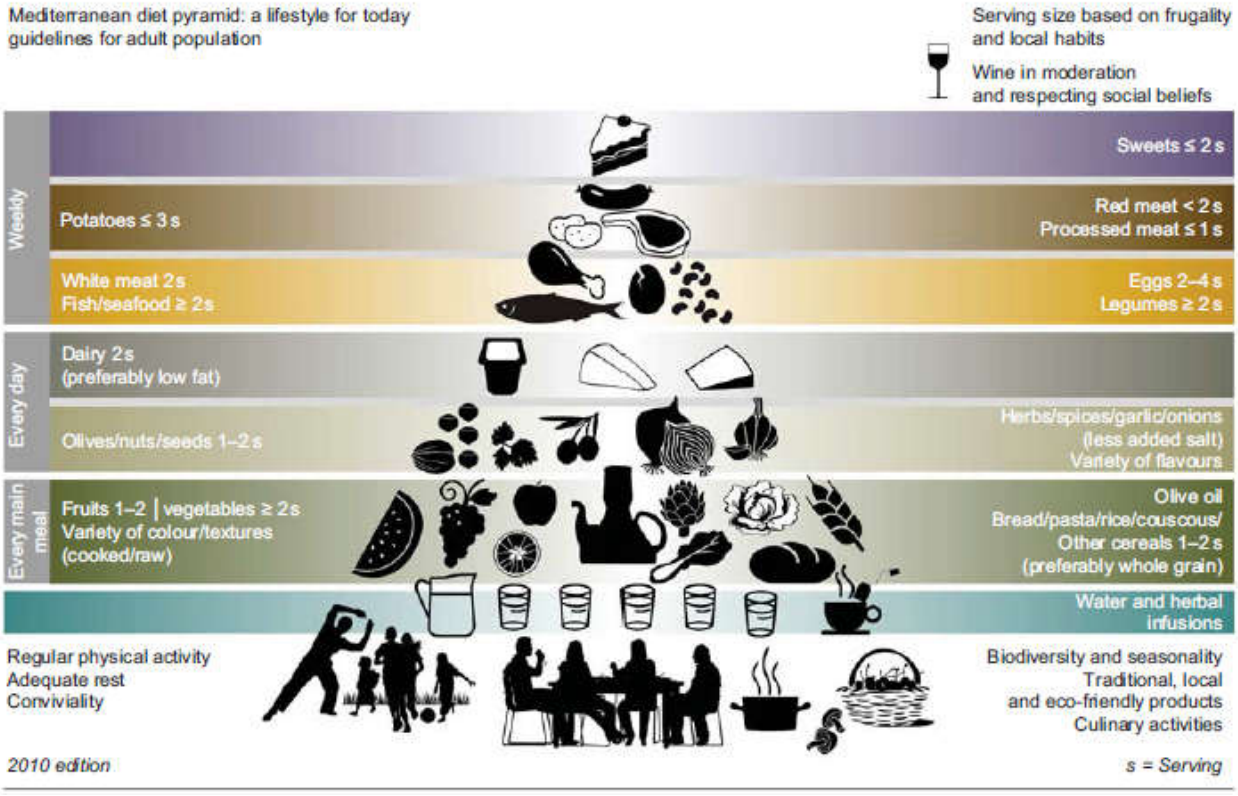
3. THE MEDITERRANEAN STYLE-DIET

3.1. INTRODUCTION

On 16 November 2010 in Nairobi, UNESCO (United Nations Educational, Scientific and Cultural Organization) inscribed on the List of Intangible World Heritage, the Mediterranean Diet. “The Mediterranean diet involves a set of skills, knowledge, rituals, symbols and traditions concerning crops, harvesting, fishing, animal husbandry, conservation, processing, cooking, and particularly the sharing and consumption of food” as reported by the Evaluation Committee. Moreover, “The Mediterranean diet includes much more than just food. It promotes social interaction, since collective meals are the cornerstone of social customs and festive events. It has given birth to a formidable body of knowledge, songs, proverbs, stories and legends” [1]. The traditional Mediterranean diet results as a combination of eating habits traditionally followed by individuals in the olive-growing areas bordering the Mediterranean Sea [2]. It is characterized by an high intake of vegetables, legumes, fruits, nuts and cereal grains (largely unrefined in the past), moderate-to-high fish intakes, high intakes of un-saturated lipids (particularly from olive oil) but low intakes of saturated fats, a low-to-moderate intake of dairy products (mostly cheese and yoghurt) and finally, a low intake of meat products [3] – Figure 1.

Figure 1. Mediterranean diet pyramid today [3].



The concept of the “Mediterranean diet” has been introduced for the first time in years Sixty by the American physiologist Ancel Keys, who noticed an inverse correlation between the traditional culinary practices of rural areas of Southern Italy, Greece and other countries of the Mediterranean basin and the onset of cardiovascular diseases. In fact, the Seven Countries Study (SCS) [4], conceived by Ancel Keys, is the first major study to investigate diet and lifestyle along with other risk factors for cardiovascular disease, across contrasting countries and cultures and over an extended period of time. It was the first prosepective trial which included 12,763 male subjects aged between 40 and 59 years divided into 16 cohorts and residents in 7 different countries (Finland, Greece, Italy, Japan, Netherlands, USA, Yugoslavia). The main hypothesis was that the rate of coronary disease in populations and individuals would vary in relation to their physical characteristics and lifestyle, particularly in fat composition of the diet and serum cholesterol levels. The SCS showed strong associations between dietary saturated fat and both serum cholesterol and cardiovascular mortality among the 16 cohorts. In contrast, eating fish once or twice a week was associated with a 50% lower 20-year fatal cardiovascular risk compared to eating no fish; moreover, the results suggested that low intake of wine may indeed protect against cardiovascular diseases. Finally, a high intake of fats rich in unsaturated fatty acids and a moderate intake of fish were typically characteristic of a Mediterranean diet. Thus, the Mediterranean dietary pattern revealed as an excellent model of healthy eating. More than 50 years after the SCS, a large number of epidemiological studies have explored the relationship between the Mediterranean diet and health, through observational, case-control, some longitudinal and a few experimental studies. The overall results, revised in a recent systematic review [5], confirmed strong evidence suggesting a protective effect of the Mediterranean diet mainly on the risk of type 2 diabetes, arterial hypertension, cardiovascular disease and certain types of cancer. The most relevant study in terms of the number of participants, the duration of the intervention and the number of publications produced was the PREDIMED (PREvencion con DIeta MEDiterranea) study which provided invaluable data on the benefits of the Mediterranean diet for a wide range of chronic diseases [6].

A greater adherence to a Mediterranean-like dietary pattern is associated with a significant improvement in health status. In adults, a Mediterranean-style diet is known to be associated with a lower risk to develop chronic diseases including cardiovascular disease, cancer, obesity, and type 2 diabetes mellitus [7, 8]. With a lower mortality [8], this dietary pattern has also been suggested to increase longevity and appears to be

associated with a better health status overall [9]. Today, in pediatrics, alarming data suggest a poor adherence to Mediterranean style-diet [10]. Also, breakfast, the first and perhaps the most important meal in the day, plays a critical role in energy balance and dietary regulation [11]. Despite this, the incidence of skipping breakfast among children and adolescents is rising as unhealthy food choice with negative consequences oh health [11, 12].

Aiming to explore the adherence to Mediterranean style-diet in school children and adolescents and the importance of dietary habits and regular mealsthe Ph.D. activity was focused on the following topics: adherence to the Mediterranean style-diet, skipping breakfast and unhealthy food behaviors associated to overweight.

The following papers have published in this regard:

- *Adherence to the Mediterranean Diet among School Children and Adolescents Living in Northern Italy and Unhealthy Food Behaviors Associated to Overweight*
- *A Systematic Review of the Association of Skipping Breakfast with Weight and Cardiometabolic Risk Factors in Children and Adolescents. What Should We Better Investigate in the Future?*
- *Mediterranean diet, nutrition transition, and cardiovascular risk factor in children and adolescents*

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3.1.1. Adherence to the Mediterranean Diet among School Children and Adolescents Living in Northern Italy and Unhealthy Food Behaviors Associated to Overweight

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Abstract

The purposes of this study were to evaluate the differences in Mediterranean diet and its components among primary and secondary school children and adolescents living in northern Italy, and the associations with the weight status. Adherence was assessed by the KIDMED (Mediterranean Diet Quality Index) questionnaire on 669 subjects (6–16 years) attending five schools of Novara. The adherence was poor in 16.7%, average in 63.7%, and high in 19.6% of the students. Poor adherence was more frequent in primary than in secondary schools (20.7% vs. 13.7%, $p < 0.04$). Some unhealthy behaviors were more prevalent in younger children. Children of other ethnic origins had a mixed behavior, choosing both traditional healthy and unhealthy foods. Besides male gender and primary school, in Italian children, the risk of overweight was directly associated with eating at fast-food restaurants (OR: 1.890, CI 95% 1.002–3.563), and inversely with consumption of vegetables more than once a day (OR: 0.588, CI 95% 0.349–0.991), and olive oil at home (OR: 0.382, CI 95% 0.176–0.826). In children of other ethnic origins, this risk was associated with skipping breakfast (OR: 16.046, CI 95% 1.933–133.266), or consuming commercial baked good or pastries for breakfast (OR: 10.255, CI 95% 1.052–99.927). The overall KIDMED score correlated with height (β: 0.108; $p < 0.005$). Poor food quality is replacing the Mediterranean dietary pattern in children and adolescents, in particular among younger children. Because the risk of overweight was associated with different components of the Mediterranean diet depending on ethnic origins, tailored nutritional programs remain a need.

Introduction

The Mediterranean diet (MD) is considered a model of a healthy diet, in particular after the publication of the first results derived by the PREDIMED study that demonstrated a reduction of cardiovascular mortality in subjects adherent to this dietary pattern [1]. MD has been associated with lower prevalence and/or incidence of several diseases, among others, type 2 diabetes, hypertension, cardiovascular diseases, and certain cancers that are all associated to overweight [2–5]. This is likely due to its composition rich in vegetables, fruits, legumes, whole cereals, as well as many sources of fiber and antioxidants, including fish, nuts, and extra-virgin olive oil. Moreover, the intake of sweets and trans fatty acids is low. Although MD is beneficial, with the urbanization of people living in the Mediterranean area, in particular children and adolescents are deviating to a “Western diet” more rich in saturated fat, refined grains, simple carbohydrates and processed foods [6]. This phenomenon has been named nutrition

transition and is one of the players implicated in the high prevalence of overweight and obesity in countries supposed to adopt a traditional MD [5,7]. A large meta-analysis of randomized controlled trials on MD reported a little but significant decrease in weight (-1.75 kg) and BMI (- 0.57 kg/m2) in those adherents to MD [8]. Although intriguing results have been obtained in trials, the dissociation between higher obesity prevalence in Mediterranean countries and lower prevalence of many of its comorbidities in subjects adherent to a MD pattern is still an issue, in particular in pediatrics. Studies investigating nutrition habits are needed to plan tailored strategies of interventions to educate on a healthy diet. In 2004, Serra-Majem et al. developed the KIDMED (Mediterranean Diet Quality Index for children and adolescents) score, a nutritional index validated in several languages that evaluates the adherence to MD and the quality of diet in children and adolescents [9]. Although it is simple to use in the clinical practice as well as in other epidemiological settings, only a few studies have been explored the adherence to the MD and to the risk of obesity in the young school population in Italy [10–13]. Most have been conducted in the southern part of Italy [14,15], where the obesity rate is higher than in other areas of the country [16], thus the generalizability has to be further demonstrated. The most extensive study using a modified version of the KIDMED score portrayed a school population of 1740 8–9-year-old children living in north Italy (Friuli, Venezia, Giulia and Liguria) and was conducted in 2009. The authors showed that only the 5.0% of the cohort was classified as high adherent to the MD, with the best rate (6.0%) in the north [17]. Although the benefits of the MD pattern can be considered as a synergistic interaction among all its components, a few studies reported protective or detrimental effects related to specific foods in adults with differences in prospective cohort or randomized controlled studies [5]. Vegetable intake was negatively associated, whereas higher intake of sweets, sugar-sweetened beverages, and fast foods was associated with obesity in a study on MD adherence conducted among adolescents living in Sicily [14]. No other Italian data have been published. Based on the above, data on MD adherence are insufficient in children living in northern Italy. The first purpose of this study was to evaluate the differences in Mediterranean diet and its components among primary and secondary school children and adolescents living in Novara, a city of northern Italy characterized by an urban community employed in both agriculture and industry. The second purpose was to evaluated the associations of MD adherence and its components with the weight status.

Subjects and Methods

Population and Anthropometric Examination

This was a cross-sectional study conducted in April and May 2017. The study is a part of a cross-sectional study on pediatric obesity approved by the Ethical Committee of the Maggiore della Carità Hospital (CE 95/12). We included 3 primary and 2 secondary schools of Novara. In 2017, the population of Novara was estimated at 104,183, with 18,634 people ≤ 14 years old. The average annual income per capita for the population is estimated around 16,132 Euro [18]. Before starting the enrollment, schools of Novara were classified according to socio-economic status based on estimates of the district's socio-economic status in which they were located. We contacted all the schools by phone; to be selected they needed not to have developed a specific structured education program on MD in the year of recruitment. Eight schools respected all the inclusion criteria and were balanced for socio-economic status. Three of them refused to participate in the survey because no scholastic days were available. For all enrolled schools, all students attending all years were invited to participate with a letter carefully explaining the purpose of the study both to them and to their parents, and written informed consent was obtained. In addition, the children and adolescents provided their verbal assent on the day of the questionnaire. To be included in the analysis, participants should write and read fluently Italian. In each school, data collection was performed by two pediatric nurses, one nutritionist, two physicians and a member of the department of the school policies who was responsible for the program. Questionnaires were completed during school hours in the classroom in the presence of a teacher, the nutritionist and at least one nurse and one physician. The staff helped with the questionnaire interpretation if needed. Questionnaires were anonymous. Students were requested to report their sex and date of birth. Nurses and physicians also performed the auxological examination after the completion of the questionnaire which returned at that moment. Anthropometric data were reported on the questionnaire form. Some days after the testing session a closing visit with a lesson on the MD and the MD food pyramid was conducted by the study staff. Anthropometric measurements were performed in duplicate for each subject, wearing light indoor clothing and without shoes. Weight was measured to the nearest 100 g with a spring scale tested daily for accuracy and calibrated against a set of standard weights (Salus, Inc., Gaggiano, Milano, Italy). Height was measured with a standard laboratory stadiometer to the nearest 0.5 cm during maximal expiration. BMI was calculated as the ratio between

weight (kg) and squared height (m^2). BMI-SDS was calculated according to the LMS methods on the Italian charts [19]. Subjects were also stratified according to BMI categories (underweight, normal weight, overweight, obesity and morbid obesity) of the International Obesity Task Force [20]. Ethnicity was defined as the country of origin of the mother, in case of the different origin of both parents.

Evaluation of Adherence to the MD

We used the Italian version KIDMED index [21], a questionnaire of 16 dichotomous (positive/negative) items appropriate for youngsters. The answers with a positive connotation in relation to the MD are assigned a value of +1 (12 items), and those with a negative connotation, a value of - 1 (4 items). The items explore the consumption of fruits, vegetables, fish, pasta/rice, cereals, yoghurt/cheese/dairy products, nuts, commercial baked and processed foods, breakfast habits and the frequency of skipping breakfast, fast-food frequency, sweet consumption, and olive oil during meals at home. The overall score can range from - 4 to 12. Total KIDMED scores were classified as follows: ≤ 3 reflects a poor adherence (very low diet quality), 4–7 an average adherence (improvement needed to adjust intake to MD patterns), and ≥ 8 a good adherence to the MD (optimal diet quality).

Statistical Analysis

Continuous data are expressed as mean, standard deviation (SD) and CI 95%. Prevalence of KIDMED, weight categories, and “yes” answers at each questionnaire item are reported as a percentage. The sample size was calculated according to the mean prevalence of low MD adherence according to the literature [8] with 95% confidence interval and an accuracy of ± 4.0% of the average value of the adherence. A sample of 585 individuals was estimated as sufficient. Because the prevalence of obesity was relatively low, overweight and obese categories were considered together in the final analysis. Data were also stratified between primary and secondary schools. Socio-demographic level was defined according to that of the district area where the school was located. Kolmogorov–Smirnov test was used to test normality of variables’ distribution. Student’s independent t-test and Mann–Whitney U-test were used for normally and not normally distributed continuous variables, respectively. Two-tailed chi-square or Fisher exact test was used to evaluate differences in categorical variables, as appropriate. Univariate and multivariate logistic regression was used to assess the association of weight status with the odds ratio (OR, 95% CI) of gender, school level, ethnicity, MD adherence, or KIDMED items, as well as of MD adherence with gender,

school level, ethnicity, and weight status. Because of several ethnic origins, ethnicity was categorized for statistical analyses as Italian and non-Italian. KIDMED items inserted in the models were those significant in the univariate analysis. Goodness-of-fit was evaluated by using the Hosmer and Lemeshow test; all the models were accepted because the χ^2 was not significant. Interactions among variables (gender, school level, and ethnicity) were also explored; when p was >0.05 data in multinomial logistic analysis were only presented together. The KIDMED score and anthropometric parameters were also tested as continuous variables through linear regression stepwise analyses and the results are represented as standardized β coefficients. The level of statistical significance for analysis was set at $p < 0.05$. Statistical analysis was performed with SPSS for Windows version 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Characteristics of the Population

The study was carried out in 669 subjects (324 males and 345 females) aged 11.2 (2.2) (CI 95% 11.0–11.4) years. All families and children gave their consent to participate in the study. Everybody completed the questionnaire and accepted the clinical visit. The analysis excluded six out of 705 subjects (0.9%) because they did not read and write fluently in Italian. All questionnaires were complete. The primary and secondary school samples were composed by 290 (138 males, 152 females) and 379 (186 males, 193 females) subjects, respectively. Six hundred twelve subjects (91.5%) were Italian. The remaining 57 subjects were born to parents from other ethnic origins. The majority of the subjects had a normal weight (n = 558; 83.4%); only 94 (14.1%) and 17 (2.5%) of them were overweight or obese, respectively. The prevalence of overweight plus obesity was higher in primary than in secondary schools (23.4% vs. 11.3%, χ^2: 17.384, $p < 0.0001$), in males than females (21.3% vs. 12.2%, χ^2: 10.047, $p < 0.0001$), and in subjects of other ethnic origins than those Italian (28.1% vs. 15.5%, χ^2: 5.932, $p < 0.001$). Sex, type of school, and ethnicity in the three-predictor model of OWB were all significant (model χ^2: 31.971, $p < 0.001$) without interactions, accounting for 7.9% of the total variance (Nagelkerke R2), and the correct prediction rate was about 83.4%. In particular, the risk to be OWB was associated with male gender (OR: 2.024, CI 95% 1.322–3.099, $p < 0.0001$), primary school (OR: 2.387, CI 95% 1.562–3.648, $p < 0.0001$), and other ethnic origins (OR: 1.947, CI 95% 1.031–3.676, $p < 0.03$) in the corrected model. Table 1 represents demographic characteristics.

Table 1. Anthropometric characteristics of the 669 subjects, by school level.

	All	Primary School	Secondary School	*p*
Gender				
M	324 (48.4%)	138 (47.5%)	186 (49.0%)	0.755
F	345 (51.6%)	152 (52.5%)	193 (51.0%)	
Age (years)	11.2 (2.2) (11.0–11.4)	9.0 (1.3) (8.8–9.2)	12.9 (1.1) (12.8–13.1)	0.0001
Ethnicity				
Italian	612 (91.5%)	258 (89.0%)	354 (93.4%)	0.03
Eastern European	22 (3.3%)	7 (2.4%)	15 (4.0%)	
African	25 (3.7%)	22 (7.6%)	3 (0.8%)	
Asian	4 (0.6%)	1 (0.3%)	3 (0.8%)	
South American	6 (0.9%)	2 (0.7%)	4 (1.0%)	
Height (cm)	147.9 (15.6) (146.8–149.2)	134.6 (10.3) (133.4–135.8)	158.2 (10.5) (157.1–159.3)	0.0001
Weight (Kg)	40.4 (12.6) (39.5 41.4)	31.3 (7.6) (30.5 32.3)	47.4 (11.2) (46.3–48.5)	0.0001
BMISDS	−0.476 (1.028) (−0.554, −0.398)	−0.309 (1.076) (−0.433, −0.185)	−0.604 (0.972) (−0.703, −0.507)	0.0001
BMI category				
Normal-weight	558 (83.4%)	222 (76.5%)	336 (88.6%)	0.0001
Overweight	94 (14.1%)	57 (19.7%)	37 (9.8%)	
Obese	17 (2.5%)	11 (3.8%)	6 (1.6%)	

Data are expressed as mean ±SD, CI 95%, absolute numbers and percentages. Differences among categorical variables were tested by Chi-square test. Associations between variables were tested by Student's independent t-test (BMISDS), or Mann–Whitney U-test (age, height, and weight). Abbreviations: F, female; M, male. BMI was stratified according to the IOTF criteria.

KIDMED

The adherence to the MD (scores ≤ 3) was poor in 16.7%, average (scores 4–7) in 63.7%, and high (scores ≥ 8) in 19.6% of the students. The overall score ranged from -1 to 11. The peak score was 6 (17.0%). The prevalence rate of the three categories of adherence was similar between males and females, normal-weight (NW) and overweight/obese (OWB) subjects. The prevalence rate of poor adherence to the MD was significantly higher in primary than in secondary schools (20.7% vs. 13.7%, $p < 0.04$), with an equal rate of high adherence (19% vs. 20.1%). The four-predictor model of MD adherence (χ^2: 8.000, $p < 0.04$) showed that only primary school was associated to a high risk of lowMDadherence (OR: 1.618, CI 95% 1.068–2.452, $p < 0.01$), accounting for 2.1% of the total variance (Nagelkerke R^2), and the correct prediction

rate was about 83.3%. Table 2 shows the distribution of the levels of adherence among subgroups.

Table 2. Distribution of the adherence to the MD of the study population and relative odd ratios among subcategories.

	KIDMED Score	*p*	KIDMED Score Low	KIDMED Score Medium	KIDMED Score High	*p*	Adj OR (CI 95%) of High Adherence
School Primary	5.4 (2.3) (5.1 5.7)	0.094	60 (20.7%)	175 (60.3%)	55 (19.0%)	0.04	1
Secondary	5.6 (2.1) (5.5–5.9)		52 (13.7%)	251 (66.2%)	76 (20.1%)		1.618 (1.068–2.452)
Weight NW	5.6 (2.1) (5.4–5.8)	0.364	91 (16.3%)	359 (64.3%)	108 (19.4%)	0.610	1
OWB	5.6 (2.4) (4.9–5.9)		21 (18.9%)	67 (60.4%)	23 (20.7%)		0.868 (0.504–1.494)
Gender M	5.6 (2.1) (5.5–5.9)	0.062	47 (14.5%)	209 (64.5%)	68 (21.0%)	0.062	1
F	5.4 (2.2) (5.2–5.7)		65 (18.8%)	217 (62.9%)	63 (18.3%)		0.725 (0.478–1.098)
Ethnicity Italian	5.5 (2.1) (5.4–5.8)	0.092	103 (16.8%)	389 (63.6%)	120 (19.6%)	0.672	1
Others	5.5 (2.2) (5.0–6.1)		9 (15.8%)	37 (64.9%)	11 (19.3%)		0.850 (0.400–1.494)
All	5.5 (2.1) (5.4–5.7)	//	112 (16.7%)	426 (63.7%)	131 (19.6%)	//	//

KIDMED score as continuous variables is expressed as mean (SD) and CI 95%. Adjusted Odd Ratios (OR) were calculated by binary logistic regression analysis with low adherence as reference category in dependent variable, and school level, gender, weight status, and ethnicity as independent variables. Medium and high adherences were considered together. The ORs were referred to secondary school, female gender, OWB, and other ethnic origin.

Abbreviations: F, female; M, male; NW, normal-weight; OWB, overweight + obese. //: not calculable.

The risk of OWB previously described was not modified by the introduction in the model of the MD adherence either as category or as continuous variable. The overall KIDMED score did not correlate with weight, BMI, and BMI SDS. Diversely, it correlated with height (β: 0.108; B: 0.015, CI 95% 0.005–0.026 $p < 0.005$) also when corrected for gender, age or school level, and ethnicity.

KIDMED Items

We analyzed the prevalence of “yes” answers in several subcategories. Subjects with the lowest adherence to theMD answered “yes” less frequently in the positive

questions, and more frequently in the negative ones ($p < 0.0001$) than those with the highest adherence to the MD. A similar distribution of answers was only reported on the daily intake of candy and sweets (Table A1). In primary schools, children ate fewer vegetables once a day (χ^2: 5.413, $p < 0.01$), fewer pulses once a week (χ^2: 4.459, $p < 0.02$), and skipped breakfast (χ^2: 5.375, $p < 0.01$). They also consumed more frequently pasta or rice almost every day χ^2: 4.4672, $p < 0.01$), ate at fast-food restaurants (χ^2: 6.585, $p < 0.007$), consumed commercial baked good or pastries for breakfast (χ^2: 17.034, $p < 0.0001$) or took sweets and candy several times every day (χ^2: 18.610, $p < 0.0001$) than in secondary schools. OWB consumed less olive oil (χ^2: 4.704, $p < 0.02$), and more frequently ate at fast-food restaurants (χ^2: 11.748, $p < 0.001$), skipped breakfast (χ^2: 3.556, $p < 0.04$) or consumed commercial baked good or pastries for breakfast (χ^2: 6.717, $p < 0.006$) than NW. Interestingly, OWB consumed more frequently vegetables once a day χ^2: 4.762, $p < 0.01$) or more than once a day (χ^2: 4.000, $p < 0.03$) than NW. Males skipped breakfast less (χ^2: 3.187, $p < 0.04$) and consumed fish more regularly several times per week (χ^2: 8.172, $p < 0.003$), but more frequently also ate at fast-food restaurants (χ^2: 4.230, $p < 0.02$), and consumed commercial baked good or pastries for breakfast (χ^2: 2.984, $p < 0.04$) than females. Children of other ethnic origins consumed more fish (χ^2: 6.460, $p < 0.008$), cereals or grain for breakfast (χ^2: 3.705, $p < 0.03$), two yoghurts and/or some cheese (χ^2: 6.083, $p < 0.01$) but more frequently also ate at fast-food restaurants (χ^2: 5.505, $p < 0.02$), skipped breakfast (χ^2: 6.621, $p < 0.01$), consumed commercial baked good or pastries for breakfast (χ^2: 4.238, $p < 0.05$), or took sweets and candy several times every day (χ^2: 6.847, $p < 0.008$) than those Italian. Table 3 shows the distribution of subjects with respect to each item among subgroups. In the crude analysis, the risk of OWB was associated with eating at fast-food restaurants (OR: 1.845, CI 95% 1.056–3.223, $p < 0.03$), frequent daily consumption of sweet and candies (OR: 1.946, CI 95% 1.238–3.059, $p < 0.004$), and in primary schools also with olive oil consumption at home (OR: 0.362, CI 95% 0.133–0.984, $p < 0.04$). In the model weighted for all the items founded significant in the descriptive analysis, the risk of OWB (χ^2: 57.393, $p < 0.0001$) was associated with the male gender (OR: 2.008, CI 95% 1.285–3.163, $p < 0.002$), the primary school (OR: 2.412, CI 95% 1.533–3.795, $p < 0.0001$), eating raw or cooked vegetables once a day (OR: 0.610, CI 95% 0.376–0.990, $p < 0.04$), olive oil consumption at home (OR: 0.521, CI 95% 0.255–0.973, $p < 0.05$), and consumed commercial baked good or pastries for breakfast (OR: 1.534, CI 95% 1.001–2.426, $p < 0.05$). The model accounted for 13.9% of the total variance (Nagelkerke R^2), and the

correct prediction rate was about 83.3%. We also split the analysis for the ethnicity due to the relative number of foreign children and significant interaction with some items. In Italian children, besides male gender and primary school, the risk of OWB (χ^2: 54.208, $p < 0.0001$) was associated inversely with eating raw or cooked vegetables more than once a day (OR: 0.588, CI 95% 0.349–0.991, $p < 0.04$), and olive oil consumption at home (OR: 0.382, CI 95% 0.176–0.826, $p < 0.01$), and directly with eating at fast-food restaurants (OR: 1.890, CI 95% 1.002–3.563, $p < 0.04$). The model accounted for 14.2% of the total variance, and the correct prediction rate was about 84.2%. Diversely, in children of other ethnic origins, besides primary school, the risk of OWB (χ^2: 22.201, $p < 0.03$) was associated with skipping breakfast (OR: 16.046, CI 95% 1.933–133.266, $p < 0.01$), or consuming commercial baked goods or pastries for breakfast (OR: 10.255, CI 95% 1.052–99.927, $p < 0.03$). The model accounted for 46.4% of the total variance, and the correct prediction rate was about 80.7%.

Table 3. Distribution of "yes" answers by school level, weight status, gender, and ethnicity.

	School Level		Weight		Gender		Ethnicity	
	Primary School	Secondary School	NW	OWB	M	F	Italian	Other
Consumption of a fruit or a fruit juice every day [1]	215 (74.1%)	292 (77.0%)	426 (76.3%)	81 (73.0%)	253 (78.1%)	254 (73.6%)	462 (75.5%)	45 (78.9%)
Consumption of a second fruit every day [1]	141 (48.6%)	180 (47.5%)	264 (47.3%)	57 (51.4%)	153 (47.2%)	168 (48.7%)	291 (47.5%)	30 (52.6%)
Consumption of raw or cooked vegetables 1 time a day [1]	**146 (50.3%)**	**225 (59.4%)**	**299 (50.3%)**	**72 (64.9%)**	181 (55.9%)	190 (55.1%)	335 (54.7%)	36 (63.2%)
Consumption of raw or cooked vegetables >1 time a day [1]	79 (27.2%)	121 (31.9%)	**158 (28.3%)**	**42 (37.8%)**	101 (31.2%)	99 (28.7%)	185 (30.2%)	15 (26.3%)
Consumption of fish regularly (at least 2–3 times a week) [1]	138 (47.6%)	177 (46.7%)	256 (45.9%)	59 (53.2%)	**171 (52.8%)**	**144 (41.7%)**	**279 (45.6%)**	**36 (63.2%)**
Eating >1 time per week to a fast-food (hamburger) restaurant [2]	**57 (19.7%)**	**47 (12.4%)**	**75 (13.4%)**	**29 (26.1%)**	**60 (18.5%)**	**44 (12.8%)**	**89 (14.5%)**	**15 (26.3%)**
Consumption of beans >1 time per week [1]	**150 (51.7%)**	**227 (56.7%)**	313 (56.1%)	64 (57.7%)	189 (50.1%)	188 (49.9%)	347 (56.7%)	30 (52.6%)
Consumption of pasta or rice almost every day (≥5 times a week) [1]	**251 (86.6%)**	**304 (80.2%)**	466 (83.5%)	89 (80.2%)	267 (82.4%)	288 (83.5%)	510 (83.3%)	45 (78.9%)
Consumption of cereals or grains (bread, etc.) for breakfast [1]	166 (57.2%)	223 (58.8%)	328 (58.8%)	61 (55.0%)	197 (60.8%)	192 (55.7%)	**349 (57.0%)**	**40 (70.2%)**
Consumption of nuts regularly (at least 2–3 times per week) [1]	75 (25.9%)	92 (24.3%)	134 (24.0%)	33 (29.7%)	80 (24.7%)	87 (25.2%)	149 (24.3%)	18 (31.6%)
Consumption of olive oil at home [1]	264 (91.0%)	354 (93.4%)	**521 (93.4%)**	**97 (87.4%)**	300 (92.6%)	318 (92.2%)	567 (92.6%)	51 (89.5%)
Skipping breakfast [2]	**43 (14.8%)**	**83 (21.9%)**	**98 (17.6%)**	**28 (25.2%)**	**52 (16.0%)**	**74 (21.4%)**	**108 (17.6%)**	**18 (31.6%)**
Consumption of a dairy product for breakfast (yoghurts, milk, etc.) [1]	225 (77.6%)	275 (72.6%)	422 (75.6%)	78 (70.3%)	248 (76.5%)	252 (73.0%)	459 (75.0%)	41 (71.9%)
Consumption of commercially baked goods or pastries for breakfast [2]	**182 (62.8%)**	**117 (46.7%)**	**287 (51.4%)**	**72 (64.9%)**	**185 (57.1%)**	**174 (50.4%)**	**321 (52.5%)**	**38 (66.7%)**
Consumption of 2 yoghurts and/or cheese (40 g) daily [1]	114 (39.3%)	170 (44.9%)	236 (42.3%)	48 (43.2%)	144 (44.4%)	140 (40.6%)	**251 (41.0%)**	**33 (57.9%)**
Consumption of sweets or candy several times every day [2]	**154 (53.1%)**	**138 (36.4%)**	**287 (51.4%)**	**72 (64.9%)**	190 (58.6%)	187 (54.2%)	**258 (42.2%)**	**34 (59.6%)**

Numbers and percentages are referred to "yes" answers. The denominators are those described in Table 1. [1] Items with a positive answer (+1). [2] Items with a negative score (- 1). Bold numbers are those significant in the univariate logistic regression. Abbreviations. F, female; M, male; NW, normal-weight; OWB, overweight + obese.

Discussion

Data on adherence to MD have been explored above all on Greek and Spanish pediatric populations. Italian data are relatively few and mainly referred to children aged 8–9 years, or adolescents living in the southern part of the country. We demonstrated that schoolchildren and adolescents, in particular primary school or overweight/obese students, are more likely to have dietary behaviors close to a Western dietary pattern. Moreover, pediatric subjects of other ethnic origins have mixed behaviors, as happens in the nutrition transition. Some specific unhealthy food choices are more prevalent, such as eating at fast-food restaurants, skipping breakfasts, consumption of commercially baked goods or pastries for breakfast, and of sweets several times every day. The risk of overweight/obesity was not associated with the overall adherence to MD, but with specific food habits different depending on ethnicity. Firstly, we observed that the prevalence of overweight and obesity (17.6%) in our cohort was quite lower than that reported by the GBD 2015 Obesity Collaborators on children and adolescents younger than 20 years [22], the IDEFICS study on children aged 2–10 years [23], and the WHO European Childhood Obesity Surveillance Initiative on primary schoolchildren [24]. This result is likely due to the different age range in our school cohort (6–15 years), beyond a geographical reason being well known that the highest rate of overweight and obesity is in the southern part of Italy. Accordingly, the last Piedmont data derived by the “OKkio alla SALUTE” project on children aged 8–9 years are comparable with the prevalence (24.0% vs. 23.4%) observed in our primary schoolchildren and with a higher risk to be obese in males [16,25]. Moreover, we recorded a higher prevalence of overweight and obesity in children and adolescents of other ethnic origins than in Italian students. This finding is in agreement with data demonstrating that the prevalence of overweight and obesity has a negative gradient with social position and income across Europe [23]. Secondly, the overall prevalence of good adherence to the MD is less than 20%. Good adherence varies in the literature from 4.3% in Greek 10–12-year-old adolescents to 53.9% in Spanish children. Most of the studies conducted in southern European countries and recently reviewed reported that about half of pediatric individuals have an average adherence, while nearly half may have poor adherence [10,11]. Our data reflect those derived by the majority of the European studies [11]. Regarding Italy, our results are similar from the Calabrian Sierras Community Study (CSCS) which investigated a population attending primary and secondary schools in a 14-town southern Italian community [26]. We also observed a lower prevalence of good adherence in students attending primary than secondary

school, suggesting that younger children are more subjected to unhealthy choices. In fact, the attendance at a primary school is the only significant risk factor related to a poor MD adherence. This result is in contrast with the majority of the data that reported a negative trend in MD adherence with age [10,11]. Unfortunately, the CSCS study did not stratify the data for the school level [27]. On the other hand, the prevalence in our secondary school sample is similar to that reported by other studies on adolescents living in southern Italy [14,25,27]. It has to been considered that other socio-demographic factors such as parents' education and income are inconsistently associated across European countries due to different demographic and education changes [11]. Although we included only those schools where no prevention programs on the diets were performed in the last year, the enlargement of the study by including all the schools of Novara, accurate data on the socio-economic level, and parental weight could explain if an unexpected selection bias occurred. Gender and overweight were not associated with the adherence to the MD. Although some studies suggest that in Western societies women tend to have better dietary habits than men [28], and MD has been associated to the prevention of obesity in adults [29], our results are in line with available European data in children recently systematically reviewed [10,11,30]. These findings suggest again that both obesity and social differentiation are complex events. Overeating, lack of physical activity, low sleep quality, and the family environment should be considered. On the other hand, the differences by gender, weight, and ethnicity on the items of the KIDMED index we recorded could help in explaining the phenomenon. Our study reported a generally better quality of the diet among those children and adolescents more adherent to the MD than those with low adherence, except for the daily intake of sweets that was somewhat common. Moreover, we observed that younger children presented more unhealthy food choices than adolescents. This result is in line with a higher rate of poor adherence to the MD in the primary schoolchildren, as discussed above. The lower intake of vegetables and pulses resembles results obtained by the ZOOM8 study in 2009 and the last report of the "OKkio alla SALUTE" study in 2016 [16,31]. A negative association between MD adherence and snacks, sweets, commercial goods, and fast-foods has frequently been reported in other European pediatric populations [10,11,16]. It is interesting that primary schoolchildren consumed a more Westernized diet, skipping breakfast, eating several times per week at fast-food restaurants, consuming commercial baked goods, and sweets. This phenomenon could be boosted by geographical reasons, being our study conducted in an urban area [25]. In line with this hypothesis, we recorded a mixed

dietary behavior in children and adolescents of other ethnic origins. In fact, the latter maintained a higher intake of more traditional foods, such as fish, cereals or grain for breakfast, and yoghurts and/or some cheese, suggesting more home-made foods in their family environment. On the other hand, contemporarily, they frequently ate at fast-food restaurants, skipped breakfast, consumed commercial baked goods for breakfast, or sweets and candy several times every day. These findings well picture the nutrition transition described in European countries including those of the Mediterranean area [7,11]. Overeating, eating anything or disliked foods, and eating at friends' home were all identified as strategies to cope with food insecurity [32]. Frequent consumption of fast food/junk calorie dense foods have been reported in several developing countries [33], and these behaviors could be replicated when low-income families move abroad, and less control over the youngest generations occurs for several reasons [11]. As previously described, the adherence to the MD did not predict the weight status. Moreover, in the literature, there is inconsistency also in the evidence about the role of specific food groups [34]. However, we recorded some associations with single KIDMED items. First, the risk of being OWB was related to eating at fast-food restaurants and daily intake of commercial baked goods or pastries for breakfast. This result is in agreement with two systematic reviews, one reporting the role of ultra-processed foods and the other that of sugar-sweetened drinks and sweets snacking with increased obesity risk also in the pediatric populations [35,36]. Direct associations between fast food availability and obesity in lower-income children have been described [37]. The adherence to MD depends on many foods with specific and synergistic activities on metabolism. It is likely that dense energy foods rich in simple carbohydrates and saturated fats have a major role in the development of obesity, in particular in younger children. Moreover, food habits associated with the risk of OWB are different depending on ethnic backgrounds. The higher risk of OWB in those of other ethnic origins related to unhealthy choices for breakfast with skipping it or consuming commercial sweets and pastries suggests that the urbanization of life may lead to a more stressful lifestyle also in migrant people with less time spent on cooking, more time out of home, and dinner as the principal meal consumed with the family. All these factors could influence the food choices of the youngest children. Interestingly, OWB subjects consumed less olive oil at home, and the risk of OWB was significantly and inversely associated with the intake of olive oil. The fact that the olive oil intake is associated with BMI is still debated. A study in children observed that the likelihood to increase their BMI was less in those who consumed only olive oil than in those who

consumed other oils [38]. A recent review did not observe an increase in weight with an enriched-olive oil diet [39], although the Food4Me study recently recorded a direct correlation with the increase in weight in adults [34]. However, these last data are a little bit ambiguous reporting at the same time an inverse relationship with the intake of monounsaturated fats. Furthermore, OWB children and adolescents consumed more frequently vegetables than NW ones. This finding could be contrasting with their other food habits. However, the risk of OWB was inversely associated with the intake of vegetables more than once a day. In addition, in the ZOOM study, OWB subjects consumed more vegetables than NW children [30], suggesting that the answers to these items hide an overeating behavior that overcomes the protective effects of healthy dietary choices [40]. Finally, we observed a direct association between the KIDMED score and height. To our knowledge, this result has not been reported by other studies on the adherence to the MD, because they have been focused on BMI, waist or waist-to-height without considering height alone [10,11], with the exception of one study limited to nine-year-old children [41]. Since children with a medium/high adherence to the MD have a more balanced diet in terms of nutrients and functional foods [10], these habits can explain our data. In particular, a diet poor in micronutrients and high-quality proteins from milk products, pork meat, and fish has been shown to negatively influence stature [42–44]. The reanalysis of data derived by HELENA and IDEFICS cohorts could confirm our findings. This study has some limitations. First, it was a cross-sectional study design. Therefore, it is a limited set to establish causal relationships between MD and health outcome, and then conclusions are indications for further prospective and experimental investigations. Second, we only used the KIDMED score, without integrating it with a food frequency questionnaire. Adherence indexes, such as KIDMED score, have been validated and used in epidemiological surveys, but their reliability and reproducibility in assessing diet quality in the single subject have not been demonstrated yet [10,11]. Indeed, data on diet composition related to KIDMED score can be only inferred from related limited literature. We cannot exclude that some nutrients are main players more than food habits we reported. On the other hand, we used the most used index of adherence in pediatric literature [10,11], and the precise and driven administration to our cohort, in particular to younger children, supports the accuracy of our data. In fact, some authors suggest that the variability of adherence to MD across studies also results from the different administration methodologies [10]. Third, data on physical activity and sleep quality are lacking. Sedentary behaviors have been demonstrated to be correlated with the risk of

OWB and low adherence to MD [10,11], and, then, co-linearity with some variable could exist. Fourth, specific data on the socio-economic level were not obtained, and schools were only stratified according to the socio-economic level of the district area they were located, and an unexpected selection bias could have occurred. Indeed, more accurate investigations of these variables, including also parental education, could have a role and improve the prediction of the OWB risk. On the other hand, this study could give a significant contribution to research since recent data on northern Italy are lacking and it could be compared with similar studies conducted in the Southern part of Italy, as well as in other urban European areas. We presented data divided for school level, gender and ethnicity and this will help in making more effective reviews on the topic. In particular, we focused on how different food habits influence the risk of OWB depending on ethnicity. These data are crucial for further investigations and description of the nutrition transition phenomenon with urbanization. Moreover, weight and height were not self-reported, and this increased the accuracy of the relationship between weight status and MD adherence. In general, our study confirms that both children and adolescents have a poor MD adherence in an urban area of northern Italy, in line with other Italian and European data [10,11]. Furthermore, the mixed food behaviors occurring in individuals of different ethnic origins suggests that tailored prevention programs are needed to mitigate in this category of people the nutrition transition resulting from urbanization and changes of lifestyle habits of their families. These programs are urgent in hopes of preserving traditional healthy food habits. Moreover, the risk of OWB seems directly and indirectly associated more with specific food categories in pediatrics. These results should be confirmed but suggest that we have to increase nutrition knowledge in children and parents, as well as nutrition researchers should work hard.

Conclusions

In conclusion, we observed a relatively low high adherence to the MD in children and adolescents, in particular in those attending primary schools. Skipping breakfast, eating at fast-food restaurants, intake of processed foods and sweets are the main unhealthy choices, in particular in those OWB or of other ethnic origins. Differences in adherence to the MD and food intake between primary and secondary school, NW and OWB subjects and ethnic groups should be taken into account. Strategies tailored explicitly to subgroups are needed. Prevention campaigns should be conducted to improve food quality and drive to consume home-made healthy foods. The phenomenon of the

nutrition transition should be accurately investigated, in particular in younger children and those in low-income brackets.

Appendix

Table A1. Comparison of the each KIDMED item in children with the lowest (score ≤ 3) and the highest adherence (score ≥ 8) to the Mediterranean diet and worst lifestyle.

	KIDMED Score Low (*N* = 112)	KIDMED Score High (*N* = 131)	*p*
Consumption of a fruit or a fruit juice every day [1]	45.5%	83.1%	0.0001
Consumption of a second fruit every day [1]	17.0%	87.0%	0.0001
Consumption of raw or cooked vegetables 1 time a day [1]	17.9%	87.0%	0.0001
Consumption of raw or cooked vegetables >1 time a day [1]	9.8%	64.1%	0.0001
Consumption of fish regularly (at least 2–3 times a week) [1]	16.1%	73.3%	0.0001
Eating >1 time per week to a fast-food (hamburger) restaurant [2]	37.5%	9.2%	0.0001
Consumption of beans >1 time per week [1]	24.1%	84.7%	0.0001
Consumption of pasta or rice almost every day (≥5 times a week) [1]	66.1%	88.5%	0.0001
Consumption of cereals or grains (bread, etc.) for breakfast [1]	23.2%	85.5%	0.0001
Consumption of nuts regularly (at least 2–3 times per week) [1]	14.3%	48.9%	0.0001
Consumption of olive oil at home [1]	77.7%	100.0%	0.0001
Skipping breakfast [2]	37.5%	5.3%	0.0001
Consumption of a dairy product for breakfast (yoghurts, milk, etc.) [1]	42.9%	95.4%	0.0001
Consumption of commercially baked goods or pastries for breakfast [2]	66.1%	37.4%	0.0001
Consumption of 2 yoghurts and/or cheese (40 g) daily [1]	32.1%	56.5%	0.0001
Consumption of sweets or candy several times every day [2]	50.0%	40.5%	0.087

[1] Items with a positive answer (+1). [2] Items with a negative score (- 1). Data are represented as percentages. The data were analyzed by univariate logistic regression.

3.1.1.i. References

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3.1.2. A Systematic Review of the Association of Skipping Breakfast with Weight and Cardiometabolic Risk Factors in Children and Adolescents. What Should We Better Investigate in the Future?

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Abstract

The incidence of skipping breakfast in pediatric subjects is rising, and a relationship with overweight (OW) and obesity (OB) has been shown. Associations with cardiovascular outcomes and skipping breakfast in adults have been reported. The purpose of this systematic review was to summarize the association of skipping breakfast with body weight and metabolic outcomes in the pediatric population. We searched relevant databases (2008–2018) and identified 56 articles, of which 39 were suitable to be included, basing on inclusion criteria (observational; defined breakfast skipping; weight and/or metabolic outcomes). Overall, 286,804 children and adolescents living in 33 countries were included. The definitions of OW/OB, skipping breakfast, and the nutrient assessment were highly heterogeneous. Confounding factors were reported infrequently. The prevalence of skipping breakfast ranged 10–30%, with an increasing trend in adolescents, mainly in girls. Skipping breakfast was associated with OW/OB in the 94.7% of the subjects. The lack of association was shown mainly in infants. Moreover, 16,130 subjects were investigated for cardiometabolic outcomes. Skipping breakfast was associated with a worse lipid profile, blood pressure levels, insulin-resistance, and metabolic syndrome. Five studies reported a lower quality dietary intake in breakfast skippers. This review supports skipping breakfast as an easy marker of the risk of OW/OB and metabolic diseases, whether or not it is directly involved in causality. We encourage intervention studies using standardized and generalizable indicators. Data on confounders, time of fasting, chronotypes, and nutrition quality are needed to establish the best practice for using it as a tool for assessing obesity risk.

Introduction

Childhood obesity (OB) is a major public health issue in both developed and developing countries across the world [1]. Overweight (OW) and OB result from a complex network in which several factors interplay, such as genetic implications, birth weight, breastfeeding, parental obesity, physical activity, socioeconomic status, age, and gender [2]. Among them, dietary habits certainly also play a role [3]; in particular breakfast, the first meal in the day, has a critical role in energy balance and dietary regulation [4]. Despite this, the incidence of skipping breakfast among children and adolescent is rising [4,5], and several studies have shown a positive relationship between breakfast skipping and OW/OB [6]. As a possible explanation, children who regularly have breakfast have been shown to be more likely to have a better diet quality

and a higher intake of key food groups, such as fruit, dairy, and dietary fibers and, furthermore, they are also more likely to meet the recommendations for micronutrients [7–9]. Children who skip breakfast, instead, tend to eat more energy-dense food such as fast food leading to excess hunger and overeating [10]. The evidence on the association of breakfast consumption with body weight in the European population has been collected in the systematic review published in 2010; collectively, the data from observational studies carried out in Europe until 2009 have consistently demonstrated that children and adolescents who eat breakfast have a reduced risk of becoming overweight or obese and have a lower Body Mass Index (BMI) compared with those who skip breakfast [6]. Moreover, a series of studies have reported that breakfast skipping is associated with hypertension, cardiometabolic disease, insulin insensitivity, diabetes mellitus, and mortality [11]. However, these metabolic outcomes have not been explored in a larger systematic review and this associated has not been confirmed. The aim of our systematic review is to analyze the association of skipping breakfast, methodologically defined based on reported questionnaires, with body weight and metabolic outcomes in the pediatric population, focusing on the studies published in the last ten years.

Methods

Literature Search

PICO methodology (Population: children and adolescents; Exposure: skipping breakfast; Comparison: not skipping breakfast; Outcomes; weight and metabolic parameters) to develop a search strategy based on medical subject headings (MeSH) and keywords were used. Guidelines of the Preferred Reporting for Systematic Reviews and Meta-Analyses (PRISMA) statement were followed, and a PRISMA checklist were followed. The Cochrane Central Register of Controlled Trials, PubMed, CINHAHL, and EMBASE databases (January 2008–December 2018) were systematically used. The reference lists of identified studies and key review articles, including previously published reviews, were also searched for all randomized and non-randomized clinical trials as well as prospective cohort and cross-sectional studies assessing the association of breakfast skipping or consumption, however, defined, with body weight and cardiometabolic aspects in children and adolescents. No country restrictions were imposed. The search terms used included “breakfast”, and “children$” (or “adolescents$”). The search strategy used both keywords and MeSH terms. No further limitations were made so the search terms would be as sensitive as possible. In

addition, we checked the references of eligible articles for further papers that were not captured by our search strategy and we corresponded with authors when the relevant information was missing in the paper.

Outcome Measures

The primary outcome measures were as follows: body weight, body weight excess (e.g., overweight, obesity), and body mass index (BMI); for cardio-metabolic aspects we considered metabolic syndrome (MetS), arterial hypertension, lipid profile, glucose levels, type 2 diabetes, insulin resistance, and uric acid. Included studies had to report at least one of these primary outcomes.

Inclusion and Exclusion Criteria

For inclusion, studies were required to (i) include children and/or adolescents aged 2–18 years (or a mean within these ranges) as subjects of study. Studies that did not state the mean age of participants were classified as child or adolescent studies depending on the ages of the majority of the sample; (ii) have a defined measure of the child's or adolescent's breakfast consumption and/or breakfast skipping; (iii) be published in peer-reviewed journals in the English language; (vii) be published in the period January 2008–December 2018. We included studies even if they did not report completely the dietary quality data. We excluded intervention studies, studies where overweight/obese subjects were the only participants, and studies focusing on eating disorders.

Identification of Relevant Studies

Potentially relevant papers were selected by reading the titles and abstracts. If abstracts were not available or did not provide enough results the entire article was retrieved and screened to determine whether it met the inclusion criteria.

Data Extraction, Synthesis, and Quality Assessment

A form was generated to register whether individual studies met eligibility criteria and to collect data regarding the study design and methodological quality. Three investigators independently reviewed and extracted data from the papers according to the predetermined criteria. Any difference in opinion about the studies was resolved by discussion between all the investigators. The following data were extracted: author, date of publication, study design, characteristics of the participants (sample size, age, gender, and country), measures of breakfast behaviors, breakfast behavior (i.e., breakfast consumption/skipping), assessment methodology and reliability and validity

of dietary measures, definition of weight excess and assessment methodology. This information is summarized in Table 1. About secondary outcomes also the following data were extracted: definition of Mets, blood pressure, lipid profile, glucose, insulin, and uric acid levels, indexes of insulin resistance. Parameters could be as continuous or dichotomous variables. If reported, data on nutrient intake quality were included. This information is summarized in Table 2.

Table 1. Summarized studies' characteristics on weight and skipping breakfast.

Reference (Author, year, n)	Study Subjects	Methods	Skipping Breakfast Definition	Breakfast Evaluation Method	OB/OW Definition	Prevalence of Breakfast Skippers	Results	Impact of Skipping Breakfast on OW/OB
Dialektakou 2008 [12]	N = 811, aged 14.9–21.2 years, M and F, Greece	Cross-sectional, self-reported questionnaires, measured height and weight	24 definitions evaluating breakfast consumption on the day of data collection, during the whole year, during the previous week, and on weekends/holidays	Not specified	Age- and sex-specific BMI cut-offs according to IOTF criteria	3.6–74.7% (according to different definitions)	Fewer breakfast-skipping variables associated with BMI than with OW/OB. Fewer associations when controlling for potential confounders. Fewer associations for variables corresponding to some definitions.	↑
Dubois 2008 [13]	N = 1549; aged 44–56 months, M and F, Canada	Longitudinal study, parent-report questionnaires, measured height and weight (cross-sectional data considered)	Frequency of breakfast eating: (1) yes, every morning; (2) regularly but not every day; (3) only on occasion; and (4) never. Categories 2 to 4 were classified as 'breakfast skippers'	Eating behavior questionnaire (Enquete sociale et de sante' aupres des enfants et des adolescents Quebecois -Health and Social Survey of Quebec Children and Adolescents) and a 24 h dietary recall interview	Age- and sex-specific BMI z-score cut-offs according to CDC criteria	10%	↑ intake of energy, carbohydrates or servings of grain products in breakfast skippers	↑
Harding 2008 [14]	N = 6599, aged 11–13 years, M and F, UK	Cross-sectional, self-reported questionnaires, measured height and weight	Number of eaten breakfasts per week (Every day; 3–4 days a week; 1–2 days a week; Never or hardly ever)	http://www.sphsu.mrc.ac.uk/studies/dash/ Food frequency questionnaire	Age- and sex-specific BMI cut-offs according to IOTF criteria	32.6–53% not eating breakfast every day	Increased risk for obesity in breakfast skippers (girls OR 1.74, 95% CI 1.30–2.34; boys OR 2.06; CI 1.57–2.70)	↑
Duncan 2008 [15]	N = 1229, aged 5–11 years, M and F, New Zeland	Cross-sectional, proxy questionnaire administered to the parents, measured height and weight, BIA	Number of eaten breakfasts in the last full week (0–7 days per week)	7-day recall	Boys and girls were classified as "overfat" if their % BF exceeded 25% and 30% (respectively)	1.3 (non-overfat)–2.6% (overfat) never eat breakfast in a week	Breakfast skippers had increased odds of overfat compared with those who had breakfast for five or more days/week.	↑
Croezen 2009 [16]	N = 25176, aged 13–16 years, M and F, The Netherlands	Cross-sectional, detailed Internet questionnaire, under supervision of instructed teachers following a standardized protocol, self-reported body weight and height	Number of eaten breakfasts per week (0–7 days per week)	Food frequency questionnaire	Age- and sex-specific BMI cut-offs according to Cole's definition	29.3–39.2%	Skipping breakfast >2 times/week associated with OW (adjusted OR 1.68 (CI 1.43–1.97) in 13–14 year-aged, and 1.32 (CI 1.14–1.54) in 15–16 year-aged subjects); skipping breakfast every day associated with OB	↑
Nagel 2009 [17]	N = 1079, aged 6.2–9.2 years, M and F, Germany	Cross-sectional, self-reported questionnaires compiled by children and parents, measured weight, height, upper arm and waist circumference, skin fold thickness	Breakfast consumption before school (yes/no)	Not specified	Age- and sex-specific BMI cut-offs according to IOTF criteria	13.4%	Breakfast skippers had increased risk for OW (OR 1.73, 95% CI 1.13–2.64) and OB (OR 2.50, 95% CI 1.19–5.29)	↑
Sun 2009 [18]	N = 5753, aged 12–13 years, M and F, Japan	Cross-sectional, self-reported questionnaires, measured height and weight	Frequency of eating breakfast: daily, almost daily, sometimes, and rarely	Food frequency questionnaire	Age- and sex-specific BMI cut-offs according to IOTF criteria	1.1% of boys and 0.7% of girls ate breakfast rarely	Skipping breakfast (i.e., eating breakfast rarely) was associated with OW (in boys only after adjustment for age, parental OW and lifestyle variables)	↑
Maddah 2010 [19]	N = 6635, aged 6–11 years, M and F, Iran	Cross-sectional, self-reported questionnaire given to the parents, measured weight and height	Breakfast skipping (yes/no)	Not specified	Age- and sex-specific BMI cut-offs according to IOTF criteria	Not reported	Higher prevalence of OW/OB in breakfast skippers than in breakfast eaters (boys: 23.6% versus 16.9%, girls: 23.5% versus 17.1%)	↑
Isacco 2010 [20]	N = 278, aged 6–10 years, M and F, France	Cross-sectional, self-reported questionnaire compiled by the parents in the presence of their child, measured weight, height, WC and skin fold thickness	Frequency of eating breakfast: every day, sometimes, never	Food frequency questionnaire	Age- and sex-specific BMI z-score cut-offs according to CDC criteria	1.4% never ate breakfast	higher BMI z-score, skinfolds and WC in breakfast skippers	↑
Deshmukh-Taskar 2010 [21]	N = 9659, aged 9–18 years, M and F, USA	Cross-sectional, self-reported data on 24-h recall methodology over two days (assisted by parent/caregivers for children aged 6 to 11 years), measured weight, height and WC	Breakfast skippers: those who consumed no food or beverages, excluding water, at breakfast	24-h recall http://www.cdc.gov/nchs/data/nhanes/dr-1-5.pdf.	Age- and sex-specific BMI z-score cut-offs according to CDC criteria	20% of children, 31.5% of adolescents	Breakfast skippers had higher BMI z-scores and a higher waist circumference than ready-to-eat cereal and other breakfast consumers. Higher prevalence of obesity in breakfast skippers than ready-to-eat cereal consumers	↑
So 2011 [22]	N = 11570, aged 9–18 years, M and F, Hong Kong	Cross-sectional, self-reported questionnaires, measured height and weight, and BIA	Breakfast skippers (ate breakfast 0–2 times/week); semi-skippers (ate breakfast 3–4 times/week); non-skippers (ate breakfast 5–7 times/week)	Rapid Dietary behavior Assessment questionnaire (daily and weekly dietary behaviors, validated against the 24 h recall nutrient intake data in a smaller sample)	Age- and sex-specific BMI cut-offs according to IOTF criteria	8% of primary school students and 14% of secondary school students	Breakfast skippers had higher BMI, BMI z-scores and BF% than their counterparts	↑
Tin 2011 [23]	N = 113457, aged 9–10 years, M and F, Hong Kong	Longitudinal, 2-year follow-up, self-reported questionnaires, measured height and weight (cross-sectional data considered)	Breakfast skippers those who chose 'no breakfast at all'	Not specified	Age- and sex-specific BMI cut-offs according to IOTF criteria	5.3% of boys, 5.2% of girls	Higher mean BMI in breakfast skippers both at baseline (β 0.77, 95% CI 0.67–0.87) and 2 years later (β 0.86, 95% CI 0.78–0.95)	↑

Table 1. Cont.

Reference (Author, year, n)	Study Subjects	Methods	Skipping Breakfast Definition	Breakfast Evaluation Method	OB/OW Definition	Prevalence of Breakfast Skippers	Results	Impact of Skipping Breakfast on OW/OB
Mushtaq 2011 [24]	N = 1860, aged 5–12 years, M and F, Pakistan	Cross-sectional, questionnaires administered to the children by senior medical students, measured height and weight	Skipping breakfast once or more in the past week	7-day recall	BMI z-scores calculated based on the WHO criteria	8%	Breakfast skippers were significantly more likely to be overweight (15% versus 9%) and obese (13% versus 7%) than breakfast eaters ($p = 0.002$). Skipping breakfast was associated with overweight among girls ($p < 0.001$). Skipping breakfast as independent predictor of OW (OR 1.82, 95% CI 1.22–2.71)	↑ (OW in girls)
Kuriyan 2012 [25]	N = 8444 (4707 aged 3–10 years; N = 3737 aged 10–16 years), M and F, Bangalore	Cross-sectional, parent/student-report questionnaires, measured height and weight, WC	Breakfast skipping (yes/no)	Not specified	Indian Academy of Pediatrics cut-off for BMI; WC > 75th percentile for classifying abdominal obesity	Not reported	-	⇔ WC in children aged 3–10 years ↑ WC in children aged 10–16 years
Kyeariazis 2012 [26]	N = 2374, aged 6–12 years, M and F, Greece	Cross-sectional, self-reported questionnaires, measured height and weight	Breakfast skipping (yes/no)	Closed format questions in the form of multiple choice Questions	Age- and sex-specific BMI cut-offs according to Cole's definition	Not reported	Skipping breakfast had a positive association with OB	↑
Van Lippevelde 2013 [27]	N = 6374, aged 10–12 years, M and F, Belgium, Greece, Hungary, the Netherlands, Norway, Slovenia, Spain, and Switzerland	Cross-sectional, self-reported questionnaires compiled by the children during school-time, measured weight and height	Breakfast frequency per week (0–7) calculated by adding up the breakfasts usually had on schooldays per week (0–5) and on weekend days per week (0–2)	http://projectenergy.eu Food frequency questionnaire	BMI z-scores calculated based on the WHO criteria	Not reported	Children's breakfast consumption negatively related to children's BMI-z-score	↑
Januszek-Trzciąkowska 2014 [28]	N = 2571, aged 7–9 years, M and F, Poland	Cross-sectional, self-reported questionnaire compiled by the parents, measured weight and height	Breakfast frequency: always, usually never	Food frequency questionnaire	Age- and sex-specific BMI cut-offs according to IOTF criteria	10.3% in girls, 9.1% in boys	Increased OB risk in girls irregularly or never eating breakfast (always versus usually, OR 2.71, 95% CI 1.33–5.51; always versus never OR 1.63, 95% CI 1.08–2.47)	↑ only for girls
Kupers 2014 [29]	T1: 2 years of age; N = 1488 T2: 5 years of age; N = 1366 M and F, The Netherlands	Longitudinal; parent-report questionnaires; measured height and weight (cross-sectional data considered)	Breakfast frequency per week (0–7), categorized as "eating breakfast daily" (7 times per week) or "not eating breakfast daily" (<7 times per week)	Food frequency questionnaire	Age- and sex-specific BMI cut-offs according Dutch reference growth charts	At T1, 3.0% of the children did not eat breakfast daily; at T2, 5.3%	No association between skipping breakfast and overweight, neither at age 2 nor at age 5	⇔
O'Neil 2015 [30]	N = 14200, aged 2–18 years, M and F, USA	Cross-sectional, self-reported questionnaires (complied by parents/guardians of 2–5 year children; by 6–11 year children assisted by an adult; older children provided their own recall), measured weight and height	24-h dietary recall: no breakfast or 11 possible breakfast patterns	24-h dietary recall interviews using an automated multiple-pass method http://www.cdc.gov/nchs/data/nhanes/nhanes_03_04/DIETARY_MEC.pdf.	Age- and sex-specific BMI cut-offs according to CDC criteria	18.7%	Mean BMI z-scores were lower among consumers of five breakfast patterns (grain/lower fat milk/sweets/fruit juice, pre-sweetened ready-to-eat cereal/whole milk, soft drinks/fruit juice/grain/potatoes, ready-to-eat cereal/whole milk, and cooked cereal/milk/fruit juice), compared to breakfast skippers.	↑
Smetanina 2015 [31]	N = 3990, aged 7–17 years, M and F, Lithuania	Cross-sectional, self-reported questionnaires (parents of younger age (7–9 years old) participants filled-in the questionnaire at home and older children and adolescents filled-in it themselves at school), measured weight and height	breakfast eating frequency per week: "Everyday" ("Everyday" and "4–6 times per week"), "1–3 times per week", and "Never"	Modified WHO questionnaires (conducted by Health behavior in School-aged Children (HBSC) and COSI study groups).Food frequency questionnaire	Age- and sex-specific BMI cut-offs according to IOTF criteria	Never eating breakfast: 6.2% in underweight, 6.5% in NW, 9.6% in OW/OB	The prevalence of subjects never having breakfast was significantly higher in OW/OB than in NW (9.6% versus 6.5%)	↑
Zakrzewski 2015 [32]	N = 6841, aged 9–11 years, M and F, Australia, Brasil, Canada, China, Colombia, Finland, India, Kenya, Portugal, South Africa, UK, US	Cross-sectional, self-reported questionnaires, measured height, weight and BF%	Breakfast frequency per week (separately for weekdays and weekend days). 1. Three-category definition: weekly breakfast frequency coded as rare (0–2 days per week), occasional (3–5 days per week) and frequent (6–7 days per week). 2. Two-category definition: weekly breakfast frequency recoded as less than daily (0–6 days per week) or daily (7 days per week).	Food frequency questionnaire	BMI z-scores calculated based on the WHO criteria	Breakfast consumption: 6.3% rarely, 27.7% less than daily	Frequent breakfast consumption was associated with lower BMI z-scores compared with occasional ($p < 0.0001$) and rare ($p < 0.0001$) consumption, as well as lower BF% compared with occasional ($p < 0.0001$) and rare ($p < 0.0001$).	↑

Table 1. Cont.

Reference (Author, year, n)	Study Subjects	Methods	Skipping Breakfast Definition	Breakfast Evaluation Method	OB/OW Definition	Prevalence of Breakfast Skippers	Results	Impact of Skipping Breakfast on OW/OB
Wijtzes 2016 [33]	N = 5913, T1: 4 years of age T2: 6 years of age, M and F, The Netherlands	Longitudinal, parent-report questionnaires, measured height and weight, percent fat mass by dual-energy X-ray absorptiometry (at age 6 years) (cross-sectional data considered)	At age 4 years: weekly consumption of breakfast ("never," "1–2 days per week," "3–4 days per week," "5–6 days per week," and "every day", coded as 1–5); At age 6 years: the number of days of breakfast consumption assessed separately for weekdays (coded as 0–5) and weekend days (coded as 0–2), and the scores were summed to calculate total weekly consumption (0–7). Breakfast skipping defined as consumption <7 days per week	Food frequency questionnaire	Age- and sex-specific BMI cut-offs according to IOTF criteria	Not reported	Breakfast skipping at age 4 years associated with increased % fat mass at age 6 years (β = 1.38; 95% CI: 0.36–2.40)	↑
Fayet-Moore 2016 [34]	N = 4487, aged 2–16 years, M and F, Australia	Cross-sectional, computer-assisted interview based on 24-h recall methodology over two days from participants or their caregivers, measured height and weight	Breakfast skippers were children who did not consume an energy containing food or beverage during breakfast on 2 recall days	24-h recall methodology	BMI z-score or centile adjusted for age and sex was calculated using the US CDC 2000 growth reference chart	4%	Higher prevalence for OW/OB in breakfast skippers than in breakfast consumers (21.2% and 23.2% versus 16.4% and 16.5%, respectively)	↑
Alsharairi 2016 [35]	T1 (2006): N = 4601, 2–3 age of years T2 (2008): N = 4381, 4–5 years of age, M and F, Australia	Cross-sectional and longitudinal study, face-to-face mother's interview, measured height and weight	Breakfast consumption in the day of interview (yes/no)	Not specified	Age- and sex-specific BMI cut-offs according to IOTF criteria	Not reported	OB boys at T1 (OR 2.38, 95% CI: 1.04–5.43) and T2 (OR 2.32, 95% CI: 1.01–5.32) and OB girls at T2 (OR 2.26, 95% CI: 1.14–4.46) were more likely to skip breakfast compared with non-overweight	↑
Fayet-Moore 2017 [36]	N = 2812, aged 2–18 years, M and F, Australia	Cross-sectional, face-to-face interviews, measured height and weight	Breakfast skipping or eating during the 24 h prior to the interview day	24-h recall methodology	Age- and sex-specific BMI cut-offs according to the WHO criteria	9%	No associations between anthropometric measures and breakfast or breakfast cereal choice were found Breakfast skippers: ↑ higher saturated fat intake ↓ intakes of dietary fibers and most micronutrients ($p < 0.001$)	⇔
Smith 2017 [37]	N = 1592, aged 2–17 years, M and F, Australia	Cross-sectional, computer-assisted interview based on 24-h recall methodology (for 2–5 year children completed by an adult; for 6–8-years an adult was interviewed with help from the child; 9–11 year children were interviewed directly with assistance from an adult; 12–17-year were interviewed directly, with the adult remaining in the room for those aged 12–14 years); measured weight and height	Breakfast skippers if they did not define an eating occasion as 'breakfast' in the 24-h recall or the energy intake for the "breakfast" occasion was <210 kJ	24-h recall methodology	Age- and sex-specific BMI cut-offs according to Cole's definition	11.8% of boys and 14.8% girls skipped on one day and 1.4% boys and 3.8% girls skipped on both days	The odds of skipping breakfast were progressively higher with increasing BMI category	↑
Gotthelf 2017 [38]	N = 2083, aged 9–13 years, M and F, Argentina	Cross-sectional, self-reported questionnaires compiled by children and parents, measured weight and height	Breakfast habit: eating breakfast on the day of the survey (yes/no). Frequency: always (6–7 days/week), sometimes (2–5 days/week), never (0–1 day/week).	Food frequency questionnaire	BMI z- scores calculated based on the WHO criteria	64.1% of students from peri-urban schools and 46.1% of students from urban schools	Among breakfast skippers, 40.7% of the girls and 54.7% of the boys were OW/OB. A higher probability of skipping breakfast was associated with obesity.	↑
Nilsen 2017 [39]	N = 2620, aged 7–9 years, M and F, Sweden	Cross-sectional, proxy questionnaire filled out by the parents or guardians, measured height and weight	Number of eaten breakfasts over a typical week (Every day; most days, i.e., 4–6 days a week; some days, i.e., 1–3 days a week; Never)	Food frequency questionnaire	Age- and sex-specific BMI cut-offs according to Swedish national growth reference	4.6%	Association between OW/OB and not having breakfast every day (OR 1.9 (CI 1.18–3.13))	↑
Kesztyus 2017 [40]	N = 1943, aged 7.1 ± 0.6 years, M and F, Germany	Cross-sectional, proxy questionnaire administered to the parents, measured height, weight and WC	4-point scale, the results were subsequently dichotomized for analyses (never, rarely versus often, always)	Food frequency questionnaire	Age- and sex-specific BMI cut-offs according to Swedish national growth reference; abdominal obesity as WHtR >0.5 or >0.47 for girls and 0.48 for boys	13.1%	Skipping breakfast associated with OW (crude OR 2.02 (CI 1.18–3.43)), OB (crude OR 1.94 (CI 1.03–3.66)), abdominal OB with WHtR >0.5 (crude OR 2.51 (CI 1.63–3.88)), abdominal OB with WHtR >0.47/0.48 (crude OR 2.20 (CI 1.58–3.07))	↑
Zalewska 2017 [41]	N = 1999, aged 18 years, M and F, Poland	Cross-sectional, self-reported questionnaires, measured height and weight	Breakfast habit: skipped, <8 AM, ≥8 AM	Not specified	BMI calculated based on the WHO criteria	25%	No difference in the prevalence of breakfast skippers between NW and OW/OB	⇔

Table 1. Cont.

Reference (Author, year, n)	Study Subjects	Methods	Skipping Breakfast Definition	Breakfast Evaluation Method	OB/OW Definition	Prevalence of Breakfast Skippers	Results	Impact of Skipping Breakfast on OW/OB
Coulthard 2018 [42]	N = 1686, aged 4–18 years, M and F, UK	Cross-sectional, 4-day food diary to be completed by the children, or their parent for those aged 11 years and under, measured weight and height	Those consuming breakfast every diary day, those consuming breakfast on at least one but not all diary days, and those not consuming breakfast on any diary day	4-day food diary	Age- and sex-specific BMI cut-offs according to Cole's definition (1990 UK charts)	19.9% of girls and 14.5% of boys	No differences in weight status by breakfast eating habits. The overall nutritional profile of the children in terms of fiber and micronutrient intake was superior in frequent breakfast consumers (micronutrients: folate, calcium, iron and iodine ($p < 0.01$)	⇔
Tee 2018 [43]	N = 8332, aged 6–17 years, M and F, Malaysia	Cross-sectional, self-administered questionnaire with assistance to children aged 10 years and above, proxy questionnaire administered to the parent for children aged 6 to 9 years; measured weight and height	Breakfast skippers (ate breakfast 0–2 days/week), irregular breakfast eaters (ate breakfast 3–4 days/week) and regular breakfast eaters (ate breakfast ≥5 days/week)	Food frequency questionnaire	BMI z-scores calculated based on the WHO criteria	9.3% in primary school children and 15.9% in secondary school children	Compared to regular breakfast eaters, the risk of being OW/OB was higher in 6–12 years boys who skipped breakfast (OR 1.71, 95% CI 1.26–2.32), in 6–12 years girls (OR 1.36, 95% CI = 1.02–1.81) and in 12–17 years girls (OR 1.38, 95% CI 1.01–1.90)	↑
Archero 2018 [44]	N = 669, aged 6–16 years, M and F, Italy	Cross-sectional, self-reported questionnaires compiled by the children during school-time, in the presence of a teachers and medical staff, measured weight and height	Breakfast skipping (yes/no)	Italian version KIDMED index, a questionnaire of dichotomous (positive/negative) items	Age- and sex-specific BMI cut-offs according to IOTF criteria	14.8% in primary school children and 21.9% in secondary school children	OW/OB skipped breakfast more frequently than NW (chi-squared 3.556, $p < 0.04$). Increased risk for OW/OB in non-Italian breakfast skippers (OR 16.05, CI 95% 1.93–133.27, $p < 0.01$)	↑
Smith 2010 [11]	T1 (1985): N = 6559; 9–15 years of age T2 (2004–2006): 26–36 years of age M and F, Australia	The Childhood Determinants of Adult Health (CDAH) study. T1: self-report questionnaires; were measured: height and weight. T2: self-report questionnaires; were measured: height, weight, waist WC and BP; a venous blood sample was collected for lipid profile and glucose metabolism	T1: Breakfast consumption was assessed by using the question "Do you usually eat something before school?" "Yes" or "no" T2: Skipping breakfast was defined as not eating between 06.00 and 09.00	T1: Not specified. T2: Food-frequency questionnaire	Age- and sex-specific BMI cut-offs according to Cole's cut-off	Skipping breakfast: 14.2% in childhood; 27.5% in adulthood	In both childhood and adulthood: ↑ WC (mean difference: 4.63 cm; 95% CI: 1.72, 7.53 cm)	↑
Shafiee 2013 [45]	N = 5625, subjects aged 10–18 years; M and F, Iran	The third survey of the national school-based surveillance system (CASPIAN-III); parent-report questionnaires; were measured: height, weight, waist WC and BP; a venous blood sample was collected for lipid profile and glucose metabolism	Subjects were classified into three groups: "regular breakfast eater" (6–7 days/week), "often breakfast eater" (3–5 days/week), and "seldom breakfast eater" (0–2 days/week)	Likert scale questionnaire	Age- and sex-specific BMI cut-offs according to the WHO growth reference standards	The % of subjects classified as: "regular"47.3%, "often" 23.7% and "seldom"29.0%, breakfast eaters	↑ ($p < 0.001$)	↑
Ho 2015 [46]	N = 2401, elementary school children; M and F, Taiwan	Elementary School Children's Nutrition and Health Survey in Taiwan (NAHSIT); self-report questionnaire; were measured: height, weight, WC and BP; a venous blood sample was collected for lipid profile and glucose metabolism	Breakfast consumption was assessed by using the question "How often do you eat breakfast in a week?" The answer could range from 0 to 7 times. The frequency was classified into three groups, including 0–4, 5–6, and 7 times per week	24-h recall; food-frequency questionnaire The Youth Healthy Eating Index for the United States of America (US—YHEI) modified to YHEI—Taiwan (YHEI—TW): indicator of dietary quality	Not reported	% Breakfast frequency (times/week): 5.4% (0–4) 5.9% (5–6) 88.7% (7)	↑ (Children who consumed breakfast daily: ↓ BMI (17.9 kg/m^2; $p = 0.009$); ↓ WC (58.6 cm; $p = 0.005$))	↑
Marlatt, 2016 [47]	N = 367, subjects aged 11–18 years; M and F, Minneapolis	Cross-sectional study; self-report survey; were measured: height, weight, % body fat, and BP; a venous blood sample was collected for lipid profile and glucose metabolism	Breakfast consumption was expressed as average number of days/week that breakfast was consumed	Self-report survey using validated questions (Nelson MC, Lytle LA, 2009. Development and evaluation of a brief screener to estimate fast-food and beverage consumption among adolescents. J Am Diet Assoc; 109, 730–734; 24-h recalls	Age- and sex-specific BMI cut-offs according to the CDC Growth Charts, (2000)	Not reported	↑ BMI and % body fat	↑

Legend: % BF = Percentage Body Fat; BIA = Bioelectric impedance Analysis; BMI = Body Mass Index; BP = Blood Pressure; CI = Confidence Interval; CDC = Center for Disease Control and Prevention; COSI = Childhood Obesity Surveillance Initiative; IOTF = International Obesity Task Force; M = Males; F = Females; NW = NormalWeight; OB = Obesity; OR = Odd Ratio; OW = Overweight; WC = Waist Circumference; WhtR = Waist-to-Height Ratio; WHO = World Health Organisation; " = Increased; # = Reduced; , = Not Variation.

Table 2. Summarized studies' characteristics on metabolic variables and skipping breakfast.

Reference (Author, Year, n)	Study Subjects	Methods	Skipping Breakfast Definition	Breakfast Evaluation Method	OW/OB Definition	Prevalence of Breakfast Skippers	Association of Skipping Breakfast with OW/OB	Association of Skipping Breakfast with Blood Pressure	Association of Skipping Breakfast with Lipid Profile	Association of Skipping Breakfast with Glucose Metabolism	Association of Skipping Breakfast with Metabolic Syndrome	Association of Skipping Breakfast with Nutrient Intake
Smith2010 [11]	T1 (1985): N = 6559; 9–15 years of age. T2 (2004–2006): 26–36 years of age. M and F, Australia	The Childhood Determinants of Adult Health (CDAH) study. T1: self-report questionnaires; were measured: height and weight. T2: self-report questionnaires; were measured: height, WC and BP; a venous blood sample was collected for lipid profile and glucose metabolism	T1: Breakfast consumption was assessed by using the question "Do you usually eat something before school?" "Yes" or "no". T2: Skipping breakfast was defined as not eating between 06.00 and 09.00	T1: Not specified T2: Food-frequency questionnaire	Age- and sex-specific BMI cut-offs according to Cole's cut-off	Skipping breakfast: 14.2% in childhood; 27.5% in adulthood	In both childhood and adulthood: ↑ WC (mean difference: 4.63 cm; 95% CI: 1.72, 7.53 cm)	Not reported	↑ Total (mean difference: 0.40 mmol/L; 95% CI: 0.13, 0.68 mmol/L) and LDL-cholesterol (mean difference: 0.40 mmol/L; 95% CI: 0.16, 0.64 mmol/L)	In both childhood and adulthood: ↑ fasting insulin (mean difference: 2.02 mU/L; 95% CI: 0.75, 3.29 mU/L)	Not reported	Not reported
Monzani 2013 [48]	N = 489, subjects aged 6.7 to 13 years; M and F, Italy	Population-based, cross-sectional study; self-reported questionnaire; were measured: height, weight, WC, and BP; a venous blood sample was collected for lipid profile, uric acid and glucose metabolism	Breakfast consumption: yes/no	Not specified	MetS according to modified NCEP-ATP III criteria of Cruz and Goran	Not reported	Not reported	Not reported	Not reported	Not reported	In school-children aged 10.1–13 years: no breakfast consumption (OR = 5.0, 95% CI = 1.5–17.2, $p = 0.02$) was ↑ in those with MetS	Not reported
Shafiee 2013 [45]	N = 5625, subjects aged 10–18 years; M and F, Iran	The third survey of the national school-based surveillance system (CASPIAN-III); parent-report questionnaires; were measured: height, weight, waist circumference (WC) and blood pressure (BP); a venous blood sample was collected for lipid profile and glucose metabolism	Subjects were classified into three groups: "regular breakfast eater" (6–7days/week), "often breakfast eater" (3–5days/week), and "seldom breakfast eater" (0–2 days/week)	Likert scale questionnaire	Age- and sex-specific BMI cut-offs according to the WHO growth reference standards Metabolic syndrome (MetS) was defined based on the Adult Treatment Panel III (ATP III) criteria modified for the pediatric age group	The % of subjects classified as: "regular" 47.3%, "often" 23.7% and "seldom" 29.0%, breakfast eaters	↑ ($p < 0.001$)	↑ ($p < 0.001$)	↑ Triglycerides, LDL-cholesterol ($p < 0.001$) ↓ HDL-cholesterol	Not reported	↑ (OR 1.96, 95% CI 1.18–3.27)	Not reported
Ho 2015 [46]	N = 2401, elementary school children; M and F, Taiwan	Elementary School Children's Nutrition and Health Survey in Taiwan (NAHSIT); self-report questionnaire; were measured: height, weight, circumference waist (WC) and blood pressure (BP); a venous blood sample was collected for lipid profile and glucose metabolism	Breakfast consumption was assessed by using the question "How often do you eat breakfast in a week?" The answer could range from 0 to 7 times. The frequency was classified into three groups, including 0–4, 5–6, and 7 times per week	24-h recall; food-frequency questionnaire. The Youth Healthy Eating Index for the United States of America (US-YHEI) modified to YHEI-Taiwan (YHEI-TW): indicator of dietary quality	MetS was defined based on criteria from Cook	% Breakfast frequency (times/week): 5.4% (0–4) 5.9% (5–6) 88.7% (7)	↑ (Children who skipped breakfast daily: BMI (17.9 kg/m^2; $p = 0.009$); WC (58.6 cm; $p = 0.005$))	↑ (Children who consumed breakfast daily: systolic BP (97.0 mmHg; $p = 0.007$); diastolic BP (57.3 mmHg; $p = 0.02$) Children who consumed breakfast daily versus children who consumed breakfast 0–4 times per week: risks of high blood pressure (OR = 0.37, 95% CI = 0.19–0.71))	HDL-cholesterol (Children who consumed breakfast daily: ↑ HDL cholesterol (59.5 mg/dL; $p = 0.03$))	⇔	↑ (Children who consumed breakfast daily: prevalence of MetS (2.89%) Children who consumed breakfast daily versus children who consumed breakfast 0–4 times per week: risks of MetS (OR = 0.22, 95% CI = 0.09–0.51))	YHEI-TW scores (Children who consumed breakfast daily versus those who consumed breakfast 0–4 times per week: ↑ intakes of: saturated fat, cholesterol, vitamins A, B1, B2, calcium, phosphorus, magnesium, and potassium; ↑ YHEI-TW scores (better dietary quality))
Osawa 2015 [49]	N = 689, subjects aged 10–13 years; M and F, Japan	Cross-sectional study; self-report questionnaire; were measured: height, weight, WC and BP; a venous blood sample was collected for lipid profile and glucose metabolism	Breakfast consumption was assessed by using the question "Do you have breakfast every day? (Yes, alone/Yes, with family/Seldom/No)	Food-frequency questionnaire designed by members of the Ichikawa Dental Association	MetS was defined based on criteria identified by the Japanese Society of Internal Medicine, the Japan Society for the Study of Obesity and the Ministry of Health, Labour and Welfare in Japan	Not reported	Not reported	Not reported	Not reported	Not reported	Not eating breakfast was associated significantly with MetS or high risk MetS (OR: 2.70, 95% CI: 1.01–7.23, $p < 0.05$)	Not reported
Marlatt, 2016 [47]	N = 367, subjects aged 11–18 years; M and F, Minneapolis	Cross-sectional study; self-report survey; were measured: height, weight, BF%, and blood pressure BP; a venous blood sample was collected for lipid profile and glucose metabolism	Breakfast consumption was expressed as average number of days/week breakfast was consumed	Self-report survey using validated questions (Nelson MC, Lytle LA, 2009. Development and evaluation of a brief screener to estimate fast-food and beverage consumption among adolescents. J Am Diet Assoc; 109, 730–734; 24-h recalls	Age- and sex-specific BMI cut-offs according to the CDC Growth Charts, (2000) MetS was defined based on the Adult Treatment Panel III (ATP III) criteria	Not reported	↑ BMI and % body fat	⇔	⇔	↑ HOMA-IR	↑ MetS cluster score	Not reported

Legend: BMI = Body Mass Index; CI = Confidence Interval; F = Females; CDC = Center for Disease Control and Prevention; M = Males; MetS = Metabolic Syndrome; OB = Obesity; OR = Odd Ratio; OW = Overweight; " = Increased; # = Reduced; , = Not Variation.

Study quality was independently assessed by three reviewers according to the Newcastle-Ottawa Scale for quality assessment of cohort studies and case-control studies [36]. The scales allocate stars, with a maximum of nine; the criteria were quality of selection (maximum, four stars), comparability (maximum, two stars), and exposure (maximum, three stars). High quality was assessed for more than eight stars.

Results

The literature search identified 239 potentially relevant articles. After reviewing the titles and abstracts and the full-length articles, 39 articles were selected for closer assessment and then included in our analysis [11–49]. The search flow-chart is represented in Figure 1. Agreement between reviewers on which studies to include was good: the K for the agreement was 85% after screening titles and abstracts and 100% after screening full-text articles. Overall, data from a total of 286,804 children and adolescents, living in 32 countries, were reported (Figure 2).

Figure 1. Flow diagram for study retrieval and selection.

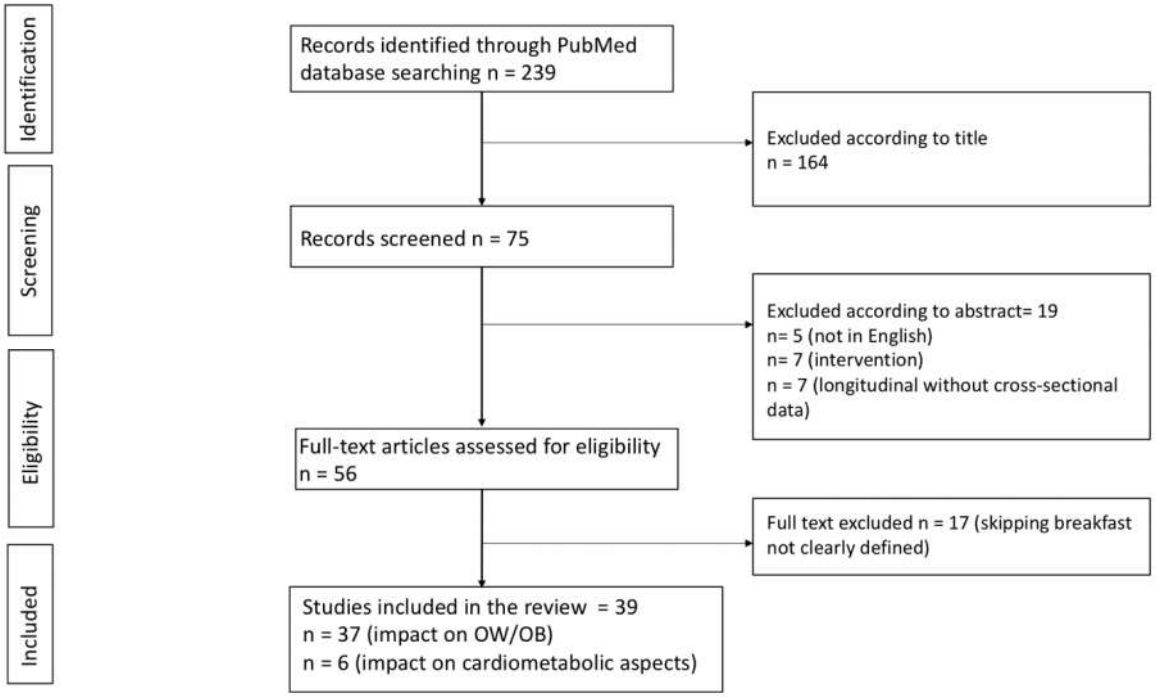
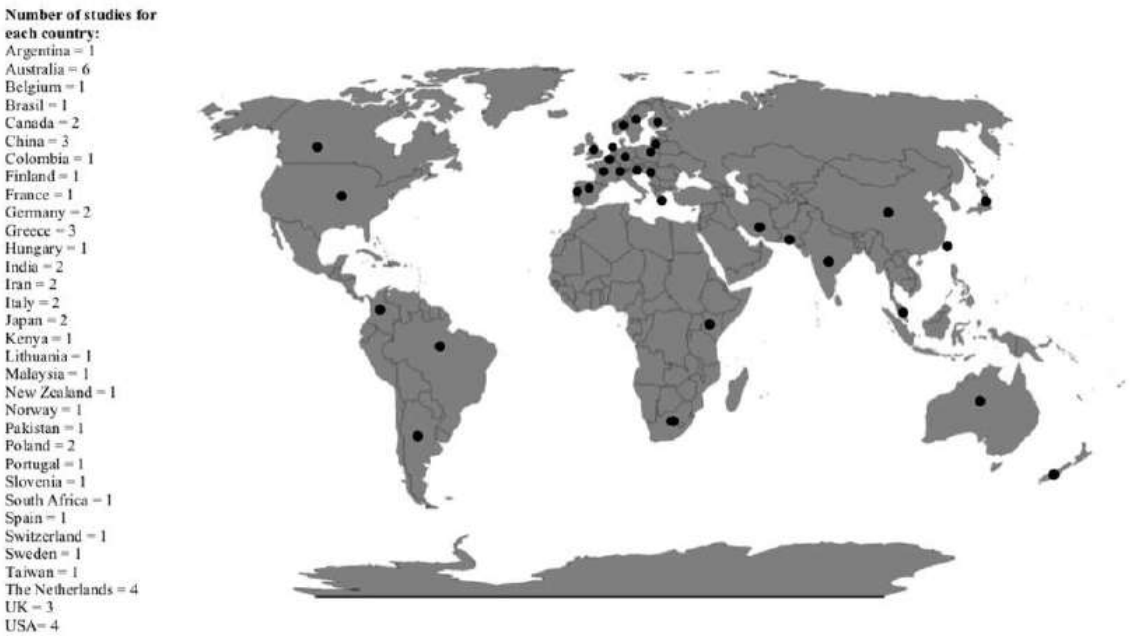


Figure 2. Countries' distribution of the 49 selected studies.



Study quality was reported in Supplemental Tables S1 and S2. The risk of bias was relatively high because (1) exclusion and inclusion criteria were not always clear; (2) some of the studies were not gender- and age-balanced; (3) data on subgroups were difficult to be extrapolated; (4) some studies did not clearly describe how the allocation was performed in cases and controls (in particular for the definition of skipping breakfast); (5) some of the studies could have a selective reporting bias (mis-reporting or under-reporting of breakfast habits due to methods used for the dietary assessment); (6) methods for the definition of skipping breakfast, and OW/OB were heterogeneous or not reported; (7) methods for the evaluation of nutrition assessment were heterogeneous; (8) confounding factors were lacking or not clearly reported in most of the studies.

Association of Skipping Breakfast with Overweight/Obesity

Thirty-seven out of 39 articles were selected for closer assessment of weight and then included in our analysis [11–47]. They are summarized in Table 1. Of the 37 selected papers, 32 were cross-sectional studies and 5 were longitudinal studies reporting cross-sectional data [11,13, 23, 29, 33]. Overall, data from a total of 285,626 children were reported. They came from 33 different countries (Figure 2). Children's age showed a wide range of variability ranging from 44 months to 21.2 years. Only one study included adolescents older than 18 years, and, although it partly failed to respect all the inclusion criteria, we did not exclude it because it was conducted in a school population; the age range depended on the repetition of grades and the population older than 18 years was only the 5.5% [12]. One of the studies included a sample of 9–15 years old, enrolled in 1985, with a follow-up of about 20 years (2004–2006) at ages 26–36 years [11]. One study included only preschool-aged children [13], 27 studies included only school-aged children and adolescents [1, 11, 14–24, 26–28, 31, 32, 38–41, 43–47], and 9 studies analyzed both preschoolaged and school-aged children and adolescents [25, 29, 30, 33–37, 42]. Most studies recorded data about breakfast skipping by food frequency questionnaires [11, 14, 16, 18, 20, 22, 27–29, 31–33, 38–40, 43–47], some on a recall-based methodology or by food diaries [13, 15, 21, 24, 30, 34, 36, 37, 42, 46], in others yes/no answers or unspecified methods were used [11, 12, 17, 19, 23, 25, 26, 35, 41,44]. Questionnaires were administered to the children/adolescents or to the parents in the case of youngsters. The subjects' weight and height were measured in most cases, whereas they were reported only in one study [16]. To define overweight and obesity, BMI z-scores or age- and sex-specific

cut-offs according to international criteria were used in most studies, whereas national-specific growth charts were referred to in 4 papers [25, 29, 39, 40]. The reported prevalence of breakfast skippers showed extremely wide variability, ranging from 0.7% to 74.7% according to the definition of breakfast skipping used. Seven studies did not report the prevalence of breakfast skippers [19, 25–27, 33, 35, 47]. Almost all the included studies concluded that skipping breakfast is associated with an increased risk or prevalence of OW/OB, in some cases with limitations: Mustaq et al. reported that skipping breakfast was associated with overweight only among girls [24], similarly to what reported by Januszek-Trzcia˛kowska et al. [28] with respect to obesity. Only 6 studies reported no association between anthropometric measures and the habit of skipping breakfast [25] for 3–10 years aged children [29, 36, 41, 42]. Overall, a positive association between skipping breakfast and OW/OB was found in 270,362 subjects (94.7%). In most studies the crude association between body fatness measures and skipping breakfast was considered, whereas in some others adjustments were performed for potential confounding factors, i.e., sex, age, ethnicity, smoking, dieting, physical activity, and parental education [12]; gender, family situation (single-parent family), ethnic background, education level and smoking [16]; eating fast food, and physical activity and sedentary lifestyle [24]. 3.2. Association of Skipping Breakfast with Metabolic and Nutritional Aspects The literature search identified 34 potentially relevant articles. After reviewing the titles and abstracts and the full-length articles, 6 articles were selected for closer assessment and then included in our analysis [11, 45–49]. They are summarized in Table 2. Of the 6 selected papers, 5 were cross-sectional studies and 1 was a longitudinal study reporting cross-sectional data [11, 45–49]. Overall, data from a total of 16,130 children were reported. They came from 6 different countries. Children's age showed a wide range of variability ranging from 6 years to 18 years. The longitudinal study included a sample of 9–15 years old, enrolled in 1985, with a follow-up of about 20 years (2004–2006) at ages 26–36 years [11]. Most studies recorded data about breakfast skipping by food frequency questionnaires [11, 45–47, 49], some on a recall-based methodology or by food diaries [46], in others yes/no answers or not specified methods were used [11, 48]. Questionnaires were administered to the children/adolescents or to the parents in the case of youngsters. In all studies, anthropometric data, such as weight, height, BMI, waist circumference, and blood pressure were collected [11, 45–49], while in one study the percentage of body fat was also reported [47]. Blood samples for the evaluation of glucose metabolism and lipid profile were collected [11, 45–49]. Metabolic syndrome (MetS) was described in 5

studies [45–49]. In some of the studies, MetS was defined using NCEP-ATP III criteria modified by Cruz and Goran for the pediatric age [45, 48] whereas in one study NCEP ATP III adult criteria [47]. Ho CY et al. used specific criteria from Cook [46], while Osawa H et al. those identified by national scientific societies and the Ministry of Health, Labour and Welfare in Japan [49]. One study included an indicator of dietary quality named “The Youth Healthy Eating Index for the United States of America (US-YHEI)” modified to YHEI-Taiwan [46]. YHEI-TW scores was calculated using the 24-hour dietary recall and FFQs, with higher scores indicating a better diet. The items included whole grains, vegetables, fruits, dairy, meat ratio, snack foods, sweetened beverages, multivitamins, fried foods outside the home, consumption of breakfast and, dinner with the family. The reported prevalence of breakfast skippers had a huge variability, ranging from 5.4% to 29.0% according to the definition of breakfast skipping used. Three studies did not report the prevalence of breakfast skippers [47–49]. Only 3 of the included studies [45–47] investigated the correlation between breakfast consumption and blood pressure, with a significant negative association in 2 of them [45, 46]. In 4 out of the 6 studies [11, 45–47], the correlation between breakfast consumption and lipid profile was evaluated. Subjects who skipped breakfast had lower HDL-cholesterol levels [45, 46], increased triglycerides [45], total and LDL-cholesterol [11,45]. Only 3 of the studies [11, 46, 47] investigated glucose metabolism. Two of them reported a higher insulin-resistance in who skipped breakfast [11, 47]. Almost all the studies concluded that skipping breakfast is associated with an increased risk or prevalence of MetS [45–49]. Ho CY et al. reported that children who consumed breakfast daily had higher intakes of saturated fat, cholesterol, vitamins A, B1, B2, calcium, phosphorus, magnesium, and potassium and better dietary quality in comparison with those who consumed breakfast <4 times per week. Diversely, breakfast skippers had the highest proportion (25.5%) of under-reporting energy intake than the controls [46].

Supplemental Table 1. Newcastle—Ottawa Quality Assessment Scale for studies on weight and skipping breakfast.

Reference (Author, year, n)	Selection	Comparability	Outcome/ Exposure
Smith KJ, 2010 (11)	***	*	*
Monzani A, 2013 (48)	***	*	*
Shafiee G, 2013 (45)	****	*	**
Ho CY, 2015 (46)	****	*	**
Osawa H, 2015 (49)	***	*	**
Marlatt KL, 2016 (47)	****	*	**

Supplemental Table 2. Newcastle—Ottawa Quality Assessment Scale for studies on metabolic variables and skipping breakfast.

Reference (Author, year, n)	Selection	Comparability	Outcome/ Exposure
Fayet-Moore 2016 (34)	***	*	*
Alsharairi 2016 (35)	***	*	*
Fayet-Moore 2017 (36)	***	*	*
Wijtzes 2016 (33)	***	*	**
Kupers 2014 (29)	***	*	**
Kuriyan 2012 (25)	***	*	*
Duncan 2008 (15)	***	*	*
Dubois 2008 (13)	***	*	**

Discussion

This systematic review confirms with more recent data and strengthens the evidence summarized in 2010 [6] indicating that children and adolescents who skip breakfast are at higher risk to be or become OW/OB. Skipping breakfast seems also associated with metabolic syndrome presence, but data are still anecdotal. The last decades are characterized by an increasing incidence of pediatric obesity, determined by the change of many lifestyle factors, including the diet. The determination of more risky

dietary habits has an impact on public health to plan prevention tailored programs. We analyzed the studies on children and adolescents published in the last ten years, not covered by the previous systematic review [6] with the purpose of analyzing trends and increasing knowledge. Unexpectedly, we failed to find in children and adolescents intervention studies or RCTs aiming to analyze the causative effect of skipping breakfast on OW/OB and related comorbidities. Firstly, we observed a prevalence of breakfast skippers ranging 1.3–74.7%, according to different definitions used for the skipping breakfast. However, most of the studies reported that at least 10–30% (mean ± SD: 16.0 ± 16.2%) of children and adolescents did not ever eat breakfast [11, 13–16, 18, 20–22, 24, 26–34, 36–40, 42–47]. The data are consistent because the studies covered 286,804 pediatric individuals living in Europe, the US, Australia, New Zealand, Asia, and Africa. They included also pre-school age individuals, although data on them are difficult to extrapolate [25, 29, 30, 33–37, 42]. On the other hand, an increasing trend in skipping breakfast from childhood to adolescence is seen [21, 22, 43, 44], as well as higher values in girls than in boys [28, 37, 42]. The prevalence is almost like to results reported in 2010 [6]. This means that, although epidemiological data on pediatric dietary habits are substantial, health plans to educate at a dietary day composed of 4–5 meals have been inefficacious, in particular in adolescents, or not portrayed yet. Furthermore, no studies investigated why breakfast was skipped, whilst some hypotheses have been widely discussed in literature including a lack of time, not feeling hungry in the morning, and weight concerns [50]. Secondly, some critical issues on methods should be underlined. In fact, the definition of skipping breakfast varied among all the studies, although the previous meta-analysis had already suggested improving this methodological topic in future studies to reach more significant results [6] and, recently, the American Heart Association proposed definitions to improve and make generalizable the research on this topic [51]. Most of the studies used a dummy (yes/no) or ordinal categories, based on the number of days without having a breakfast during the week [11, 12, 14–17, 19, 22–27, 29–39, 41–44, 48, 49]. On the other hand, some others used qualitative and unspecific categories (usually, often etc.) [13, 18, 20, 21, 28, 40, 45–47] making a comparison among the studies difficult. We can speculate that the metabolic balance reflects likely daily circadian rhythms influenced by prevalent meal schedules [52], but rigorous methods are needed to ascertain this hypothesis. Furthermore, the dietary history was collected with validated semi-quantitative or qualitative methods only in some studies [11, 13–16, 18, 20–22, 24, 27–40, 42–47 ,49]. The nutritional assessment is always difficult with varying degree of reliability and

needs carefully handling, even more in children when parents or caregivers should be engaged. The lack of accuracy may increase the already in itself high prevalence of mis-reporting and under-reporting biases, mainly in obese individuals [53]. Despite several methodological limitations, skipping breakfast in children and adolescents is associated with OW/OB in most of the studies [12–27, 30–35, 37–40, 43, 44]. Only 6 studies did not report an association or reported it only in specific categories of subjects [24, 28, 29, 36, 41, 42]. Even if these studies are comparable to the others with respect to the administration of questionnaires (self-reported completed by children or parents, or face-to-face interviews) and are not restricted to a specific country, several hypotheses could be considered. Firstly, most of them used a definition of skipping breakfast more general or referred only to the day before the study [24, 29, 36, 41]. Since Coulthard JD and colleagues used the UK chart published in 1995 to define OW or OB, the cut-offs different from WHO or recent IOTF growth charts could have significantly contributed [42]. An interesting point refers to the lack of association with OW/OB in infants compared to older children. Kupers LK et al. failed to observe a higher risk of OW/OB in children aged 2 or 5 years [29], and Kuriyan R et al. a higher waist circumference in those younger than 10 years, but the association became significant only in those older than 10 years [25]. Moreover, in 3 out of 6 studies without an association with the risk of OW/OB breakfast skippers are mainly adolescent girls older than 14 years [36, 41, 42]. Coulthard JD and colleagues observed that most of the breakfast skippers were adolescent girls and that a higher proportion of girls in the 11-18-year-old age group stated that they were currently dieting than boys [42]. Moreover, 77.6% of the cohort published by Zalewska M et al. was composed of girls of 18 years old beyond the dietary assessment with a non-standard questionnaire [41]. Similarly, 61% of breakfast skippers of the studies of Fayet-Moore F et al. were again in the 14–18-year-old group [36]. On the other hand, two studies reported a higher risk of OW/OB only in females who skipped breakfast, but these populations are younger (5–12 years) [24, 28] than the latter [36, 41, 42]. These outwardly contrasting data could be secondary to the fact that the time of puberty is characterized by the starting peak of OW/OB incidence and females enter before males in this period. Even so, in all, these findings suggest that in studies focusing mainly on infants or adolescents more attention to subgroups and related age-specific diseases is needed. The weight-control behaviors and puberty should be carefully considered, and this group should be analyzed separately. It is known that chronotype varies with age and environmental factors [52, 54–56]. We can hypothesize that desynchronization of circadian rhythms

does not still occur in infants and it is not so hitting on weight balance in infants. Conversely, in very young children the reason of skipping breakfast could vary including breastfeeding during the night, loss of appetite in a failure to thrive due to several diseases (malabsorption, sleep apnea etc.). On the other hand, none of these studies can explain why skipping breakfast is associated with OW/OB. Some authors suggest that breakfast skipping is affected by collinearity with confounding factors such as sleep duration and quality, circadian rhythms with more eating in the evening, length of night fasting, and lower physical activity levels [6, 42, 52, 55, 56]. Some of the selected studies corrected for confounders [12, 16, 24], butmore tailored study designs are needed. This suggestion is strengthened if we observed that Fayet-Moore and colleagues reported an association in a study [34] and failed to confirm it in another one [36]. The authors used in both the occasions data fromthe National Nutrition and Physical Activity Survey, conducted in 2007 [34], and 2011–2012 [36], respectively. Because the period between the two surveys is relatively short, not captured confounders seem to be the more realistic hypothesis. All the aspects discussed (growth charts, the definition of skipping breakfast, age-dependent responses, gender, and confounding factors) are critical issues for the planning of future intervention trials.

As already anticipated, our review is not able to clarify if the OW/OB phenotype is secondary to a higher energy intake during the following hours in children who skipped breakfast. The data in the literature on this topic are few and controversial [6,42,52,57]. However, in the paper we selected, it seems that children and adolescents eating breakfast have a better nutritional profile in terms of micro- and macronutrients [13, 36, 42, 44, 46] and is in line with data on adolescents consuming 5 meals including breakfast [58]. How this aspect impacts on metabolism is still a matter of investigation.

Lastly, we aimed to evaluate if children and adolescents who were skippers had different metabolic phenotype. Unexpectedly, the published papers are few, counting only 6 studies, but enclosed about 16,000 children. This is surprising if we consider the long-lasting impact of OW/OB on metabolic and cardiovascular diseases. All the studies included reported that breakfast skippers had a worse lipid profile and/or a higher prevalence of MetS [11,45–49]. Two studies also reported higher insulin [11,47] and another higher blood pressure levels [46] than those that ate breakfast regularly. All these results mirror mournfully those in adults [51]. The findings on higher cholesterol, triglycerides, and blood pressure levels have been mainly linked to higher insulin resistance in the morning [11,51]. Regarding cholesterol, higher fasting insulin levels may trigger higher hydroxyl methyl glutaryl Co-A (HMG-CoA) reductase

expression, resulting in increased circulating concentrations [11]. On the other hand, because the nutrition quality of breakfast skippers seems worse, the role of higher intake of saturated fats, simple carbohydrates and salt in the other meals is another plausible explanation. The increasing risk of MetS is likely associated again to higher insulin-resistance in breakfast skippers, but also to increased fat oxidation and low-grade inflammation [59, 60]. Moreover, it could be partly mediated by a higher BMI, as recently shown in a meta-analysis of prospective cohorts for the risk of type 2 diabetes in adults [61]. On the other hand, Mets is strictly associated with other unhealthy lifestyles such as poor physical activity. However, it has been demonstrated that skipping breakfast adversely modulates clock and clock-controlled gene expression resulting in increased postprandial glycemic response in both healthy individuals and individuals with diabetes and MetS [62]. Some clock genes are associated also with lipid metabolism and the development of MetS [51], making the picture more colored and intriguing. Although findings from observational studies cannot establish causality, more prospective and intervention studies from childhood to adulthood with the assessment of clinical end-points, including cardiovascular and metabolic diseases could provide insight into the associations we reviewed. In adults, contrary to observational data, skipping breakfast has not been found to be causal for obesity, metabolic alterations or hungry perception under the conditions so far examined in RCTs [57, 63, 64]. RCTs are needed to test the association in children and adolescents and confirm our recommendations. Moreover, authors should pay attention to all the confounders suggested and skipping breakfast, as well as nutritional assessment, should be defined and evaluated by using definitions and tools with an international agreement, as suggested by the American Heart Association [51]. The better investigation of all these aspects could help in validating “skipping breakfast” as a marker of risk of OW/OB. The “chrono-nutrition” has been investigated within the context of obesity since studies have shown that eating at the “wrong” time of day can induce weight gain, despite a similar caloric intake [55]. In this context, the role of skipping meals, in particular, breakfast that stops the night fasting, caught the eyes of researchers. On the other hand, many other players have been still poorly investigated, and in the future could explain some of the controversial results. Among the others, also the nutrient content of the daily meals could have an impact on the desynchronization of circadian rhythms implicated in the obesity increase [52]. Moreover, food timing seems to have a higher heritability, also for breakfast [65]. The length of night fasting, the presence of time-restricted feeding, the composition of

nutrients of the last meal before sleeping, the chronotype of individuals, the food timing behaviors of parents and sibling should be all investigated with respect to breakfast skipping or not in further studies to reduce residual confounders beyond sleeping habits, daily food quality and physical activity. Since high plasticity and reprogramming of most metabolic patterns in pediatric age has been described [66], filling this gap is an unmet need. With these limitations, our study provides data supporting skipping breakfast as a potential “marker” of lifestyle behaviors (yet to be elucidated) in children and adolescents that promote OW/OB and metabolic diseases. Additional studies would be helpful to standardize the definition and assessment method for breakfast skipping in order to establish the best practice for using it as a tool for assessing obesity risk.

3.1.2.i. References

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3.1.3. Mediterranean diet, nutrition transition, and cardiovascular risk factor in children and adolescents

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Abstract

Nutrition is one of the main factors influencing growth, maturation and the risk of diseases. In adults, the Mediterranean-style diet (MD) has been shown to decrease the risk of non-communicable diseases (NCDs), including cancers. Studies on MD in the paediatric age are relatively few. Behaviour, social and physiological players influencing eating habits change very quickly in children and adolescents. Contemporary, the phenomenon of nutrition transition has been occurred. MD or traditional diets has been progressively replaced by diets rich in simple sugars and saturated fats. Thus, adherence to the MD is poor in Mediterranean countries in younger populations. Unhealthy food choices impact on cardiometabolic health of children and adolescents. In this scenario, this chapter reviews factors influencing eating habits from infancy to adolescence, the strategies adopted to improve food choices, the adherence to the MD in the paediatric age, and the role of MD in prevention and development of metabolic diseases and obesity.

Introduction

Development is a continuous process that does not stop from birth. Nutrition is one of the main factors influencing growth, maturation and the risk of diseases, mainly non-communicable diseases (NCDs) [1-9]. Nutrition acts in several manners, and apart from the storing of nutrients, it plays directly the modulation of metabolic functions, as well as indirectly through epigenetic mechanisms. Growth is dynamic and complex, and some critical windows have been described, as the first 1,000 days, the adiposity rebound period, puberty, and the transition to young adulthood. However, the role of the interplay among nutrients, lifestyle, and metabolic mechanisms is largely unexplored and exciting. The most studied food in the paediatric age is milk, by comparing the effects of breastfeeding respect to formula feeding on growth and the risk of developing several diseases, including obesity in late childhood [6-9]. Diversely, the style of weaning and the consolidation of later nutrition habits did not attract the same scientific attention since the last years. In adults, the Mediterranean-style diet (MD) has been shown to decrease the risk of NCDs, including cancers. These data derive from efforts to characterize also clinical tools aiming to measure and quantify as accurately as possible the adherence to MD [1-5]. The validation of similar tools, mainly questionnaires, in children took up to some years later [10]. However, the picture is quite complex and motley than that described in adults, because environmental, behaviour, and physiological players change very quickly in children and adolescents.

Contemporary, MD or similar style-diets have been progressively replaced by diets rich in simple sugars and saturated fats, due to ultra-processed foods, mainly in the paediatric age, following changes in family and population lifestyle habits [11]. In this view, this chapter aims to describe factors and educational models influencing food habits in the paediatric age, connecting them with data on adherence to MD and the risk of later NCDs.

Factors influencing food choices in childhood

Childhood is an important stage in which food preferences and eating habits take shape also influencing style diet later in life. If understanding how children's food choices develop is crucial for health, there is still limited knowledge of modifiable food choices in children. Deciphering which are the main triggers driving children and adolescents to make food choices can inform to improve eating habits and promote healthy behaviours among them. During childhood, food choice is mostly determined by the sensory perception and liking of foods, mainly those sweet [12]. However, growing evidence suggests that foods preferred by mothers during pregnancy influence the later acceptance of solid foods in their infants. Many flavours of the maternal diet are also present in the amniotic fluid. Because taste and smell are already functional during foetal life, the first experiences with flavours occur before the birth [13]. Undoubtedly, parents are reference figures playing the role of health promoters and educators for sons. The mother is perceived as an important model for food choice very soon. Parental behaviours correlate with food consumption behaviours of their children, either in term of promotion and prevention [14]. Parental modelling affects both healthy and unhealthy food habits, including also fruits, vegetables, and sugar-sweetened beverage consumption. Since children develop food preferences through continuous exposure to foods, the availability of food at home resulted in a predictor of children's food preferences. Moreover, the efficacy of some parenting practices seems to be correlated to the type of foods and the age of the child. For healthy foods, education might be more effective, while for unhealthy foods, restrictive guidance might be more successful, mainly in children older than 7 years. Diversely, in children younger than 6 years, rewarding with verbal praise could better promote healthy eating and prevent unhealthy eating. Furthermore, using food as a reward was more elated to unhealthy eating habits among children, likely since reward foods are often unhealthy, such as snacks rich in sugars and saturated fats [14]. Moreover, the IDEFICS study recently demonstrated that children of parents who trust advertisements and not avoid additives

had a high consumption frequency of sweet, fatty, processed foods and beverages, reaching a low healthy diet adherence score. These habits were associated with a low education background, suggesting also a low socioeconomic status [15]. Besides, children use gender schemas also to make food choices. Thus, children at the peak age of cognitive gender rigidity (age 3-6 years) are more likely to choose gender-consistent packaging than gender-inconsistent packaging (pink vs blue coloured packaging as an example) when the food inside is the same, suggesting again a role of trust advertisements [16]. Moreover, also popular cartoon characters and brand mascots have been shown to modify children's food choices and eating behaviours [17-19]. Furthermore, in children older than 8 years, friends' preferences start influencing nutrition behaviours and food choice of their peers, making the social environment more complex [19]. This is more pronounced in adolescents [20]. If trust advertisements have been already identified as influencer factors, also TV cooking shows, including the children' version, have a role being very popular even among the youngest. Food portions shown on TV may affect the amount of food that children select by triggering food cravings, mainly during adolescence. For example, it seems that the amount of sugar sprinkled on pancakes was influenced by observing the food cues in these TV shows even beyond the effects of hunger [21]. Children's food experience at school is also important to develop healthy eating habits. So, the school environment could be an optimal setting in which to encourage, from the early years, to adopt healthy eating habits [22].

Healthy food education models

As described above, a multi-faced environment exerts a strong influence on children' and adolescents' food choices. Successful strategies could be applied in school settings where children spend much of their time from early childhood and can also experience and learn healthy habits. A wide variety of school-based programs on MD or other healthy diets have been implemented aiming to increase fruits and vegetable intake among primary school-aged children. However, a recent meta-analysis of school-based obesity prevention programs found that they are mildly effective, and results depend on the duration, the presence of environmental supports, and local scenarios [22, 23]. Interventions that provide access to or education on fruits and vegetables do not produce sustained lifestyle changes that are required to strongly impact public health [24]. Despite these review findings, multi-component programs, based on the engagement of children to change their eating behaviours, were more

effective than single-component studies, mainly focused on free or subsidized fruit- and vegetable-distribution [24]. However, multi-component interventions may often require the purchase of materials (for example, stickers, videos, tangible rewards) and always some amount of teachers' work, being cost- and time-consuming. Thus, other intervention strategies could be approached, including gamification which is based on a social learning theory [25]. Their goal is to impact on children's dietary habits with fun methods that also minimize direct costs [26, 27]. As an example, in the game-based intervention designed by Jones BA et al. schoolchildren play together a game in which, by consuming fruits and vegetables, they help hero characters to search and capture a gang gaining virtual rewards. The study was successful, and fruit and vegetable intake increased by 66% and 44% also at home, respectively, above baseline levels [27]. These results are encouraging for further long-term studies in different settings [26]. In line with this strategy and to educate children and young people about the benefits of a healthy MD diet, an educational project known as "Mediterranean diet and enhancement of traditional foodstuff - MedDiet" has been founded by the European Union in the framework of the ENPI CBC Mediterranean Sea Basin Programme [28]. Firstly, a training activity has been developed to allow 120 directors and 1,200 teachers to acquire knowledge and competences concerning how to promote nutrition education initiatives on MD. The program includes many tools to distribute to schoolchildren. One of these is a game known as "The Mediterranean Goose". By the cooperation of trained directors and teachers, about 120 pilot projects have been implemented in primary and secondary schools involving almost 5,000 students, and epidemiological results are expected. Therefore, the MedDiet project also involves local authorities as key players in stimulating strategies and policies promoting knowledge, skills, and habits aiming to increase the adherence to the MD [28]. Interactive cooking and food activities have been shown to have a valuable impact on children's food awareness, eating and cooking enjoyment, as well as food preferences. They provide the opportunity to gain food skills and feel greater ownership of food. A recent review concluded that cooking in education programs hosted in the community or school settings may promote positive changes in children's food-related preferences, attitudes, and behaviours. However, because study results varied widely, determining best practices is still difficult [29]. A healthy school environment helps students in making more healthy food decisions, encouraging many European countries to take actions in this regard. For example, English chef Jamie Oliver struggled against unhealthy diets and poor cooking habits in United Kingdom schools since 2005 when he launched his *"Feed Me Better"*

campaign. Since 2006, junk food is forbidden in British schools and new law school food standards have been brought in [30]. Regulations related to school lunches are also present in Portugal [31], whereas automatic vending machines and energy drinks are forbidden in France since 2005 and 2008, respectively [31]; therefore, the sale in schools of food and drinks with high amounts of saturated fat, salt or sugar is banned in Spain [32]. Despite these findings, substantial evidence whether healthy eating habits, including those on MD, are maintained over time if acquired in the paediatric age.

MD Adherence in school children and adolescents

Adherence to the MD is poor in children and adolescents living in the Mediterranean countries. With the urbanization of people living in the Mediterranean area, children and adolescents are deviating to a Western diet rich in saturated fat, refined grains, simple carbohydrates, and processed foods [33]. This phenomenon is called nutrition transition or “Westernization” of the diet and is one of the players implicated in the high prevalence of overweight and obesity in countries supposed to adopt a traditional MD [4, 11]. This process is particularly evident among the younger generations. Modernization of the society implies several unhealthy lifestyle habits, not just limited to the modification of food preferences toward “junk” foods, but also relative to sedentary activities (computer and TV use), leading to an overall imbalance between energy intake and expenditure [34]. Data on MD adherence in the first two decades of life have mainly been provided by studies using the KIDMED score. It was developed in 2004 by Serra-Majem et al. and is a nutritional index validated in several languages; it evaluates the adherence to MD and the quality of the diet in children and adolescents [10]. The rationale behind the KIDMED index relied on the principles characterizing the Mediterranean dietary pattern. The index ranged from 0 to 12 points based on a 16-question test. Questions indicating food components, not in line with the MD (fast-food, skip breakfast, sweets, baked goods) were assigned a value of −1, meanwhile those indicating foods that characterize MD (fruit and vegetable, fish, pulses, pasta, cereals, nuts, olive oil, and dairy products) were assigned + 1. The sums obtained were classified into three levels: (1) > 7, optimal MD; (2) 4–7, improvement needed to adjust intake to MD patterns; and (3) < 4, very low diet quality. In the systematic review by Iaccarino Idelson et al. the adherence to the MD, as estimated by KIDMED index, widely varied in the Mediterranean countries for both children and adolescents [35].
Good adherence varies in the literature from 4.3% in Greek 10–12-year-old adolescents to 53.9% in Spanish children. Most of the studies conducted in southern

European countries reported that about half of pediatric individuals have an average adherence, while nearly half may have poor adherence also in the southern countries close to the Mediterranean Sea [35-37]. Only one study [38] reported higher levels of adherence to the MD in Italian children compared with other countries, as well as with other studies conducted in Italy [35-37]. However, depending on the regional (north versus south) and environmental (urban versus rural) areas of investigation, results may vary with a great extent and the general trend is shifting away from traditional dietary patterns. The “identification and prevention of dietary- and lifestyle-induced health effects in children and infants (IDEFICS)” study, a population-based cohort of 16,228 children aged 2–9 years, was conducted in eight European countries (Sweden, Germany, Hungary, Italy, Cyprus, Spain, Belgium, and Estonia) in 2007–2008 [39]. During two examinations, parents completed a self-administered questionnaire to assess behavioral and sociodemographic factors and a computer-based 24 h dietary recall, namely, Self-Administered Children and Infant Nutrition Assessment (SACINA). The highest adherence to the MD was found in Italy followed by central and northern European countries. Spain and Cyprus reported the lowest level of adherence. A low prevalence of high consumers of vegetables and legumes among Italian children, and high consumers of both dairy and meat products among Sweden children were found. Italy and Cyprus were also characterized by a high prevalence of children consuming high unsaturated: saturated fat ratio, whereas the high consumption of cereal grains and potatoes characterized both the Swedish and the Estonian diets [39]. There is no clear and immediate explanation for the wide variations in the MD adherence even within the same country, which might only partially be due to some factors such as gender, age, socioeconomic status, and urbanization. Consistent findings from the literature have indicated that in modern Western societies women often tend to have better dietary habits. On the contrary, recent reviews on MD adherence in children and adolescents reported no difference between genders in most studies [35, 40]. According to a recent Italian study, a lower prevalence of good adherence was found in students attending primary than secondary school, suggesting that younger children are more subjected to unhealthy eating habits and the attendance at a primary school is the only significant risk factor related to a poor MD adherence [37]. Interestingly, primary schoolchildren consumed a more Westernized diet, skipping breakfast, eating several times per week at fast-food restaurants, consuming commercial baked goods, and sweets [37]. This phenomenon could be boosted by geographical reasons [41]. This result is in contrast with most of the data that reported a negative trend in MD

adherence with age [35, 36]. However, paying more attention to single studies, in the systematic review by Iaccarino Idelson et al unfavorable trend with age was observed only in 9 out of 24 studies, thus most studies did not support the idea that MD adherence extensively changes with age [35]. Moreover, a mixed dietary behavior in children and adolescents of other ethnic origins is reported in the literature. The latter maintained a higher intake of more traditional foods, such as fish, cereals or grain for breakfast, and yogurts and/or some cheese, suggesting more home-made foods in their family environment. On the other hand, contemporarily, they frequently ate at fast-food restaurants, skipped breakfast, consumed commercial baked goods for breakfast, or sweets and candy several times every day. These findings well portray the nutrition transition described above [11, 36, 37]. Overeating, eating anything or disliked foods, and eating at friends' home were all identified as strategies to cope with food insecurity [42]. Frequent consumption of fast food/junk calorie-dense foods have been reported in several developing countries [43], and these behaviors could be replicated when low-income families move abroad, and less control over the youngest generations occurs for several reasons [36]. Overall, the social and cultural environment makes more complex the derangement from MD reference models.

Mediterranean-style diet and cardiovascular risk factors in children and adolescents

In adults, MD has been associated with a low prevalence and/or incidence of several chronic non-communicable diseases, including type 2 diabetes, hypertension, cardiovascular diseases, and cancers. All these diseases have been also associated with overweight. The beneficial effects of MD are attributed to its composition rich in vegetables, fruits, legumes, whole cereals, fish, nuts, and extra-virgin olive oil; most of them are sources of fibers and antioxidants. The intake of sweets and trans fatty acids is low [1-4]. Evidence on the health effects of MD has been recently strengthened by findings of the Global Burden Diseases Study. In 2017, 11 million deaths and 255 million disability-adjusted life-years were attributable to dietary risk factors of a suboptimal diet, mainly high intake of sodium, and low intake of whole grains, fruits, nuts and seeds, vegetables and omega-3 fatty acids [5]. Each of the leading dietary factors accounted for more than 2% of global deaths [5], suggesting that synergic interaction among foods but also specific foods have protective or detrimental effects.
Despite growing evidence of the protective effect of MD on health in adulthood, findings in children and adolescents are quite anecdotal or limited. Although MD has been

associated with the prevention of obesity in adults, in children and adolescents, the adherence to the MD did not predict the weight status in most of the studies conducted in European countries [35-37, 44]. These findings suggest that low physical activity and sleep quality, family and peers, and other drivers of food behaviours discussed above all influence the risk of obesity in this age. However, some unhealthy eating habits far away from MD have been related to the obesity risk. It seems that dense energy foods rich in simple carbohydrates and saturated fats have a leading role, being associated with skipping breakfast, frequent meals at fast-food restaurants, daily intake of commercial baked goods or pastries for breakfast, ultra-processed foods, sugar-sweetened drinks, and sweets snacking [35, 45-47]. This is even more true for youngest children and ethnic background associated with a low-income status [35-37]. If data on unhealthy food choice are rather clear, data on EVOO are still debated with studies indicating a high intake as protective and others as not significant or unfavourable for the weight status in adulthood [37, 48-50]. If studies on weight are few, even fewer findings are present on other cardiovascular risk factors. Some results derive from the HELENA study. The Consortium showed that low adherence to MD is associated in adolescents with subtle inflammation and stress, evaluating circulating biomarkers as interleukins and cortisol [51, 52]. Specific foods typical or not of the MD were associated with anti- and pro-inflammatory mediators [53], and these findings were confirmed also in the IDEFICS study on children younger than 10 years [54]. Furthermore, obese adolescents more adherent to the MD had a lower risk to have a metabolically unhealthy phenotype and the metabolic syndrome cluster [47, 55, 56]. In agreement with these data, children adherent to MD presented a lower risk to have liver steatosis confirmed by biopsies, insulin resistance, subtle inflammation, and high blood pressure levels [57]. This seems true also in children with other inflammatory diseases, like asthma or diabetes mellitus [58-59]. Very recently a Chilean study on more than 1,100 children aged 8-12 years, demonstrated that children with high adherence to the MD have lower systolic, diastolic and mean arterial blood pressure than those with low-medium adherence, although this association is mostly dependent on age, sex, BMI, and handgrip strength, a measure of physical activity [60]. The relationship between the adherence to MD and the level of physical activity was suggested by another study in about 2,200 Brazilian adolescents. The high cardiovascular risk due to low adherence to the MD was blunted by high cardiorespiratory and muscular fitness [44]. Furthermore, also the adherence of the mother during pregnancy seems to reduce some cardiovascular risk factors in the

offspring in the mid-childhood, strengthening the concept that diet should be a leading factor for prevention considering its epigenetic role [7-9, 61].

Conclusions

In conclusion, adherence to the MD is almost poor in European countries. Both MD and physical activity are leading health factors in the paediatric age and programs aiming to improve both could guarantee the best cardiometabolic health. More efforts are needed to design strategies to improve, from early childhood, good nutritional habits with successful long-term impact on eating habits. However, nutrition education should work with interdisciplinary approaches and health policy aiming to modify age-dependent social, environmental and cultural factors. Studies on the later risk of NCDs starting from the paediatric age in wide cohorts geographically representative and accurate in the characterization of the dietary intake are warranted.

3.1.3.i. References

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4. THE MEDITERRANEAN STYLE-DIET, OBESITY, AND MICROBIOTA IN PEDIATRICS

4.1. INTRODUCTION

4.1.1. Obesity and treatments: the gamification

As previously described, obese children are more prone to become obese adults, with an enhanced risk of obesity-related complications and cardiovascular diseases [1-4]. Alongside efforts in public health and policy to reverse the childhood obesity epidemic, medical providers seek to play effective roles in its treatment [5]. Limited interventional studies with effective long-term maintenance of weight loss in children are available in the literature [6]. Certainly, family-based lifestyle interventions, including dietary modifications and increased physical activity, are the cornerstone of the treatment of childhood obesity. However, lifestyle interventions resulted only in a modest effect on weight loss, particularly in children with severe obesity. Moreover, there is limited information on the long-term efficacy and safety of pharmacological treatments for weight loss in children. Bariatric surgery seems to be effective in decreasing excess weight and improving comorbidities in adolescents with severe obesity. However, there are limited data on the long-term also on this treatment choice [7].

Education to a healthy lifestyle is the fist undeniable step, and new approaches are needed to strenghten the results obtained until now. One of them, could be the gamification. Gamification is defined in the Oxford English Dictionary as the application of game design elements to traditionally non-game contexts. The appeal of gamification is that it “promises to make the hardstuff in life fun” [8]. Accordingly, it is being used in medicine and public health as a powerful tool in medical education to motivate behavior changes. Clinicians should have sometimes to convince their patients to behave in healthy lifestyle choices, for example to perform regular exercise, or eat a healthy diet. Normally, many patients receive these indications as an obligation or a duty. In this context, reframing the same behaviors as fun and challenging might be more motivating [9]. Creating ludic interventions that are fun and enjoyable is still a challenge, although the game is a natural way to learn and grow in childhood and adolescence. The intervention in gamified elements is generally viewed positively and deemed acceptable. Results suggest that gamification may have an important role in encouraging children and adolescents to be more engaged in modifying their lifestyle [10]. Knowledge, attitudes, behaviours, and skills developed through effective health programs may result in a better quality of life and empower children to make correct choices to promote the health of the individual, the family, and the community [11].

Although gamification is increasingly used in interventions, the efficacy of them in improving nutrition habits in healthy and/or obese children have not been proven yet and remains sparse [9]. A wide variety of school-based programs have been implemented with the goal of increasing fruits and vegetables consumption among elementary school aged children, reaching promising results [12- 14]. In this context, the MedDiet Project has been developed [15], as a strategic project funded by European Union, characterized by severaltools focused on Mediterranean-style Diet directed to several stakeholders, including schools. Promising results will also describe in the study protocol by Kang J. et al. [16] who proposed an exercise intervention model with gamification and social incentives theory through a mobile technology, in a sample of overweight and obese children who have at least one marker of metabolic syndrome. Gamification could be an opportunity for clinicians to promote an healthy lifestyle as an engaging, fulfilling, and fun activity, although evaluation of its effectiveness lacks and further studies are mandatory [9].

4.1.2. The Mediterranean Diet, obesity and microbiota

In 406 B.C., Hippocrates, the father of medicine, stated: *"Αφήστε το φαγητό να είναι το φάρμακο και το φάρμακό σας να είναι η τροφή σας" – "Let food be your medicine and medicine be your food"*. About twenty centuries after, the Mediterranean style-Diet was inscribed in 2013 on the Representative List of the Intangible Cultural Heritage of Humanity (http://www.unesco.org/culture/ich/RL/00884). The proof of the indissoluble link between diet and health, advocated by Hippocrates in ancient times, comes from the current, striking evidence in the literature that specific dietary patterns can modulate risk factors for non communicable chronic diseases, such as diabetes, arterial hypertension and hyperlipidemia [17].

One of the explanations could reside in the gut microbiota function. Our body is colonized by a huge number of micro-organisms with an amount of more than 1 kg of weight. Most of them live in our gut, constituting the so-called gut microbiota [18]. The human gastrointestinal tract harbours more than 100,000 billion microorganisms, representing 10–100 times the number of human cells. Bacteria are classified according to phyla, classes, orders, families, genera, and species. Only a few phyla are represented in the gut, accounting for more than 160 species [19]. As gut microbiota and human beings are in a symbiotic relationship, the hypothesis of a combined "super-organism" has been recently developed [20]. The gut microbiota seems to play a relevant role, mainly being involved in the development and growth of immunity and the

regulation of several fundamental metabolic pathways [21-23]. There is no singular optimal gut microbiota composition since it is different for everyone. Indeed, the human gut microbiota is characterized by an inter-individual variability due to genetic and environmental factors, including delivery method, breastfeeding, age, the use of prebiotics and/or probiotics, and antibiotics. Therefore, among the environmental ones, dietary habits play a key role in the modulation of gut microbiota composition. Specific changes in the composition of gut microbiota have been demonstrated among subjects according to a different dietary intake. These, and possibly other, factors can lead to an imbalance in the composition and function of the gut microbiota observed in childhood obesity and are hypothesized to be involved in many phases of the obesity pathogenesis [54 – Figure 3].

The exact mechanisms of action are not yet fully understood, and various hypotheses have proposed to explain how the gut microbiota may contribute to host obesity. Most of the results come from pre-clinical studies. Furthermore, several human studies that replicate these findings are increasing, although in-depth studies are still lacking [24]. Also, data in the literature concerning microbiota and obesity in childhood and adolescence are lacking and sometimes contrasting. The principal mechanisms proposed through which the gut microbiota could affect the host obesity, concern the energy metabolism, the integrity of the gut barrier, insulin resistance and the metabolism of bile acids [25 – Figure 4].

Figure 3. Factors that may modify infant gut microbiota (A); mechanisms in microbially-induced obesity (B) [24].

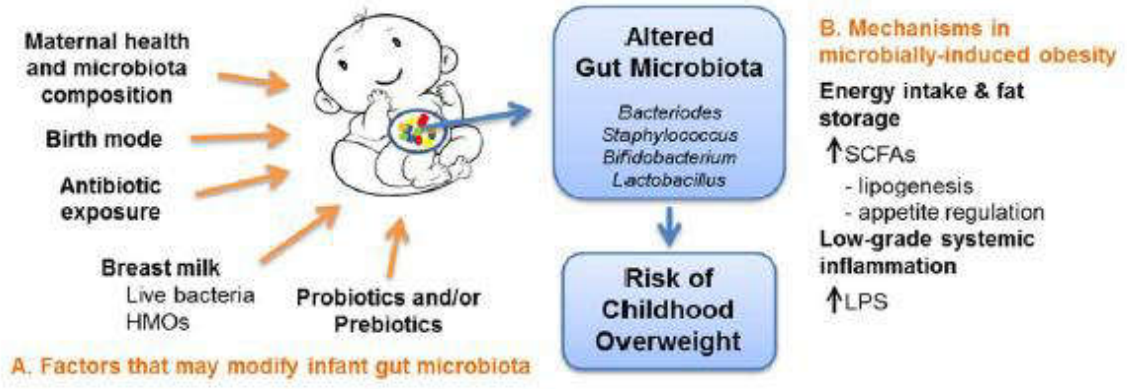
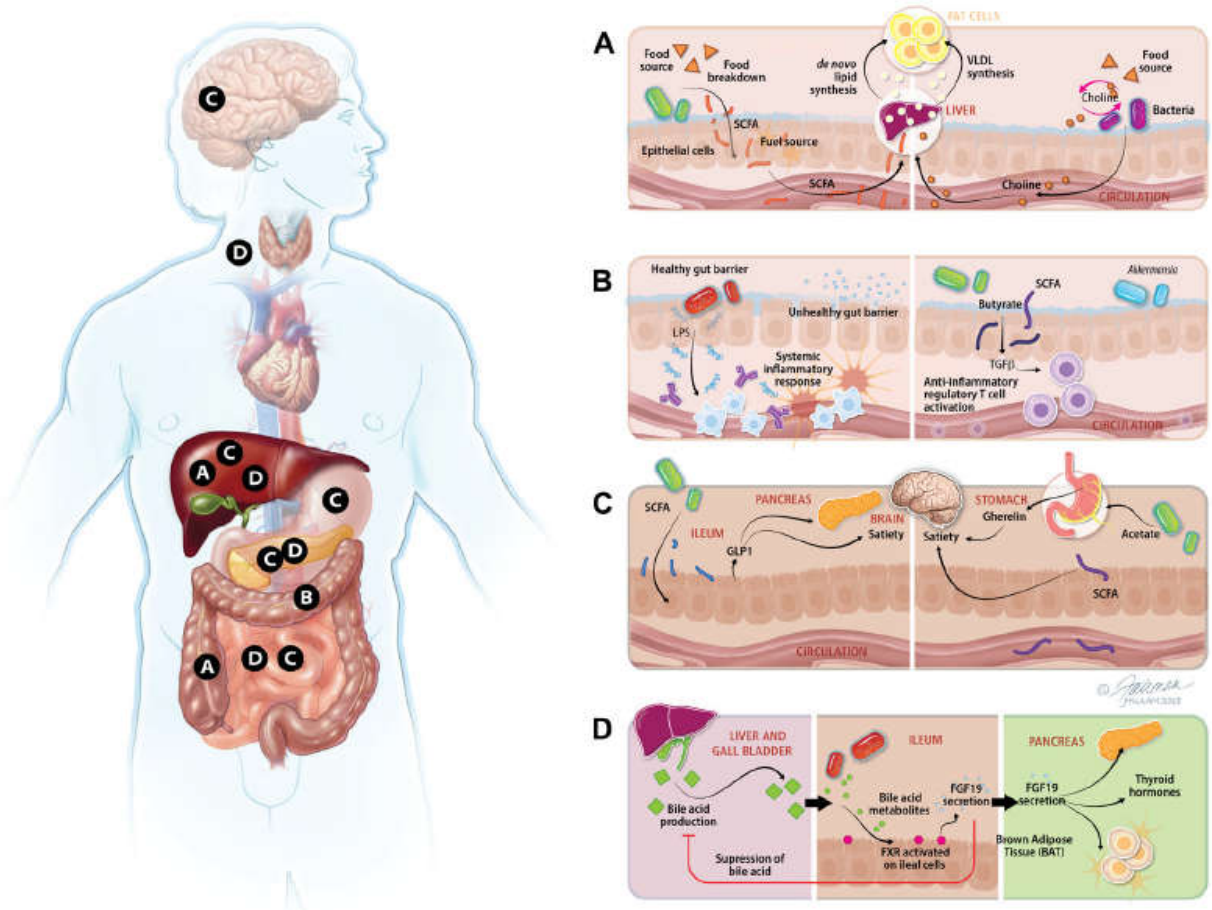


Figure 4. Proposed mechanisms through which the gut microbiota affects the host obesity: (A) energy metabolism, (B) gut barrier health, (C) insulin resistance, and (D) bile acid metabolism [25].

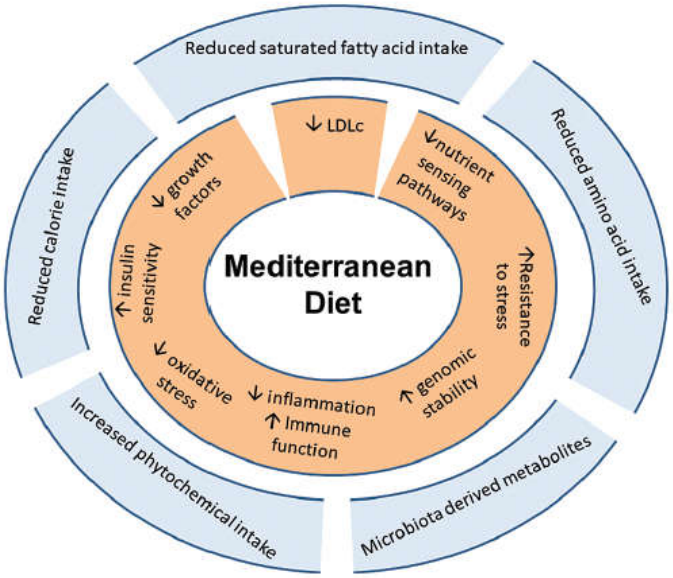


A rich and diverse microbial community leads to a well-balanced and healthy gut microbiota composition [26]. Certainly, our dietary habits are the result of a specific mixture of micro and macronutrient amounts, as well as carbohydrates, proteins and fats. Carbohydrates can be categorized into digestible and indigestible substrates. Digestible carbohydrates such as glucose, fructose, and galactose are enzymatically degraded in the small intestine and rapidly released as glucose in the bloodstream. Conversely, indigestible carbohydrates, also called “dietary fibres” are resistant to digestion in the small intestine and reach the large intestine. Moreover, dietary fibers may be categorized according to their fermentability (fermentable or non-fermentable) in the colon [27]. Fermentable dietary fibres undergo saccharolytic fermentation, essentially under the action of gut bacteria, which in turn yield monosaccharides, short-chain fatty acids - SCFAs (i.e., butyrate (15%), acetate (60%), and propionate (25%)) and gases (i.e., methane and carbon dioxide). Furthermore, acetate and propionate are used as substrates for lipid and cholesterol metabolism, and glucose metabolism, respectively. On the other hand, butyrate plays a crucial role in maintaining tissue barrier function and regulating gene expression and immunoregulation. SCFAs are also involved in the colonic homeostasis and can exert other beneficial effects, acting as anti-cancer agents and increasing transit time and satiety by activating hormones such as glucagon-like peptide 1, peptide YY, and leptin via their endogenous receptors,

throughout the well-known “gut–brain axis” [26]. The effects of proteins on gut microbiota composition vary according to the protein type (animal or vegetal based). The proteolytic fermentation produces less SCFAs than saccharolytic fermentation, but also branched-chain fatty acids BCFAs (e.g., isobutyrate, 2-methyl butyrate, isovalerate) and potentially toxic substrates such as ammonia, the amines of which include nitrosamines and trimethylamine N-oxide (TMAO), compound known for its proatherogenic potential. Dietary fatty acids can be divided into saturated (SFAs), monounsaturated (MUFAs) and polyunsaturated fatty acids (PUFAs) according to the presence of double bonds between carbon molecules. The consumption of a high-SFA diet can stimulate the production of sulfate-reducing bacteria (SRB), causing a defective mucus layer and increasing gut inflammation [28]. PUFAs are subdivided into omega-3 PUFAs (including linolenic acid) and omega-6 PUFAs (including linoleic acid). PUFAs are also called “essential fatty acids” since they cannot be synthesized by the human body and need to be obtained from the diet. Omega-3 PUFAs can exert a positive action by restoring a healthy microbiota composition and increasing the production of anti-inflammatory compounds throughout SCFA butyrate [26]. However, a high omega-6/omega-3 PUFA ratio, predominant in the Western diet, has been related to an enhanced gut barrier permeability and metabolic endotoxemia through a gut-microbiota-driven mechanism [29]. The Mediterranean style-diet is based on the regular consumption of MUFAs and PUFAs, polyphenols and other antioxidants, a high intake of prebiotic fibres and low-glycemic carbohydrates, and greater consumption of plant proteins than animal proteins. The consumption of complex polysaccharides and plant protein could be associated with an increase of beneficial bacteria quantity, stimulating SCFA production. Thus, it appears to have the potential to confer health benefits via modulation of the gut microbiota promoting its diversity and richness [30-33, Figure 5]. On the one hand, the Westernization of the diet, characterized by high intake of animal proteins, saturated fat, sugar, and salt seems to stimulate the growth of pathogenic bacteria to the detriment of beneficial bacteria, leading to potential alterations of the intestinal barrier [26].

Future studies concerning the interactions between food compounds and specific intestinal bacteria would lead to a better understanding of both positive and negative interactions with dietary habits, also in childhood and adolescence. A novel nutritional approach may be adopted by building a personalized diet after microbiota analyses, to modulate and promote a healthy gut microbiota.

Figure 5. The effectors of the Mediterranean style-diet [33].



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4.2. EXPERIMENTAL STUDY

G☺☺D-DAY Trial is a longitudinal study of two years. COVID-19 pandemic resulted in a temporary stop of the trial due to changes in the lifestyle conditions of the enrolled subjects because of lock-down restrictions. Therefore, we performed an *interim* analysis concerning baseline variables obtained before the COVID-19 pandemic event.

4.2.1. Aims

1) *Aims of Protocol G☺☺D-DAY Trial (on-going):*

In a cohort of pediatric subjects with visceral adiposity, the aims of the study were to assess the efficacy of an educational training to the Mediterranean style-Diet based on gamification with respect to conventional treatment on weight loss and improvement of cardiometabolic risk factors; to investigate the relationship among metabolic profile, dietary composition and microbiome in children and adolescents enrolled in different intervention groups.

2) Aim of the baseline study:

The aim of the first study on the G☺☺D-DAY Trial, was to characterize at baseline the relationship among metabolic profile, dietary composition, and microbiome in pediatric subjects with visceral adiposity

4.2.2. Methods

G☺☺D-DAY Trial Protocol (on-going)

The G☺☺D-DAY (GamificatiOn of an educatiOnal training to meDiterranean Diet on weight and metabolic control in pAediatric obesity) is a single-centre pilot open-label randomized control Trial, which started from 2017, with a follow-up over of one year. The study is ongoing at the Paediatric Endocrine Service of Division of Paediatrics, Department of Health Sciences, University of Piemonte Orientale, in Novara. The G☺☺D-DAY study has been approved by the Local Ethics Committee of A.O.U. Maggiore della Carità (CE 50/17) and registered on ClinicalTrials.gov (ID NCT03154255).

Population: The study involves subjects of both sexes, between 10 and 18 years of age, overweight or obese, according to the IOTF criteria *[Cole TJ, et al. 2012]* and with visceral adiposity, like waist circumference ≥ 90th percentile *[Cook S, et al. 2003; Cruz ML, et al. 2004; de Ferranti SD, et al. 2004; Xi B, et al. 2020]*, diet naïve or with failure of weight loss (defined as -1 kg/m^2 BMI in 1 year).

Inclusion criteria:

1) overweight or obesity, according to the IOTF criteria;
2) waist circumference ≥ 90th percentile;
3) diet naïve or with failure of weight loss (defined as -1 kg/m^2 BMI in 1 year).

Exclusion criteria:

1) Specific causes of endocrine or genetic obesity;
2) type 1 diabetes;
3) previous kidney diseases.

Intervention: Patients are randomized in an open-label, into two groups homogeneous for the number and sex of the subjects. One group (group SD) has received Standard Diet according to routine care and practice, and one group (group SD+MSD) has received a standard diet according to routine clinical practice + a 30 minutes Mediterranean Style Diet educational training with the explanation of Mediterranean pyramid + gamification to Mediterranean diet inside the Hospital and at home throughout “The Mediterranean Goose” (“Il Gioco dell’Oca Mediterranea”).

Dietary restriction: The standard diet is distributed with 55-60% of carbohydrates (45-50% complex and no more than 10% refined and processed sugars), 25-30% lipids and 15% proteins, and is performed in accordance with the calories of an isocaloric balanced diet calculated throughout the Italian LARN Guidelines for age and gender *[LARN, 1996]*, inspired to the Mediterranean pyramid. The standard diet is given as diet plans in writing, after explanation by a well-trained and experienced clinical paediatric endocrinologist.

Education in Hospital: As well as receiving the aforementioned recommendations, the SD+MSD group also received a simple visual guide of Mediterranean food pyramid explained by a dietitian/nutritionist for 30 minutes - see Figure 1 *[Bach-Faig A, et al. 2011]*.

Gamification: The MSD group is involved inside the Hospital in an educational and playful training playing to “The Mediterranean Goose” (“Il Gioco dell’Oca Mediterranea”

- see Appendix I) in the ludic area of the Pediatric Department; this game has been proposed in an educational project known as “Mediterranean diet and enhancement of traditional foodstuff - MedDiet”, founded by European Union in the framework of the ENPI CBC Mediterranean Sea Basin Programme *[www.med-diet.eu]*. This material is given to the family to continue to play also at home.

Physical activity: all subjects received general recommendations about performing physical activity. Exercises are conducted daily, consisting of 30 minutes of aerobic physical activity.

Randomization: Alternately per week, participants are randomly assigned in a 1:1 to one of the two dietary intervention groups.

Timing: Patients are evaluated firstly at time of enrollment (V0) and, during the first 2 weeks of study (V1), biochemical and ultrasound evaluations have been completed. During the year of intervention, patients have been evaluated after 3 months, 6 months, 9 months and 12 months (V2, V3, V4, V5). The following anthropometric measures, biochemical and ultrasound evaluations and questionnaires have been obtained (see Appendix II):

1) *Anthropometric measures:*

- height (V0, V2, V3, V4, V5);
- weight (V0, V2, V3, V4, V5);
- Body Mass Index (BMI; Kg/m^2) (V0, V2, V3, V4, V5);
- waist and hip circumferences (V0, V2, V3, V4, V5) for the calculation of the following ratios: waist/hip, waist/height;
- Tanner stage (V0, V2, V3, V4, V5) *[Tanner JM, 1961]*;
- blood pressure and heart rate (V0, V2, V3, V4, V5).

2) *Biochemical evaluations* (after a 12-h overnight fast):

- CBC with formula, serum insulin-like growth factor 1 (IGF1, ng/mL), 25-hydroxy (OH) vitamin D (ng/mL), uric acid (mg/dL), alkaline phosphatase (U/L), ACTH (pg/mL), cortisol (microg/dL), TSH (uuI/mL), fT4 (ng/dL) (V1, V3, V5);
- aspartate aminotransferase (AST, IU/L), alanine aminotransferase (ALT, IU/L); AST-to-ALT ratio has been calculated as the ratio of AST (IU/L) and ALT(IU/L) (V1, V3, V5);
- serum creatinine concentration (mg/dL) has been measured with the enzymatic method; according to the NKF-K/DOQI Guidelines for CKD in children and

adolescents [8], the eGFR has been calculated using updated Schwartz's formula [9]: eGFR (mL/min/1.73 m2) = [0.413 x patient's height (cm)] / serum creatinine (mg/dL) (V1, V3, V5);

- glucose (mg/dL), insulin (μUI/mL); insulin-resistance (IR) has been calculated using the formula of Homeostasis Model Assessment (HOMA)-IR: (insulin [mU/L] x glucose [mmol/lL) / 22.5) (V1, V3, V5);
- lipid profile: total cholesterol (mg/dL), High-Density Lipoprotein (HDL)-cholesterol (mg/dL), triglycerides (mg/dL); Low-Density Lipoprotein (LDL)-cholesterol has been calculated by the Friedwald formula and non-HDL (nHDL)-cholesterol has been also calculated (V1, V3, V5);
- oral glucose tolerance test (OGTT: 1.75 g of glucose solution per kg, maximum 75 g) and samples have been collected for the determination of glucose and insulin every 30 min. The area under the curve (AUC) for parameters after OGTT has been calculated according to the trapezoidal rule. Insulin sensitivity at fasting and during OGTT has been calculated as the formula of the Quantitative Insulin-Sensitivity Check Index (QUICKI) and Matsuda index (ISI). The stimulus for insulin secretion in the increment in plasma glucose as insulinogenic index has been calculated as the ratio of the changes in insulin and glucose concentration from 0 to 30 min (InsI). Beta-cell compensatory capacity has been evaluated by the disposition index defined as the product of the ISI and InsI (DI) *[Khan UI, et al. 2015]* (V1, V5);
- a collection at rest of first-morning urine sample. Physical and chemical urinalysis; urine albumin (mg/L) has been determined by an advanced immunoturbidimetric assay and urine creatinine (mg/dL) has been measured using the enzymatic method. Urine albumin to creatinine ratio (u-ACR – mg/g), has been calculated using the following formula: [urine albumin (mg/dL) / urine creatinine (mg/dL)] x 1000. For these calculations both albumin and creatinine were in the same unit. The subjects whose urine were positive, they collected two more samples and the u-ACR mean value of these has been considered (V1, V3, V5).
- Stool samples for microbiota analyses (sequencing, and metaproteomic analyses) have been collected (V1, V3, V5).

  All blood samples have been measured evaluated using standardized methods in the Hospital's Chemistry Laboratory, previously described *[Prodam F, et al. 2014; 2016]*.

3) *Ultrasound evaluations:*

- liver ultrasound (V1, V5).

4) *Nutritional and physical activity measurements:*

- KIDMED questionnaire for children and adolescents; the index comprises 16 yes or no questions *[Serra-Majem L, et al. 2004]*, and the total score of the KIDMED index ranged from -4 to 12 and is classified into 3 levels: 1)≥8: optimal Mediterranean diet; 2) 4-7: improvement is needed to adjust intake to the Mediterranean diet; 3) ≤3: very low diet quality; the Italian version is reported and approved by Istituto Superiore Sanità in Rapporti ISTISAN 12/42 *[Rapporti ISTISAN 12/42, 2012]* (V0, V2, V3, V4, V5);
- the Food Frequency Questionnaire section of the Children's Eating Habits Questionnaire (CEHQ-FFQ), performed by Identification and prevention of Dietary and lifestyle induced health Effects In Children and infantS (IDEFICS) study, on which parents or other caregivers are asked to report the number of meals the children usually consumed at home or other people's homes, such as of grandparents and friends, in a typical week of the previous month *[Huybrechts I, et al. 2011]* (V0, V2, V3, V4, V5). The Italian version of CEHQ-FFQ has been validated and kindly given through a signed agreement between the IDEFICS Consortium and the Principal Investigator (Assoc. Prof. Flavia Prodam).
- 24-hour recall and a list of all foods and constituents included in the Dietary Inflammatory Index (DII) along with the respective adjusted score has been evaluated. Food- and constituent-specific scores has been multiplied by the intake for each subject and then has been summed to create the overall Inflammatory Index score *[Cavicchia PP, et al. 2009; Shivappa N, et al. 2014]* (V0, V2, V3, V4, V5);
- the Mediterranean Adequacy Index (MAI) has been obtained by dividing the sum of the percentage of total energy from typical Mediterranean food groups by the sum of the percentage of total energy from non-typical Mediterranean food groups, according to the following equation *[Alberti-Fidanza A, et al. 2004]* (V0, V2, V3, V4. V5):

$$MAI = \frac{(\%\text{en cereals} + \text{legumes} + \text{potatoes} + \text{vegetables} + \text{fruit fresh and dry} + \text{fish} + \text{wine} + \text{virgin olive oil})}{(\%\text{en milk} + \text{cheese} + \text{meat} + \text{eggs} + \text{animal fats and margarines} + \text{sweet beverages} + \text{cakes/pies} + \text{cookies})}$$

- the International Physical Activity Questionnaire (IPAQ) developed, validated for Italian adolescents (IPAQ-A) has been developed; It covers four domains of physical activity: (1) school-related physical activity, including activity during physical education classes and breaks, (2) transportation, (3) housework and (4) leisure time *[Hagstromer M , et al. 2008; Mannocci A, et al. 2018]* (V0, V2, V3, V4, V5).

Good Clinical Practice: The protocol has been conducted in accordance with the declaration of Helsinki. Informed consent has been obtained from all parents before the evaluations after careful explanations to each patient.

Study Design at baseline (V0, V1)

This Thesis focuses on the baseline data at recruitment of Protocol G☺☺D-DAY Trial.

Clinical evaluations and biochemical measurements

All subjects underwent a clinical evaluation by a trained research team. Pubertal stages were determined by physical examination, using the criteria of Marshall and Tanner *[Tanner JM, 1961]*. Height was measured to the nearest 0.1 cm using a Harpenden stadiometer, and body weight with light clothing to the nearest 0.1 kg using a manual weighing scale. Body mass index (BMI) was calculated as body weight divided by squared height (kg/m^2). The BMI standard deviation score (BMISDS) was calculated by the least median squares (LMS) method *[Cacciari E, et al. 2006]*. Waist circumference (WC) was measured at the high point of the iliac crest around the abdomen and was recorded to the nearest 0.1 cm. Waist/height ratio was calculated as the ratio of WC (cm) and height (cm). Acanthosis has been evaluated according to Burke JP, et al. scale *[Burke JP, et al. 1999]*. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured three times at 2-minute intervals using a mercury sphygmomanometer with an appropriate cuff size after participants were seated quietly for at least 15 minutes, with their right arm supported at the level of the heart and feet flat on the floor, prior to other physical evaluations, and at least 30 minutes after blood sampling, using a standard mercury sphygmomanometer. Mean values were used for the analyses. Hypertension was determined if BP values recorded on enrollment day (V0) and on blood samples day (V1) are always elevated.

After a 12-h overnight fast, blood samples for glucose (mg/dL), insulin (µUI/mL), uric acid (mg/dL), serum insulin-like growth factor 1 (IGF-1) (ng/mL), 25-OH-Vitamin D serum levels (ng/mL), total cholesterol (mg/dL), High-Density Lipoprotein (HDL)-

cholesterol (mg/dL) and triglycerides (mg/dL) were measured, using standardized methods in the Hospital's Laboratory previously described *[Prodam F, et al. 2014; 2016].* Low-Density Lipoprotein (LDL)-cholesterol was calculated by the Friedwald formula and non-HDL (nHDL)-cholesterol was also calculated. Uric acid (mg/dL) was measured by Fossati method reaction using uricase with a Trinder-like endpoint. Subjects also underwent an OGTT (1.75 g of glucose solution per kg, maximum 75 g) and samples were drawn for the determination of glucose and insulin every 30 min. IR was calculated using the formula of homeostasis model assessment (HOMA)-IR. Insulin sensitivity at fasting and during OGTT was calculated as the formula of the Quantitative Insulin- Sensitivity Check Index (QUICKI) and Matsuda index (ISI). Glucose was expressed in mg/dL (1 mg/dL: 0.05551 mmoL/Ll) and insulin in µUI/mL (1 µUI/mL=7.175 pmoL/L) in each formula. Assays and formulas used are described previously *[Prodam F, et al. 2011]*.

Definitions

Subjects were classified as overweight or obese according to IOTF criteria *[Cole TJ, et al. 2012]*. WC percentiles were stratified according to sex and age *[*Xi B, et al. 2020*]*, identifying visceral adiposity in the presence of WC ≥ 90th percentile, as suggested by NCEP-ATP III *[Cook S, et al. 2003; Cruz ML, et al. 2004; de Ferranti SD, et al. 2004]*, or in a cut-off of 0.5 for waist-to-height ratio *[McCarthy HD, et al. 2006]*. According to criteria of Marshall and Tanner *[Tanner JM, 1961]*, pubertal stage was defined in the presence of testicular volume of 4 mL for males and breast at the stage 2 for females. SBP and DBP values were evaluating according to percentiles for age, sex and height, of National High Blood Pressure Education Program (NHBPEP) Working Group *[Flynn JT, et al. 2017]*. Arterial hypertension was defined according to the following different criteria: (1) > 95th percentile as suggested by the (NHBPEP) Working Group of American Academy of Pediatrics (AAP) *[National High Blood Pressure Education Program Working Group on High Blood Pressure in Children and Adolescents, 2004]*.
Triglycerides, LDL- and HDL-cholesterol percentiles for age and sex were classified according to the Lipid Research Clinic Pediatric Prevalence Study *[Daniels SR, et al. 2008]* and was considered dyslipidemia if triglycerides ≥ 90th percentile or HDL-cholesterol ≤ 10th percentile as suggested by NCEP-ATP III *[Cook S, et al. 2003; Cruz ML, et al. 2004; de Ferranti SD, et al. 2004]*. HOMA-IR percentiles for age and sex were classified according to standardised reference values for an international European children's population *[Peplies J, et al. 2014]* and was considered insulin-

resistance if HOMA-IR ≥ 90th percentile *[Ahrens W, et al. 2014]*. Impaired fasting glucose and impaired glucose tolerance were defined by a fasting plasma glucose of ≥ 5.6 to 6.9 mmol/L (100-125 mg/dL), and, as 2-h post-OGTT, glucose of ≥ 7.8 to 11.0 mmol/L (140-199 mg/dL), respectively, according to American Diabetes Association *[Zimmet P, et al. 2007].*

Anamnestic evaluations

The study population has been characterized by familiarity known for gestational diabetes, gestational age and weight at birth, the order of birth (firstborn or second child). Last data have also been used to classify subjects in AGA (Adequate for Gestational Age), SGA (Small for Gestational Age) or LGA (Large for Gestational Age) according to the Italian neonatal growth curves *[Bertino E, et al. 2010]*.

Nutritional evaluations

Information concerning nutritional intake were collected by trained dietician or nutritionist in an adequate and calm setting during interviews that lasted about 1-hour. The dietary 24-hours recall involved an in-depth interview where the previous 7 day's intake is described. The interviewer assigned average weights to the foods and children and parents estimated portion sizes using photographs *[Istituto Scotti Bassani, 2005]*. Daily mean intake of macronutrients like percentage of carbohydrates, proteins, fats and fibres were calculated. The level of adherence to the Mediterranean dietary pattern was assessed using the KIDMED questionnaire (Mediterranean Diet Quality Index for children and teenagers) and the Mediterranean Adequacy Index (MAI) – see above.

Physical Activity evaluations

Data on physical activity were collected using the International Physical Activity Questionnaire (IPAQ) developed and validated for Italian adolescents (IPAQ-A). It covers four domains of physical activity: (1) school-related physical activity, including activity during physical education classes and breaks, (2) transportation, (3) housework and (4) leisure time *[Hagstromer M, et al. 2008; Mannocci A, et al. 2018]*. The level of physical activity was measured in MET (Metabolic Equivalent Task), a unit of metabolic equivalent consumption calculated considering the amount of O_2 consumed at rest in 1 minute; if the consumption of O_2 at rest is about 3.5 mL/min/Kg of body weight, thus 1 MET = 3.5 mL VO_2/min/Kg ≈ 1 KcaL/Kg/h. The energy cost of various activities can be expressed as a multiple of the MET, multiplying by the minutes and days of the week

used to perform a given activity. In particular, 3.3 MET are considered for minor activities, 4 MET for moderate activities and 8 MET for intense activities (for example, MET intense activity = minutes per day x days a week x 8 MET). The MET was then calculated daily (MET/day), considering all activities of different intensity carried out by the subject in a day. According to the obtained results different grades of performed activity were defined: 1) ≤ 700 MET: inactive; 2) ≥ 701 and ≤ 2519: sufficiently active; 3) ≥ 2520: active.

The MET/day was then converted into KcaL: $\left(\frac{(\text{MET day} * 3.5) * \text{weight in Kg}}{1000} * 5\right)$

To calculate the total daily metabolism we summed the MET in KcaL with the basal metabolic rate in KcaL obtained with the Schofield formula *[Henry CJK, 2005]*.

DNA extraction and sequencing

For each enrolled subject, 4 faecal samples have been collected, and fastly frozen at -80°C. Genomic DNA was extracted using DNeasy® PowerSoil® Kit (Qiagen) from 0.25 g of stool following the manufacturer's instructions. Obtained DNA was quantified by the fluorimetric method according to the Qubit® 2.0 Fluorometer protocol. The preparation of the bacterial 16S DNA libraries was performed using the "Microbiota solution B" kit (amplifying the hypervariable regions V3-V4-V6) provided by Arrow Diagnostics Srl (Genoa, Italy). This kit provided two distinct PCRs: the first (PCR target) allows to select the V3-V4-V5 regions, the second (PCR index) which inserted the bar-codes and adapters compatible with the Illumina MiSeq platform. Sequencing procedures were performed using the MS-103-100 MiSeq Reagent Nano Kit v2 (500-cycles) kit (Illumina Inc.) and Phix (Illumina Inc.) was used as the internal standard. Raw sequences obtained were processed with the MicrobAT software (SmartSeq Srl). MicrobAT is based on the RDP database which provides bacterial and Archaea 16S rRNA sequences. MicrobAt does not produce OTUs (operational taxonomic units), but aligns the individual sequences with the references of the database, allowing not to add errors to the identification of microorganisms.

Bioinformatics and statistical analysis for the baseline study

A sample of 60 individuals (30 in each group) has been estimated to be sufficient to demonstrate a difference of 0.4kg/m^2 in BMI-SDS with an SD of 0.65 *[Prodam F, et al. 2013]* with 90% power and a significance level of 95% and a drop-out rate of 20% using the Student test. Statistical significance will be assumed at $P< 0.05$.

From MicrobAT, three files can be generated which were used for statistical analyzes regarding variations within the bacterial communities using the Microbiome-Analyst software (Comprehensive Statistical, Visual, and Meta-Analysis of Microbiome data). Before data analysis, a data integrity check has been performed to make sure that all the necessary information has been collected.

The sample variable should contain at least two groups to perform most of the comparative analysis. First data filtering was used to identify and remove features that are unlikely to be of use when modelling the data. No phenotype information has been used in the filtering process, so the result can be used with any downstream analysis. This step can usually improve the results. Features having low count and variance can be removed during the filtration step. Features having very few counts are filtered based on their abundance levels (minimum counts) across samples (prevalence). The normalization procedures implemented below are grouped into three categories. Data rarefaction and scaling based methods deal with uneven sequencing depths by bringing samples to the same scale for comparison. Heat tree method is used to compare the abundance of different taxonomic levels for each pair of factors in a metadata variable. Heat Tree uses a hierarchical structure of taxonomic classifications to quantitatively (median abundance) and statistically (non-parameter Wilcoxon Rank Sum test) depict taxon differences among communities. It generates a differential heat tree to show which taxa are more abundance in each treatment. Heat tree analysis is performed using the R package metacoder package, according to Foster S, 2017 *[Foster S, 2017]*. Alpha diversity can be characterized via the total number of species (richness), the abundances of the species (evenness) or measures that considered both richness and evenness. There are indices such as Shannon and Simpson in which along with the number (richness), the abundance of organisms (evenness) is also measured to describe the actual diversity of a community. Alpha diversity analysis is performed using the phyloseq package *[MacMurdie PJ, et al. 2013]*. The results are plotted across samples and reviewed as box plots for each group or experimental factor. Further, the statistical significance of grouping based on experimental factor is also estimated using either parametric or non-parametric test. Beta diversity analysis method provides a way to compare the diversity or composition between two samples or microbial communities. These methods compare the changes in the presence/absence or abundance of thousands of taxa present in a dataset and summarize these into how ’similar’ or ’dissimilar’ two samples. Each sample gets compared to every other sample generating a distance or dissimilarity matrix. Two

parameters need to be considered when performing beta diversity analysis. The first one is how similarity or distance between sample is measured which includes non-phylogenetic (Bray-Curtis distance, Shannon index) and phylogenetic-based (weighted and unweighted UniFrac) distances. The other parameter is how to visualize such a dissimilarity matrix in lower dimensions. Ordination-based methods such as Principle Coordinate Analysis (PCoA) and non-metric multidimensional scaling (NMDS) are used to visualize this matrix in 2 plot where each point represents the entire microbiome of a single sample. Each axis reflects the percentage of the variation between the samples with the X-axis representing the highest dimension of variation and the Y-axis representing the second-highest dimension of variation. Further, each point or sample displayed on PCoA plots is coloured based on either sample group, features alpha diversity measures, or the abundance levels of a specific feature. Also, the statistical significance of the clustering pattern in ordination plots can be evaluated using Permutational ANOVA (PERMANOVA). Beta diversity analysis is performed using the phyloseq package *[MacMurdie PJ, et al. 2013]*. Hierarchical cluster analysis was performed: in this analysis, each sample begins as a separate cluster and the algorithm proceeds to combine them until all samples belong to one cluster. Two parameters need to be considered when performing hierarchical clustering. The first one is how similarity or distance between sample is measured which includes Bray-Curtis distance, Shannon index, Jaccard index, weighted and unweighted UniFrac. The other parameter is clustering algorithms, including average linkage (clustering uses the centroids of the observations), complete linkage (clustering uses the farthest pair of observations between the two groups), single linkage (clustering uses the closest pair of observations) and Ward's linkage (clustering to minimize the sum of squares of any two clusters). In MicrobiomeAnalyst, the result of clustering analysis is supported using Heatmap and dendrogram. Hierarchical clustering is performed with the hclust function in package stat. Finally, the LDA Effect Size (LEfSe) method was applied. This method is specifically designed for biomarker discovery and explanation in high-dimensional metagenomic data (Segata 2013). It incorporates statistical significance with biological consistency (effect size) estimation. It performs non-parametric factorial Kruskal-Wallis (KW) sum-rank test to identify features with significant differential abundance concerning experimental factor or class of interest, followed by Linear Discriminant Analysis (LDA) to calculate the effect size of each differentially abundant features. The result consists of all the features, the logarithmic value of the maximum mean among all the groups or classes, and if the features are differentially significant, the group with

the highest mean and the logarithmic LDA score (Effect Size). Features are considered to be significant based on their adjusted p-value. The default is adjust P-value cutoff = 0.05. We decided to select some of the variables to perform the PCA: gender, age, puberty grade, BMI IOTF, waist circumference, tanner, acanthosis, total cholesterol, HDL (high density lipoprotein cholesterol), LDL (low density lipoprotein cholesterol), c-not HDL, triglycerides, uric acid, IGF-1, SDS IGF1, fasting and post-OGTT glucose levels, fasting and post OGTT insulin levels, mean IRI (insulin resistance index), HOMA-IR (homeostatic model assessment of insulin resistance), ISI during OGTT (insulin sensitivity index), QUICKI (quantitative insulin-sensitivity check index at fasting), HOMA percentile, waist/height ratio, BMI-SDS, MAI, total energy, carbohydrates % and grams, proteins % and grams, fats % and grams, systolic and diastolic blood pressure, Met day (metabolic equivalent task per day), physical activity level, and some voices of the KIDMED questionnaire (legumes, oil, breakfast, bakery, simple sugars intake, total KIDMED score, kidmed adherence level as low, average, high).

4.2.3. Results

The object of this thesis is to analyze the baseline characteristics of all subjects who performed at least one follow-up visit before COVID-19 lifestyle restrictions.

Study population

Fifty-five patients have been enrolled. Six patients were excluded because stool samples were not available The randomization was balanced respect to gender and puberty (Table 1); the age range was 10 -18 years (12.9±2.1 years).

Clinical, biochemical and lifestyle characteristics of the population

Clinical, biochemical, metabolic, and lifestyle parameters are reported in Tables 1, 2 and 3 (see Appendix) according to gender and puberty. Considering the whole sample, 100% of subjects had visceral adiposity. Arterial hypertension was diagnosed in 61.2% of the subjects. At V0, 34.1% had hypertriglyceridemia and 36.7% had low HDL-cholesterol. None had impaired fasting glucose or diabetes mellitus, only one subject had impaired glucose tolerance. Of the total cohort, only 9.6% of the subjects had a high adherence to MSD, while 32.7% and 57.7% had low and average adherence to MSD, respectively. 25.9% of the total subjects reported high level of physical activity,

while 24.1% and 50.0% low and average level of physical activity, respectively, according to IPAQ questionnaire.

Microbial distribution in the whole population

From the 49 stool samples, sequences belonging to the rRNA16S have been extracted and used to identify the microbiome population. The Phylum level showed 51.0% of *Bacteroidetes*, 39.5% of *Firmicutes*, 4.9% of *Actinobacteria*, 2.8% of *Proteobacteria*, and 1.3% of *Verrucomicrobia*. Furthermore, going deep at the taxonomic level of genus, *Bacteroides* were *27.6%*, followed by *Unclassified Lachnospiraceae* (5.8%), *Unclassified Ruminococcacea* (6.8%), *Faecalibacterium* (4.6%), *Alistipes* (5.1%), and *Prevotella* (4.7%). Despite differences in the inter-individual microbiome, a *core microbiome* emerged and was represented in Figure I (All Figures are reported in Appendix).

Correlation between weight, metabolism, and microbiota

Considering all variables, either clinical, biochemical, and microbial ones, the PCA analysis (Figures II a, b and c) revealed that the majority of the sample was homogeneous and only a few subjects contributed to the variability of the same. Moreover, males were each other much more similar independently to puberty, whereas higher heterogeneity was described between starting puberty and end-puberty females (Figures III a, b and c).

Contribution percentage of each variable to the different dimensions, described in Figure IV, revealed that the major contributor to the overall sample derived from BMI-SDS, total cholesterol, LDL-cholesterol, HOMA-IR, insulin sensitivity (like ISI and QUICKI) and physical activity level.

To identify variables with the most significant contribution to the microbiota ordination, it was built a PCA-BiPlot in different dimensions (Figures V a, b and c). ISI, QUICKI and Tanner stage were associated with *Verrucomicrobia*, while glucose-insulinemic metabolism to *Bacteroidetes*, and intake of proteins and cholesterol level to Firmicutes.

There were significant α-and β-diversity differences among weight subgroups, such as for overweight, obesity and morbid obesity. Overweight group resulted to have a consistent difference compared with the other two groups (Figures VI, VII). The heat tree showed an increase in *Suttarella* and a decrease of *Bacteroides* in morbid obesity versus obesity (Figure VIII a and b). Furthermore, BMI-SDS had a U-shape distribution and was positively correlated in 1° and 3° tertile with the abundance of *unclassified*

Actinobacteria (Table 4). No significant differences were reported concerning waist circumference and waist-to-height ratio and microbiome since the majority of the sample recognised status of visceral adiposity (see Table 1).

No significant differences were reported about neither lipid profile, uric acid, glucose profile at fasting and during 2-hours oral glucose tolerance test and microbiome. Only total-cholesterol seemed to be positively correlated with the abundance of *Parabacteroides merdae* (Figure IX). However, in subjects with insulin-resistance, defined as HOMA≥95° percentile, we showed a significant increase in *Proteobacteria* (*Sutterella*) and a decrease in *Rikenellaceae* and *Veiollenaceae* (*Dialister invisus*) (Figure X). Specularly, in subjects with higher insulin-sensitivity expressed as QUICKI, we showed a decrease in *Proteobacteria* (*Sutterella*) and an increase in *Rikenellaceae* and *Veiollenaceae* (*Dialister invisus*) (Figure XI a and b). Regarding ISI, significant differences in α-diversity was also found demonstrating a higher number of gut microorganisms in the presence of higher insulin-sensitivity (Figure XII).

Correlation between diet and microbiota

The α- diversity showed an increase in biodiversity (either using Shannon and Simpson index) and numerosity (using Observed index) associated with an intake of carbohydrates > 45% (Figure XIII). Also, β-diversity resulted in significative differences among different percentage intakes of carbohydrates (Figure XIV). The heat tree revealed a higher abundance of *Faecalibacterium* in those subjects consuming a higher amount of carbohydrates (Figure XV).

No significant differences were shown on the percentage intake either of proteins and lipids and microbiome. Only the heat tree analysis showed an increase in *Rikenellaceae* in those subjects reporting an intake of proteins lower than 15% (Figure XVI). Therefore, a prevalence of *Parabacteroides distasonis* was seen in those subjects who consumed an amount of fats lower than 30%, whereas an increase in *Erysipelotrichaceae* was observed in those consuming a percentage intake of fats > 35% (Figure XVII).

Considering the adherence to the MSD, expressed as KIDMED score, differences in the composition of microbiota have been seen especially comparing high versus low level of adherence. Besides, β-diversity showed that the microbial population was more diversified in those with high adherence and less diversified in average and low levels of adherence (Figure XVIII). Also, α-diversity (using Observed index) showed increased microbial numerosity associated with better adherence to the MSD (Figure XIX).

Concerning the individual KIDMED items, we studied even correlations between extra-virgin oil consumption, skipping breakfast, bakery consumption and microbiota. Since, in those who did not consume extra-virgin olive oil was reported a significative prevalence of *Rikenellacae*, *Porphyromonadaceae*, *unclassified Bacteroidales*, *Barnesiella* (Figure XX), as also confirmed in the Lefse analysis (Table 5). Subjects who skipped breakfast recognised an abundance in *Barnesiella* (Table 6), also described in those who did not consume bakery (Figure XXI).

Correlation between physical activity and microbiota

Looking at the α-diversity analysis, using Shannon index, representing the diversity of species within the sample, resulted evident how the microbial diversity increased together with the level of physical activity (Figure XXII). However, a certain microbial prevalence has not been demonstrated in different levels of physical activity.

4.2.4. Discussion

In this study, we explored associations between faecal microbiota composition, diet, physical activity, and metabolic characteristics, in particular insulin-sensitivity and insulin-resistance, in a well-characterized sample of 49 overweight and obese children and adolescents recruited for the GOOD-Day Trial.

Recently, there is increasing evidence that gut microbiota plays a key role in the maintenance of host health. The gut microbiota is influenced by a variety of factors and the healthy microbiota is diverse, however, compositionally it is affected by geographical and ethnic factors *[Prideaux L, et al. 2013]*. At the Phylum level, we described 51.0% of *Bacteroidetes*, 39.5% of *Firmicutes*, 4.9% of *Actinobacteria*, 2.8% of *Proteobacteria* and 1.3% of *Verrucomicrobia*. Thus, our cohort recognized microbiota characteristics distinguishing with Caucasian ethnicity. Although obesity was initially related to increased *Firmicutes/Bacteroidetes* ratio, either in adults *[Ley RE, et al. 2006; Turnbaugh PJ, et al. 2009]* and paediatrics *[Bervoets L, et al. 2013; Ferrer M, et al., 2013; Riva A, et al. 2017; Xu P, et al. 2012]*, these findings have not been confirmed in a recent meta-analysis in adults *[Walters WA, et al. 2014]* and other pediatric studies *[Abdallah Ismail N, et al. 2011; Payne et al., 2011]*, as also reported in our study. These inconsistencies could be explained by methodological differences or population differences such as host genetics, geography, and diet *[Lozupone CA, et al. 2013]*. Moreover, this large variation, as well as the contradicting results from previous studies, suggests that the *Firmicutes/Bacteroidetes* ratio may not be a robust marker for

obesity. Therefore, we found that the α-diversity of the gut microbiota was negatively correlated with BMI, describing a consistent difference in the overweight group compared with those subjects with obesity and morbid obesity. This finding confirms the hypothesis that richness in the microbial community is associated with a well-balanced and healthy status *[Rinninella E, et al. 2019]*. Also, BMI-SDS recognized a U-shape distribution and was positively correlated in 1° and 3° tertile with the abundance of *unclassified Actinobacteria*. In general, the *Actinobacteria* phylum is proportionally less abundant and mainly represented by the *Bifidobacterium* genus *[Rinninella E, et al. 2019]*. Among the *Actinobacteria* phylum, *Bifidobacterium* genus resulted related to improved glucose metabolism, insulin resistance and low-grade inflammation from previous murine and human studies *[Philippe D, et al. 2011; Verdam FJ, et al. 2013; Moreno-Indias et al., 2016]*. Besides, specific trains of *Bifidobacterium* are also associated with fibre intake *[Garcia-Mantrana I, et al. 2018]*. However, recently, Rampelli S, et al. *[Rampelli S, et al. 2018]* showed the concomitant presence of *Prevotella*, *Bacteroides* and *Bifidobacterium* in bacterial clusters characterized by lower diversity and associated with pre-obese and obese children. These clusters showed also positive associations with inflammation, which is a common aspect both in obesity, insulin resistance and diabetes, plausibly being a common cause in driving microbiota changes.

Interestingly, we observed specularly differences in several faecal microbial communities at the family and genus taxonomic levels when insulin-sensitive and insulin-resistant subjects were compared at fasting. Insulin-resistance was positively associated with *Proteobacteria* (*Sutterella*) and negatively with *Rikenellaceae* and *Veiollenaceae* (*Dialister invisus*). Inversely, insulin-sensitivity was negatively correlated with *Proteobacteria* (*Sutterella*) and positively with *Rikenellaceae* and *Veiollenaceae* (*Dialister invisus*). A higher abundance of genus *Dialister* has been previously reported among overweight or obese African-American men with impaired glucose tolerance and in overweight or obese adults with insulin-resistance in Australia *[Ciubotaru I, et al. 2015; Naderpoor N, et al. 2019]*. Its abundance was also higher in patients with coronary artery disease who were diabetic compared to non-diabetic *[Sanchez-Alcoholado L, et al. 2017]*. Furthermore, in a small cohort of patients with type 2 diabetes who underwent bariatric surgery, the abundance of *Dialister* was reduced *[Graessler J, et al. 2012]*. However, *Dialister* abundance was not different in overweight versus obese individuals indicating that *Dialister* abundance may be more reflective of insulin sensitivity rather than BMI. Therefore, *Dialister* is a Gram-negative

coccobacillus, which seems to be capable of producing acetate, lactate, and propionate but not butyrate *[Wade WG, 2015]*. Thus, *Dialister* could potentially have both negative (inflammatory) and positive (via SCFAs) effects on the host and the overall effect may be determined through interactions with other bacteria. In particular, no data are nowadays available concerning pediatrics, so this relationship awaits further studies. Consistent with our results, a recent study found an increased concentration of *Sutterella* in pre-diabetic individuals *[Allin KH, et al. 2019]*. Moreover, in a cohort of pregnant with gestational diabetes mellitus a positive correlation between either Lipopolysaccaride (LPS) biosynthesis either protein C reactive with *Sutterella* was observed *[Ferrocino I, et al. 2018]*. *Sutterella* is a proteobacteria, Gram-negative bacteria, with known pro-inflammatory capacity, which could produce inflammatory LPS triggering a pro-inflammatory state, a condition characterizing both type 2 diabetes and obesity *[Allin KH, et al. 2015].*

Recognizing the key role of diet as a gut microbiota effector, we investigated associations between dietary habits and microbial characteristics. In our study, an intake of carbohydrates > 45% has been positively associated with an increase in numerosity and microbial biodiversity with a higher abundance of *Faecalibacterium* and *Roseburia*. The latter is a member of the *Clostridium coccodis* cluster of the phylum *Firmicutes*. *Roseburia spp.* are known to ferment complex polysaccharides entering the colon to butyrate as a terminal product. Butyrate is the preferred energy source of colonocytes in the human large intestine as well as a known histone deacetylase inhibitor and immunomodulatory signal. Recently, it has been suggested that butyrate production by gut microbes and, specifically, *Roseburia spp.*, may confer health benefits to humans, including prevention of type 2 diabetes, ulcerative colitis and colon cancer. Moreover, it has been suggested that B vitamin biosynthesis genes in *Roseburia spp.* might play a large role in their ecology in the gut environment *[Hillman ET, et al. 2020]*. *Faecalibacterium*, a group of major butyrate producers in the colon was also positively correlated with acetate and butyrate *[Louis P, et al. 2009]*, SCFAs involved in maintaining tissue barrier function and colonic homeostasis, modulating immunoregulation and decreasing inflammation *[Rinninella E, et al. 2019]*. However, literature data are conflicting about the role of *Faecalibacterium*, in particular *F. prausnitzii*, in obesity with studies showing positive *[Balamarugan R, et al. 2010]*, negative *[Borgo F. et al, 2016]* or no association *[Feng J, et al. 2014]*. These contradictory results may be due to experimental factors such as small cohort sizes or the use of different primer sets or may be explained by the existence of multiple *F.*

prausnitzii phylotypes *[Louis P, et al. 2009; Hippe B, et al. 2016]*. In contrast, it was recently reported data which may reflect its proven anti-inflammatory capacities. *Faecalibacterium* showed a negative association with blood pressure in adults *[Quevrain E, et al. 2015]*. Besides, patients with metabolic syndrome showed a reduction in *Faecalibacterium prausnitzii* compared to healthy individuals, which was restored upon a dietary intervention with a Mediterranean-type diet *[Haro C, et al. 2015].*

The richness and microbial biodiversity resulted positively associated with higher adherence to Mediterranean style-diet expressed as KIDMED score. De Filippis F, et al. in 2016, provided the first concrete evidence for the interconnection between Mediterranean dietary patterns, gut microbiota and microbial metabolites in adults. Consumption of fruit, vegetables, and legumes by subjects with satisfactory adherence to the Mediterranean style-diet associated with an increase in faecal SCFAs levels, and this is likely boosted by certain bacteria belonging to both *Firmicutes* and *Bacteroidetes* that can degrade carbohydrates not digestible by the host. By contrast, lower adherence to the Mediterranean style-diet is linked to higher urinary TMAO *levels [De Filippis F, et al. 2016].* These findings were also confirmed with positive correlations between adherence to Mediterranean style-diet and increase of total bacteria, *Bifidobacteria/E. coli* ratio, the relative share of *Bacteroides*, *C. albicans*, and total SCFAs, as well as decrease of *E. coli* levels. All these results demonstrated a link between adherence to the Mediterranean style-diet and improvements to the diversity and richness of gut microbiota *[Mitsou EK, et al. 2017; Rinninella E, et al. 2019].* Concerning items in the KIDMED questionnaire, we reported in subjects who did not consume extra virgin oil and those who skipped breakfast an abundance of *Barnesiella*. *Barnesiella*, a member of *Bacteroidetes*, in a recente meta-analysis, has been identified only in populations living in developed countries, suggesting that its presence was promoted by the urbanization or industrialization process *[Mancabelli L, et al. 2017].* Therefore, it has been considered as a marker for Westernized style-diet because it seems to result from animal fat- and protein-based diets *[Zuluaga J, et al. 2018]*. This could also be explained with the plausible use of other types of fats, different from olive oil, used to cook such as saturated, polyunsaturated or trans fats typically associated with Westernized style-diet. In contrast, it is well known that extra virgin olive oil consumption, recognized as one of the main components of the Mediterranean style-diet, has been demonstrated to have cardioprotective properties, reducing the risk of coronary heart disease *[Colica C, et al. 2017].* However, its antioxidant and anti-

inflammatory properties do not seem to be as related to the MUFA oleic acid as to its phenolic compounds *[Bulotta S, et al. 2014]*. At the same time, skipping breakfast is associated with Westernized style-diet and, subsequently, obesity, unhealthy metabolic profile and cardiovascular risk from pediatric age *[Monzani A, et al. 2019]*. In our study, an abundance in *Barnesiella* was also described in those who did not consume commercially baked goods or pastries for breakfast. This finding would seem to be in contrast with those above discussed. However, a possible explanation is that who answer negatively to this item are subjects who skipped breakfast.

Lastly, about physical activity, the level of activity has been positively associated with microbial biodiversity, but further investigation is needed to explore which is the prevalence of certain genus. Previously published cross-sectional studies performed on human subjects showed that increased gut microbiome diversity is common amongst healthy physically fit or otherwise physically active humans, in comparison to sedentary or otherwise fewer active individuals *[Clarke SF, et al.2014; Estaki M, et al. 2016].* Physical activity was not associated with α-diversity changes in any of included studies (intervention or longitudinal studies) in a recent systematic review *[Shahar RT, et al. 2020]*, while β-diversity changes were positively correlated with physical activity intervention in three of the five studies included, enrolling young and adults. Possible mechanisms involved in the link between physical activity and gut microbiota related changes composition seem to be the following three: an increase of the number and volume of mitochondria; an increase in energy demands related to growing SCFA-producing bacterial types that provide an energy source; and the possibility to reduce systemic inflammation and the subsequent translocation of gut microorganisms from the intestinal lumen to the blood circulation *[Mailing LJ, et al. 2019; Shahar RT, et al. 2020]*.

Our study recognized some limitations. First, our sample had a high homogeneity, being all cohort characterized by high visceral adiposity. However, these data are obtained from an intervention trial, in which a selected pediatric population with obesity was enrolled. Second, we did not reach species, thus further investigations should be planned. Third, metabolome data were not still available and it could help us to depict some associations. Lastly, fibre intake, as a crucial dietary pattern of Mediterranean style-diet, should be inserted to have more precise data on our sample. However, we so far conducted only preliminary results. The study will continue for obtaining more complete information concerning its relationship with microbiota.

4.2.5. Conclusions

In conclusion, these first baseline findings correlated gut microbiota to dietary pattern and adherence to the Mediterranean style-diet, suggesting that dietary intervention would have enormous potential in modulating the microbial composition and promoting a more health-associated metabolic profile. A novel nutritional approach may be adopted by building a personalized diet after to microbiota analyses, to modulate a healthy gut microbiota and contrast obesity, insulin-resistance, and cardiovascular risk.

4.2.i. Appendix

Appendix I. **"**The Mediterranean Goose" game *[www.med-diet.eu]*.

Appendix II. Study design G☺☺D-DAY Trial.

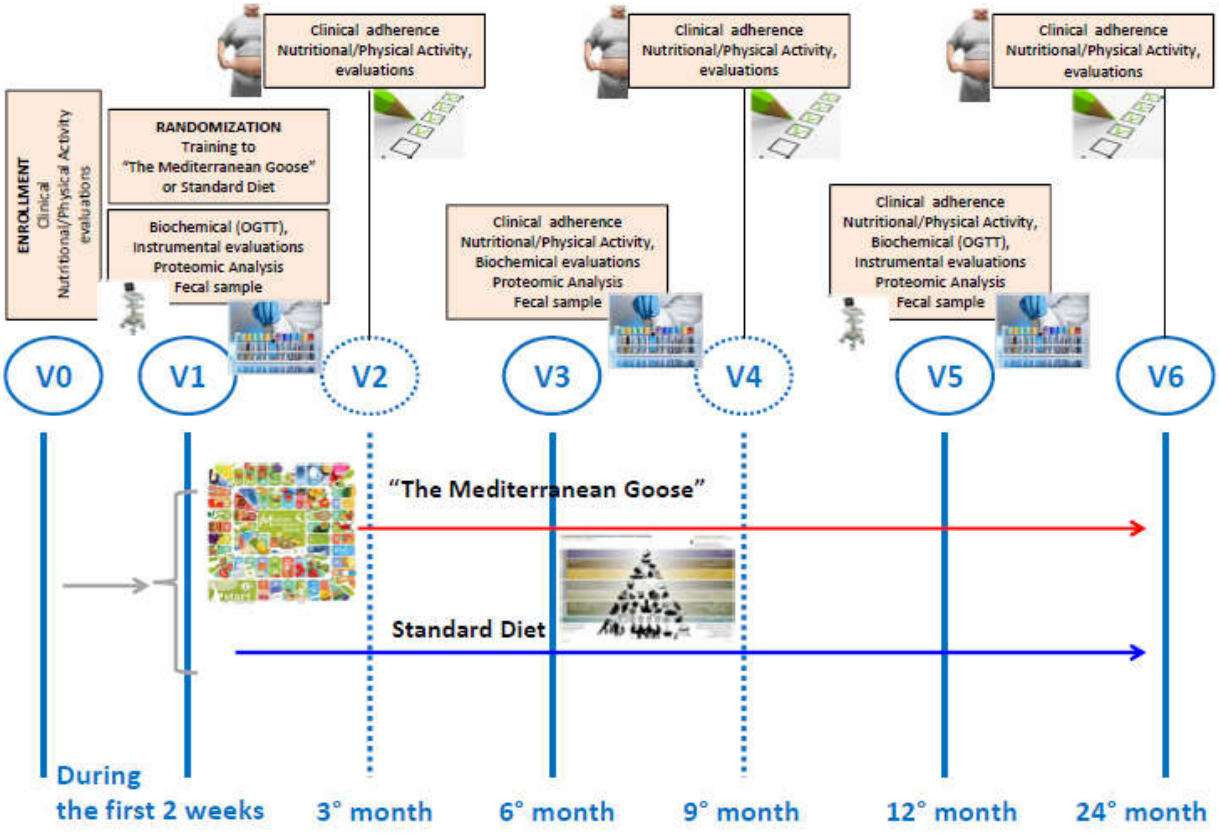


Table 1. Auxological-clinical characteristics of the whole sample.

	Females	Males	Prepubertal	Pubertal
Subjects	24	25	17 (F8/M9)	32 (F26/M16)
Age	13.7±2.2	12.2±1.7	11.1±0.9	14.1±1.8
Weight at birth (g)	3237.6± 466.0	3428.9± 419.2	3410±396.0	3291.9±479.5
Classification				
AGA	96(%)	92.9(%)	95.2(%)	93.8 (%)
SGA	0.0(%)	3.6(%)	0(%)	3.6(%)
LGA	4.0(%)	3.6(%)	4.8(%)	3.6(%)
GDM	0.0(%)	6.7(%)	4.5(%)	3.0(%)
WC (cm)	96.2±10.6	96.5±8.2	92.3±7.0	99.1±9.7
WC >=90° percentile	100%	96.55%	95.23(%)	100(%)
Waist/height ratio	0.61±0.05	0.62±0.04	0.62±0.04	0.61±0.05
BMI SDS	2.31±0.49	2.22±0.40	2.1±0.28	2.36±0.50
Acanthosis grade				
0	16(%)	10(%)	18.2(%)	9.1(%)
1	16(%)	26.6(%)	22.7(%)	21.2(%)
2	32(%)	43.4(%)	31.8(%)	42.4(%)
3	24(%)	10(%)	18.2(%)	15.15(%)
4	12(%)	10(%)	9.10(%)	12.12(%)
SBP or DBP < 95° percentile	29.2(%)	48(%)	47.0(%)	34.4(%)
SBP or DBP ≥ 95° percentile	70.8(%)	52(%)	52.9(%)	65.6(%)

All data are expressed as media ± SD (Standard Deviation).
Abbreviations: F: Females, P: Pubertal, PP: Prepubertal, Classification: AGA: Adequate for gestational age, SGA: Small for Gestational Age, LGA: Large for Gestational Age, GDM: Gestational Diabetes Mellitus, M: Males, WC: Waist Circumference BMI: Body Mass Index, SBP: Systolic Blood Pressure, DBP: Diastolic Blood Pressure.

Table 2. Metabolic and biochemical characteristics of the whole sample.

	Females	Males	Prepubertal	Pubertal
Subjects	24	25	17 (F8/M9)	32 (F26/M16)
Total cholesterol (mg	155.8±27.7	145.9±32.1	149.7±37.4	150.8±26.3
HDL -c (mg/dL)	44.4±7.7	43.3±11.1	45.5±11.1	42.8±8.6
LDL-c (mg/dL)	95.4±26.9	85.0±26.1	84.8±32.1	92.6±23.5
C NON HDL (mg/dL)	102.6±42.1	95.5±37.7	89.3±49.4	104.8±31.2
Triglycerides (mg/dL)	85.1±49.8	89.7±36.7	100.7±44.4	80.3±40.7
Uric acid (mg/dL)	5.2±0.9	5.1±1.0	4.8±0.7	5.3±1.0
IGF 1 (ng/ml)	369.8±122.5	289.6±111.7	238.0±83.8	376.2±112.8
SDS IGF1	0.16±0.7	-0.06± 0.4	-0.13±0.3	0.14±0.6
VIT D (ng/mL)	19.7±7.9	21.7±6.3	20.7±7.0	20.8±7.2
GLC 0 (mg/dl)	79.8±7.02	84.3±7.1	83.9±7.9	81.2±6.9
GLC 30 (mg/dL)	116.8±20.1	122.0±29.4	121.6±32.8	118.4±19.9
GLC 60 (mg/dL)	105.2±18.1	116.5±19.4	114.2±15.0	109.3±21.8
GLC 90 (mg/dL)	100.3±17.4	109.6±20.3	106.6±17.7	104.6±20.7
GLC 120 (mg/dL)	96.0±20.1	107.1±16.6	106.8±16.5	98.8±20.0
AUC GLC (mg/dL)	12300.6±1649.2	13306.1±1969.7	13120.5±1832.5	12668.0±1918.1
INS 0 (mcUI/mL)	13.5±7.3	17.2±13.1	19.7±13.9	12.7±7.2
INS 30 (mcUI/mL)	86.7±70.6	105.3±65.3	125.9±59.7	78.5±66.8
INS 60 (mcUI/mL)	62.5±54.9	92.00±85.8	110.9±91.7	58.1±52.5
INS 90 (mcUI/mL)	63.5±55.9	80.3±63.6	98.00±75.04	56.7±42.7
INS 120 (mcUI/mL)	60.4±61.3	79.1±87.0	110.0±105.1	45.7±33.3
IRI	58.2±40.4	75.3±55.2	92.9±59.0	50.4±32.
HOMA	2.7±1.5	3.7±3.0	4.2±3.2	2.6±1.5
ISI	5.49±3.22	4.30±2.34	3.67±2.37	5.64±2.84
QUICKI	0.34±0.03	0.33±0.03	0.32±0.03	0.34±0.02
HOMA%B	377.5±344.7	303.0±192.1	353.2±201.8	326.7±315.1

All data are expressed as media ± SD (Standard Deviation).

Abbreviations: F: Females, HDL-c: High-Density Lipoprotein, LDL-c: Low Density Lipoprotein, C NON HDL: Non HDL Cholesterol, IGF-1: Insulin like growth factor, VIT D: Vitamin D, GLC: Glucose, AUC: Area Under the Curve, INS: Insulin, IRI: Total Insulin during OGTT, HOMA: Homeostatic Model Assessment of Insulin Resistance, ISI: Insulin Sensitivity Index, M: Males, QUICKI: Quantitative Insulin-Sensitivity Check Index.

Table 3. Nutritional data and physical activity level of the whole sample.

	Females	Males	Prepubertal	Pubertal
Subjects	24	25	17 (F8/M9)	32 (F26/M16)
MAI	1.9±1.6	1.6±0.8	1.7±1.5	1.7±1.0
Total energy (KCal)	2093.1±482.2	2464.8±511.1	2347.2±566.2	2261.7±506.6
Carbohydrates %	46.2±5.7	50.4±4.5	49.5±5.0	47.8±5.7
Carbohydrates (g)	244.2±67.9	311.2±79.2	288.1±82.2	275.9±81.0
Fat %	38.9±5.3	35.0±4.0	36.5±4.6	37.0±5.3
Fat (g)	90.1±22.7	94.5±19.8	93.9±23.3	91.5±19.7
Proteins %	14.9±2.0	14.3±2.0	14.0±2.2	15.0±1.8
Proteins (g)	74.5±16.4	84.9±16.5	78.5±16.3	81.3±17.8
KIDMED total score	4.0±2.5	5.1±2.1	5.1±2.0	4.3±2.5
KIDMED adherence score				
low	40.0(%)	25.0(%)	30.0(%)	33.33(%)
medium	56.0(%)	60.7(%)	55.0(%)	60.61(%)
high	4.0(%)	14.28(%)	15.0(%)	6.06(%)
Met day	191.3±148.2	330.1±329.8	271.3±345.7	264.2±211.7
Level				
0	32.0(%)	17.2(%)	22.7(%)	25.0(%)
1	48.0(%)	51.7(%)	59.1(%)	43.8(%)
2	20.0(%)	31.0(%)	18.2(%)	31.3(%)

All data are expressed as media ± SD (Standard Deviation) or percentage.
Abbreviations: F: Females, M: Males, MAI: Mediterranean Adherence Index, Met: Metabolic Equivalent of Task. Level of physical activity have been categorized as 0: 1° tertile; 1: 2° tertile; 2: 3° tertile.

Table 4. Lefse analysis BMI SDS (A: 1° tertile, B: 2° tertile; C: 3° tertile).

Lefse BMI

	Pvalues	FDR	A	B	C
unclassified_Peptostreptococcaceae	0.003442	0.16897	23995	10697	27105
Bifidobacterium_bifidum	0.00476	0.16897	24681	10848	58196
unclassified_Actinobacteria	0.020491	0.48495	19002	11821	39794

Table 5. Lefse analysis OIL (0: who didn't consume extra-virgin olive oil, 1: who consumed extra-virgin olive oil).

Lefse OIL

	Pvalues	FDR	0	1	LDAscore
unclassified_Alistipes	0,0039182	0,27819	219990	65403	4,89
unclassified_Barnesiella	0,0095266	0,3382	138510	60684	4,59
unclassified_Bacteroidales	0,019442	0,34509	715760	383460	5,22
Alistipes_putredinis	0,019442	0,34509	389610	165780	5,05
unclassified_Gemmiger	0,031211	0,37175	114490	65819	4,39
Odoribacter_splanchnicus_DSM_220712	0,04461	0,37175	33985	13917	4
unclassified_Collinsella	0,048627	0,37175	38117	19473	3,97

Table 6. Lefse analysis breakfast (0: breakfast consumed, -1: skipping breakfast).

Lefse BREAKFAST

	Pvalues	FDR	0	-1	LDAscore
Barnesiella	0.028105	0.97848	88880	178670	-4.65

Figure I. Heatmap of core microbiome. The heat map graphically represented the overall abundance of taxonomic groups in the sample. The colour scale represented the scaled abundance of each variable with red indicating high abundance and blue indicating low abundance.

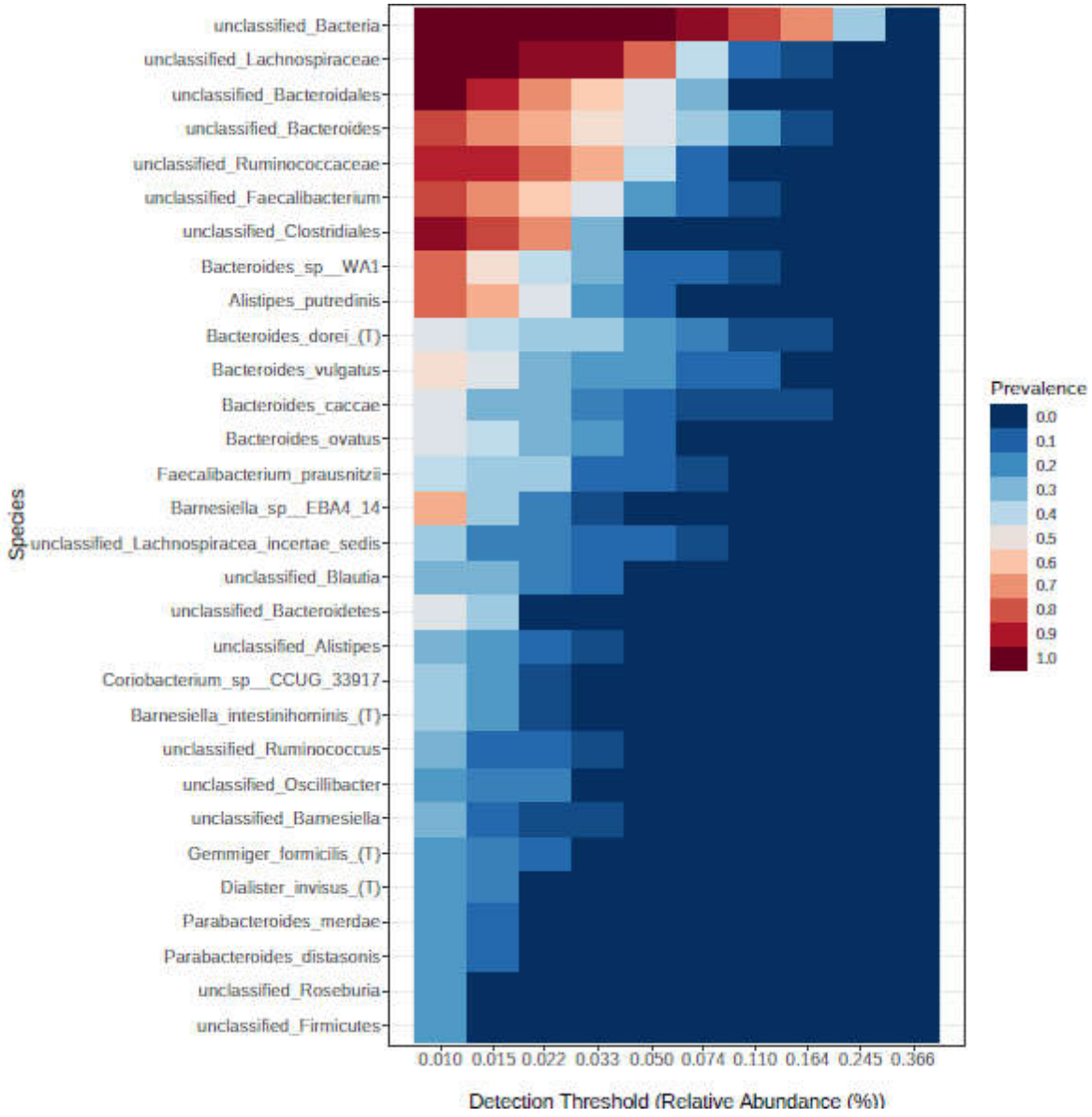


Figure II, Panel a, b and c -PCA. Contribution of each subject (represented as a dot) on the whole variability in different dimensions (Dm1-Dm5). The colour scale represented the scaled contribution with red indicating high contribution and blue indicating low contribution.

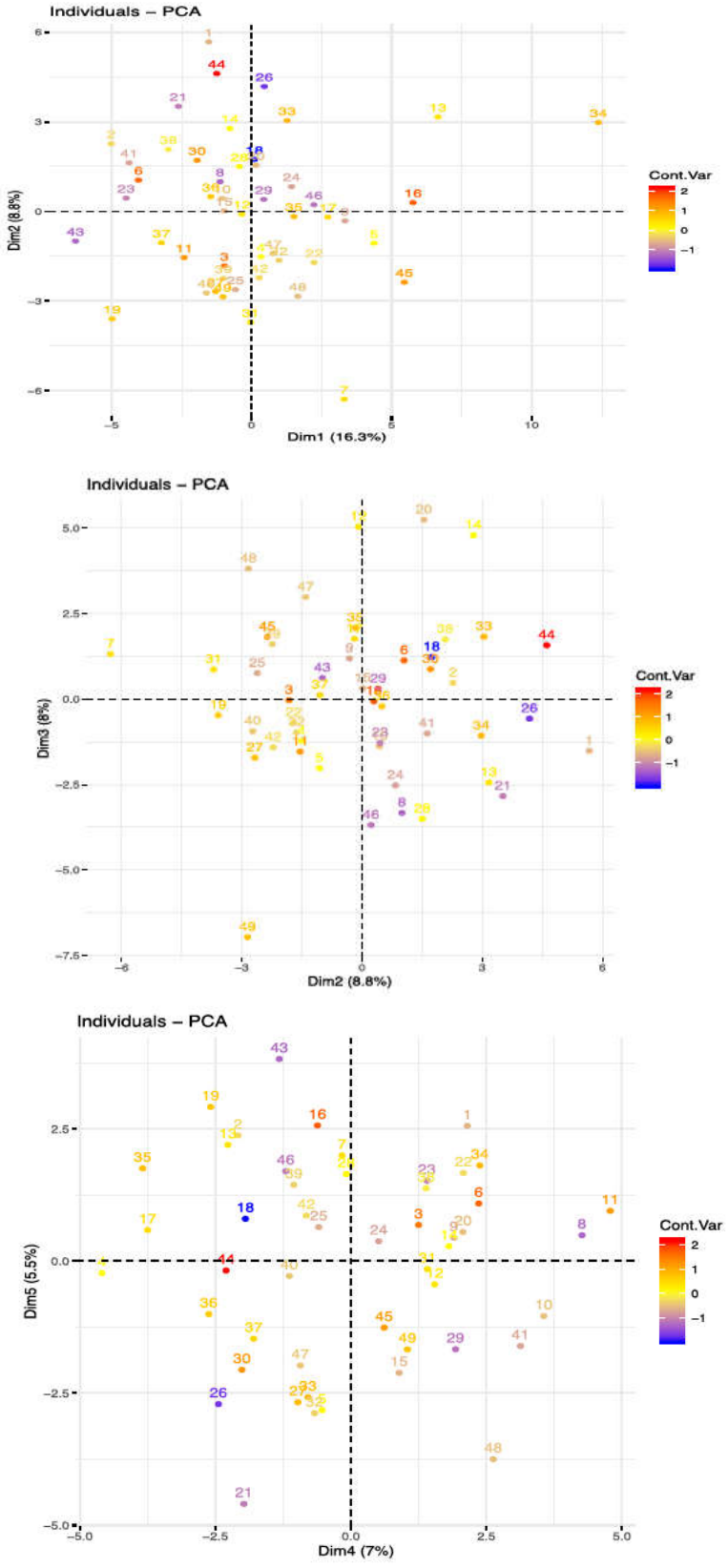


Figure III, Panel a, b and c –PCA. Contribution of each subject clusterized according to puberty and gender on the whole variability in different dimensions (Dm1-Dm5). F1 – M1: starting-puberty (F: females; M: males); F2 – M2: end-puberty (F: females; M: males).

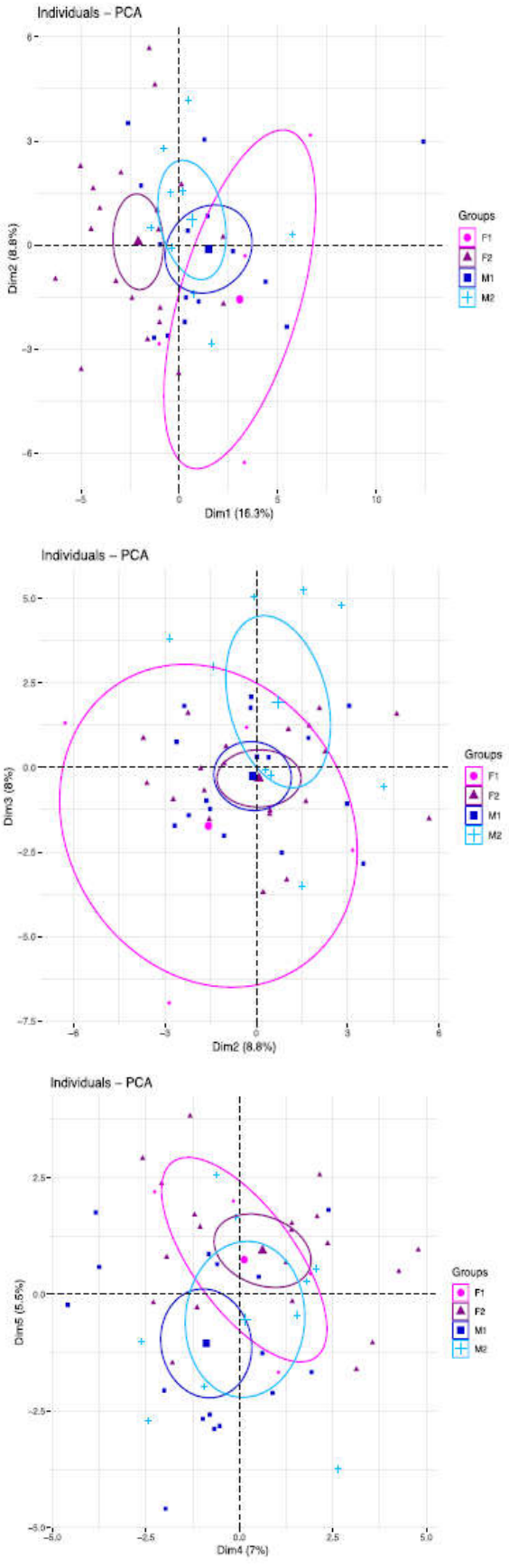


Figure IV. Contribution of each parameter on the whole variability in different dimensions (Dm1-Dm5). Larger size of the dot represented high contribution.

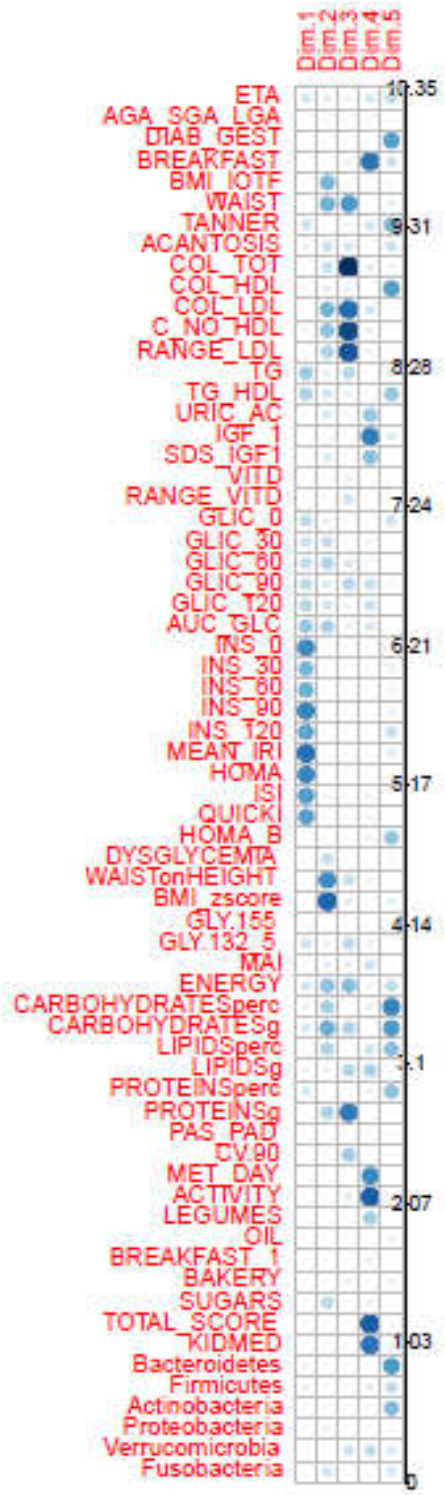


Figure V, Panel a, b and c PCA analysis at Phyla level in different dimensions (Dm1-Dm5). The larger dots represented the maiority distribution of sample, while the small dots indicated each subject, both clusterized according to puberty and gender. Red arrows represented the anthropometric, biochemical and nutritional variables; blu arrows represented Phyla. This information combined showed which variables are more influent on different bacterial Phylum. F1 – M1: starting-puberty (F: females; M: males); F2 – M2: end-puberty (F: females; M: males).

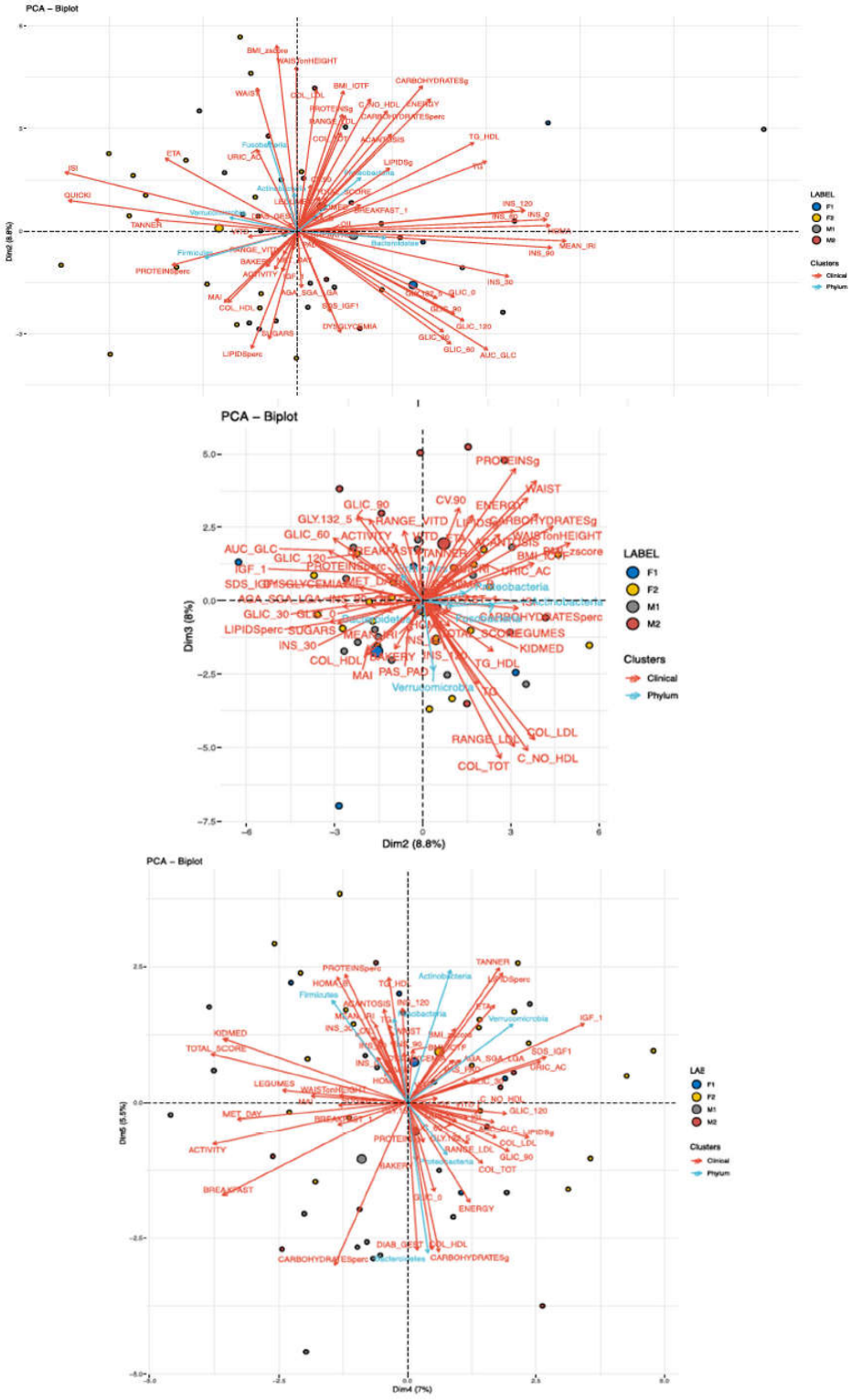


Figure VI. α-diversity (index Observed) BMI according to IOTF criteria. 2: overweight; 3: obesity. 4: morbid obesity. *P value = 0.03*.

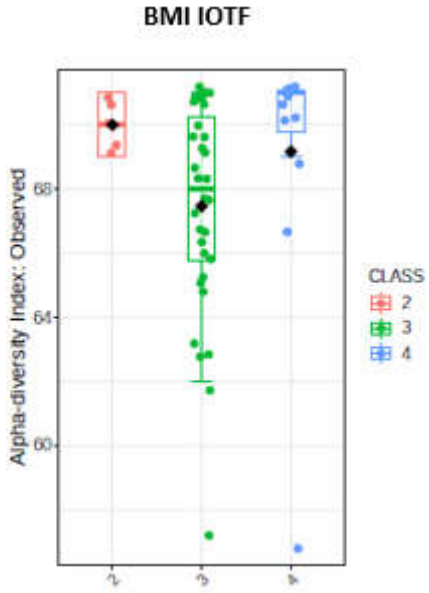


Figure VII. β-diversity (index Observed) BMI according to IOTF criteria IOTF. 2: overweight; 3: obesity. 4: morbid obesity. *P value < 0.045.*

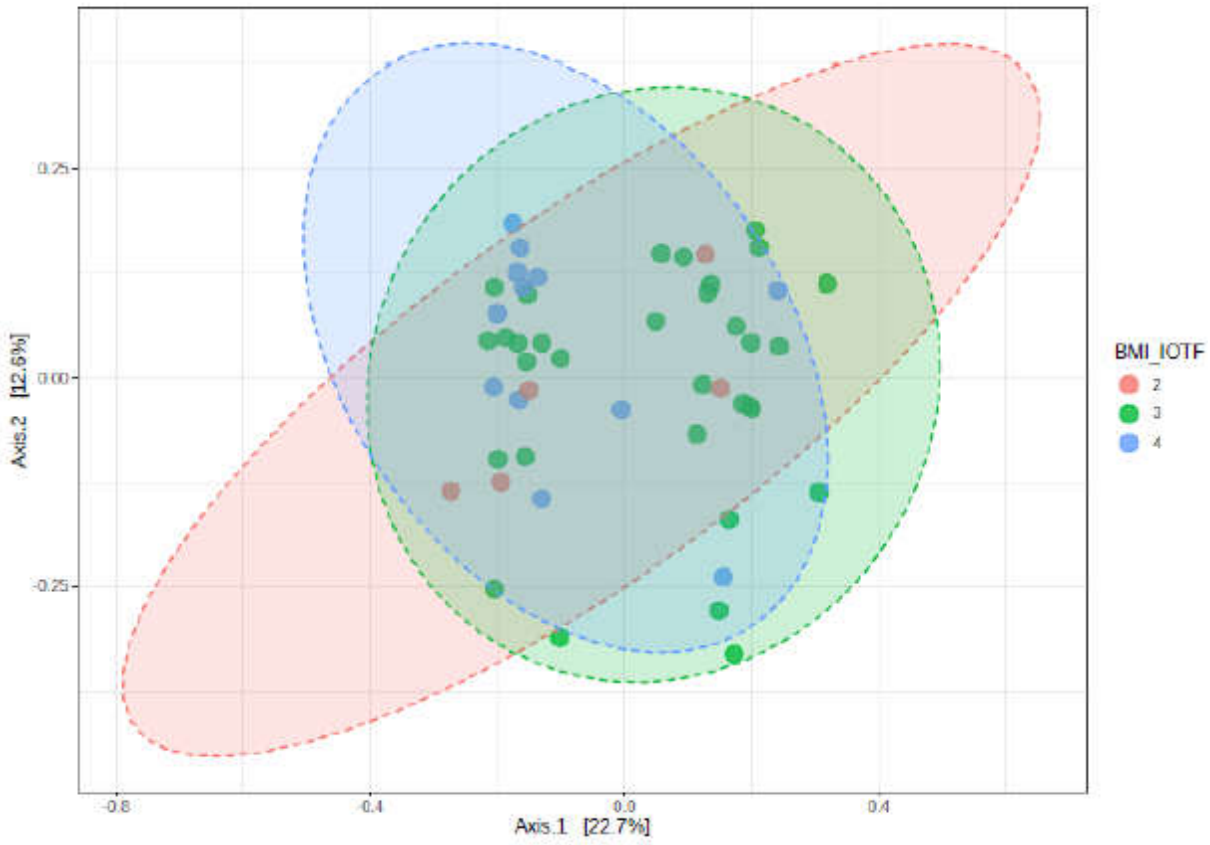
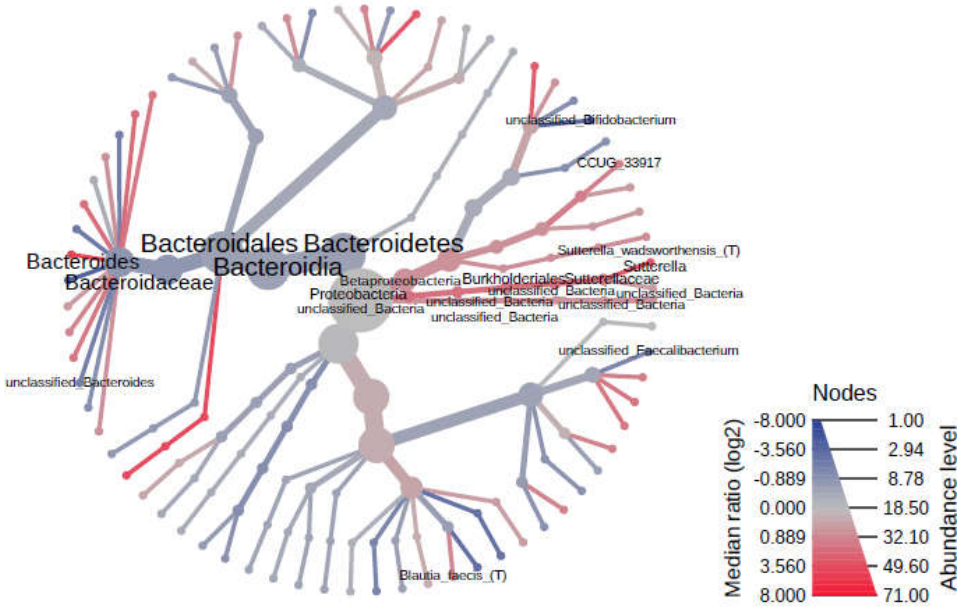


Figure VIII, Panel a and b. Heat tree BMI according to IOTF criteria; (a) heat tree BMI SDS (b).

Panel a. Morbid obesity versus obesity. The colour scale represented the scaled contribution with red indicating high abundance in morbid obesity and blue indicating high abundance in obesity.



Panel b. BMIS SDS: 3° tertile versus 1° tertile. The colour scale represented the scaled contribution with red indicating high abundance in 3° tertile and blue indicating high abundance in 1° tertile.

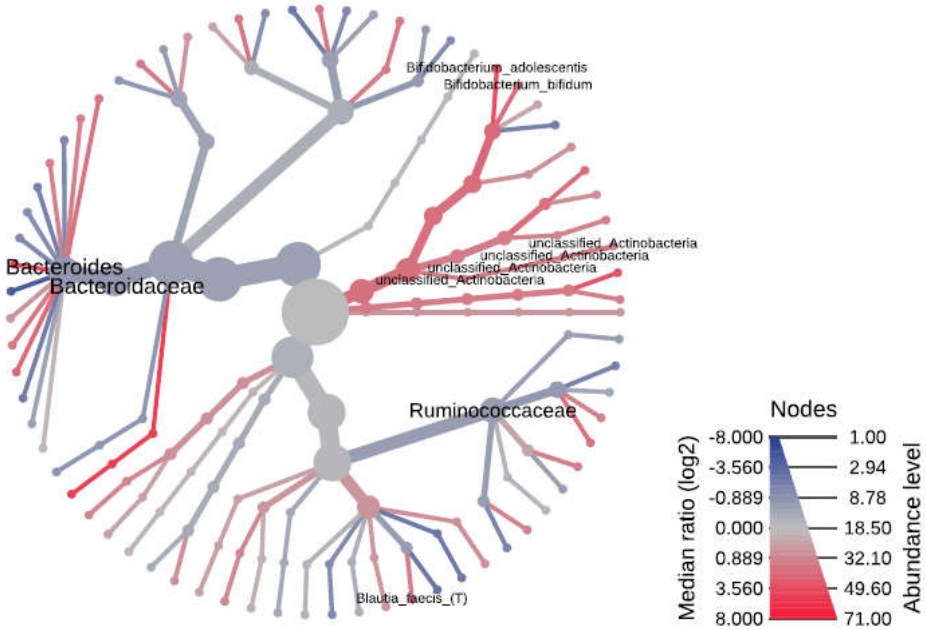


Figure IX. Heat tree Total cholesterol (1° tertile versus 3° tertile). The colour scale represented the scaled contribution with red indicating high abundance in 1° tertile and blue indicating high abundance in 3° tertile.

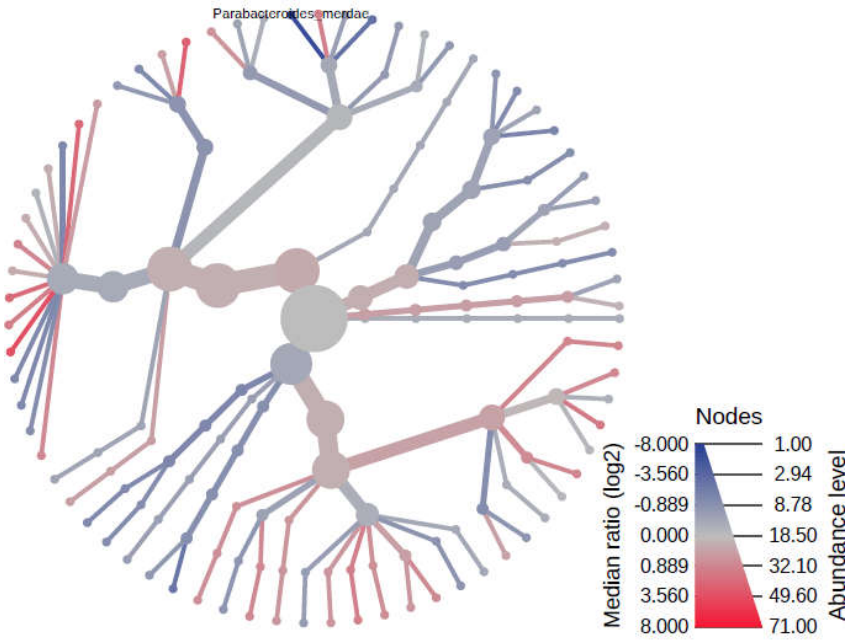


Figure X. Heat tree HOMA percentile (HOMA < 95° versus HOMA ≥ 95°). The colour scale represented the scaled contribution with red indicating high abundance in HOMA < 95° percentile and blue indicating high abundance in HOMA ≥ 95° percentile.

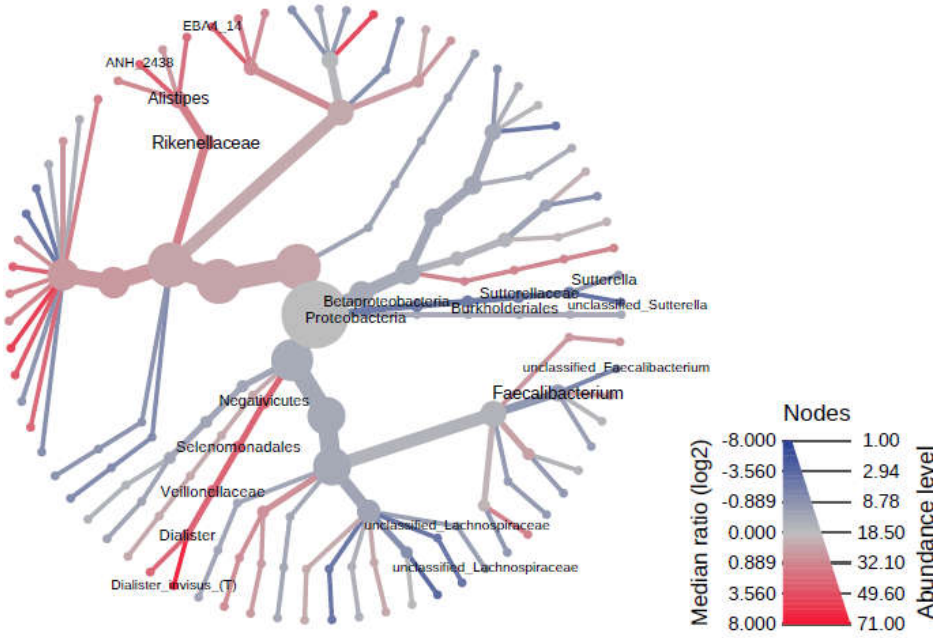
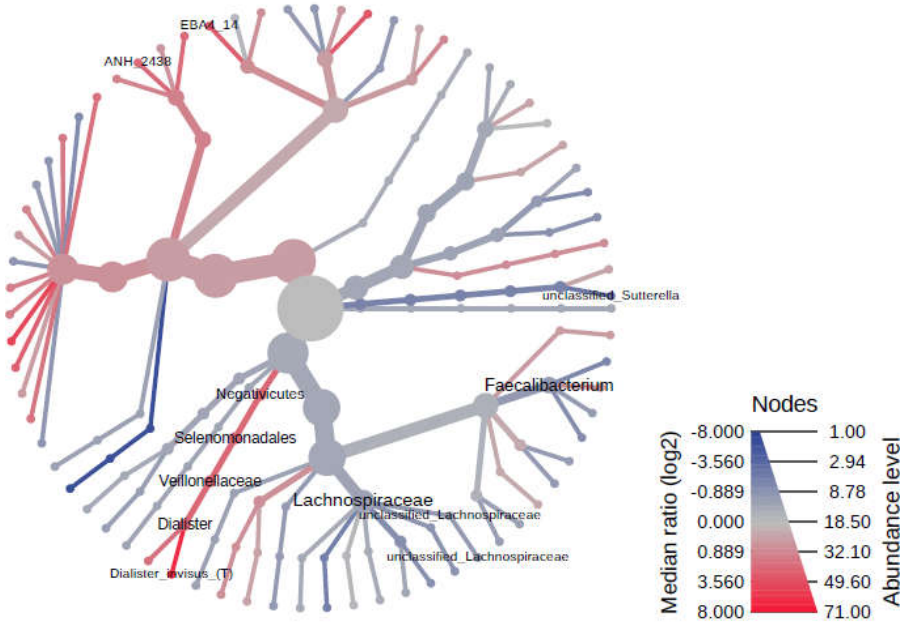


Figure XI. Panel a and b. Heat tree QUICKI.

Panel a. QUICKI: 2° tertile versus 1° tertile. The colour scale represented the scaled contribution with red indicating high abundance in 2° tertile and blue indicating high abundance in 1° tertile.



Panel b. QUICKI: 3° tertile versus 1° tertile. The colour scale represented the scaled contribution with red indicating high abundance in 3° tertile and blue indicating high abundance in 1° tertile.

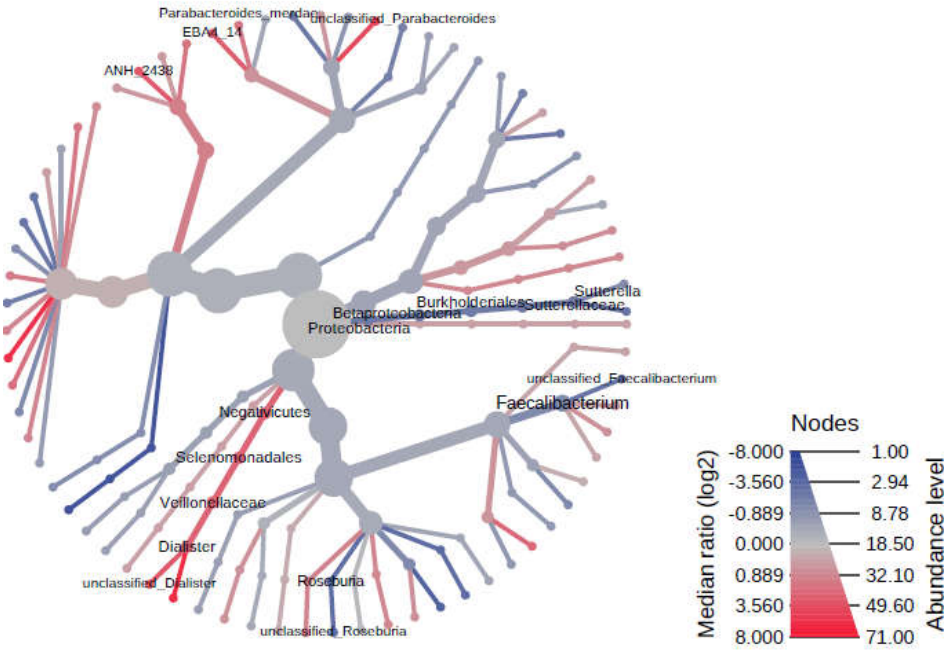


Figure XII. α-diversity (index Observed) ISI. A: 1° tertile; B: 2° tertile; C: 3° tertile.
P value = 0.03.

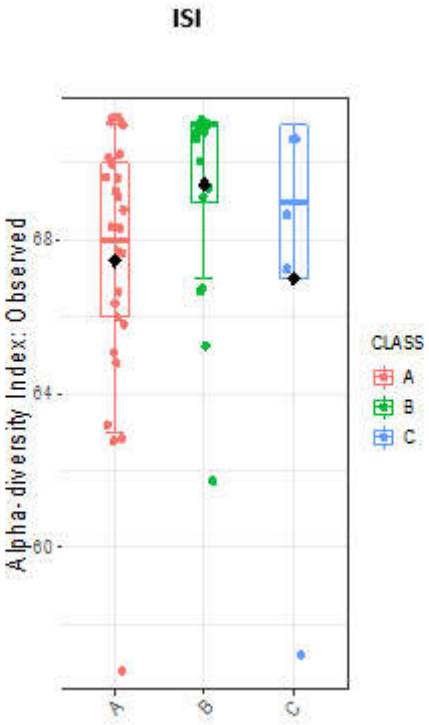


Figure XIII. α-diversity carbohydrates (CHO) percentage (index Observed at the left; index Simpson in the middle; index Shannon at the right). A: < 45% CHO; B: 45-55% CHO; C: ≥ 55% CHO.
P value index Observed = 0.04; P value index Shannon =0 .02; P value index Simpson = 0.004.

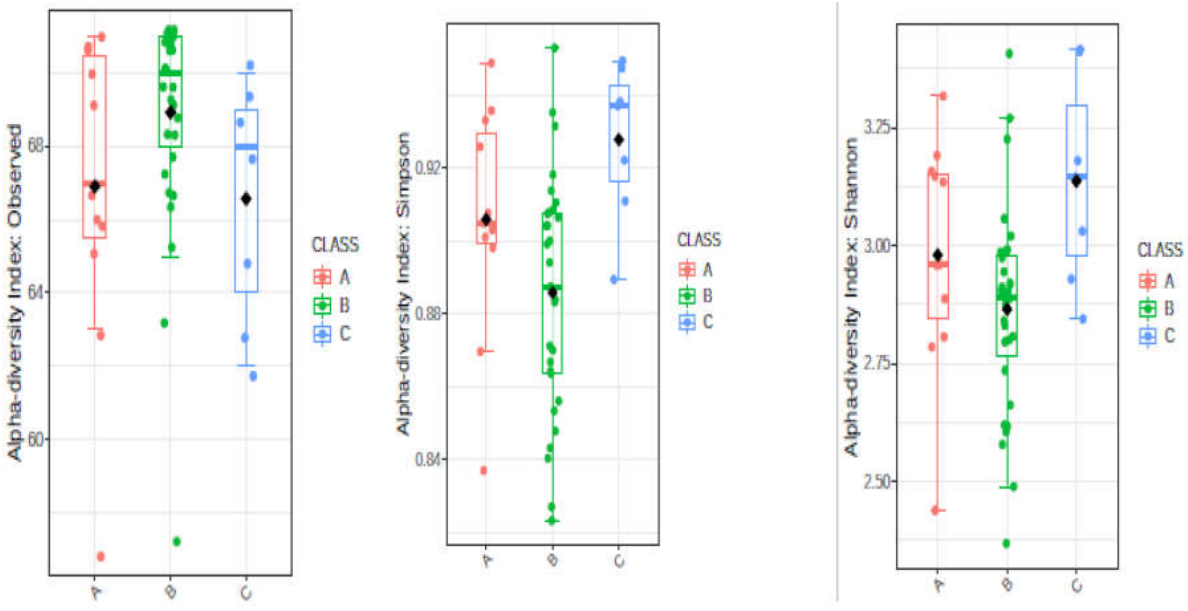


Figure XIV. β-diversity carbohydrates (CHO) percentage. A: < 45% CHO; B: 45-55% CHO; C: ≥ 55% CHO.

P value < 0.001.

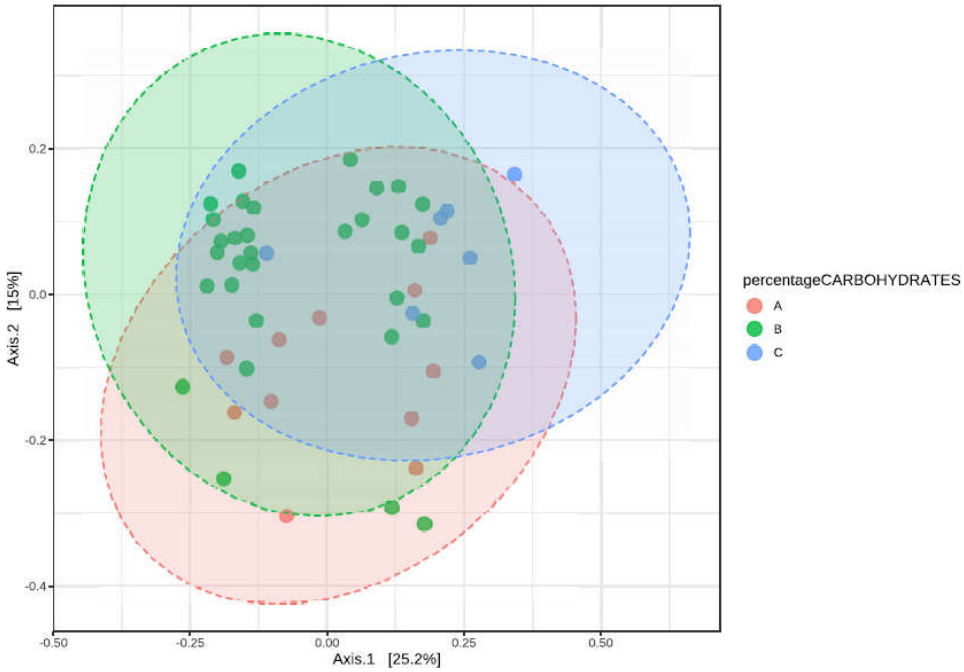


Figure XV. Heat tree carbohydrates (CHO) percentage (< 45% CHO versus ≥ 55% CHO). The colour scale represented the scaled contribution with red indicating high abundance in < 45% CHO and blue indicating high abundance in ≥ 55% CHO.

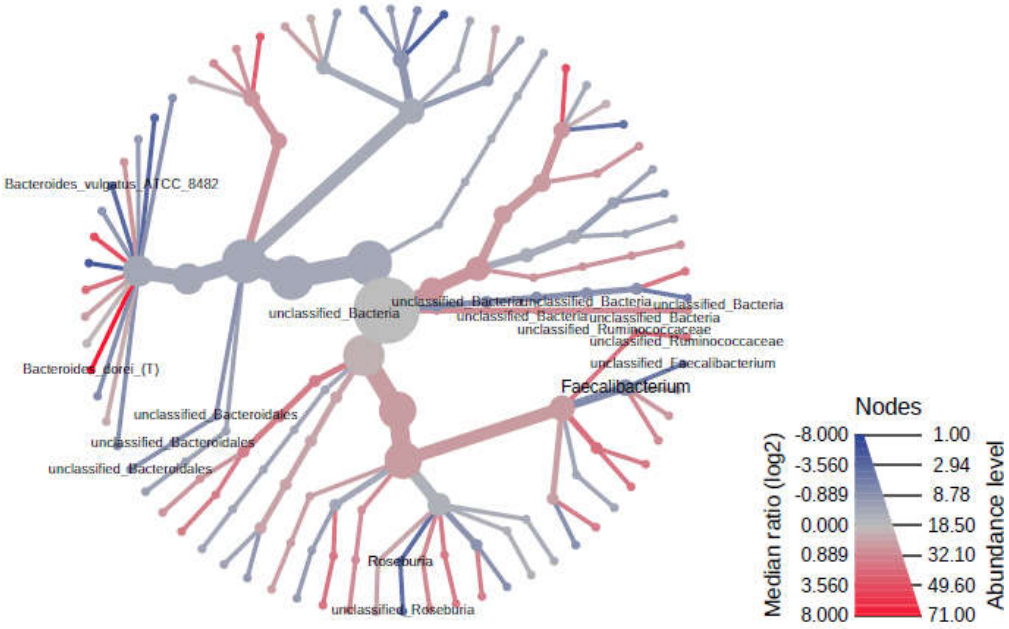


Figure XVI. Heat tree proteins percentage (< 15% versus ≥ 15% proteins). The colour scale represented the scaled contribution with red indicating high abundance in < 15% proteins and blue indicating high abundance in ≥ 15% proteins.

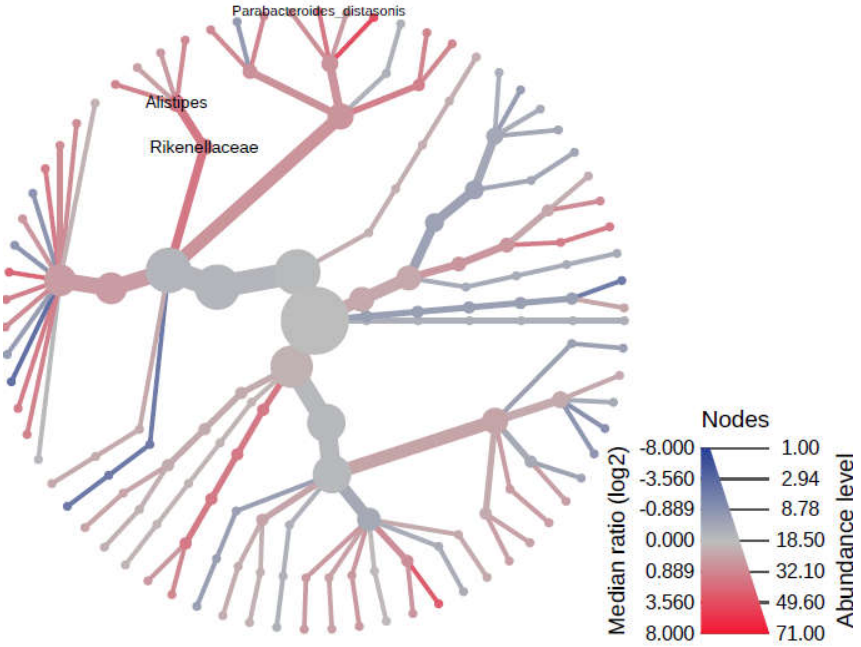


Figure XVII. Heat tree fats percentage (> 35% versus < 30% fats). The colour scale represented the scaled contribution with red indicating high abundance in > 35% fats and blue indicating high abundance in < 30% fats.

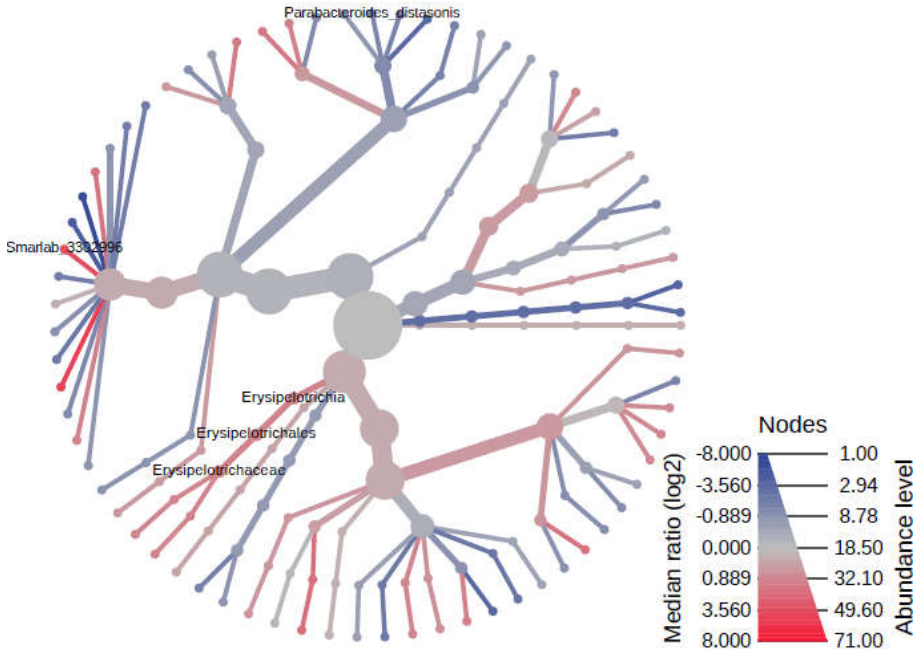


Figure XVIII. β-diversity KIDMED score. 0: low adherence; 1: medium adherence; 2: high adherence.

P value < 0.02.

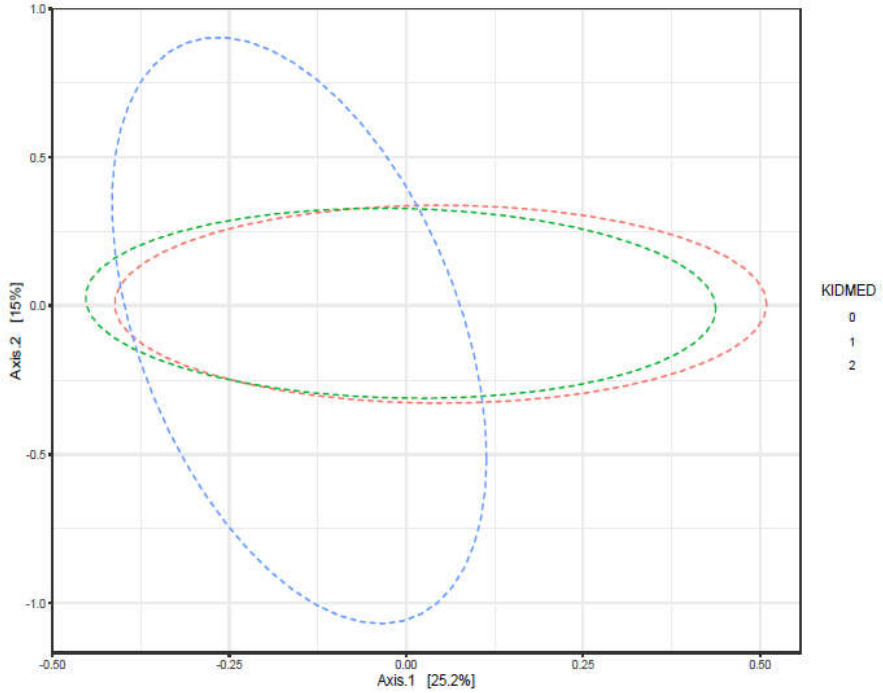


Figure XIX. α-diversity (index Observed) KIDMED score. 0: low adherence; 1: medium adherence; 2: high adherence.

P value = 0.04.

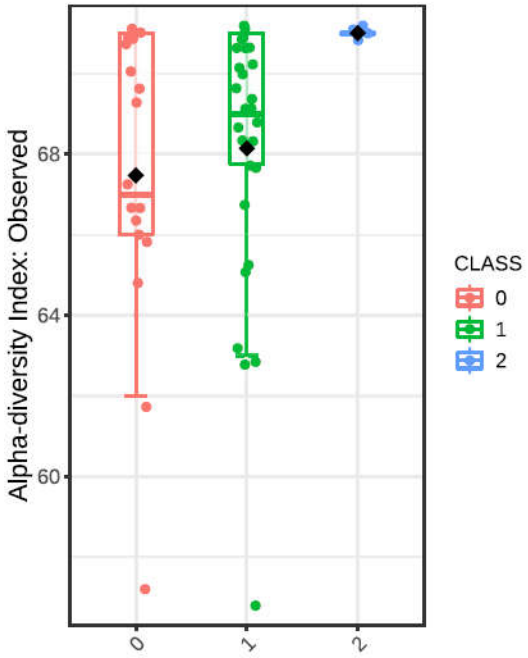


Figure XX. Heat tree KIDMED item OIL (who consumed extra-virgin olive oil versus those who did not consume it). The colour scale represented the scaled contribution with red indicating high abundance in those who consumed extra-virgin olive oil and blue indicating high abundance in those who did not consume it.

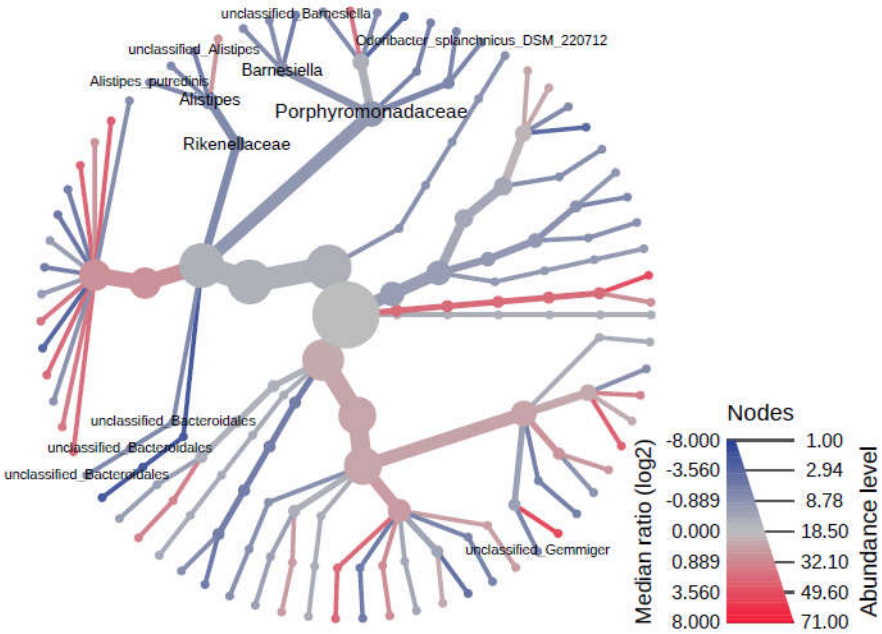


Figure XXI. Heat tree KIDMED item BAKERY (who consumed baked goods or pastries for breakfast versus those who did not consume it). The colour scale represented the scaled contribution with red indicating high abundance in those who consumed baked goods or pastries for breakfast and blue indicating high abundance in those who did not consume it.

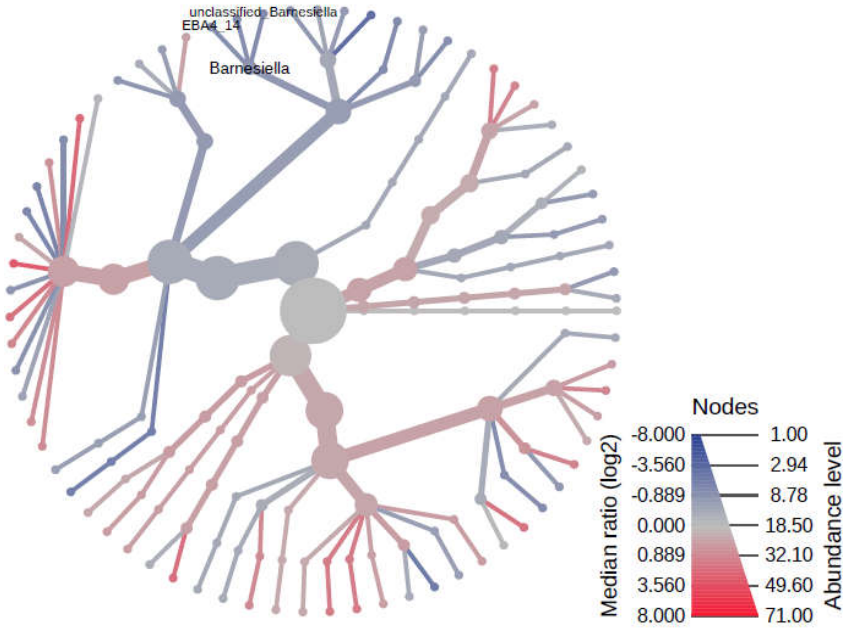
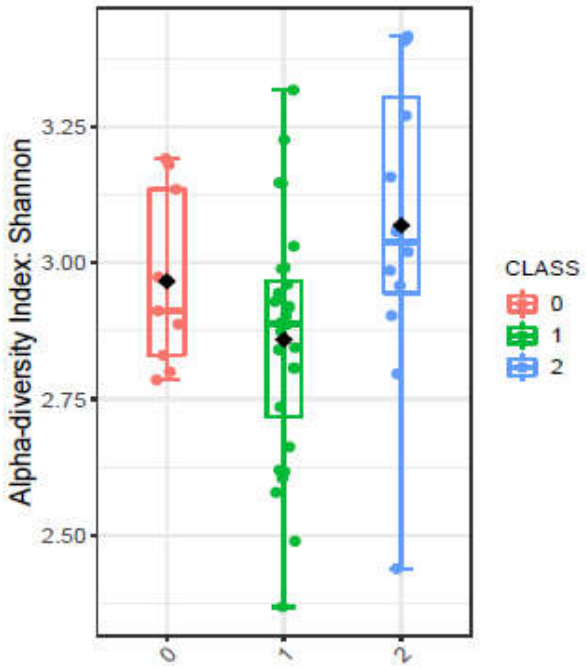


Figure XXII. α-diversity (index Shannon) physical activity intensity level. 0: low; 1: medium; 2: high.

P value = 0.04.



4.2.ii. References

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di origine
D.O.P.
Prodam