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CHILDHOOD OBESITY AND RELATED COMORBIDITIES:
FROM UNHEALTHY DIET TO A FOOD-BASED APPROACH

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ABSTRACT

The childhood obesity epidemic, that is rapidly increased in most high-income, low- and middle-income countries, is considered as one of the most serious global public health challenges for the 21st century. It may be associated with adverse health effects during childhood and an increased risk of premature morbidity and mortality later in life. Investigating possible therapeutic strategies able to counteract negative effects on child health and the risk of more severe comorbidities during adulthood is considered as a major priority. Intensive lifestyle modifications, involving diet, physical activity and behaviors are fundamental to achieve this goal. However, the characteristics of all intervention components as well as the length, the intensity, and the effectiveness of lifestyle interventions may vary largely among studies.

Additionally, recently a great deal of attention has been focused on the gut microbiota as "environmental factor" playing an important role in the development of obesity and its complications and several mechanisms able to explain this association have been proposed. This evidence needs to be further elucidated because it may have a relevant role in prevention and treatment of childhood obesity.

Lastly, diets high in fruits and vegetables are widely recommended for their health-promoting properties, as they are important sources of dietary fiber, vitamins, especially vitamins C and A, minerals and phytochemicals, especially antioxidants and polyphenols. It has been suggested that, among phytochemicals, salicylic acid may have an important role, being involved in the regulation of inflammation, oxidative stress and glucose metabolism.

The present PhD thesis tried to further elucidate these topics through three different tasks. The primary aim of the present PhD thesis was to evaluate whether a 1-year lifestyle intervention, based on normocaloric diet, promotion of physical activity and behavior changes, may improve obesity, metabolic profile and obesity-related comorbidities, as glucose metabolism alterations, hyperlipidemia, prehypertension/hypertension, increased liver echogenicity and metabolic syndrome, in a cohort of obese children. Secondary aims were to evaluate qualitatively and quantitatively gut microbiota biodiversity in obese and normal-weight children and to compare gut microbiota profiles with SCFAs and BMI z-scores to gain insights into the structure and activity of the microbiota in pediatric obesity. The tertiary aim was to determine the concentrations of serum salicylic acid in a group of obese children, compared to normal-weight children, and to evaluate if an association may exist between serum salicylic acid and fruit and vegetable consumption.

Our results confirmed that obesity is associated with detrimental effects on health already during pediatric age, thus children may show prehypertension/hypertension, insulin resistance, pre-diabetes, hyperlipidemia, liver steatosis and metabolic syndrome.

Moreover, childhood obesity may be associated with changes of some core microbial species, preexisting or diet-induced, and these changes may be involved in the etiology of obesity. Among these, an alteration of the gut microbiota composition of obese children, characterized by an increased abundance of Firmicutes and a decreased abundance of Bacteroidetes, was observed.

Although beneficial effects of fruit and vegetable consumption are well-known, results from our study showed that fruit and vegetable consumption in children was very low, about 50% lower of the minimum recommended value by WHO (400 g daily). Furthermore, obese children had lower levels of serum salicylic acid than normal-weight children. These results suggest that nutrition education towards an adequate fruit and vegetable consumption should be stressed in children. Moreover, although the relationship of serum salicylic acid with fruit and vegetables consumption did not reach statistical significance both in obese and normal-weight children, promotion of fruit

and vegetables with higher content of salicylic acid might be considered as part of the nutrition counseling for obese children.

Finally, findings from our longitudinal study clearly highlighted the importance of a 1-year lifestyle intervention, based on a normocaloric Mediterranean balanced diet for pediatric age, promotion of physical activity and behavior changes, in the improvement of cardio-metabolic risk factors and in the reduction of the prevalence of some obesity-related comorbidities, as insulin resistance, pre-diabetes, prehypertension/hypertension, hypertriglyceridemia, higher liver echogenicity and metabolic syndrome.

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RIASSUNTO

La cosiddetta epidemia di obesità in età pediatrica, che è rapidamente aumentata nei Paesi ad alto, medio e basso reddito, è considerata uno dei più gravi problemi di salute pubblica del 21° secolo a livello mondiale. Questa può difatti associarsi ad effetti negativi sulla salute del bambino e ad un aumentato rischio di morbidità e mortalità prematura in età adulta. Pertanto, una delle principali priorità della nostra epoca è quella di analizzare le possibili strategie terapeutiche, in grado di contrastare gli effetti negativi sulla salute dei bambini e il rischio di comorbidità più gravi durante l'età adulta. Per raggiungere questo obiettivo sono fondamentali delle modifiche dello stile di vita, che prevedano quindi cambiamenti dell'alimentazione, del livello di attività fisica e dei comportamenti. Tuttavia, le caratteristiche specifiche, la durata, l'intensità e l'efficacia degli interventi sullo stile di vita possono variare ampiamente tra gli studi.

Inoltre, recentemente, molta attenzione è stata posta al microbiota intestinale come "fattore ambientale" in grado di giocare un ruolo importante nello sviluppo dell'obesità e delle sue complicanze e sono stati proposti diversi meccanismi in grado di spiegare questa associazione. Questo aspetto dovrebbe essere maggiormente approfondito poiché potrebbe avere un ruolo rilevante nella prevenzione e nel trattamento dell'obesità pediatrica.

Infine, le diete che comprendono un'elevata assunzione di frutta e verdura sono ampiamente raccomandate per i loro effetti positivi sulla salute, in quanto frutta e verdura rappresentano un'importante fonte di fibra alimentare, vitamine, soprattutto vitamina C ed A, sali minerali e sostanze fitochimiche, antiossidanti e polifenoli. È stato suggerito che, tra le sostanze fitochimiche, l'acido salicilico potrebbe avere un ruolo rilevante, essendo coinvolto nella regolazione dell'infiammazione, dello stress ossidativo e del metabolismo glucidico.

La presente tesi di dottorato ha cercato di chiarire ulteriormente questi argomenti attraverso tre differenti studi. L'obiettivo primario è stato quello di valutare se un "lifestyle intervention" della durata di un anno, basato su dieta normocalorica, promozione dell'attività fisica e cambiamenti comportamentali, possa determinare un miglioramento dello stato di obesità, un miglioramento del profilo metabolico e delle comorbidità associate all'obesità, tra cui eventuali alterazioni del profilo glucidico e lipidico, stato di preipertensione/ipertensione, iperecogenicità epatica e sindrome metabolica, in una coorte di bambini obesi. Obiettivi secondari sono stati valutare qualitativamente e quantitativamente la biodiversità del microbiota intestinale di bambini obesi e normopeso e confrontare il profilo degli acidi grassi a corta catena (SCFA) in relazione al BMI z-score, per ottenere informazioni sulla composizione e l'attività del microbiota intestinale associato all'obesità in età pediatrica. Il terzo scopo è stato quello di determinare le concentrazioni di acido salicilico sierico in un gruppo di bambini obesi, rispetto ad un gruppo di controllo rappresentato da bambini normopeso, e valutare l'eventuale presenza di un'associazione tra acido salicilico sierico e consumo di frutta e verdura.

I nostri risultati hanno confermato che l'obesità in età pediatrica si associa ad effetti negativi sulla salute dei bambini, nei quali si possono già manifestare complicanze quali: preipertensione/ipertensione, insulino-resistenza, pre-diabete, iperlipidemia, steatosi epatica e sindrome metabolica.

Inoltre, l'obesità in età pediatrica si può associare a cambiamenti della composizione del microbiota intestinale, preesistenti o indotti dalla dieta, e questi cambiamenti potrebbero essere coinvolti nell'eziologia dell'obesità. Tra questi è stata osservata una alterazione della composizione del microbiota intestinale, caratterizzata da aumentati livelli di Firmicutes e ridotti livelli di Bacteroidetes.

Sebbene gli effetti benefici legati al consumo di frutta e verdura siano ben noti, i risultati del nostro studio hanno mostrato che il consumo di frutta e verdura nei bambini era molto basso, circa il 50%

in meno del valore minimo raccomandato dall'OMS (400 g al giorno). Inoltre, i soggetti affetti da obesità hanno mostrato livelli inferiori di acido salicilico sierico rispetto ai bambini normopeso. Questi risultati suggeriscono la necessità di promuovere maggiormente un adeguato consumo di frutta e verdura in età pediatrica. Inoltre, sebbene l'associazione tra acido salicilico sierico e consumo di frutta e verdura non abbia raggiunto la significatività statistica in entrambi gruppi, la promozione di frutta e verdura con un maggiore contenuto di acido salicilico potrebbe essere considerata parte integrante dell'educazione nutrizionale in caso di obesità essenziale.

Infine, i risultati del nostro studio longitudinale hanno sottolineato l'importanza del "lifestyle intervention", basato su una dieta equilibrata, normocalorica e di tipo Mediterraneo, sulla promozione dell'attività fisica e sui cambiamenti comportamentali, nel miglioramento dei fattori di rischio cardio-metabolici e nella riduzione della prevalenza di alcune comorbidità associate all'obesità essenziale in età pediatrica, come la resistenza insulinica, il pre-diabete, la preipertensione/ipertensione, l'ipertrigliceridemia, l'iperecogenicità epatica e la sindrome metabolica.

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1. INTRODUCTION

1.1. CHILDHOOD OBESITY

1.1.1. How big is the problem?

The so-called "Globesity", global epidemic of obesity and being overweight is rapidly becoming a major public health problem in many parts of the world, since it represents a risk factor for serious noncommunicable diseases (NCDs), including diabetes mellitus, cardiovascular disease, hypertension and stroke, and certain forms of cancer [1].

Of global concern is especially the childhood obesity epidemic that is rapidly increased in most high-income as well as low- and middle-income countries [2]. Indeed, the World Health Organization (WHO) considers childhood obesity as one of the most serious global public health challenges for the 21st century [3]. In absolute numbers more overweight and obese children live in low- and middle-income countries than in high-income countries. For example, in Africa the number of children who are overweight or obese has nearly doubled since 1990, increasing from 5.4 million to 10.3 million [2]. On the other hand, although the rise in obesity prevalence in several high-income countries might be reaching a plateau, prevalence is historically high and is considered as a "time bomb" for future demands on health services [4]. Estimates suggest that one in five children in Europe is overweight and that 400,000 children become overweight each year [5].

In recent years, the worldwide prevalence of childhood overweight and obesity among preschool children increased from 4.2% in 1990 to 6.7% in 2010 and is expected that, in 2020, this trend will reach 9.1%, or about 60 million of children [6]. However, from the WHO Childhood Obesity Surveillance Initiative (COSI) [7] resulted that in some European countries efforts and interventions to decrease the prevalence of childhood obesity may have some positive results, although small. Effectively, a recent paper, aiming to explore changes in overweight among 6–9 year-old children, within and across nine countries, from school years 2007/2008 (Round 1) to 2009/2010 (Round 2), showed that between rounds, countries with higher prevalence of overweight in Round 1, for example Italy and Portugal, showed a decrease in prevalence. On the other hand, however, countries with lower prevalence in Round 1, for example Latvia and Norway, showed an increase in prevalence [7]. However, the prevalence of obesity in these children ranged from 6% to 31% in boys and 5% to 21% in girls [7]. The Health Behaviour in School-aged Children study in the WHO European Region in 2009–2010 showed that the

prevalence of overweight and obesity was 11–33% for children aged 11 years, 12–27% for children aged 13 years and 10–23% for those aged 15 years [8]. Recently, in Italy data from a national surveillance system promoted by the Ministry of Health in 2007, named "OKkio alla SALUTE", showed that although in last years there was a slight and gradual reduction of obesity prevalence, it remained still high: in 2014 prevalence of overweight and obesity among Italian children aged 8-9 years was 20.9% and 9.8%, respectively [9].

1.1.2. The economic burden of obesity

The ancient concept that "bigger is better", with a "chubby" child considered as a healthy child is now outdated. Indeed, nowadays it is well-known that childhood obesity may be associated with adverse health complications and an increased risk of premature morbidity and mortality later in life [6]. The increased incidence of comorbidities in obese children is likely to lead to increased health-care utilization and expenditures even during the school-age years and adolescence [10]. However, the rise in health-care expenditure and utilization also represent a small subset of burden that can be attributed to childhood obesity. It is difficult to quantify indirect costs such as lost school days and workdays associated with comorbidities of childhood obesity [10]. Since obese children are at greater risk of adult obesity [11,12] a portion of economic costs associated with adult obesity may also be attributable to childhood obesity [10]. An analysis of the 2001–2003 Medical Expenditure Panel Survey recognized that obese children had annual total health-care expenditures $220 higher than children with a normal BMI [13].

1.1.3. Definition

Overweight and obesity are defined as "abnormal or excessive fat accumulation that presents a risk to health" [14]. A practical and simple approach, providing an acceptable approximation for assessment of total body fat, is represented by the body mass index (BMI). BMI is defined as a person's weight in kilograms divided by the square of his height in meters (kg/m2). Since during childhood bodies undergo several physiological changes as they grow, different methods for the measurement of overweight and obesity are available, depending on the age [14].

0-24 months of age

Up to 24 months of age, overweight and obesity are identified using weight-for-length WHO child growth standards [15], according to the following cut-offs:

Risk of overweight	>85° percentile	>1 SD
Overweight	>97° percentile	>2 SD
Obesity	> 99° percentile	>3 SD

2-5 years of age

Up to age of 5, overweight and obesity are identified using BMI-for-age WHO child growth standards [16], according to the following cut-offs:

Risk of overweight	>85° percentile	>1 SD
Overweight	>97° percentile	>2 SD
Obesity	> 99° percentile	>3 SD

5-18 years of age

Up to age of 18, overweight and obesity are identified using Italian BMI charts [17] or BMI-for-age WHO Growth references [18], according to the following cut-offs:

	Cacciari, 2006	WHO 2007	
Overweight	≥75° percentile	>85° percentile	>1 SD
Obesity	≥95° percentile	>97° percentile	>2 SD

1.1.4. Causes

In most cases, obesity is the consequence of a chronic imbalance between energy intake and energy expenditure, involving many environmental and life-style factors, such as easy access to energy-dense foods, increased portion sizes, reduced physical activity and increased time spent in sedentary activities. A chronic exposure over time to these adverse factors may potentiate weight gain over many years [19].

Many children today are growing up in an obesogenic environment that stimulates weight gain and obesity. Moreover, the behavioral and biological responses of a child to this environment can be shaped by processes even before birth, which can increase the risk of obesity if the child faced

with an unhealthy diet and low physical activity [2]. Among factors that should be considered as potentially involved in later risk of obesity there are: maternal prepregnancy weight and nutritional status, diet and weight changes in pregnancy, gestational diabetes, placental function, markers of fetal growth, breastfeeding and general infant feeding, and infant growth [20]. Therefore, early nutrition may program obesity and its comorbidities through three hypothesized mechanisms that are not mutually exclusive and could have a greater or lesser impact in different circumstances: the fuel mediated 'in utero' hypothesis; the accelerated postnatal weight gain hypothesis and the mismatch hypothesis [21].

The concomitant increased prevalence of obesity in almost all countries seems to be determined mainly by changes in the global food system. Indeed, the economic transition observed in the world, from the richest countries to those most disadvantaged, also carried with it several transitions: demographic transition (younger to older population distribution, rural to urban); epidemiological or health transition (infectious diseases to NCDs); technological transition (low to high mechanisation and motorisation); and nutritional transition (traditional foods to more processed energy-dense foods). Recently, the rate of these transitions has increased so rapidly that many countries are faced with the so-called "double burdens of disease" [22]. An example is given by the double burden of malnutrition, characterized by the coexistence of undernutrition and overweight and obesity, or diet-related noncommunicable diseases, within individuals, households and populations, and across the life course [23]. Indeed, undernutrition and overnutrition may coexist in the same population as well as within the same subject, sometimes, because fetal and infant undernutrition may be followed by adult overnutrition with a double effect on the later burden of NCDs [22].

While economic growth is especially important for low-income countries, for high-income countries, economic growth and higher gross domestic product determine greater consumption of all products, often leading to overconsumption and obesity. Indeed, cheapest food with lower nutritional quality, together with the economic forces that drive consumption, contribute greatly to the expansion of childhood obesity, while undernutrition remains unresolved [22].

1.1.5. Consequences

First of all, the main problem of childhood obesity is that an obese child is at greater risk of adult obesity, with consequent higher risk of obesity complications and increasing the public health burden of adult obesity [19]. However, it is important to note that the obese child is exposed to

obesity detrimental effects on health already during childhood, both in the short and long term, with an increased risk of insulin resistance, non-alcoholic fatty liver disease (NAFLD), type-2 diabetes mellitus (T2DM), dyslipidemia, metabolic syndrome, hypertension, obstructive sleep apnea, joint problems, gallstones and psychosocial problems [24]. Moreover, obesity is often associated with a chronic low-grade inflammation status, a key mechanism linking obesity to its systemic complications. Indeed, adipose tissue may be considered as an endocrine organ able to secrete and produce inflammatory mediators and especially visceral fat is characterized by increased inflammatory profile [25].

1.1.6. What can "we" do

With the Action Plan on Childhood Obesity 2014-2020 [26], the European Union Member States (EU) want to contribute to halting the rise in overweight and obesity in children and young people (0-18 years) by 2020. It is based on eight key areas for action: support a healthy start in life; promote healthier environments, especially in schools and pre-schools; make the healthy option the easier option; restrict marketing and advertising to children; inform and empower families; encourage physical activity; monitor and evaluate; increase research. Likewise, by adopting the WHO European Food and Nutrition Action Plan 2015–2020, Member States took a further decisive step towards promoting healthy diets and addressing the alarming rates of obesity and noncommunicable diseases across Europe [27]. However, as recognized by the Commission on Ending Childhood Obesity [2], established in 2014, "it is only by taking a multisectoral approach through a comprehensive, integrated package of interventions that address the obesogenic environment, the life-course dimension and the education sector, that sustained progress can be made. This requires government commitment and leadership, long-term investment and engagement of the whole of society to protect the rights of children to good health and well-being. The Commission believes that progress can be made if all actors remain committed to working together towards a collective goal of ending childhood obesity" [2].

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1.2. METABOLIC AND CARDIOVASCULAR COMPLICATIONS OF CHILDHOOD OBESITY

In parallel with increasing prevalence of childhood obesity adverse implications on health are becoming more common in children. Several metabolic and cardiovascular complications of obesity start during childhood and are strictly associated with insulin resistance/hyperinsulinemia, the most common abnormality of obesity [1].

1.2.1. Insulin resistance

The number of children and adolescents with clinical signs of insulin resistance (IR) has increased significantly, concomitant with the rise in childhood obesity [2]. From the analysis of the US NHANES 1999-2002, involving 1802 adolescents without diabetes, it has resulted that insulin resistance prevalence was of 52% among obese adolescents [3] while according to a European cohort study involving 232 children with excessive body weight, mean aged 11 years, prevalence of insulin resistance was 32% among obese children [4]. Indeed, insulin resistance is considered as the most frequent metabolic disorder associated with obesity, representing also an important link between obesity and other metabolic abnormalities and cardiovascular complications [5]. Among these, insulin resistance plays a major role in development of T2DM as the hyperinsulinemic subject may develop impaired glucose tolerance (pre-diabetes) and, when the pancreatic β-cell reserve diminishes, T2DM [2]. The term "insulin resistance," refers to a whole-body decrease in the ability of insulin to stimulate the use of glucose by muscles and adipose tissue and to a reduced ability to suppress hepatic glucose production and output [5].

Several mechanisms have been described in the pathogenesis of insulin resistance [6]. A first mechanism is represented by an altered partitioning of fat between subcutaneous and visceral or ectopic sites. Indeed, hypertrophic adipocytes which characterized visceral adipose tissue are highly lipolytic, resulting in greater free fatty acids (FFA) release and impaired secretion of adipokines into the circulation. According to the "portal theory" these FFAs reach the liver through the portal vein, developing hepatic insulin resistance, which could be, according to some authors, also a consequence of a higher release of inflammatory cytokines by visceral fat into the portal vein [6]. Indeed, adipose tissue produces several inflammatory cytokines: e.g. tumor necrosis factor-α (TNF-α) which can alter insulin action at different levels in the intracellular pathway, as well as interleukin-6 (IL-6), another inflammatory cytokine, that stimulates the

hepatic production of C-reactive protein, increasing obesity-related inflammation [5]. Another theory, the so-called "spillover hypothesis" suggests that a reduced ability of adipose tissue to expand, in response to a positive energy balance, could lead to the deposition of FFA in visceral fat and ectopic non-adipose tissues, such as liver, muscle, pancreas, kidney, bone. Since these tissues are unable to oxidize FFA, the consequence could be insulin resistance, cell lipotoxicity and apoptosis [6]. Moreover, it should be considered that insulin blood levels are due to insulin production by pancreas and insulin clearance, mainly by the liver. This process is characterized by the uptake and degradation of insulin and is regulated by several factors, including high levels of FFAs that can have an inhibitory effect. In obesity, hyperinsulinemia may be the result of an increased production of insulin, induced by high fatty acids and glucose, as well as by a decreased clearance of insulin by the liver. Indeed, hyperinsulinemia promotes downregulation of insulin receptors leading to a decrease of insulin clearance from circulation. The sympathetic nervous system (SNS) may have a role too: visceral obesity increases SNS activity and adrenergic outflow increasing lypolisis and FFAs influx to the cell [6].

The gold standard technique to determine whole-body insulin sensitivity is the hyperinsulinemic-euglycemic clamp, but this method is expensive and requires considerable time and expertise to be performed. Therefore, several surrogate measures have been developed to estimate insulin sensitivity and they are usually based on measuring a fasting insulin concentration and glucose, supposing that in the euglycemia, insulin secretion will compensate for insulin resistance [7]. The homeostatic model assessment of insulin resistance (HOMA-IR) is the most widely used surrogate measure of insulin resistance while the quantitative insulin sensitivity check index (QUICKI), more difficult to calculate, is considered as a surrogate measure of insulin sensitivity [7]. From a study involving pubertal obese children and adolescents, it has been suggested that as a measure of insulin resistance among children and adolescents, HOMA is more reliable than QUICKI and that, while for adults the HOMA cut-off point is > 2.5, in children and adolescents the HOMA cut-off point for diagnosis of insulin resistance is 3.16 [8]. Moreover, among healthy Italian children and adolescents percentiles of HOMA-IR and QUICK indexes, grouped by sex and pubertal Tanner's Stage, have been defined [9].

Beyond obesity, several risk factors are associated with the development of insulin resistance in children and adolescents [10]. First of all, ethnicity and puberty may have a key role: African-American, Hispanic, Pima Indian, and Asian children are less insulin sensitive compared with Caucasian children and during puberty there is a 25–50% decline in insulin sensitivity.

Concerning the role of visceral obesity, although some authors [5,10,11] have suggested that also in the pediatric population visceral fat was associated with insulin level, insulin resistance and inversely correlated with insulin sensitivity, in children this association is not so clear and further studies are needed. Indeed, from a study involving 30 overweight and obese children, has resulted that insulin sensitivity was negatively correlated with subcutaneous adipose tissue and liver fat content while, contrary to what is observed in adults, insulin sensitivity was not correlated with visceral fat tissue [12]. Genetics and heritability may play a role too: children with a family history of T2DM are more likely to be insulin resistant with an impaired balance between insulin sensitivity and secretion; genetic heritable variants associated with insulin sensitivity have been also discovered [10]. Moreover, it has been observed that intrauterine exposure to poorly controlled maternal gestational diabetes increases the risk of obesity, insulin resistance, and impaired glucose tolerance in childhood [10]. In the same way, maternal obesity and excessive gestational weight gain may increase the risk of obesity and obesity-related metabolic disorders later in life [2]. Among postnatal factors, weight at birth may play a key role: children born small for gestational age or large for gestational age are both associated with an increased risk of lower insulin sensitivity and of T2DM than their peers of normal birth weight. Rapid postnatal weight gain is also associated with an increased risk of obesity and insulin resistance in children and adolescents [2].

It is well known that lifestyle and diet have a key role in the prevention of obesity and insulin resistance. It has been observed that sedentary lifestyle is associated with decreased insulin sensitivity in children and adolescents and that after interventional studies, increased physical activity is associated with improvement of insulin sensitivity independent of weight change [2]. Concerning dietary factors, the quality of the diet plays an important role in the pathogenesis of insulin resistance: diets high in total fat, as well as high intake of sugars, especially from sugar-sweetened beverages, are related to lower insulin sensitivity. Moreover, a low intake of whole grain carbohydrate or dietary fiber is also associated with lower insulin sensitivity. In general, evidence has suggested that a "Western" dietary pattern, high in total fat, saturated fatty acids, refined grains, and added sugars, is associated with a greater risk of obesity and insulin resistance compared with a "Mediterranean" pattern, including high consumption of vegetables, fruits, legumes, fish, and whole grains [2].

Insulin resistance is associated with other comorbidities (Figure 1) and clinical manifestations. Among clinical manifestations, acanthosis nigricans is a thickened and pigmented skin lesion in

the flexural areas, usually armpit, posterior region of the neck, groins elbow, and knuckles, and its severity correlates well with the degree of insulin resistance. Moreover, insulin resistance is present in most cases of polycystic ovary syndrome (PCOS) in adolescent girls, characterized by features of ovulatory dysfunction, hyperandrogenism (acne, hirsutism, or alopecia), and polycystic ovarian morphology [2]. Insulin resistance is considered as the driving force of fat accumulation in the liver and therefore plays a key role in the development of nonalcoholic fatty liver disease (NAFLD), which is associated with both central and peripheral insulin resistance [2].

It is well known that insulin resistance is a key factor for the development of glucose metabolism disorders, dyslipidemias and high blood pressure, components of the metabolic syndrome, which in turn is a risk factor for the development of T2DM and cardiovascular disease [13]. From a cross-sectional study, recruiting 466 obese children and adolescents between 11-13 years of age, has resulted that higher levels of IR were associated with a greater degree of alterations in the components of the metabolic syndrome. This study suggested that increased degree of insulin resistance was associated with higher risk of metabolic syndrome among obese children and adolescents [13]. Moreover, for what concern insulin resistance, a recent study published on Pediatrics [14], has evaluated the association between childhood fasting insulin levels and later T2DM. This longitudinal study, based on fasting insulin values of 2478 children and adolescents, age 3 to 18 years, and data on adult T2DM, has shown that elevated insulin values in children 3- to 6-year-olds were associated with a higher risk for later type 2 diabetes. Instead, in 9- to 18-year-olds, elevated BMI, but not insulin, was associated with later type 2 diabetes [14].
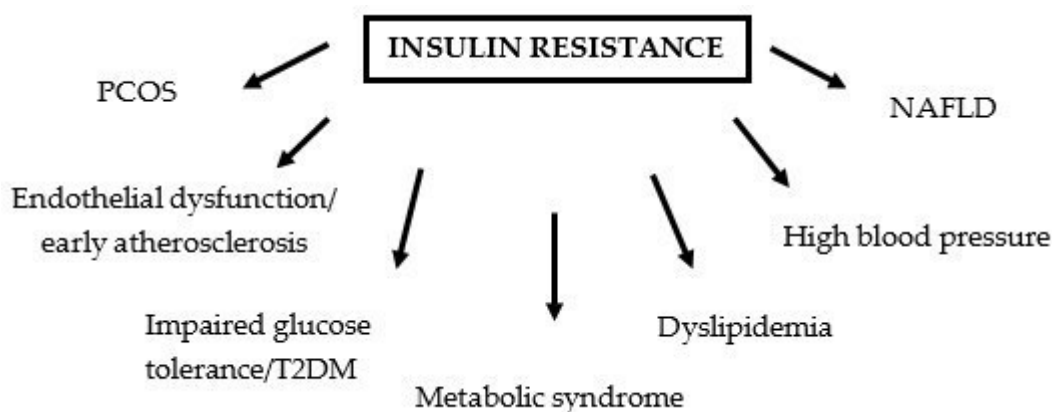

Figure 1. Insulin resistance comorbidities

1.2.2. Impaired fasting glucose (IFG), impaired glucose tolerance (IGT) and type 2 diabetes mellitus

Insulin resistance is a risk factor for impaired fasting glucose, impaired glucose tolerance and T2DM in childhood, whose prevalence is increasing among obese children and adolescents both in United States and Europe [2]. Impaired glucose tolerance and impaired fasting glucose are intermediate stages of glucose metabolism alterations between normal glucose homeostasis and diabetes. IFG is a measure of impaired glucose metabolism in the fasting state, whereas IGT is a dynamic measure of carbohydrate intolerance after a standardized oral glucose tolerance test (OGTT) [15]. In obese adolescents, prediabetes is often transient: around 60% of subjects return to normal glucose tolerance within 2 years. However, continuous weight gain is a predictor of persistent prediabetes and progression to diabetes [15].

The prevalence of prediabetes varies greatly between populations. From a cross-sectional study investigating the prevalence of IFG in two nationwide cohorts of obese children in Germany and Sweden the total prevalence of IFG among obese children according to the American Diabetes Association (ADA) criteria was 5.7% and 17.1% in Germany and Sweden, respectively. This study also showed that the prevalence increased with age, although IFG was common also among young obese children, was higher in boys than girls, and increased with higher degree of obesity [16].

With regard to diagnosis of IFG and IGT, in 1997 and 2003, The Expert Committee on Diagnosis and Classification of Diabetes Mellitus [17,18] recognized the existence of subjects whose glucose levels do not meet criteria for diabetes, although are higher than normal. Therefore, impaired fasting glucose is identified for fasting plasma glucose levels of 100 mg/dl (5.6 mmol/l) to 125 mg/dl (6.9 mmol/l)] while impaired glucose tolerance is characterized by values of 140 mg/dl (7.8 mmol/l) to 199 mg/dl (11.0 mmol/l)] after 2-h in the OGTT [19] Diagnosis of pre-diabetes can be made also by glycated hemoglobin (HbA1c) values between 5.7%-6.4% (39–47 mmol/mol) [19].

The mechanism behind IFG and IGT is still not fully understood. However, IFG may be considered as a prediabetic state characterized by disorder of glucose-sensing organs (β-cells and liver), thus associated to alterations in glucose sensitivity of first-phase insulin secretion and in insulin sensitivity of liver glucose output. On the other hand, IGT seems to be characterized by impaired peripheral insulin sensitivity and a compensatory increase in basal glucose clearance. Finally, a great insulin resistance, associated with an additional defect in β-cells may explain the combined phenotype IFG/IGT [20]. Longitudinal studies evaluating if insulin resistance predicts the development of IGT and T2DM are limited [10]. In this regard, it has been suggested that obese

adolescents progressing to IGT show primary defects in β-cell function, then exacerbated by a progressive decline in insulin sensitivity [21].

In parallel with childhood obesity epidemic, the prevalence of type 2 diabetes, once thought to be an adulthood metabolic disorder, is significantly increased in the pediatric population. From epidemiologic studies has resulted that incidence of T2DM in children and adolescents have a range of 1-51/1000, depending upon ethnicity [22]. Until 15 years ago, type 2 diabetes was associated with less than 3% of all new-onset diabetes in adolescents while at present around 45% of cases are ascribed to it [23]. Diabetes may be diagnosed based on the fasting plasma glucose (FPG) or the 2-h plasma glucose (2-h PG) value after an OGTT [in children 1.75 g/kg body weight (up to a maximum of 75 g)] or the glycated hemoglobin (A1C) (Table 1) [19].

Table 1. Criteria for the diagnosis of diabetes [19]

FPG ≥126 mg/dL (7.0 mmol/L). Fasting is defined as no caloric intake for at least 8 h.*
OR
2-h PG ≥200 mg/dL (11.1 mmol/L) during an OGTT. The test should be performed as described by the WHO, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.*
OR
A1C ≥6.5% (48 mmol/mol). The test should be performed in a laboratory using a method that is NGSP certified and standardized to the DCCT assay.*
OR
In a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose ≥200 mg/dL (11.1 mmol/L).
*In the absence of unequivocal hyperglycemia, results should be confirmed by repeat testing.

Diabetes mellitus is a metabolic disease characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both [24]. T2DM arises when insulin secretion is inadequate to meet the increased request due to insulin resistance, leading to relative insulin deficiency [25]. Therefore, type 2 diabetes development is progressive and is the consequence of insulin resistance and β-cell dysfunction. Initially, peripheral insulin resistance is compensated by hyperinsulinemia (insulin secretion increases to maintain a normal glucose tolerance) [22]. The ability of the β-cell to secrete sufficient levels of insulin depends on several factors, including β-cell mass and secretory capacity, which are influenced by genetic and environmental factors. Afterwards, pancreatic β-cells fall to produce adequate insulin over time, thus leading to hyperglycemia [22,23].

While in adulthood the transition from prediabetes to type 2 diabetes is usually a gradual process that occurs over 5–10 years, it has been suggested that in pediatric age this process is

shorter and that the deterioration in β-cell function in youth with type 2 diabetes is more accelerated than that observed in adults. This observation has also suggested a more aggressive course in the development of T2DM in children and adolescents than in adults [23].

According to "ISPAD Clinical Practice Consensus Guidelines 2014 Compendium" [25] characteristics of individuals with youth-onset T2DM may include:

- onset occurring often during the second decade of life, usually concomitant with the peak of physiologic pubertal insulin resistance;
- onset rarely occurring before puberty;
- family history of type 2 diabetes (first and second degree relatives);
- ethnicity: much greater prevalence in those of non-White European descent, e.g., those of Black African descent, native North American, Hispanic (especially Mexican)-American, Asian, South Asian (Indian Peninsula), and Native Pacific islanders;
- overweight/obesity (not true among Asian population).

For what concern comorbidities, since their development is time-dependent, the presence of T2DM from childhood will probably determine a greater increase of long-term morbidities. It should be underlined that the chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of several organs, especially the eyes, kidneys, nerves, heart, and blood vessels [24]. Indeed, poor glycemic control eventually results in serious health complications such as retinopathy, neuropathy, nephropathy and cardiovascular disease [22]. In this regard the Treatment Options for type 2 Diabetes in Adolescents and Youth (TODAY) study [26], designed to primary examine the effect of three different treatments on the durability of glycemic control on 699 obese participants (10–17 years old) with T2DM, has also observed and described the complications and comorbidities of T2DM in these youths. Findings from this study show that hypertension was present in 11.6% of the population at baseline and increased to 33.8% by the end of the study. Prevalence of high-risk LDL-cholesterol increased from 4.5% at baseline to 10.7% at the end of the study. Microalbuminuria was found in 6.3% of the cohort at baseline and increased to 16.6%. Retinopathy was observed in 13.9% of the population at the end of the study. These data have suggested that in pediatric age complications and comorbidities are similar to that seen in adults but their development and onset is more rapid and that these youths may be probably early burdened with the consequences of cardiovascular disease, nephropathy, and retinopathy in the third and fourth decades of life [26].

1.2.3. Atherogenic dyslipidemia

As a consequence of childhood obesity epidemic, during the last decades the prevalence of atherogenic dyslipidemia is increasing among children and adolescents. The atherogenic dyslipidemia consists of a combination of hypertriglyceridemia, increased very-low-density lipoprotein (VLDL), small dense LDL (sdLDL) particles, and reduced levels of HDL cholesterol, thus showing components of the metabolic syndrome [27]. Data from the National Health and Nutritional Examination Survey (NHANES III) showed that the prevalence of abnormal lipid levels among youths aged 12–19 years was 20.3%. This prevalence was different according to BMI: 14.2% of normal weight youths, 22.3% of overweight and 42.9% of obese had at least one abnormal lipid level [28].

Acceptable, borderline, and high plasma lipid, lipoprotein, and apolipoprotein concentrations (mg/dL) for children and adolescents, according to the Expert Panel on Integrated Guidelines for Cardiovascular Health and Risk Reduction in Children and Adolescents, are shown in table 2 [29].

Table 2. Acceptable, borderline-high, and high plasma lipid, lipoprotein, and apolipoprotein concentrations for children and adolescents [29]

Category	Low, mg/dL[a]	Acceptable, mg/dL	Borderline-High, mg/dL	High, mg/dL[a]
TC	—	<170	170–199	≥200
LDL cholesterol	—	<110	110–129	≥130
Non-HDL cholesterol	—	<120	120–144	≥145
Apolipoprotein B	—	<90	90–109	≥110
Triglycerides				
0–9 y	—	<75	75–99	≥100
10–19 y	—	<90	90–129	≥130
HDL cholesterol	<40	>45	40–45	—
Apolipoprotein A-1	<115	>120	115–120	—

Although the pathogenetic mechanism of dyslipidemia is multifactorial and still unknown, visceral obesity and insulin resistance play a key role one in its development and progression [27]. An excessive caloric intake with a consequent excessive weight gain may determine a great increase in visceral adiposity, reflecting the inability of the subcutaneous fat to expand its storage capacity and resulting in ectopic fat deposition, primarily in the viscera but also in the liver, heart, and skeletal muscle [30]. As explained, visceral adipose tissue may be considered as an endocrine organ able to produce significant amounts of proinflammatory cytokines, which may interfere with normal insulin action in fat and muscle cells, and release great amounts of free fatty acids to the liver via the portal vein [30]. Indeed, when insulin resistance is established, fatty acid esterification and increased lipolysis are defective. This condition is probably due to the reduced

suppression of hormone-sensitive lipase (HSL), usually mediated by insulin, leading to an increased mobilization of fatty acids from the visceral adipose tissue. Furthermore, there is a decreased clearance of triglycerides (TG)-rich lipoproteins in the circulation due to lipoprotein lipase dysfunction. Therefore, the fatty acids flux to the liver is enhanced and associated with insulin-stimulated hepatic TG synthesis, leading to increased production of triglycerides and VLDL secretion [27]. Insulin resistance alters function of lipoprotein, increasing the risk of atherogenesis. Indeed, an entropic mechanism involves TG-rich particles exchanging their TG for cholesterol ester via cholesterol-ester transfer protein (CETP) thereby enriching LDL and HDL with TG (this process is increased by insulin resistance). Both LDL and HDL become substrates for hepatic TG lipase (HTGL), which is upregulated, leading to the formation of small dense LDL and small HDL susceptible to degradation (Figure 2) [31].
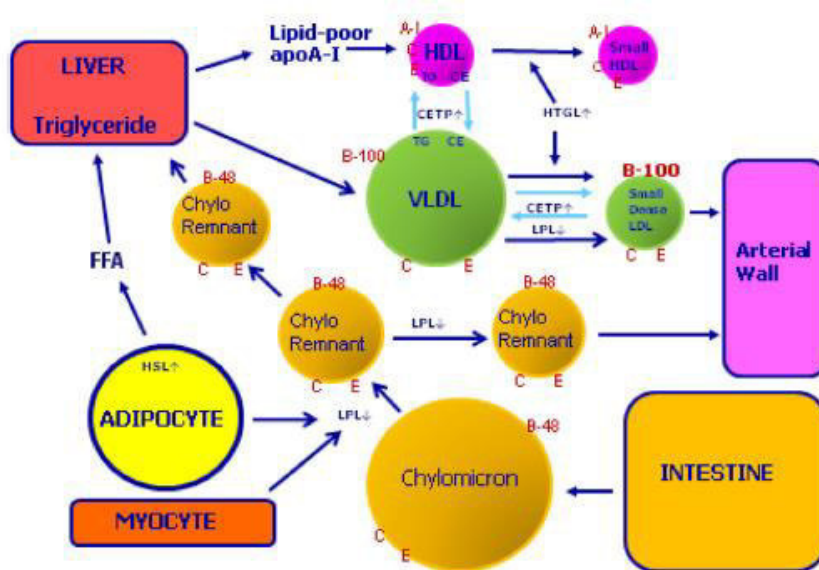
Figure 2. Lipoprotein metabolism in insulin resistance [31]

The combined dyslipidemia associated with obesity is particularly atherogenic because small dense LDL particles are inefficiently cleared by LDL receptors, because elevated circulating LDL particles increase the risk of binding at the arterial wall, with higher oxidation susceptibility, and because decreased large HDL particles reduce reverse cholesterol transport [32]. In childhood obesity the atherogenic dyslipidemia may be associated with structural and functional vascular changes, as increased carotid intima-media thickness (cIMT) and increased arterial stiffness [32]. Therefore, the atherosclerotic process may begin during childhood thus increasing the risk of early cardiovascular events in adulthood. Indeed, it has been observed that high non–HDL cholesterol and low HDL cholesterol were associated with autopsy evidence of premature atherosclerosis, as

well as high triglycerides and low HDL cholesterol in youth were associated with increased cIMT, higher pulse wave velocity, and increased carotid artery stiffness [30]. Clinical events such as myocardial infarction, stroke, peripheral arterial disease, and ruptured aortic aneurysm, that occur during adulthood, are the end stage of a long vascular process of atherosclerosis. Pathologically, the process begins with the accumulation of abnormal lipids in the vascular intima, a reversible stage, progresses with the covering of a core of extracellular lipid by a fibromuscular cap, and culminates in thrombosis, vascular rupture, or acute ischemic syndromes [29].

1.2.4. Hypertension

Hypertension is the leading cause of premature death among adults throughout the world, both in developed and developing countries, being associated with increased risk of myocardial infarction, stroke, and cardiovascular mortality [33]. Changes in health-related behaviors, including the childhood obesity epidemic, are also associated with increasing rates of elevated blood pressure among children and adolescents [34]. Indeed, as observed in a NHANES pediatric cohort, a strong relationship between blood pressure and BMI exists [35]. Since blood pressure varies according to sex, age, ethnicity and degree of obesity, the prevalence of high blood pressure levels, and in particular hypertension is highly heterogeneous (7-33%), ranging from 4%–14% in overweight children to 11%–33% in obese children [35,36]. According to the "Fourth Report on the Diagnosis, Evaluation, and Treatment of High Blood Pressure in Children and Adolescents" realized by the "National High Blood Pressure Education Program Working Group on High Blood Pressure in Children and Adolescents" the definition of hypertension in children and adolescents is based on the normative distribution of blood pressure in healthy children. Blood pressure is considered as normal if systolic blood pressure (SBP) and diastolic blood pressure (DBP) are below the 90th percentile for gender, age, and height. Prehypertension is defined as average SBP or DBP levels that are ≥ 90th percentile but < 95th percentile. However, adolescents with BP levels >120/80 mmHg but < 90th percentile should be considered prehypertensive. Finally, hypertension is defined as average systolic or diastolic blood pressure ≥ 95th percentile for gender, age, and height on at least 3 separate occasions. In this case, hypertension should be staged: stage 1 if BP is ≥ 95th percentile but < 99th percentile plus 5 mmHg; stage 2 if blood pressure is ≥ 99th percentile plus 5 mmHg. If a patient has blood pressure levels ≥ 95th percentile in a physician's office or clinic, but is normotensive outside a clinical setting, has "white-coat hypertension" [33].

Potential factors involved in the pathophysiology of hypertension in overweight and obese children have been widely discussed in a recent systematic review [37]. It has been underlined that obesity can determine increased sympathetic nervous system activity and decreased vagal activity, thus probably leading to the development of hypertension in obese children. Moreover, there seems to be a role for: endocrine determinants, such as renin–angiotensin–aldosterone system that plays an important role in blood pressure regulation by influencing the regulation of vascular tone and sodium homeostasis; corticosteroids, such as cortisol; and adipokines, such as adiponectin, retinol binding protein 4 and visfatin, whose levels can be reduced or increased in subjects with obesity [37]. Concerning insulin resistance, although this review [37] did not show a clear role of insulin in the development of hypertension, other studies demonstrated that insulin resistance per se may determine hypertension influencing sympathetic nervous system activity, sodium retention by kidney, and vascular smooth-muscle growth stimulation [38]. Moreover, in obese children, disturbed sodium homeostasis may influence blood pressure regulation and thus the risk of hypertension. Also oxidative stress, inflammation and endothelial dysfunction may play a role in the pathogenesis of hypertension although their role is complex and it is often difficult to establish what is the cause and the effect in this relationship. Finally, an association between variants of obesity-associated genes and hypertension has been observed [37]. There is increasing evidence that maternal malnutrition, gestational diabetes and the exposure to an adverse environment during intrauterine life will impact organogenesis, with future consequences for adult health. Therefore, higher blood pressure may be also the consequence of an unfavorable programming of the child [39].

Among obese children with prehypertension/hypertension, vascular abnormalities and early target organ damage may occur. Indeed, it has been observed that in obese children and adolescents, higher blood pressure may be associated with left ventricular hypertrophy (LVH), which is the most prominent evidence of target-organ damage, structural changes in forearm vessels, carotid intima-media thickness and arterial stiffness. Moreover, higher blood pressure in childhood may be associated with alteration in the microvasculature and impaired cognitive function [34,39]. Finally, it should be considered that, although uncommon, adolescents with severe elevation of blood pressure have increased risk of adverse outcomes, including hypertensive encephalopathy, seizures, and even cerebrovascular accidents and congestive heart failure [33].

1.2.5. Non-alcoholic fatty liver disease (NAFLD)

NAFLD, considered as hepatic manifestation of metabolic syndrome, is the most common chronic liver disease in children of industrialized countries, reaching a prevalence up to 40%-70% in obese children [40]. NAFLD includes different diseases ranging from 'simple' hepatic steatosis, with pathological accumulation of fat in excess of 5% of liver weight, to non-alcoholic steatohepatitis (NASH), a liver disease characterized by steatosis and periportal and lobular inflammation, with or without fibrosis. The main complication of NAFLD is represented by progression to hepatic fibrosis and cirrhosis, characterized by advanced fibrosis with disruption of hepatic architecture and regenerative nodules. It should be noted that 'NAFLD' refers to both one sub-group of the spectrum and the whole spectrum of disease [41,42].

NAFLD is associated with both hepatic and peripheral IR [10]. Moreover, it seems that hepatic steatosis is both caused by and exacerbates insulin resistance [41]. This relation between insulin resistance and NAFLD seems to be, in part, explained by abdominal fat content [10]. Indeed, in almost all cases, the initiating factor is the development of excess visceral fat [41], secreting high quantity of FFAs in the blood, especially in the presence of a condition of insulin resistance. Then, these FFAs are absorbed by liver cell, representing one of the mechanisms that possibly affect triglycerides deposition in the liver [43].

Transformation from NAFLD to NASH has been explained by the so-called "two hits" hypothesis: the "first hit" is characterized by intrahepatic accumulation of fatty acids, which augments hepatocytes susceptibility to secondary insults, such as oxidative stress, mitochondrial dysfunction, overproduction and release of pro-inflammatory cytokines, and endotoxin-mediated activation of the innate immune response. This step represents the "second hit", explaining the progression of NAFLD to NASH [44] (Figure 3).
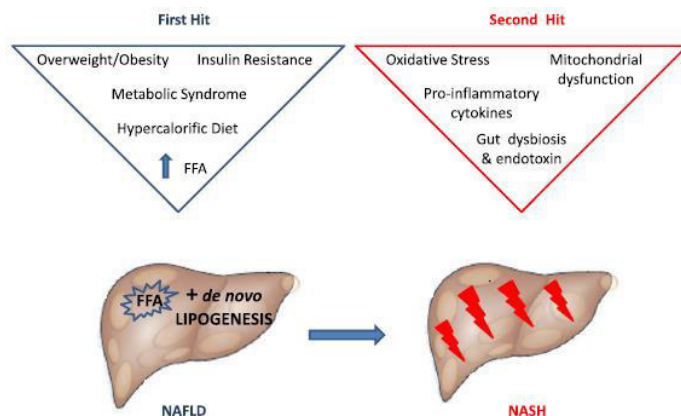

Figure 3. The "two hits" hypothesis [44]

Nowadays NAFLD pathogenesis and progression is largely explained by the "multiple-hit" hypothesis, involving several factors, deriving at the same time from adipose tissue and gut [40]. As explained, overeating causes an overload in hepatic fat accumulation that worsens hepatic insulin resistance, dysregulating insulin signaling pathways and hormone-sensitive lipase. Moreover, the activity of lipoprotein lipase (LPL) in peripheral tissue is inhibited thus leading the hepatic uptake of TGs-rich chylomicrons remnants. In this process adipose tissue is a crucial player, releasing free fatty acids and secreting pro-inflammatory cytokines, which lead to progressive liver damage. Excessive FFA influx to the liver overwhelms the mitochondria and causes the accumulation of fatty acids, ceramide and diacylglycerides. Increased beta-oxidation may lead to the accumulation of electrons, ROS (reactive oxygen species) production, and cellular damage, thus increasing the oxidative stress [40].

Beyond glucose and saturated fatty acids, that may regulate de novo lipogenesis, high fructose intake plays a key role in NAFLD pathogenesis. Fructose, a highly lipogenic sugar metabolized almost totally in the liver via GLUT5, is relatively unregulated by insulin but it can ultimately increase insulin resistance. Moreover, it determines increased VLDL production and hepatic fat storage. Finally, it may alter the microbiome, which increases the movement of endotoxins into the portal system, increasing liver inflammation and IR via Toll-like receptor (TLR)-4 activation. Moreover, it has been suggested that Farnesoid X receptor (FXR), a nuclear bile acid receptor, is involved in the regulation of insulin sensitivity and NAFLD pathogenesis, as well as, endogenous ethanol produced by gut microbiota or resulting from an insulin-dependent impairment of alcohol dehydrogenase activity in liver tissue. Others factors involved in the progression of NAFLD are: oxidative stress, due to perturbations of iron and copper homeostasis; the ghrelin-ghrelin O-acyltransferase system, which is involved in IR, lipid metabolism dysfunction, and inflammation; low levels of vitamin D; and obesity-related obstructive sleep apnea syndrome (OSAS), a sleep disorder that may stimulate progression to steatohepatitis, by a chronic intermittent hypoxia that promotes liver inflammation and fibrosis. Finally, liver steatosis may be the result of mutations in several genes involved in lipid and glucose metabolism, redox cellular state and inflammation [40].

In clinical practice the diagnosis of NAFLD is usually suggested by elevated serum hepatobiliary enzymes and/or evidence of a bright liver on ultrasound (US), usually among overweight/obese children. However, the sensitivity of alanine aminotransferase (ALT) as biochemical marker is low and does not permit to exclude liver steatosis. Accurate noninvasive imaging techniques have been developed: US which is safe but unable to quantify steatosis or

fibrosis; MRI, not cost-effective, even if it could allow rapid, reproducible measurements of steatosis and fibrosis and fibroscan, not yet suitable for widespread use [42].

According to ESPGHAN guidelines, liver biopsy is required for definitive diagnosis of NAFLD. However, indications for liver biopsy are still discussed and it is not proposed for screening, because invasive and expensive. Therefore, for clinical purposes, the diagnosis of NAFLD is at present usually based on presence of one features of the metabolic syndrome, ultrasound imaging of the liver showing liver brightness, and eventually increased transaminase activity. Exclusions of other steatotic or nonsteatotic liver diseases are mandatory in pediatrics [42].

"Chronic" excessive food intake and sedentary lifestyle, resulting in obesity and insulin resistance, are important environmental risk factors associated also with NAFLD pathogenesis [45]. In this regard, energy-dense diet of high fat and high fructose in association with a reduced physical activity but also sugar-sweetened beverages consumption, especially within a low fiber diet, have a highly relevant role [41]. Beyond obesity and diet and sedentary lifestyle, obstructive sleep apnea may be another relevant risk factors. NAFLD development is associated also with non-modifiable risk factors as being male, Hispanic origin, family history of NAFLD or T2DM, parental (maternal) obesity, low birth weight and genetic polymorphisms [41].

In adults NAFLD is often associated with abdominal obesity, insulin resistance and dyslipidemia, all of which are components of metabolic syndrome. Indeed, NAFLD is, nowadays, considered as the liver manifestation of metabolic syndrome [46]. Emerging data suggest that also in children metabolic syndrome may be associated with NAFLD. Indeed, a study including 254 children and adolescents, aged 6-17 years, has shown that metabolic syndrome is prevalent among children with NAFLD and is associated with severity of steatosis, hepatocellular ballooning, NAS, NAFLD pattern, and the presence of advanced fibrosis, evaluated through liver biopsies [47]. However, the concept that NAFLD is the liver manifestation of the metabolic syndrome may be outdated [48]. Indeed, in pediatric age it has been suggested that a vicious circle between NAFLD and metabolic syndrome exists [49] (Figure 4).
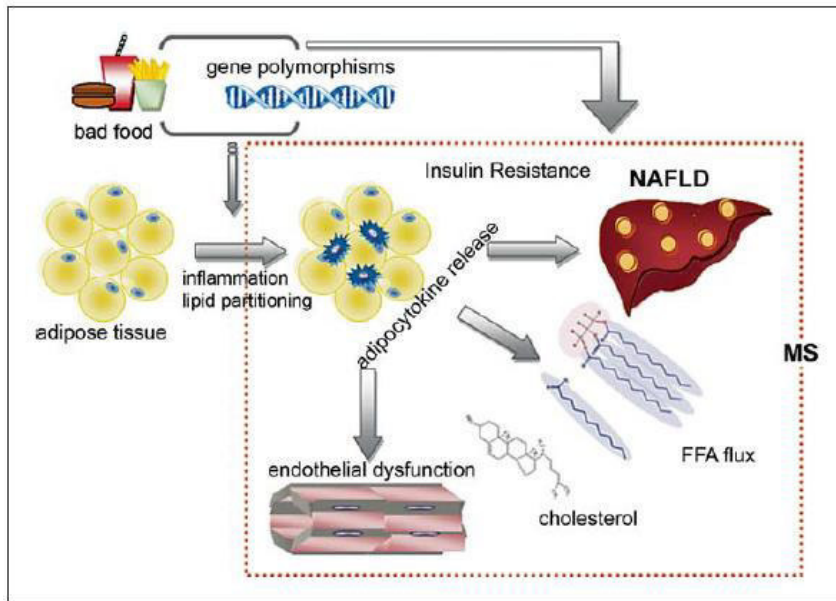
Figure 4. The vicious circle between NAFLD and metabolic syndrome [49]

1.2.6. Metabolic syndrome

Metabolic syndrome is a cluster of cardiovascular risk factors, as hypertension, altered glucose metabolism, dyslipidemia, and abdominal obesity, which increases the risk for cardiovascular disease (CVD) and type 2 diabetes mellitus [50]. First defined as ''a link between insulin resistance, hypertension, dyslipidemia, impaired glucose tolerance and other metabolic abnormalities associated with an increased risk of athero-slerotic cardiovascular diseases in adults'' [38] metabolic syndrome prevalence is increased also among children and adolescents during last decades. Evaluating the prevalence of metabolic syndrome in children is controversial because more than 40 definitions have been proposed and none of these is "universally accepted" [51]. Differences among definitions consist of different components measured, different threshold values and essential criterion used [52]. However, some common features include the estimation of obesity (usually by body mass index (BMI) or waist circumference), the measurement of blood pressure, blood lipids (usually triglycerides, HDL cholesterol or LDL cholesterol], and risk factor associated with diabetes (fasting glucose, glucose tolerance or insulin) [51]. Among different criteria, those more used (with or without modification) are the International Diabetes Federation (IDF), National Cholesterol Education Program's Adult Treatment Panel III (ATP), and World Health Organization (WHO) criteria [52].

The IDF definition of metabolic syndrome [53] is different according to ages. Indeed, there are three age groups (6 to < 10, 10 to < 16, and ≥ 16 yr) and in all of them abdominal obesity is the 'sine qua non' criterion. Below the age of 10 years, the metabolic syndrome is not diagnosed, from 10 to

16 years the diagnosis of metabolic syndrome requires the presence of abdominal obesity (waist circumference greater than or equal to the 90th percentile or adult cut off if lower) plus the presence of two or more of the other 4 criteria: triglycerides ≥ 150 mg/dL, HDL-cholesterol < 40 mg/dL, systolic blood pressure ≥ 130 or diastolic blood pressure ≥ 85 mmHg, and fasting plasma glucose ≥ 100 mg/dL or or known T2DM. Finally, in adolescents aged ≥ 16 years the IDF adult criteria can be used. Another relevant criterion used in children and adolescents aged between 12 and 19 years is the National Cholesterol Education Program/Adult Trial Panel III as modified for adolescents [54]. The diagnosis of metabolic syndrome requires the presence of three or more of the criteria: waist circumference greater than or equal to the 90th percentile, level of triglycerides ≥ 110 mg/dL, HDL-C level ≤ 40 mg/dL, systolic or diastolic blood pressure ≥ the 90th percentile for age, sex, and height, and fasting plasma glucose ≥ 110 mg/dL [54]. Finally, considering WHO diagnostic criteria, the diagnosis of metabolic syndrome requires the presence of impaired glucose tolerance or impaired fasting glucose or insulin resistance plus the presence of two or more of the other criteria: waist circumference or BMI above the 95th or 97th percentile, HDL cholesterol level <5th percentile, level of triglycerides > 95th percentile and blood pressure above the 95th percentile [52].

On the basis of these three criteria (with or without modifications) a systematic review [52], including 85 papers, highlighted that the median prevalence of metabolic syndrome among overweight and obese children was 11.9% and 29.2%, respectively. Moreover, the median metabolic syndrome prevalence was higher in older children (5.6 %) than younger children (2.9 %) and was higher in boys (5.2 %) than girls (3.1 %) [52], as confirmed also by the 2014 NHANES report [55] on the metabolic syndrome in adolescents showing a prevalence of metabolic syndrome of 7.9 % in boys compared with 6.7 % in girls. Therefore, concerning gender, the prevalence of metabolic syndrome tends to be higher in boys than in girls, irrespective of the criteria used; while, concerning pubertal status, metabolic syndrome prevalence seems to be higher in pubertal children than prepubertal [51]. However, it should be considered that the prevalence of metabolic syndrome in children is highly variable according to the criteria used. Nowadays there is not agreement on this topic, although some promote the use of IDF criteria.

As previously explained insulin resistance is highly involved in the pathogenesis of the metabolic syndrome and specifically may have a direct effect on the single components of the syndrome [13]. Along with insulin resistance, metabolic syndrome in children is associated with a proinflammatory state, although it is not yet known if this state is a consequence of metabolic

syndrome and insulin resistance or if, viceversa, the increase release of inflammatory cytokines from adipose tissue may be partly responsible for insulin resistance and metabolic syndrome [38]. Furthermore, it has been recently suggested that probably the partitioning of adipose tissue, which refers to the distribution of body fat, is the major link between insulin resistance, NALD and metabolic syndrome in obese children [38]. Indeed, usually, excess fat is stored in subcutaneous depots but it may also be stored in intra-abdominal (visceral) adipose tissue, muscle and liver (altered lipid partitioning). Lipid accumulation in these tissues is associated with a metabolic profile characterized by elevated free fatty acids and inflammatory cytokines with reduced levels of adiponectin. This combination can independently lead to peripheral insulin resistance and to endothelial dysfunction, thus driving the development of altered glucose metabolism and of cardiovascular disease (Figure 5) [50].
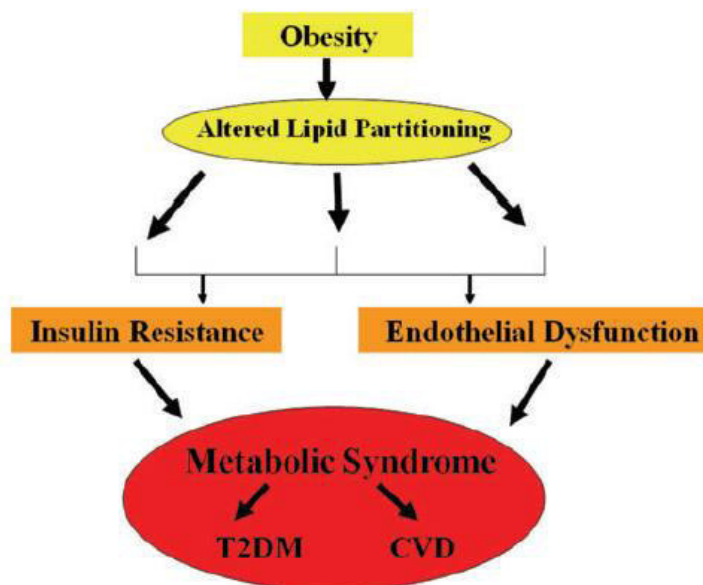
Figure 5. A hypothesized mechanism linking obesity and metabolic syndrome [50]

As explained for single components, the quality of the diet plays a key role in the pathogenesis of the metabolic syndrome by increasing hepatic insulin resistance and/or increasing ROS formation [50]. It has been observed that higher intake of nonroot vegetables, higher consumption of sugar-sweetened beverages, higher consumption of low-fat vegetable-oil-based margarine, and lower consumption of vegetable oils were associated with a higher metabolic risk [51]. Moreover, it has been observed that rapid BMI gain in childhood and adolescence and earlier adiposity rebound are associated with adult metabolic syndrome [56,57].

Finally, there are some issue about the concept of and the application of metabolic syndrome to pediatric age. Firstly, cut points may be difficult to apply in the pediatric population given the

fluctuations associated with growth and puberty [51,58]. Moreover, ethnic differences make a single definition questionable for metabolic syndrome. Finally, the clinical utility of the metabolic syndrome in children continues to be debated, especially if the patients are still treated for the individual risk factors. However, it has been recognized that modeling the metabolic syndrome allows clinicians to see how the risk factors cluster together differently in different populations of children, thus better understanding the underlying pathophysiologic processes [51].

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1.3. THE ROLE OF DOCOSAHEXAENOIC ACID IN OBESITY AND RELATED DISEASES

The role of docosahexaenoic acid (C22:6 n-3, [DHA]), a long chain polyunsaturated fatty acid, in obesity and obesity-related metabolic comorbidities has been largely discussed in the following published reviews, considering its possible association with obesity and metabolic syndrome and its role in NAFLD treatment.

Review

Docosahexaenoic Acid Levels in Blood and Metabolic Syndrome in Obese Children: Is There a Link?

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Abstract: Prevalence of metabolic syndrome is increasing in the pediatric population. Considering the different existing criteria to define metabolic syndrome, the use of the International Diabetes Federation (IDF) criteria has been suggested in children. Docosahexaenoic acid (DHA) has been associated with beneficial effects on health. The evidence about the relationship of DHA status in blood and components of the metabolic syndrome is unclear. This review discusses the possible association between DHA content in plasma and erythrocytes and components of the metabolic syndrome included in the IDF criteria (obesity, alteration of glucose metabolism, blood lipid profile, and blood pressure) and non-alcoholic fatty liver disease in obese children. The current evidence is inconsistent and no definitive conclusion can be drawn in the pediatric population. Well-designed longitudinal and powered trials need to clarify the possible association between blood DHA status and metabolic syndrome.

1. Introduction

Childhood obesity is one of the most pressing public health issues [1] and a major risk for adult obesity and related comorbidities [2], which may already develop during pediatric age, such as insulin resistance, non-alcoholic fatty liver disease (NAFLD), type-2 diabetes mellitus (T2DM), dyslipidemia, hypertension, metabolic syndrome (MS), obstructive sleep apnea, joint problems, gallstones, and psychosocial problems [3].

There has been a marked increase in the prevalence of both obesity and metabolic syndrome in children over the past decades [4]. Metabolic syndrome is defined as a cluster of cardiovascular and type-2 diabetes risk factors, as hypertension, altered glucose metabolism, dyslipidemia, and abdominal obesity [5]. More than 40 definitions have been proposed to define metabolic syndrome and none of these is "universally accepted" [6]. However, some common features include the assessment of obesity (usually through body mass index (BMI) or waist circumference), the measurement of blood pressure and blood lipids (usually triglycerides (TG), high-density lipoprotein (HDL) cholesterol or low-density lipoprotein (LDL) cholesterol), and evaluation of risk factors associated with diabetes (fasting glucose, glucose tolerance and insulin resistance) [6].

Among different criteria to define metabolic syndrome in children, International Diabetes Federation (IDF), National Cholesterol Education Program's Adult Treatment Panel III (ATP), and World Health Organization (WHO) criteria (with or without modification) are the most used [7]. Although there is no agreement on what criteria to use, recently the use of IDF-based criteria has been suggested [7]. The IDF definition of metabolic syndrome [8] in childhood is different according to age (6 to <10, 10 to <16, and ≥16 years). Below the age of 10 years the metabolic syndrome is not diagnosed; from 10 to 16 years the diagnosis requires the presence of abdominal obesity (waist circumference greater than or equal to the 90th percentile) plus the presence of two or more of: blood level of triglycerides ≥150 mg/dL, level of HDL-cholesterol <40 mg/dL, systolic blood pressure ≥130 or diastolic blood pressure ≥85 mmHg, and fasting plasma glucose ≥100 mg/dL or known T2DM. In adolescents aged ≥16 years the IDF adult criteria can be used [8].

The prevalence of metabolic syndrome in children is highly variable according to the criteria used. A systematic review of studies using one of the main three criteria (IDF, ATP III, WHO) to define MS [7], highlighted that the median prevalence of metabolic syndrome was 3.3% in the whole population of children while it was 11.9% and 29.2% in overweight and obese children, respectively. Moreover, the median metabolic syndrome prevalence was higher in older (5.6%) than younger children (2.9%) and was higher in boys (5.2%) than girls (3.1%) [7]. Similar results were published in National Health and Examination Survey (NHANES) report [9] showing a metabolic syndrome prevalence in adolescents of 7.9% in boys compared to 6.7% in girls. Moreover, the prevalence of metabolic syndrome tends to be higher in pubertal than prepubertal children [6].

Finally, the link between metabolic syndrome and NAFLD should be evaluated. Indeed, in parallel to the rising epidemic of metabolic syndrome, also the prevalence of NAFLD has increased [10]. NAFLD is also often associated with clinical and biochemical features of metabolic syndrome [11] in children [12]. Although NAFLD might be considered the liver manifestation of the metabolic syndrome, this concept may be outdated [11]. A vicious cycle between NAFLD and metabolic syndrome could exist in pediatric age [13]. A recent review suggested that NAFLD is a determinant for

the onset of the metabolic syndrome and, therefore, a precursor [11]. Therefore, although to date NAFLD is not a component of the diagnostic criteria for metabolic syndrome its importance needs to be stressed [11,14].

2. Docosahexaenoic Acid: Metabolism and Properties

Long chain n-3 polyunsaturated fatty acids (n-3 LCPUFAs) are long chain fatty acids (20 carbons or more), with the first double bond located after the third carbon from the methyl end [15]. Together with eicosapentaenoic acid (C20:5 (n-3), EPA) and docosapentaenoic acid (C22:5 (n-3), DPA), docosahexaenoic acid (C22:6 (n-3), DHA) is a main n-3 LCPUFAs in food sources [16]. Alfa-linolenic acid (C18:3 (n-3), ALA), an essential dietary fatty acid that cannot be synthesized in humans, is the precursor of all n-3 LCPUFAs [4]. Humans can convert ALA to EPA and DHA, but, since conversion efficiency is low, an adequate dietary intake is required [16].

Significant amounts of EPA and DHA characterize fish and derivative fish oil, especially salmon, tuna, mackerel, anchovy, and sardines, while ALA can be found in vegetable oils [4]. Moreover, DHA is found in human milk and it is necessary for optimal development of the brain and the retina of the infant [16]. However, DHA content in human milk varies substantially depending on the maternal intake of DHA, genetics, and other environmental factors [17], such as maternal smoking during pregnancy [18].

EPA and DHA intake through the diet increases the n-3 LCPUFA content of phospholipids, the main component of the cell membrane, also reducing arachidonic acid (AA) levels [15]. Fatty acids in erythrocyte are considered as the most reliable markers of habitual dietary intake of n-3 LCPUFAs, reflecting the intake over several months [19]. Fatty acids in plasma phospholipids reflect the intake over a relatively short period [19]. However, plasma phospholipids may reflect the fatty acids composition of erythrocyte lipids [20] and, in turn, erythrocyte fatty acids composition may reflect the fatty acids (especially PUFAs) composition of muscle membrane phospholipids [21].

n-3 LCPUFAs are associated with health benefits. EPA and DHA are essential for optimal fetal development and healthy aging [22], constitute phospholipids of most biological membranes with a relevant role in structure and function [16], have anti-inflammatory properties and modulate viscosity of cell membranes [22], and contribute to membrane fluidity, which can influence the function of membrane receptors [16]. Moreover, EPA and DHA are the precursors of numerous metabolites that function as lipid mediators with a plausible beneficial role in the prevention or treatment of several diseases [22]. Series D resolvins and protectins, two active metabolites derived from DHA, may modulate the inflammatory response by decreasing cytokine production and promoting the resolution of inflammation [23]. These metabolites could have a potential and important role in metabolic syndrome since a low-grade inflammation characterizes this condition [24]. It has been suggested that reducing the ratio of n-6/n-3 PUFA in diet (currently estimated about 10:1 in the Western diet), the risk factors of metabolic syndrome could be reduced [25]. However, the evidence about the relationship of n-3 PUFAs and components of the metabolic syndrome is inconsistent [26].

Topic of Review

This paper reviews the literature published in the last decade and discusses the relationships of blood DHA with each component of IDF criteria for metabolic syndrome (obesity, alteration of glucose metabolism, blood lipid profile and blood pressure) and NAFLD in obese children.

3. Childhood Obesity: Relationship between DHA Content in Plasma and in Erythrocytes and Metabolic Syndrome Criteria and NAFLD

3.1. Obesity

Abdominal obesity, rather than obesity, can predict the presence of insulin resistance and related metabolic syndrome [27]. A systematic review and meta-analysis [28], including a total of 21 studies (11 conducted in childhood) for a total of 1575 participants, was performed in order to evaluate LCPUFA status in blood in overweight/obese subjects. Compared with healthy controls, overweight/ obese subjects showed lower DHA levels in total plasma lipids but no difference was found in plasma phospholipid and plasma cholesteryl ester fraction, suggesting that DHA deficiency might be not systemic [28].

Only a few studies have been conducted considering DHA status in blood among the obese pediatric population [29–36]. A case-control study on 67 normolipidemic obese children, aged 8–12 years, and 67 age- and sex-matched normal-weight children, observed that obese children showed significantly lower levels of DHA/ALA ratio in total plasma fatty acids compared to normal-weight controls [29]. Moreover obese children in the highest quartile of BMI z-score showed lower levels of DHA, DHA/AA, and DHA/ALA ratios than normal-weight children, despite a higher dietary PUFAs intake, suggesting a metabolic dysfunction in the synthetic pathway of the n-3 series in severely obese children [29]. Saito *et al.* [30] assessed the analysis of fatty acid composition of plasma phospholipids in 32 obese children and found an inverse association (almost statistically significant) of DHA content with BMI. Similarly, a study found that 60 overweight adolescents had lower total n-3 PUFA and DHA concentrations in plasma phospholipids, compared to normal-weight controls [31]. Another study conducted on adolescents, showed that obese girls, but not boys, had lower concentrations of n-3 PUFAs, including DHA in plasma phospholipids compared to normal-weight controls, and that DHA was inversely associated with all fat depots, measured by magnetic resonance imaging, except visceral adipose tissue, both in girls and in boys [32]. Furthermore, in another study, obese children showed after one year of nutritional-behavioral intervention a decreased BMI z-score of 12.3% and increased plasma levels of DHA and DHA/AA ratio, compared to baseline, with a consequent disappearance of the difference for DHA/AA ratio between obese children and normal-weight controls [33]. It should be interestingly noted that in this study, whereas the plasma PUFA increased after one year, the dietary PUFA intake decreased [33]. However, further studies are needed to better clarify the role of dietary change on specific plasma fatty acid in obese children. On the contrary, a study performed on obese prepubertal children with metabolic syndrome showed higher levels of DHA in total plasma lipids, compared to normal-weight controls while no difference was observed in plasma phospholipid and plasma cholesteryl ester fraction [34]. Another study found no

difference in total plasma lipid levels of DHA between obese children and normal-weight controls [35].

Moreover, in a recent study, 33% of obese children showed an n-3 index (calculated by adding EPA% and DHA% (weight/weight) values) <4.0 (associated to high risk of cardiovascular disease) in erythrocytes compared to 17% of non-obese children, suggesting that obese children may have an altered erythrocyte fatty acid composition [36].

As a whole, several discussed studies found blood DHA may be lower in obese children and negatively associated with the degree of obesity, but further studies are needed to better understand the relationship between DHA status and obesity.

3.2. Glucose Metabolism Alterations

An important key factor in the pathogenesis of metabolic syndrome is insulin resistance [37], a whole-body decrease in the ability of insulin to stimulate the use of glucose by muscles and adipose tissue and to suppress glucose production in the liver [38]. Prevalence of insulin resistance has increased significantly in children in the last three decades [39]. Indeed, the analysis of the US NHANES 1999–2002, involving 1802 adolescents without diabetes, has shown that insulin resistance prevalence was 52% among obese children [40]. A marked increase of the prevalence of pre-diabetic stages' conditions and type 2 diabetes mellitus among obese children and adolescents has also been observed [39].

Low levels of LCPUFAs, especially DHA, and a high n-6/n-3 LCPUFA ratio in skeletal muscle membrane phospholipids have been associated with insulin resistance in adults [41]. Moreover, membrane flexibility, determined by the polyunsaturated fatty acid/saturated fatty acid (PUFA/SFA) ratio, could impact on the effectiveness of glucose transport by insulin-independent glucose transporters (GLUTs) and the insulin-dependent GLUT4 [42].

Literature concerning blood DHA status in the pediatric obese population is scanty [30–33,43,44]. In obese children, DHA content in plasma phospholipids was not associated with parameters of glucose metabolism as fasting glucose, fasting insulin, and homeostasis model assessment-insulin resistance (HOMA-IR) [30]. The lack of association between plasma DHA levels and HOMA-IR was confirmed in other different studies [31,33]. On the contrary, a study found that DHA in plasma phospholipids was inversely associated with serum insulin and HOMA β-cell function [32] and other studies conducted on obese children showed that HOMA-IR was negatively associated with DHA in plasma phospholipids [43,44].

On the whole, the evidence from existing literature is not conclusive about the association between DHA status in blood and glucose metabolism alterations in obese children. However, it should be pointed out that breastfeeding, as the best feeding practice in early life, could have a protective role on glucose metabolism derangements [45,46], possibly also involving DHA in breast milk [18,45]. Indeed, fatty acids composition of breast milk, including DHA, may increase LCPUFAs in skeletal muscle membranes protecting against insulin resistance, β-cell failure, and type-2 diabetes [18,45].

3.3. Abnormal Blood Lipid Profile

The alterations of blood lipid profile associated with metabolic syndrome are usually characterized by increased triglycerides, very-low-density lipoproteins (VLDLs), small dense LDL particles, and reduced HDL cholesterol levels [47,48]. Visceral obesity and insulin resistance could be key factors involved in the promotion of atherogenic dyslipidemia by increasing the synthesis of TG-rich VLDLs in the liver [4].

In adults, increased plasma levels of EPA and DHA might be inversely associated with the risk of the progression of coronary atherosclerosis, sudden cardiac death, and coronary heart disease, clinical conditions related to risk factors for cardiovascular disease, including dyslipidemia [49].

The possible relationships of DHA with blood lipid profile have been poorly investigated in the pediatric population [30,31,33]. A study performed on 32 obese children showed that plasma phospholipids' DHA content was negatively associated with VLDL-triglyceride, a major factor involved in the development of metabolic syndrome [30]. A cross-sectional study did not find any associations of DHA in both plasma phospholipids and cholesteryl esters with parameters of blood lipid profile in overweight adolescents, while the PUFA/SFA and linoleic acid levels in plasma phospholipids were positively associated with HDL cholesterol [31]. Another study, analyzing total plasma fatty acids on 57 normolipidemic obese children, concluded that after one year of nutritional-behavioral intervention changes in plasma DHA and DHA/AA ratio (both increased) were inversely associated with changes in plasma total TGs [33].

In conclusion, association between plasma DHA levels and blood lipid profile alterations in pediatric obese population is inconsistent.

3.4. Blood Pressure Alterations

The prevalence rates of hypertension and obesity are increasing worldwide in children [50]. The blood pressure lowering effect of DHA, observed in adults, could be mediated by the adenosine triphosphate (ATP) release from the endothelium, which increases vasodilation by stimulating the release of nitric oxide, and by the decrease in noradrenaline levels [51].

To our knowledge, only one study evaluated the association between DHA status in blood and blood pressure in obese children. This study, analyzing plasma fatty acid composition in 60 overweight adolescents found that DHA status was not associated with systolic blood pressure [31].

Regarding breastfeeding, a systematic review stated that breastfeeding has a small protective effect against high systolic blood pressure, although residual confounders had to be eliminated [45]. One of the plausible mechanisms that has been suggested to explain this protective effect is represented by the presence of LCPUFAs, including DHA, which are important structural components of the vascular endothelium [45]. In a multicenter, randomized, controlled trial, children fed with a formula supplemented with LCPUFAs (mainly DHA and EPA) showed at age 6 years lower blood pressure than children fed with a formula without LCPUFAs [52].

In conclusion, while in adults an association of DHA status with blood pressure has been observed, in obese children the literature is limited and further longitudinal studies would be desirable.

3.5. NAFLD (Non-Alcoholic Fatty Liver Disease)

In children of industrialized countries, NAFLD is the most common chronic liver disease, reaching a prevalence up to 80% in obese or overweight children [53]. NAFLD includes different diseases ranging from "simple" liver steatosis, with pathological accumulation of fat in excess of 5% of liver weight, non-alcoholic steatohepatitis (NASH), with different degree of inflammation and fibrosis, to end-stage liver disease with cirrhosis and hepatocellular carcinoma [54].

Obese adults with NAFLD showed lower levels of n-3 LCPUFAs, EPA, DHA, and a higher n-6/n-3 ratio in liver than controls [55]. Lower n-3 LCPUFA levels in liver have also been associated with lower levels in erythrocyte phospholipids [56]. The low n-3 LCPUFA levels in liver, by promoting the synthesis of fatty acids and triglycerides with parallel imbalance in the oxidation of fatty acids and export of triglycerides from the liver, could determine fat accumulation and promote liver steatosis [55,57]. To our knowledge there are no studies investigating the association between fatty acids composition of liver phospholipids, and especially liver levels of DHA, and NAFLD in obese children. Only one study showed that in obese children with single-nucleotide polymorphism (SNP), 276G>T at adiponectin gene, the increased liver echogenicity could be associated with higher levels of n-6 PUFA in plasma phospholipids (unpublished results, presented at 44th ESPGHAN Annual Meeting, Sorrento) [58]. However, some trials evaluated the effect of DHA supplementation on pediatric NAFLD [59,60]. A reduced liver hyperechogenicity was observed in children with NAFLD after DHA supplementation for 6, 12, 18, and 24 months [59]. After 18 months of DHA treatment an improvement of histo-pathological parameters (NAFLD activity score, ballooning, and steatosis) has been also observed [60].

Only one study has evaluated the association between breastfeeding and NAFLD in children. This retrospective study suggested that breastfeeding might be protective against NASH and liver fibrosis, suggesting a long-lasting effect of breast milk DHA [61]. The authors speculated that DHA, supplied by breast milk, could be protective, acting as a peroxisome proliferator-activated receptors (PPAR)-agonist, a transcription factor involved in protection against fibrosis [61,62].

In conclusion, further studies are needed to evaluate the existence of a relationship between DHA status in blood and NAFLD in children and to confirm the protective role of DHA in breast milk against NAFLD progression.

4. Discussion and Conclusion

The metabolic syndrome, considered in the past as an adulthood disorder, also affects children with increasing prevalence [4,5].

DHA has been associated with beneficial effects on health and in treatment of several diseases [22], such as cardiovascular disease, cancer, inflammatory, thrombotic and autoimmune disease, coronary heart disease, hypertension, and type-2 diabetes, in adults [16]. The reduction of dietary n-6/n-3 PUFA ratio could reduce risk factors associated with metabolic syndrome [25,63].

Table 1 summarizes the observed relationship between DHA content in plasma and erythrocytes and components of IDF criteria for metabolic syndrome in obese children. The current evidence is inconsistent and no definitive conclusion can be drawn in the pediatric population. Further

well-designed studies are needed to evaluate a possible role of DHA supplementation as a prevention strategy of obesity-related comorbidities in childhood.

Table 1. DHA status in blood and components of IDF criteria for metabolic syndrome in obese children.

Metabolic Syndrome Components [Ref]	Blood DHA Status
Obesity [29–36]	DHA content is lower in obese children and negatively associated with the degree of obesity, except for two studies [34,35]
Glucose metabolism alterations [30–33,43,44]	Inconsistent results
Abnormal blood lipid profile [30,31,33]	Inconsistent results
Blood pressure alterations [31]	None association with systolic blood pressure

Author Contributions

Carlotta Lassandro had primary responsibility for manuscript management, and contributed to the writing of the manuscript. Giuseppe Banderali, Giovanni Radaelli, Elisa Borghi, Francesca Moretti performed critically the literature research about this issue and contributed to the writing of the manuscript. Elvira Verduci supervised the review project and contributed to the writing of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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EDITORIAL

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Docosahexaenoic acid and non-alcoholic fatty liver disease in obese children: a novel approach?

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Abstract

Non-alcoholic fatty liver disease represents the most common chronic liver disease in obese children of industrialized countries. Nowadays the first line of treatment of pediatric non-alcoholic fatty liver disease is based on dietary and lifestyle intervention; however compliance to these interventions is very difficult to maintain in long term period. This editorial discusses about docosahexaenoic acid treatment as possible novel approach for non-alcoholic fatty liver disease in obese children. Docosahexaenoic acid may modulate the inflammatory response, improve insulin sensitivity and could be effective in enhancing intestinal barrier integrity, essential to protect a healthy gut-liver axis. Indeed alteration of gut microbiota composition and increased intestinal permeability may rise the exposure of liver to gut-derived bacterial products, causing activation of signalling pathways implicated in liver inflammation and fibrogenesis. This mechanism has been observed in vitro and animal models of non-alcoholic fatty liver disease but also in a clinical study in adults. While evidence suggests that n-3 long-chain polyunsaturated fatty acids supplementation may decrease liver fat in adults, in pediatric population only a study examined this topic. In obese children with non-alcoholic fatty liver disease well designed randomized controlled trials are needed to better clarify the possible efficacy of docosahexaenoic acid treatment, and underlying mechanisms, to identify the optimal required dose and to evaluate if the docosahexaenoic acid effect is limited to the duration of the treatment or it may continue after the end of treatment.

Keywords: DHA, Childhood obesity, NAFLD, n-3 LCPUFA

Background

Non-alcoholic fatty liver disease (NAFLD), considered as liver manifestation of metabolic syndrome, represents the most common chronic liver disease in obese children of industrialized countries, with a reported prevalence of 3% to 10% in the general pediatric population and reaching a prevalence of 80% in obese or overweight children [1]. NAFLD is characterised by the pathological accumulation of liver fat without relation to alcohol intake, ranging from 'simple' liver steatosis to non-alcoholic steatohepatitis (NASH). Significant complications of NAFLD are represented by progression to liver fibrosis and cirrhosis [2]. Excess food intake and sedentary lifestyle, resulting in obesity and insulin resistance, are important environmental risk factors associated with NAFLD [1]. However the complete pathogenesis remains unexplained and seems to involve several factors. Recently, a great deal of attention

has been focused on the gut-liver axis malfunction [small intestinal bacterial overgrowth (SIBO), intestinal dysbiosis, and increased intestinal permeability ("leaky gut")], considered as another key factor involved in development and progression of NAFLD [2]. Indeed intestinal epithelium, gut microbiota and dietary pattern are linked in different ways. For example, a high fat diet can stimulate a proinflammatory microbiota and interfere with intestinal permeability [2]. Alteration of gut microbiota composition and increased intestinal permeability may rise the exposure of the liver to gut-derived bacterial products, as lipopolysaccharides (LPS), which could determine endotoxemia. Then, endotoxemia can stimulate Toll-Like Receptors (TLR), causing activation of signalling pathways implicated in liver inflammation and fibrogenesis [3]. This mechanism has been observed in vitro and animal models of NAFLD but also a clinical study has shown that NAFLD in adults is associated with increased intestinal permeability and SIBO, related to severity of liver steatosis [4]. Moreover, Giorgio et al. [3] showed that intestinal permeability is increased

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also in pediatric population with NAFLD, according to severity of liver disease, suggesting its important role in NAFLD progression. Therefore it appears that intestinal barrier integrity is essential to protect a healthy gut-liver axis.

Nowadays the first line of treatment of NAFLD in obese children is represented by dietary and lifestyle intervention; however compliance to these interventions is very difficult to maintain in long term period, especially in pediatric population [1]. The aim of this editorial is to discuss the possible role of DHA in treatment of NAFLD in childhood obesity with respect to current evidence.

Main text

Several studies showed that NAFLD is characterized by a low total level of n-3 fatty acids, in turn associated with steatosis, increased oxidative stress and NASH [5]. Moreover, from studies in NAFLD experimental models, it has been shown that n-3 fatty acids may alter liver gene expression (switching intracellular metabolism from lipogenesis and storage to fatty acid oxidation and catabolism), improve insulin sensitivity, have anti-inflammatory properties and reduce tumor necrosis factor levels [5].

Supplementation with n-3 long-chain polyunsaturated fatty acids (LCPUFA), and in particular DHA, has been experienced as potential treatment for obesity-related NAFLD especially in adult population. Indeed, a recent systematic review showed that n-3 LCPUFA (eicosapentaenoic acid and docosahexaenoic acid) supplementation may decrease liver fat in adults [6]. Currently, there is in literature only a study examining this topic in pediatric age [7]. Indeed a randomized controlled trial reported that DHA supplementation was associated with improved liver steatosis in obese children with NAFLD [7]. This study showed that DHA supplementation may reduce liver hyperechogenicity in children with NAFLD after 6 months of treatment, with comparable effect using a dose of 250 mg/day or 500 mg/day of DHA. The improvement in echogenicity observed at 6 months remained unchanged also after 24 months of treatment. Hovever, it should be pointed out that in this study the results were obtained using liver ultrasound sonography test (US) only and caution has to be paid in inferring any definitive conclusion. Indeed Chemical shift magnetic resonance imaging (MRI) (opposed-phase imaging) should be also considered for its recognized its ability to quantify hepatic fat content accurately, and in turn to identify fat regression or accumulation over time in children with NAFLD [8,9].

The potential protective effect of DHA has been suggested also from a retrospective study evaluating early type of feeding (breastfed versus formula-fed and duration of breastfeeding) in a cohort of children with NAFLD [10]. It has been speculated that DHA, delivered by human milk after prolonged lactation, could be protective against progression of the liver disease from simple steatosis to steatohepatitis and fibrosis (NASH) acting as peroxisome proliferator-activated receptors agonist (PPAR-agonist), transcription factor involved in protection against fibrosis [11]. Recently it has been also suggested that DHA, and in particular two active metabolites derived from it, resolvins and protectins, can modulate the inflammatory response not only by decreasing cytokine production but also with promotion of the resolution of inflammation [12]. Indeed, animal models showed that these mediators might reduce inflammation induced by excessive adipose tissue and improve insulin resistance, stimulating adiponectin expression [12].

Furthermore, considering the importance of the gut-liver axis in the development and progression of NAFLD, a significant result, derived from animal models, suggests that DHA could be effective in enhancing intestinal barrier integrity, by increasing, for example, protein expression of intestinal tight junction proteins [12]. It seems to be a bi-directional relationship between DHA and gut microbiota: DHA may alter the gut microbial populations, and some microbial species such as *Bifidobacterium* may improve the tissue distribution of DHA [12].

Conclusions

In conclusion, DHA might exert a positive role in treatment of NAFLD in pediatric age but it remains to be demonstrated. Further well designed randomized controlled trials are needed to better clarify the possible efficacy of DHA treatment, and underlying mechanisms, in obese children with NAFLD, to identify the optimal required dose and to evaluate if the DHA effect is limited to the duration of the treatment or it may continue after the end of treatment.

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1.4. THE ROLE OF THE GUT MICROBIOTA IN OBESITY AND OBESITY-RELATED DISEASES: A BRIEF FOCUS

Recently, a great deal of attention has been focused on gut microbiota as "environmental factor" playing an important role in the development of obesity and its complications.

1.4.1. Introduction

Humans may be considered as supra-organisms that carry two sets of genes, those encoded on their own genome and those encoded in genome of microorganisms living in it. Indeed, the human microbioma, that is the genome of the entire population of microorganisms associated to the human body, is composed by about 3 million genes, that means over 100 times the number of human genes [1]. Moreover, on average humans harbor more bacterial cells than their own cell numbers (10^{14} *vs.* 10^{12}). Considering the existing high inter-individual variability, the microbiota (the entire population of microorganisms associated to human body) of each subject can be considered as a specific "fingerprint", although a "core" consisting by at least 57 bacterial species, common to all individuals, exists [2].

A great number of human-associated microorganisms are distributed throughout the gut [3]. The gut microbiota is an ecosystem formed by many ecological niches, with different bacterial species (about 300-500) and a large amount of strains [2]. The gut microbiota is mostly represented by anaerobic bacteria of the phyla Firmicutes and Bacteroidetes. Other bacterial phyla identified in the human gut include Actinobacteria, Proteobacteria, Verrucomicrobia, Fusobacteria, Cianobacteria and Spirochaeta spp [4]. However, it has been suggested that the gut microbiota of most individuals can be categorized into one of three variants or "enterotipi", on the basis of the dominant genera (Bacteroides, Prevotella or Ruminococco) [5].

The gut microbiota plays numerous metabolic and enzymatic activities that can also compensate functions that human organism is unable to do, and exert numerous protective, structural and metabolic effects on the epithelium. Specifically, the gut microbiota is involved in the functioning of mucosal barrier, in normal development and function of the mucosal immune system, in enterocyte tropism, in resistance to pathogens, in the extraction of energy from the fermentation of non-digestible polysaccharides with the production of short chain fatty acids (SCFA), in the production of vitamins B and K and ion absorption, and in degradation of xenobiotics (Figure 1) [6]. SCFAs provide an additional source of energy for the body: propionate

is taken up by the liver and used as a precursor for liponeogenesis, gluconeogenesis and protein synthesis; acetate is used as a substrate for cholesterol synthesis; and butyrate is the main energy supply for colonic epithelial cells [7].
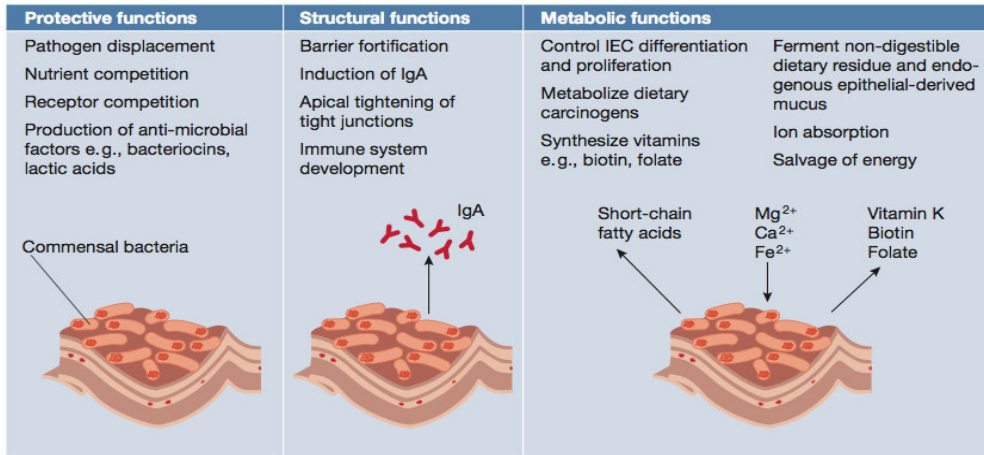
Figure 1. Protective, structural and metabolic effects of commensal bacteria on the intestinal mucosa [6]

The composition of the gut microbiota may change over time. Specifically, during the first period of life, which is considered as a critical window for the development of gut microbiota, the mode of infant delivery, antibiotic exposure, nutrition and other extrinsic factors influence microbial ecology. Therefore, microbial diversity increases during the first few years of life and then stabilizes when the child is around 2–4 years of age, with a composition similar to that of an adult [8] (Figure 2).
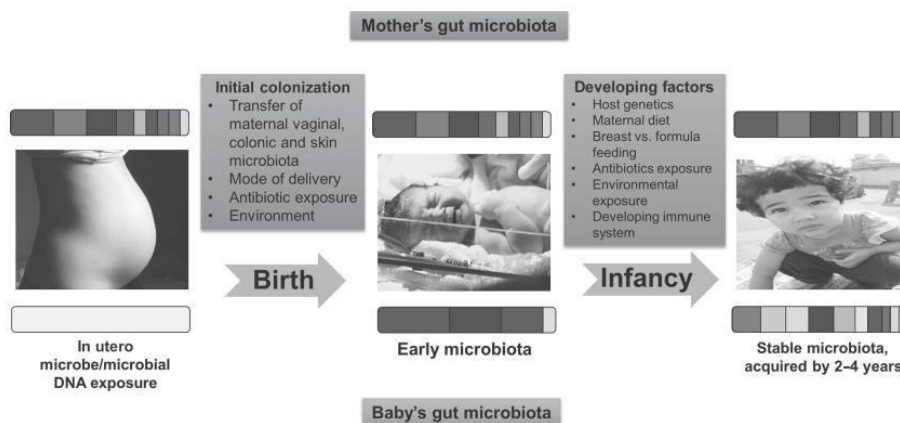
Figure 2. Development of gut microbiota [8].

Afterwards, although the intestinal microbiota is relatively stable throughout life, stress, alcohol consumption, exercise and diet may determine changes in the composition and function of the microbiota [8].

1.4.2. Diet and gut microbiota composition

Diet is a major driver of gut microbiota composition: any major change in lifestyle or diet may affect microbial stability. From the first stages of life, diet results as a main determinant in the development of the microbiota colonization pattern [9]. Indeed, gut microbiota composition is different among breastfed infants and formula-fed infants: in breastfed, microbiota is enriched in bifidobacteria and lactobacilli, which results in a more acidic intestinal content with a higher abundance of short-chain fatty acids, whereas formula-fed infant microbiota has more enterococci and enterobacteria [9,10]. This difference may be partially explained by the differences in composition between human milk and standard infant formula: breast milk is rich in prebiotic oligosaccharides, which act as substrates for fermentation in the distal gut and promote the growth of beneficial microbes as bifidobacteria. Indeed, the human milk oligosaccharides (HMOs) are not directly digested by the host, but instead serve as an energy source for colonic bacteria [9,10,11]. During the weaning, the introduction of solid food leads to a large compositional shift into the intestinal microbiota composition, as observed by comparing gut microbiota composition between Burkina Faso and Italian children during the weaning [12]. In mice it has been observed that diet changes explained 57% of the total variation in gut microbiota, while genetic mutation accounted for no more than 12% [13]. Moreover, in conventional mice the shift from a diet with low fat and high polysaccharides to a "western diet" is associated with a significantly higher relative abundance of the Firmicutes (especially Mollicutes) and a significantly lower relative abundance of the Bacteroidetes [14]. In addition, this change seems to take place within 24 hours [15]. Similarly, in humans, the transition from a "high-fat / low-fiber" to a "low-fat / high-fiber" diet determines significant changes in the gut microbiota within 24 hours. It was also noted that different diets may be associated with specific enterotypes that seems to remain stable over time: the Bacteroides enterotype was associated with animal protein and saturated fats while the Prevotella enterotype was associated with carbohydrates and simple sugars [16].

1.4.3. Gut microbiota, obesity, insulin resistance and NAFLD

As anticipated, the gut microbiota has been suggested as a driving force in the development of obesity and related comorbidities, although underlying mechanisms are not fully understood. This assumption stems from the study performed by Ley et al. who, for the first time, showed that, compared to wild-type lean mice, ob/ob mice (genetic obese mice homozygous for a mutation in the leptin gene) had a different composition of the gut microbiota, with 50% reduction in the abundance of Bacteroidetes and a proportional increase in Firmicutes [17]. Moreover, it was observed that transplantation of gut microbiota from ob/ob mouse to germ-free mice and thus colonization of germ-free mice with an 'obese microbiota' induces a significantly greater increase in total body fat than colonization with a 'lean microbiota' (47% vs. 27%) [18].

In humans, some studies have reported an increased Firmicutes/Bacteroidetes ratio in both obese adults [9,19,20] and children [21,22], but other studies did not find consistent results, thus showing a reduced Firmicutes/Bacteroidetes ratio [23] or no differences between groups [24] as also recently confirmed by meta-analyses [25]. Moreover, it has been observed that subjects with low bacterial richness gained more weight and had increased inflammation, insulin resistance and dyslipidemia than subjects with high bacterial richness suggesting that microbial diversity may have a relevant role in obesity and comorbidities [26]. Similarly, it has been shown that, in obese adults, microbiota cluster was characterized by diminished bacterial diversity, decreased ratio of Bacteroidetes to Firmicutes, and was associated with intestinal and systemic inflammation [27]. Moreover, since intestinal permeability was not altered in obesity nor associated with inflammation, it has been suggested that the "obese" microbiota may modulate intestinal and systemic inflammation independently of gut permeability [27].

Several mechanisms able to explain the association between gut microbiota and obesity have been proposed [28,29]. A first mechanism is represented by the extraction of energy from dietary fiber. Indeed, gut microbiota is able to "break" indigestible polysaccharides (fiber) to SCFA, butyrate, propionate, acetate, providing 80 to 200 kcal/daily and increasing hepatic lipogenesis. In addition, gut microbiota, may be able to modulate the expression of host genes, suppressing fasting-induced adipocyte factor (FIAF) in the gut, which in turn increases lipoprotein lipase (LPL) activity in adipocytes with consequent increased energy storage as fat. A third mechanism could be represented by the inhibition of adenosine monophosphate-activated protein kinase (AMPK), normally involved in fatty acids oxidation. By interfering negatively with the AMPK activity, the gut microbiota determines an increased accumulation of fatty acids. Finally, an obese microbiota

may favor higher gut permeability with higher plasma lipopolysaccharide (LPS) level, known as metabolic endotoxemia, promoting the state of "low-grade inflammation" with a consequent reduction of insulin sensitivity [28,29] (Figure 3).
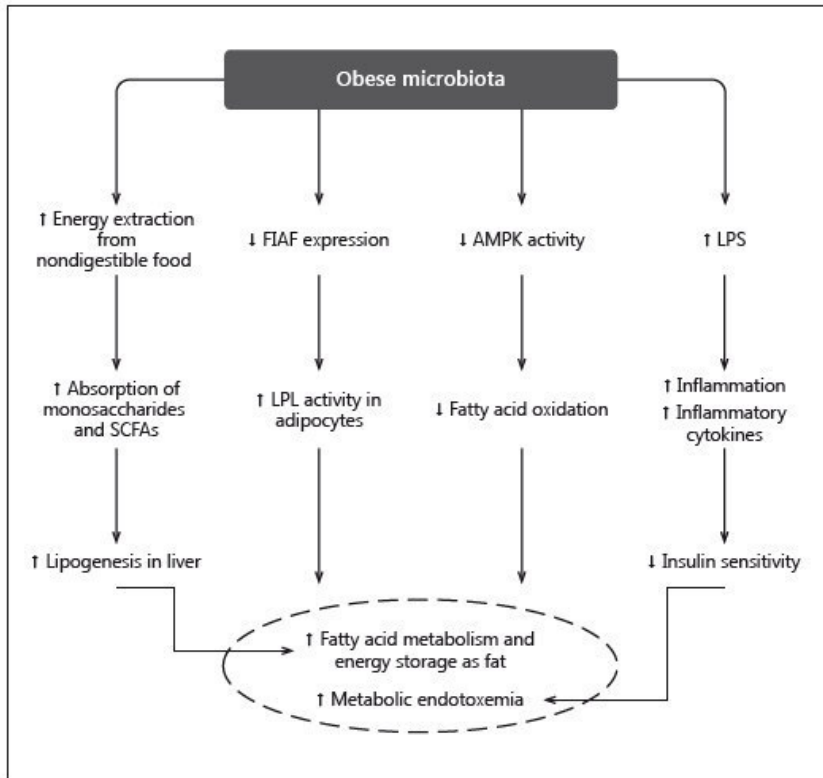
Figure 3. Hypothesized mechanisms linking gut microbiota to obesity [28]

However, the theory of "increased energy harvest" from fiber seems to contradict health benefits associated with high fiber intake and SCFA production. Indeed, especially butyrate, seems to have, through several presumed mechanisms of actions, beneficial effects on obesity and related comorbidities. It may increase satiety and decrease energy intake and postprandial glycemia via modification of gut peptide production. In addition, butyrate is the main energy source for enterocytes, and therefore, regulates cell proliferation and differentiation and induces glucagon-like peptide (GLP)-2 production, strengthening the gut barrier function. Butyrate also reduces oxidative damage and inflammation by inhibiting histone deacetylases and the activation of the transcription factor nuclear factor-κB and the associated cytokine production. However, obesogenic diets, like the western diet, may promote disbiosis and the growth of potential pathogens, which could trigger an inflammatory response, thus leading to a leaky gut, translocation of microbial molecules (especially LPS), and overall, promotion of systemic

inflammation. Therefore, considering the relevant role of inflammation in obesity-related comorbidities, a reduced abundance of some butyrate-producing bacteria with an increased abundance of opportunistic pathogens may favor the development of insulin resistance and type-2 diabetes [30].

Gut microbiota is highly involved also in the pathogenesis of non-alcoholic fatty liver disease. Indeed, recently, gut-liver axis malfunction [small intestinal bacterial overgrowth (SIBO), intestinal dysbiosis, and increased intestinal permeability (leaky gut)], has emerged as another key factor involved in development and progression of NAFLD [31]. High fat diet and high fructose consumption seem to affect microbiota composition and intestinal permeability [31]. Indeed, this kind of diet may disrupt intestinal barrier and increase intestinal permeability to gut-derived products (LPS, DNA, RNA, etc.), known as pathogen-associated molecular patterns (PAMPs). The final effect is the activation of the signaling cascade triggered by specific immune receptor resulting in the expression of pro-inflammatory cytokine genes including TNF-α and several interleukins that may exacerbate hepatocyte damage. Therefore, endotoxemia causes activation of signalling pathways implicated in liver inflammation and fibrogenesis [32]. In this regard, Giorgio et al showed that, intestinal permeability is augmented in pediatric population with NAFLD, as in adults, according to the severity of liver disease, suggesting its important role in NAFLD progression [32]. Therefore, gut microbiota can contribute to the development and maintenance of liver steatosis and hepatic inflammation, as observed in animal models of NAFLD/NASH and patients, through several mechanisms: the altered microbiome may produce more SCFAs and alcohol, and carry more Gram-negative bacteria that supply LPS to the portal circulation and to the liver [33].

1.4.4. Future directions

The recent evidence that gut microbiota may be involved in the development of obesity and related diseases, through several underlying mechanisms that need to be further elucidated, suggests future prospective of prevention and treatment. Indeed, in the future, gut microbiota manipulation through probiotics and prebiotics could, theoretically, change microbial composition, thus promoting host health.

1.4.5. References

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1.5. THE TREATMENT OF CHILDHOOD OBESITY AND RELATED COMORBIDITIES

Guidelines for prevention and treatment of childhood obesity recommend intensive lifestyle modifications, involving diet, physical activity and behaviors, for the entire family and the child, in an age-appropriate manner [1]. However, compliance to multifactorial interventions (behavioral/ dietary/ physical) may be difficult to maintain in long term period, especially in school and adolescence age. In this regard, a meta-analysis of randomized trial performed in children showed a significant 1.5 kg/m^2 decrease in body mass index (BMI) when lifestyle modifications were implemented with family support, but showed only a 0.4 kg/m^2 decrease in BMI in the absence of family support [2]. In confirmation of this, a Cochrane systematic review stated that in children family-based lifestyle interventions aimed at changing dietary, behavioral and physical activity patterns can determine a reduction in overweight, compared to standard care or self-help [3].

Pharmacotherapy, associated with lifestyle modifications, is supported for obese children who failed intensive lifestyle intervention and with severe comorbidities that persist also after intensive lifestyle modification, especially if they have a strong family history of T2DM or cardiovascular disease [1]. The only obesity medication approved in children is Orlistat, that acts locally in the stomach and intestine to inhibit the action of gastric and pancreatic lipases, decreasing fat absorption by up to 30%. Adverse effects are usually localized and transient, but frequent gastrointestinal-related events may cause therapy discontinuation and low compliance [4]. Finally, for adolescents with BMI >50 kg/m^2 or BMI >40 kg/m^2 with severe comorbidities who failed lifestyle modifications and/or pharmacotherapy, bariatric surgery could be an option [1,5].

1.5.1. The Italian Consensus

According to the Italian consensus [6] about prevention, diagnosis and treatment of pediatric obesity, published in 2006, the first goal should generally be the "achievement" of a healthy diet and life-style, through the involvement of parents and the entire family.

Specifically, in overweight children and adolescents, the goals will be the weight maintenance until the BMI reaches the range of normality and an improvement or disappearance of complications (dyslipidemia, insulin-resistance, hypertension, liver steatosis, OSAS, joint pain). Similarly, in obese children and adolescents without complications the goal will be firstly the weight maintenance over time, while in obese children and adolescents older than 6 years and

with complications, weight loss can be pursued until the BMI reaches the range of normality and obesity-related complications improve or disappear. To set up weight loss program the energy expenditure should be estimated and calorie surplus should be calculated on the basis of weight gain in the last months (7000 Kcal/kg body weight). Follow-up visits should be scheduled every 2 months (at maximum) during the phase of "weight loss" and every 6 months (at maximum) during the "maintenance" phase.

Concerning diet, a nutrition education program is an essential element to modify bad eating habits and promote lifelong healthy diets in the child and its family. Daily energy intake should be divided into five meals (3 main meals + 2 snacks). As a first approach, a diet, rather than nutrition education, is not recommended, especially if unbalanced (very low calorie diet, increased-protein diet, low-carbohydrate diet). The caloric restriction can be reached by the limitation and elimination of high-calorie foods also with the aid of "traffic light" approach, which is characterized by the categorization of foods as GREEN, YELLOW, and RED, on the basis of their energy density. Healthier food choices include that low-glycemic index foods should be preferred (cereals such as pasta, barley and whole cereals (2 times a day), legumes (4 times a week), fruits and vegetables (5 portions a day), rather than high-glycemic index foods (bread, rice, potatoes, sugar sweetened beverages, desserts, candies).

In obesity treatment programs, increasing the time spent in physical activities, together with the reduction of sedentary behaviors, is essential, as well as nutrition education and counseling, to achieve the goal. Parents should be motivated to an active lifestyle involving the child: time spent watching TV or playing video games should be highly reduced, the child should be involved in physical activity including play, games, sports, physical education, or planned exercise. The type of exercise mainly recommended is aerobic exercise, like swimming, biking or walking. The duration of exercise should be initially of 30 minutes, which can be gradually increased in subsequent session.

Cognitive-behavioral therapy may be useful for obese children but requires a specific training of involved personnel and the collaboration with a psychologist team. The cognitive-behavioral techniques include: dietary record for self-monitoring, stimulus control, positive reinforcement, cognitive restructuring and training on contingency planning.

Pharmacological therapy in childhood obesity may be hypothesized only if intensive lifestyle intervention (dietary intervention plus physical activity plus cognitive-behavioral therapy) is

failed and obesity is associated with severe comorbidities. Similarly, bariatric surgery may be considered as an option only in adolescents with highly refractory obesity [6].

1.5.2. Multifactorial intervention for childhood obesity: a revision of the literature

Nowadays, there are not randomized clinical trials examining the effects of diet only on weight and body composition independently from intensity of the treatment, behavioral intervention and physical activity, in pediatric age. However, recently a systematic review, comparing effectiveness of diets with different macronutrient distribution on BMI and cardio-metabolic profile in overweight and obese children, has been performed [7]. According to defined inclusion criteria, 14 studies were included: 7 studies compared a low-carbohydrate diet to a conventional low-fat diet, 6 studies compared an increased-protein diet to an isocaloric standard-protein diet and 1 study compared an increased-fat to an isocaloric standard-fat diet. It has been observed an improvement in weight-related outcomes irrespective of the macronutrient distribution, as well as improvements in blood lipids, glucose and insulin homeostasis, and blood pressure. However, it has been underlined that a specific macronutrient distribution may be relevant to target specific cardiometabolic risk factors. These results suggest that a dietary intervention should firstly determine a reduction of total energy intake [7]. However, very-low calorie diet may be ineffective in the long term because are associated with higher risk of drop-out and, as a consequence, the worsening of obesity and related complications.

As already mentioned, it is very difficult to differentiate diet from physical activity. In this regard, a recent systematic review and meta-analysis [8] about lifestyle interventions in obese children and adolescents, incorporating different types of dietary interventions (traffic light or modified traffic tight diet, hypocaloric diet or a calorie restriction approach, general healthy eating educational approach or undefined dietary intervention), exercise and/or behavioral intervention, has been performed. From this review has resulted that lifestyle interventions may lead to significant weight loss compared with no treatment, significant reduction on BMI and BMI z-score, compared with written information only, over a 6- to 12-months intervention period, and improvements in LDL cholesterol, triglycerides, fasting insulin and blood pressure up to 1 year from baseline.

The importance of both dietary intervention and physical activity has been highlighted also by another systematic review and meta-analysis [9], comparing the effect of diet-only intervention

and diet plus physical activity or physical activity-only on weight loss and cardiometabolic outcomes. Results from this meta-analysis showed that although both diet-only and combined interventions (diet plus exercise) may result in weight loss and metabolic improvement in the overweight/obese pediatric population, diet plus exercise determine greater improvements of HDL cholesterol, fasting glucose and fasting insulin that diet only, over 6 months.

Other two systematic reviews evaluated the effect of exercise-only intervention, showing that exercise intervention is associated with the reduction of BMI [10,11], as well as the reduction of body weight, body fat percentage and waist circumference among overweight/obese adolescents [11]. Moreover, although the evidence was limited exercise intervention may be associated with the improvement of some cardiometabolic risk factors (capacity to regulate glucose and insulin during an OGTT, HOMA index and systolic blood pressure) [11].

Another key element in the treatment of pediatric obesity is the reduction of sedentary behaviors. Indeed, systematic reviews indicate that sedentary behaviors (especially TV viewing and screen-time) may be important determinants of health, independently of physical activity, thus being associated with obesity [12]. Indeed, it has been underlined that watching TV for more than 2 hours/day is associated with unfavorable body composition and decreased fitness and that the risk for obesity increased in a dose response manner with increased time spent in sedentary behaviors [13]. Therefore, interventions to reduce sedentary behaviors have resulted in BMI reduction in overweight/obese children, especially in the range from 5 to 12 years of age [14]. In this regard, exergames, a new generation of active video games, may be considered as an additional strategy to reduce physical inactivity of overweight/obese children and adolescents. Indeed, a systematic review showed that exergaming may lead to a more active lifestyle by increasing the level of physical activity, energy expenditure, and cardiorespiratory function and by reducing body fat and sedentary behaviors [15], although according to another systematic review the evidence remains unclear due to design problems, measurement issues and other methodology concerns, thus highlighting the needs for further studies about this topic [16].

Lifestyle intervention should include also behavioral components and cognitive skills training to target weight-related behaviors [17]. Indeed, cognitive-behavioral therapy (CBT) may be essential to achieve results in the short, medium and long term. The most effective techniques include stimulus control (e.g., restructuring the home to encourage healthy behaviors and limit unhealthy behaviors associated with eating and activity) and self-monitoring of weight, eating, and physical activity. Moreover, in family-based interventions, parents should become as models

for their children by monitoring and modifying their own behaviors; they are also encouraged to use a behavioral "rewards" system, in which the achievement of a goal, as weight loss, reduced caloric intake, increased physical activity, is positively reinforced with rewards that are interpersonal and/or promote healthy behavior (e.g., family outings, bike riding, ice skating) [17]. A meta-analysis of family-based interventions targeting childhood obesity, including studies published between the years 2000-2009, showed that this intervention based on family active involvement is successful in producing weight loss in the short and long-term, although future research is needed [18].

1.5.3. The importance of the Mediterranean diet

The traditional Mediterranean diet has been described as the dietary pattern found in the olive-growing areas of the Mediterranean region between the late 1950s-early 1960s, when the consequences of World War II had been overcome but before fast-food culture had reached this area. This diet is characterized by a high intake of vegetables, legumes, fruits and nuts, and whole cereals, and a high intake of olive oil but a low intake of saturated lipids, a moderately-high intake of fish, a low-to-moderate intake of dairy products, a low intake of meat and poultry, and a regular but moderate intake of wine, generally during meals [19]. Recently a new graphic representation of the Mediterranean diet pyramid, that can be adapted to the different nutritional and socio-economic contexts of the Mediterranean region, has been realized [20] (Figure 1).

Figure 1. The new Mediterranean diet pyramid [20]

Since the early 1970s many investigators have reported the effects on health of the Mediterranean diet, as originally reported by Keys in the seven countries study [21]. In this regard, a meta-analysis by Sofi et al. [22] showed that, in an overall analysis comprising more than 1.5 million healthy adults and 40000 fatal and non-fatal events, greater adherence to a Mediterranean diet is significantly associated with a reduced risk of overall mortality, cardiovascular mortality, cancer incidence and mortality, and incidence of Parkinson's disease and Alzheimer's disease, thus systematically assessed, for the first time, the possible association between adherence to this diet, mortality, and the occurrence of chronic diseases in the general population [22]. A recent review also showed a strong evidence of association between adherence to a Mediterranean diet and cardiovascular disease, T2DM, metabolic syndrome and obesity, supporting the role of this diet in the prevention of cardiodiabesity in adults, a hybrid term used to define and describe the well-known relationship between T2DM, obesity, metabolic syndrome and cardiovascular disease [23]. Moreover, from another review, examining data on the association between the Mediterranean diet and NAFLD in adults, has resulted that greater adherence to the Mediterranean diet may be

associated with reduced liver enzymes at 6 and 12 months of intervention, reduced liver steatosis and improved insulin sensitivity at 6 weeks of intervention [24]. The therapeutic role of Mediterranean diet has been observed also in obese children with NAFLD [25]. The beneficial effects of this diet on NAFLD may be due to several intrinsic factors, such us the low glycemic index, the olive oil, the high content in n-3 PUFA e phytonutrients [24] (Figure 2). Recently, it has been suggested that the adherence to the Mediterranean diet is also associated with a 'normalization' trend of gut microbiota, characterized by an increase of Bacteroidetes and Clostridium clusters and reduction in Proteobacteria and Bacillaceae bacterial phyla [26].

Concerning the relationship between Mediterranean diet and obesity, several mechanisms that can explain the protective role of Mediterranean diet have been suggested [27]. This diet is rich in dietary fiber that may increase satiety and satiation through mechanisms, such as prolonged mastication, increased gastric detention and enhanced release of cholecystokinin. Moreover, the Mediterranean diet is characterized by low energy density, low glycemic load and high water content that may lower total calorie intake, helping to prevent weight gain. Among the other positive characteristics, it should be considered that the fatty acids profile of Mediterranean diet has important health benefits, as it is low in cholesterol-rising fats (saturated and trans fats) and high in monounsaturated fats (approximately 67% of fat energy) as that found in olive oil. Diets with higher monounsaturated fatty acids have been associated with improved glucose metabolism, and increased postprandial fat oxidation, diet-induced thermogenesis and overall daily energy expenditure, compared with diets with higher saturated fats. Furthermore, the habitual use of olive oil in salads and vegetable and legume dishes enhances palatability of these foods and of the overall diet thus possibly increasing the compliance. Finally, this type of diet provides a high intake of both non-nutritional factors and micronutrients (especially antioxidants), with additional health benefits [27] (Figure 2).
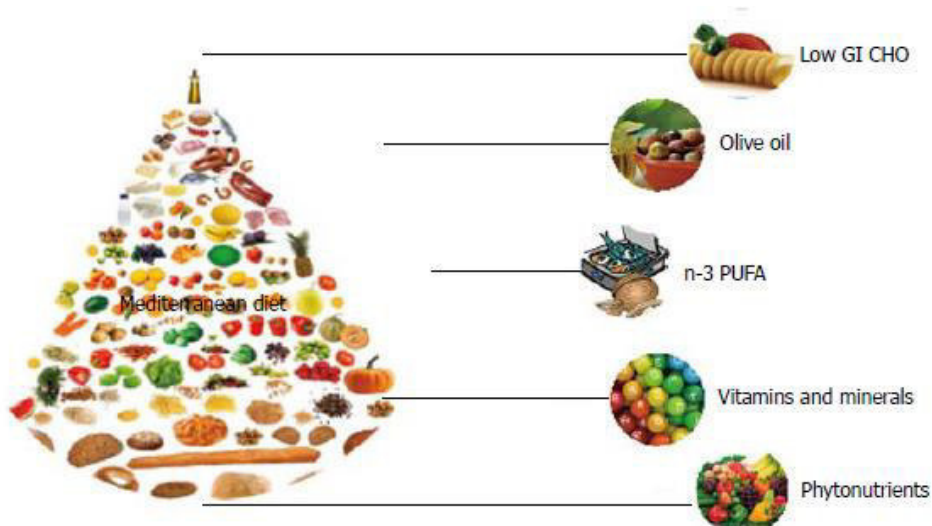
Figure 2. Beneficial properties of Mediterranean diet [24]

The association of the Mediterranean diet with childhood obesity has been evaluated in a cohort of children from the IDEFICS study. It has been observed that a Mediterranean-like dietary pattern, evaluated by a food frequency-based Mediterranean Diet Score, was inversely associated with overweight including obesity and fat mass percentage, independently of confounding factors. Furthermore, a high adherence at baseline was protective against increases in BMI z-score, waist circumference and waist-to-height ratio with a similar trend observed for percent fat mass [28]. Unfortunately, the Mediterranean pattern seems to be uncommon among children living in the Mediterranean region, with a prevalence of high adherence ranging from 55.9%, among the Italian pre-school boys, to 26.0%, among the Spanish school-aged girls [28,29].
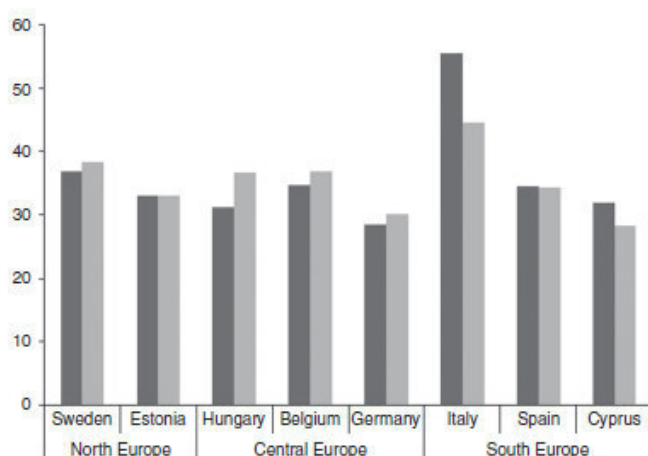
Figure 3. Prevalence of high adherence to a Mediterranean-like dietary pattern among pre-school (dark grey) and school children (light grey), stratified by country, participating at the IDEFICS study [29]

To our knowledge, only one study has evaluated the effect of a Mediterranean-like diet on cardiovascular risk factors in obese children and adolescents. Forty-nine obese children were randomized to receive a Mediterranean diet rich in polyunsaturated fatty acids, fiber, flavonoids and antioxidants or a standard diet. At the end of the intervention period (16 weeks) the Mediterranean diet group showed a significant decrease in BMI, lean mass, fat mass, glucose, total and LDL cholesterol and triglycerides, concomitant with a significant increase in HDL cholesterol. Moreover, in the group of children who followed the Mediterranean-like diet, the prevalence of several components of the metabolic syndrome also significantly decreased, as well as the prevalence of the metabolic syndrome at the end of the intervention period. In the other group, only glucose levels and the frequency of glucose >100 mg/dL significantly improved, thus supporting the positive effect of the Mediterranean diet in the treatment of childhood obesity and related comorbidities [30].

Health benefits of fruit and vegetables: is there a role for salicylic acid?

Fruit and vegetables are the basis of the Mediterranean diet pyramid [20]. A WHO/FAO report recommends a minimum of 400g of fruit and vegetables per day (excluding potatoes and other starchy tubers) for the prevention of chronic diseases such as heart disease, cancer, diabetes and obesity, as well as for the prevention and improvement of several micronutrient deficiencies [31]. Therefore, diets high in fruits and vegetables are widely recommended for their health-promoting properties. Fruits and vegetables are important sources of dietary fiber, vitamins, especially vitamins C and A, minerals, especially electrolytes and phytochemicals, especially antioxidants and polyphenols [32]. All these nutrients provide support for the biological plausibility that fruits and vegetables play a role in health [32]. It has been suggested that, among phytochemicals, salicylic acid (SA) may have an important role, at least in part, in the explanation of the beneficial effects associated with diets rich in fruit and vegetable [33,34].

Salicylic acid (SA) and its acetylated form, Aspirin™, have a long history of therapeutic and disease-preventative use, especially when inflammation and oxidative stress are involved. Indeed, the use of salicylates as anti-inflammatory and antipyretic treatments may date back to the third millennium before Christ. SA is produced by plants as part of their defense systems against pathogen attack and environmental stress. Being widely distributed throughout the plant kingdom, the systemic presence of salicylic acid in humans may arise from fruits and vegetables, wines, tea, fruit juices, herbs and spices [35].

Actually, information about the salicylate content of foods is difficult to obtain, since it is influenced by numerous factors, including plant varieties, seasonality, growing conditions, storage and cooking [35]. To date the most definitive estimate of the salicylate content of foods is represented by a systematic review of the literature [36], where included data derived from food items randomly selected and purchased from various commercial outlets during different seasons of the year. Therefore, this review provided the first comprehensive and systematic assessment of the salicylate content of commonly consumed foods [36] (Table 1).

Table 1. Examples of total salicylate content of food items [35]

Food item	Salicylates/mg kg^{-1}	Food item	Salicylates/mg kg^{-1}
Fruits		*Vegetables*	
Blackberries	0.81	Asparagus	1.29
Blueberries	0.57	Carrots	0.16
Gala melon	0.62	Celery	0.04
Grapefruit	0.44	Green bean	0.07
Green apple	0.55	Mange tout	0.20
Kiwi fruit	0.31	Mushroom (button)	0.13
Nectarine	3.29	Onion (white)	0.80
Strawberry	0.61	Tomato	0.13
Drinks		*Spices and herbs*	
Apple	0.83	Black cumin	25.05
Cranberry	0.99	Cumin	29.76
Grapefruit	0.10	Chat masala	5.74
Orange	0.68	Garam masala	12.85
Pineapple	4.06	Paprika	28.25
Tomato	1.32	Turmeric	20.88
White wine	0.44	Thyme	28.60
Red wine	0.50	Mint	54.20
Tea	1.06	Fennel	14.00

Concerning salicylate intake, to date published estimates of daily SA intake vary markedly, ranging from 0.4 to 200 mg/day (generally considered as an overestimation) so it is unclear whether the Western diet can provide sufficient amount of salicylates to exert beneficial effects on health. According to the review by Wood et al [36], estimated salicylate intakes in a Scottish population were of 4.42 and 3.16 mg/day for males and females, respectively. Primary food sources of salicylates were alcoholic beverages (22%), herbs and spices (17%), fruits (16%), non-alcoholic beverages including fruit juices (13%), tomato-based sauces (12%) and vegetables (9%) [36].

Fruit and vegetables intake may influence serum salicylic acid concentration. Indeed, in 2007 Spadafranca et al. [37] observed a positive association between circulating levels of SA and fruit and vegetables intake of both the last week and the day before the sampling, in heathy adults. Previously, Blacklock et al. [38] reported that vegetarian adults may exhibit higher serum concentration of SA than nonvegetarians.

SA has anti-inflammatory properties and maybe the ability to modulate activity and/or expression of components involved in oxidative stress processes [35]. Studies performed on adults

have reported that treatment with salicylates may improve glycemic control and reduce glycated hemoglobin levels, in patients with type 2 diabetes [39,40,41]. The exact mechanism by which salicylate acts on glucose metabolism is not entirely clear. Furthermore, in a longitudinal double-blind, placebo-controlled trial, conducted on obese nondiabetic adults, administration of 4 g/day of salsalate (a dimer of salicylic acid) for 1 month was related to reduced c-reactive protein levels and increased adiponectin concentration, suggesting that salsalate might modulate inflammatory cardiovascular risk indexes in obese adults [42].

Obviously, the amount of salicylic acid introduced with the diet cannot be compared to therapeutic doses introduced as Aspirin. Therefore, caution has to be paid, also because, besides salicylates, other different phenolic compounds with recognized anti-inflammatory and redox-related bioactivity are widely distributed through the plant kingdom.

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2. AIMS OF THE PhD THESIS

The global epidemic of childhood obesity as well as the increasing diffusion of related comorbidities among children and adolescents underlines the need to investigate possible therapeutic strategies able to counteract negative effects on child health and the risk of more severe comorbidities during adulthood. Intensive lifestyle interventions, including diet, physical activity and behaviors, are fundamental to achieve this goal. However, as previously discussed, the characteristics of all intervention components as well as the length, the intensity, and the effectiveness of lifestyle interventions may vary largely among studies. Additionally, the role of gut microbiota in the development of obesity and related-comorbidities needs to be further investigated, to identify potential future therapeutic options. Finally, it is well-known that higher intakes of fruit and vegetable are associated with beneficial effects on health, also due to the presence of phytochemicals. Among phytochemicals, salicylic acid may have an important role being involved in the regulation of inflammation, oxidative stress and glucose metabolism.

The present PhD thesis tries to further elucidate these important topics through three different tasks.

The primary aim of the present PhD thesis was to evaluate whether a 1-year lifestyle intervention, based on normocaloric diet, promotion of physical activity and behavior changes, may improve obesity, metabolic profile and obesity-related comorbidities, as glucose metabolism alterations, hyperlipidemia, prehypertension/hypertension, increased liver echogenicity and metabolic syndrome, in a cohort of obese children.

Secondary aims were to evaluate qualitatively and quantitatively gut microbiota biodiversity in obese and normal-weight children and to compare gut microbiota profiles with SCFAs and BMI z-scores to gain insights into the structure and activity of the microbiota in pediatric obesity.

The tertiary aim was to determine the concentrations of serum salicylic acid in a group of obese children, compared to normal-weight children, and to evaluate if an association may exist between serum salicylic acid and fruit and vegetable consumption.

3. EFFECTIVENESS OF 1-YEAR LIFESTYLE INTERVENTION, BASED ON MEDITERRANEAN DIET, ON OBESITY AND RELATED COMORBIDITIES

The aim of this study was to evaluate whether a 1-year lifestyle intervention, based on normocaloric diet, promotion of physical activity and behavior changes, may improve obesity, metabolic profile and obesity-related comorbidities, as glucose metabolism alterations, hyperlipidemia, prehypertension/hypertension, increased liver echogenicity and metabolic syndrome, in a cohort of obese children.

3.1. EXPERIMENTAL SECTION

A cohort of 125 obese children (61 boys and 64 girls) was consecutively recruited among those admitted with diagnosis of obesity by primary care pediatricians to the Department of Pediatrics, San Paolo Hospital, Milan, between March 2014 and August 2015, according to the following eligibility criteria:

Inclusion criteria:

- age ≥6 years;
- obesity according to Italian BMI charts [1];
- weight at birth ≥2500 g and <4000 g;
- gestational age 37–42 weeks;
- single birth;
- Italian children residing in Milan or neighborhood (<30 km).

Exclusion criteria:

- severe malformations interfering with nutrition, physical activity and/or growth;
- syndromic, organic and hormonal conditions besides obesity.

The parents of eligible children or their legal guardian received detailed explanation about the aim of the study, and signed a consent form. The Hospital Ethics Committee approved the study protocol and gave ethical clearance.

3.1.1. Anthropometry and Blood Pressure

A medical history was collected at recruitment from parents by a standardized questionnaire during a personal interview conducted by the pediatrician. Moreover, the pediatrician made a general examination of the child and evaluated the Tanner stage of puberty [2]. The pediatrician also took anthropometric measurements and blood pressure of children both at baseline and at the end of intervention, assisted by an experienced operator.

Anthropometric measurements included:

- body weight;
- height;
- triceps skinfold thickness;
- waist circumference (WC).

Body weight and height were measured using a mechanical column scale (seca 711; seca GmbH & KG, Hamburg, Germany) with integrated measuring rod (seca 220; seca GmbH & KG). BMI was calculated from the ratio of weight to height squared (kg/m2). BMI were transformed to age- and sex-specific z-scores according to WHO reference data 2007 [3]. Waist circumference was measured using the measuring tape seca 203 (seca GmbH & KG) to the nearest 0.1 cm at the mid-point between the iliac crest and the lower edge of the ribs at the end of a normal expiration. The waist/height ratio was calculated and a cut-off ≥ 0.6 was used to identify children with higher cardiometabolic risk [4]. Triceps skinfold thickness was measured on the left side of the body, using the Harpenden Skinfold Caliper (Chasmors Ltd, London, UK) halfway between the acromion process and the olecranon process [5]. Body composition was determined by bioelectrical impedance analysis, using the BC-418 Segmental Body Composition Analyzer (Tanita Corporation of America, Inc, Arlington Heights, Illinois, USA).

Blood pressure was measured according to recommendations of the National High Blood Pressure Education Working Group [6]. Systolic or diastolic blood pressure $\geq 90^{th}$ percentile for gender, age, and height were considered as indicative of prehypertension/hypertension [6].

3.1.2. Biochemistry

Biochemical measurements were performed at baseline and one year (±5 day) after starting intervention (end of intervention). Fasting blood samples were taken at 8 h ± 30 min a.m. and immediately analyzed at the hospital laboratory of biochemistry for total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, apolipoprotein A1 (ApoA1), apolipoprotein B (ApoB), insulin, glucose and glycated hemoglobin (HbA1c) on the cobas® 6000 analyzer series, c501 and e601 modules (Roche Diagnostics GmbH, Hoffmann-La Roche ltd, Mannheim, Germany). Furthermore, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma glutamyltransferase (γGT) were determined to test liver function, and high sensitivity C-reactive protein (hs-CRP) and erythrocyte sedimentation rate (ESR) were determined as inflammation markers. Finally, vitamin D was measured out. Oral glucose tolerance test was performed: flavored glucose at a dose of 1.75 g ∕ kg body weight (up to a maximum of 75 g) was given orally after fasting blood sample, and additional blood samples were taken for measurements of plasma glucose at 120 min.

3.1.3. Glucose metabolism

Several glucose metabolism indices have been calculated. Specifically, insulin resistance was evaluated by:

- the homeostatic model assessment of insulin resistance (HOMA-IR), calculated as [7]:
  HOMA-IR: (fasting glucose (mmol/L) x fasting insulin (μU/mL))/22.5. Insulin resistance was defined as HOMA-IR >3.16 [8];
- the triglyceride glucose index (TyG index), calculated as [9,10]:
  TyG index: ln [fasting triglycerides (mg/dL) × fasting glucose (mg/dL)/2].

Insulin sensitivity was evaluated by:

- the quantitative insulin sensitivity check (QUICK) index, calculated as [11]:
  QUICK: 1/(log10 fasting plasma insulin in μU/mL + log10 glucose in mg/dL).

Pancreatic β-cell function was evaluated by:

- HOMA-β%, calculated as [7]:
  HOMA-β%: (20 × fasting insulin in μU/mL)/(fasting glucose in mmol/L−3.5).

The ratio triglycerides/HDL cholesterol was used to identify obese children at risk of IGT with a cut-off value of 2.2 [12].

Impaired fasting glucose was identified for fasting plasma glucose levels of 100 mg/dl (5.6 mmol/l) to 125 mg/dl (6.9 mmol/l)] while impaired glucose tolerance by values of 140 mg/dl (7.8 mmol/l) to 199 mg/dl (11.0 mmol/l)] after 2-h in the OGTT. Pre-diabetes was also identified by HbA1c values between 5.7%-6.4% (39–47 mmol/mol), according to ADA criteria [13]. Diabetes was diagnosed according to ADA criteria, too [13] (Table 1. Criteria for the diagnosis of diabetes, subchapter 1.2.2.).

3.1.4. Lipid profile

ApoB/apoA ratio, LDL/HDL cholesterol ratio, total cholesterol/HDL cholesterol ratio and triglycerides/HDL cholesterol ratio were calculated. It has been observed that triglycerides/HDL cholesterol ratio may be useful to identify children with atherogenic dyslipidemia, insulin-resistance and preclinical signs of early organ damage [14,15]. Therefore, triglycerides/HDL ratio ≥2.2 was also considered as a biomarker of atherogenic dyslipidemia and altered cardiometabolic risk [14,16]. Furthermore, the logarithm of the ratio of plasma triglycerides to HDL-cholesterol (Log [Triglycerides/HDL]), called atherogenic index of plasma (AIP), was calculated as it is considered a marker of plasma atherogenicity [17].

Regarding hyperlipidemia, the prevalence of children with borderline-low/high concentration of plasma lipids was determined according to proposed cut-off by the Expert Panel on Integrated Guidelines for Cardiovascular Health and Risk Reduction in Children [18] (Table 1).

Table 1. Acceptable, Borderline-Low/High Plasma Lipid and Lipoprotein for children and adolescents [18]

Category	Acceptable (mg/dL)	Borderline-Low/High (mg/dL)
Total cholesterol	<170	≥170
LDL-cholesterol	<110	≥110
Triglycerides		
0-9 years	<75	≥75
10-19 years	<90	≥90
HDL-cholesterol	>45	≤45

3.1.5. Abdominal ultrasonography (US)

Abdominal US was performed according to a randomized sequence. The same radiologist (AR) performed US by a GE Logiq 9 (General Electric Healthcare Medical Systems, Milwaukee, WI, United States) using a 3.5 MHz convex array transducer.

3.1.6. Metabolic Syndrome

Metabolic syndrome was defined in accordance with the International Diabetes Federation (IDF) criteria for children and adolescents (Table 2) [19,20]. As IDF suggests that below the age of 10 years metabolic syndrome cannot be diagnosed, in this study it was evaluated only in children of 10 years or older.

Table 2. IDF definition of metabolic syndrome in children and adolescents aged 10-16 years [19,20].

Years	Obesity (WC)	Triglycerides	HDL-C	Blood pressure	Glucose
10 - < 16 years	WC ≥ 90th pc or adult cut-off if lower	≥ 150 mg/dL (1.7 mmol/L)	< 40 mg/dL (1.03 mmol/L)	Systolic BP ≥ 130 or diastolic BP ≥ 85 mm Hg	FPG ≥ 100 mg/dL (5.6 mmol/L)** or known T2DM

HDL, high density lipoprotein; BP, blood pressure; FPG, fasting plasma glucose

3.1.7. Dietary Habits

Dietary habits of children were assessed at baseline and at the end of intervention by a food frequency questionnaire (FFQ) originally developed at our Department in 1990's on the original Block FFQ [21] and then revised and updated in 2008 on the basis of the full-length Block 2005 FFQ © (NutritionQuest, Berkeley, CA, USA) and the 2007 national food composition tables [22], to appropriately adjust for cultural food/beverage items of the Italian pediatric population. Parents completed the FFQ about their children's habits during an interview of approximately 50 min, conducted at hospital by the same nutritionist. Each meal was analyzed to find out which food was eaten and how often. Usual portion sizes were estimated using household measures and the weight (e.g., pasta) or unit (e.g., fruit juice) of the purchase. A 24-h recall was additionally recorded at the end of the interview to standardize the usual serving size. Quantification and analysis of the energy intake and nutrient composition were performed with an *ad hoc* PC software program developed by a consultant.

3.1.8. Intervention

The intervention was based on the promotion of a normocaloric diet, balanced for the macronutrient distribution, in accordance with the national guidelines for treatment of childhood obesity [23] and the reference intake levels for nutrients and energy, defined by the Italian Society of Human Nutrition [24]. Specifically, it was recommended that children follow, for a 1-year period, a normocaloric diet (daily caloric intake by age and sex) consisting of protein (population reference intake (PRI): 0.94 – 0.99 g/kg×die, according to age and sex), carbohydrates (45%–60% En), fat (20%–35% En; <10% En from saturated fatty acids, 5-10% En from polyunsaturated fatty acids, ≤15% En from monounsaturated fatty acids) and fiber (8.4 g/1000 kcal) [24]. Additionally, it was recommended that children engage in at least 60 min of moderate- to vigorous-intensity physical activity (MVPA) daily [25], based on walking, and tailored to individual preferences. MVPA was estimated by a physical activity recall.

During 1-h educational session (at least) held at hospital a pediatrician and a nutritionist provided counseling and instructed parents and children about the intervention to be performed and actions to maintain throughout a 1-year period. Education was based and focused on regulation of energy expenditure, body composition, physical activity, consequences of sedentary lifestyle, principles of nutrition, food sources, glycemic index and glucose metabolism, to continuously enhance and maintain parental and self-efficacy for dietary change. This educational session also took into account a range of behavior change techniques from the revised CALO-RE taxonomy (items 1, 2, 5, 6, 8, 16, 21, and 26) [26]. In particular, written educational guidelines were given to the parents, including general nutritional advice, food choice lists, a Mediterranean diet pyramid for the pediatric age (**ATTACHMENT 1**), and recommended average servings for principal food categories, according to updated Italian Dietary Reference Values [24] (**ATTACHMENT 2**). The Mediterranean diet pyramid was developed *ad hoc* at our Department, on the basis of the Mediterranean diet pyramid for adults [27]. General nutritional advice included increasing fruit and vegetable intake, increasing legume and fish intake while decreasing meat consumption, using more whole grain, avoiding sugary beverages and limiting sweets, according to a Mediterranean pattern [27].

An educational and illustrated brochure about potential benefits of daily physical activity, about the importance of accumulate at least 60 minutes of MVPA daily and to include "programmed" physical activity 3 times per week, was also given to the child and the parents (**ATTACHMENT 3**). A progressive increase in physical activity to gradually achieve the target

was recommended, especially for inactive children: "it may be appropriate to start with smaller amounts of physical activity and gradually increase duration, frequency and intensity over time." The concept that, during childhood, physical activity includes walk, bike ride, play, games, sports, physical education, or planned exercise, in different contest was strongly stressed, as well as, the importance to reduce sedentary activities, as watching TV, playing videogames, etc.

Medical examinations were scheduled every 6 months during the intervention period. At each visit, parents of children or their legal guardians filled out a 24-hour recall (plus FFQ at 12 months) and physical activity recall to evaluate adherence to lifestyle recommendations.

CONSIGLI PER UNA SANA ALIMENTAZIONE
-AREA MEDITERRANEA-

L'organismo umano consuma continuamente energia, gli alimenti forniscono le sostanze necessarie per garantire il fabbisogno energetico e le necessità dei diversi organi.

Per mantenersi in forma si consiglia di:

☑ fare **attività fisica quotidiana** per almeno 1 ora. Alimentazione corretta ed attività fisica costante sono una coppia inseparabile per vivere bene ed in salute. Impara a mantenerti in esercizio! Quando si parla di attività fisica non si deve pensare solo a sport o fatica. Le attività che favoriscono l'utilizzazione del tessuto adiposo sono quelle che possiamo praticare ogni giorno: andare in bicicletta, con pattini o skate, camminare, fare una passeggiata con il cane, ballare, salire le scale anziché prendere l'ascensore, saltare con la corda, giocare con amici a bandiera, a nascondino ….. Per il tempo libero, quindi, non solo computer, televisione, videogiochi.

☑ **alimentarsi in modo variato** ed equilibrato;

☑ mantenere l'abitudine di una **prima colazione sostanziosa** che è il carburante per la mattinata, l'attività scolastica e il gioco.

☑ evitare periodi di digiuno prolungati e distribuire ogni giorno gli alimenti in 4-5 pasti:

    ☑ **COLAZIONE: 15% delle calorie**

Un'abitudine importante da mantenere è quella di fare una buona prima colazione: è il carburante per la mattinata, l'attività scolastica e per il gioco. La prima colazione può essere molto semplice ma deve essere completa:

    ► Latte parzialmente scremato o yogurt bianco
    + Cereali pronti o fette biscottate o pane integrale con marmellata o muesli o plum cake
    + Frutta fresca o eventualmente spremuta di arancia

    ☑ **SPUNTINO AL MATTINO: 5% delle calorie**
    ☑ **MERENDA A METÀ POMERIGGIO 10% delle calorie**

La merenda di metà mattina e quella del pomeriggio sono momenti importanti della giornata perché servono a non lasciare un lungo intervallo tra i pasti, evitando di arrivare a pranzo o a cena affamati, con il rischio di mangiare troppo.

    ► Frutta di stagione. Saltuariamente sorbetto di frutta;
    ► Yogurt bianco;
    ► Pane integrale (ad esempio pane e olio o pane e pomodoro).

    ☑ **PRANZO 40% delle calorie, CENA 30% delle calorie**

Per assicurare un corretto apporto di tutti i nutrienti devono essere presenti carboidrati, proteine, lipidi e fibre. Due possono essere gli schemi da seguire:

1. <u>piatto unico</u>: primo piatto, pasta o riso o orzo o farro o polenta conditi con carne o pesce o legumi o formaggio o uova **+** <u>verdura</u> **+** <u>frutta</u>.

2. <u>primo piatto:</u> pasta o riso orzo o farro o polenta conditi con verdura **+** <u>secondo piatto</u> (carne o pesce o legumi o formaggio o uova) **+** <u>verdura</u> **+** <u>frutta</u>.

I 14 pasti settimanali possono essere così suddivisi:

🌿 **FRUTTA E VERDURA**	3-5 PORZIONI AL GIORNO
🌿 **PASTA** o **RISO** o **ALTRI CEREALI**	3-5 PORZIONI AL GIORNO
🌿 **TUBERI**	1-2 VOLTE LA SETTIMANA
🌿 **LATTE E YOGURT**	1-2 PORZIONI AL GIORNO
🌿 **NOCI E SEMI**	1 PORZIONE AL GIORNO (es 3-4 noci)
🌿 **CARNE**	MASSIMO 3 VOLTE LA SETTIMANA (≤ 2 PORZIONI A SETTIMANA DI CARNI BIANCHE, < 2 PORZIONI DI CARNI ROSSE, ≤ 1 PORZIONE A SETTIMANA DI CARNI LAVORATE es prosciutto cotto e bresaola)
🌿 **PESCE**	3-4 VOLTE LA SETTIMANA
🌿 **LEGUMI**	4-5 VOLTE LA SETTIMANA
🌿 **FORMAGGI**	2 VOLTE LA SETTIMANA
🌿 **UOVA**	1-2 VOLTE LA SETTIMANA

<u>COME ORIENTARSI NELLA SCELTA DEGLI ALIMENTI</u>

LATTE parzialmente scremato

YOGURT bianco

CEREALI privilegiare cereali integrali e riso parboiled (quest'ultimo massimo 2 volte alla settimana). Si raccomanda la cottura al dente. Variare la scelta considerando anche altri cereali quali orzo, farro, sorgo, grano saraceno, miglio, quinoa, amaranto.

PANE 1 panino al giorno, preferire quello preparato con farine integrali o con farina tipo 1.

CARNE pollo, coniglio, tacchino, vitello, manzo magro, maiale magro.

PESCE fresco o surgelato, preferire il pesce azzurro (sarde, alici). Merluzzo, nasello, sgombro, spigola o pesce persico meglio non più di 1 volta a settimana. Cefalopodi (calamari, polpo) non più di 1 volta a settimana. Crostacei e molluschi bivalvi saltuariamente. Da evitare pesce di grossa taglia (pesce spada e tonno).

LEGUMI freschi, secchi o surgelati vanno sempre associati nello stesso pasto ai cereali (pasta, riso, orzo). Non sono verdure, ma una alternativa a carne, pesce, uova e formaggi.

FRUTTA 2-3 volte al giorno. Da preferire quella di stagione. Non frullata o passata. Da assumere non più di 2-3 volte alla settimana: kiwi o uva o banana o ananas o papaya o melone. Da limitare (≤ 1 volta alla settimana): datteri o avocado o tamarindo o anguria.

VERDURA fresca o surgelata, non frullata o passata, 2 volte al giorno. Da preferire verdura di stagione (le patate sono da limitare).

CONDIMENTI privilegiare olio extravergine di oliva, aceto e limone.

SALE da limitare

COTTURA   in umido, al vapore, al forno, al cartoccio.

LA PIRAMIDE DELLA DIETA MEDITERRANEA PEDIATRICA

utilizzo giornaliero

Limitare al minimo

1-2 porzioni settimanali

2 porzioni settimanali

Max 3 porzioni settimanali — pollo, coniglio, tacchino, vitello, manzo magro, maiale magro,...

3-4 porzioni settimanali — sarde, alici, triglia, calamari, polpo, merluzzo, sgombro, spigola, gamberi,...

4-5 porzioni settimanali — fagioli, piselli, lenticchie,..

1 porzione al giorno

1-2 porzioni al giorno

3-5 porzioni al giorno — grano duro, grano tenero, orzo, farro, mais, segale, grano Khorasan (meglio se integrali), riso parboiled

3-5 porzioni al giorno (Varietà di colori e di consistenza) — mele, pere, arance, ciliegie, albicocche, pomodori, insalata, zucchine, broccoli,...

DOLCI SNACKS
CONDIMENTI
UOVA — TUBERI
FORMAGGI
CARNE
PESCE
LEGUMI
NOCI — SEMI
LATTE — YOGURT
CEREALI
FRUTTA — ORTAGGI
ACQUA

- Attività fisica
- Adeguato riposo
- Convivialità
- Biodiversità e stagionalità
- Prodotti locali ed ecologici

Porzioni e frequenze alimentari per la fascia di età 4-6 anni

ALIMENTI	QUANTITA' CONSIGLIATA*	FREQUENZE DI CONSUMO
Pasta/riso *(anche integrali)*, polenta, orzo, farro, ecc.	40-50 g	2 volte al giorno
Pastina in brodo/ minestra d'orzo	30 g	
Gnocchi	120 g	
Tortellini in brodo	50 g	
Pane *(anche integrale)*	30 g	2-3 volte al giorno
Pizza	150 g	1 volta alla settimana *(in sostituzione di pasta, riso, pane, patate, ecc.)*
Prodotti da forno per l'infanzia e cereali da colazione *(anche integrali)*	4 biscotti o 4 fette biscottate o 40 g cereali da colazione	1 volta al giorno
Patate	150 g	1 volta alla settimana *(in sostituzione del pane)*
Verdure	50 g crude *(es.: pomodori, lattuga, ecc.)* o 130 g da cuocere *(es.: bieta, spinaci, ecc.)*	2 volte al giorno
Frutta fresca	100 g frutta	2-3 volte al giorno
Legumi	20-25 g secchi o 60-70 g lessi	4-5 volte alla settimana
Latte	200 mL	1 volta al giorno
Yogurt da latte intero	125 g *(1 vasetto)*	4 volte alla settimana
Formaggio	40 g fresco *(es.: mozzarella)* o 30 g semistagionato *(es.: caciotta)* o 20 g stagionato *(es.: parmigiano)*	2 volte alla settimana
Pesce**	50 g	3-4 volte alla settimana
Carne***	40-50 g	Max 3 volte alla settimana (incluso massimo 1 volta settimana carni trasformate)
Carni trasformate	30 g *(es.1 fetta e ½ di prosciutto cotto)*	
Uova	60 g (1 uovo)	1-2 volta alla settimana
Olio	10 g extra vergine di oliva 5-10 g semi di girasole	2 volte al giorno 1 volta al giorno
Dolci	30 g dolci da forno *(es.: crostata, ciambellone, ecc.)* o 10-15 g cioccolato, marmellata, ecc.	Limitare al minimo (Max 1 volta alla settimana)

	o 100 g dolci a cucchiaio *(es.: gelato, budino, ecc.)*	
Acqua	200 mL *(un bicchiere medio)*	6 bicchieri (circa 1200 mL) *nell'arco della giornata*

* Le quantità si riferiscono all'alimento crudo, al netto degli scarti o, in alcuni casi, pronto per il consumo (es.: latte e derivati, pane, ecc.).

**Limitare il consumo di pesce di taglia grande (es.: tonno e pesce spada, meglio variare).

*** Preferire tagli magri e carne bianca (pollo, tacchino e coniglio).

Porzioni e frequenze alimentari per la fascia di età 7-11 anni

ALIMENTI	QUANTITA' CONSIGLIATA*	FREQUENZE DI CONSUMO
Pasta/riso (anche integrali), polenta, orzo, farro, ecc.	70-80 g	2 volte al giorno
Pastina in brodo/ minestra d'orzo	40 g (pastina/orzo)	
Gnocchi	150 g	
Tortellini in brodo	50 g	
Pane (anche integrale)	50 g	2-3 volte al giorno
Pizza	200 g	1 volta alla settimana (in sostituzione di pasta, riso, pane, patate, ecc.)
Prodotti da forno e cereali da colazione (anche integrali)	4 biscotti o 4 fette biscottate o 40 g cereali da colazione	1 volta al giorno
Patate	150 g	1 volta alla settimana (in sostituzione del pane)
Verdure	50 g crude (es.: pomodori, lattuga, ecc.) o 150 g da cuocere (es.: bieta, spinaci, ecc.)	2-3 volte al giorno
Frutta fresca	100 g frutta	2-3 volte al giorno
Legumi	30-35 g secchi o 90-100 g freschi	4-5 volte alla settimana
Latte	200 mL	1 volta al giorno
Yogurt	125 g (1 vasetto)	4 volte alla settimana
Formaggio	70 g fresco (es.: mozzarella) o 50 g semistagionato (es.: caciotta) o 30 g stagionato (es.: parmigiano)	2 volte alla settimana
Pesce**	80 g	3-4 volte alla settimana
Carne***	70-80 g	Max 3 volte alla settimana (incluso massimo 1 volta settimana carni trasformate)
Carni trasformate	40 g (es. 2 fette prosciutto cotto)	
Uova	60 g (1 uovo)	1-2 volte alla settimana
Olio	10 g extra vergine di oliva 10 g semi di girasole	2 volte al giorno 1 volta al giorno
Dolci	30-50 g dolci da forno (es.: crostata, ciambellone, ecc.) o 20-30 g cioccolato, marmellata, ecc. o 100-125 g dolci a cucchiaio (es.: gelato, budino, ecc.)	Limitare al minimo (Max 1 volta alla settimana)

Acqua	200 mL *(un bicchiere medio)*	6 bicchieri (circa 1200 mL) *nell'arco della giornata*

* Le quantità si riferiscono all'alimento crudo, al netto degli scarti o, in alcuni casi, pronto per il consumo
   (es.: latte e derivati, pane, ecc.).

** Limitare il consumo di pesce di taglia grande (es.: tonno e pesce spada, meglio variare).

*** Preferire tagli magri e carne bianca (pollo, tacchino e coniglio).

Porzioni e frequenze alimentari 12-15 anni

ALIMENTI	QUANTITA' CONSIGLIATA*	FREQUENZE DI CONSUMO
Pasta/riso (anche integrali), polenta, orzo, farro, ecc.	90-100 g	2 volte al giorno
Pastina in brodo/ minestra d'orzo	50 g (pastina/orzo)	
Gnocchi	220-250 g	
Tortellini in brodo	100-110 g	
Pane (anche integrale)	50 g	2-3 volte al giorno
Pizza	300 g	1 volta alla settimana (in sostituzione di pasta, riso, pane, patate, ecc.)
Prodotti da forno e cereali da colazione (anche integrali)	4 biscotti o 4 fette biscottate o 40 g cereali da colazione	1 volta al giorno
Patate	150 g	1 volta alla settimana (in sostituzione del pane)
Verdure	80 g crude (es.: pomodori, lattuga, ecc.) o 200 g da cuocere (es.: bieta, spinaci, ecc.)	2-3 volte al giorno
Frutta fresca	150 g frutta	2-3 volte al giorno
Frutta secca	20-30	3-4 volte alla settimana
Legumi	40-45 g secchi o 120-130 g lessi	4-5 volte alla settimana
Latte	250 mL	1 volta al giorno
Yogurt	125 g (1 vasetto)	4 volte alla settimana
Formaggio	80 g fresco (es.: mozzarella) o 50 g semistagionato (es.: caciotta) o 50 g stagionato (es.: parmigiano)	2 volte alla settimana
Pesce**	100 g	3-4 volte alla settimana
Carne***	90 g	Max 3 volte alla settimana (incluso massimo 1 volta settimana carni trasformate)
Carni trasformate	50 g (es. 2 fette prosciutto cotto)	
Uova	120 g (2 uova)	1 volte alla settimana
Olio	10 g extra vergine di oliva 10-15 g semi di girasole	2 volte al giorno 1 volta al giorno
Dolci	30-50 g dolci da forno (es.: crostata, ciambellone, ecc.) o 20-30 g cioccolato, marmellata, ecc. o 100-125 g dolci a cucchiaio (es.: gelato, budino, ecc.)	Limitare al minimo (Max 1 volta alla settimana)

| Acqua | 200 mL *(un bicchiere medio)* | 6 bicchieri (circa 1200 mL) *nell'arco della giornata* |

* Le quantità si riferiscono all'alimento crudo, al netto degli scarti o, in alcuni casi, pronto per il consumo
  (es.: latte e derivati, pane, ecc.).
** Limitare il consumo di pesce di taglia grande (es.: tonno e pesce spada, meglio variare).
*** Preferire tagli magri e carne bianca (pollo, tacchino e coniglio).

PROMOZIONE DELL'ATTIVITA' FISICA IN BAMBINI E ADOLESCENTI

LE REGOLE D'ORO PER STARE IN FORMA

- ✓ Prendi le scale al posto dell'ascensore
- ✓ Fai una passeggiata con il cane per 30 minuti al giorno
- ✓ Vai a scuola a piedi o in bici
- ✓ Quando senti la musica, balla
- ✓ Gioca al parco con gli amici
- ✓ Aiuta la mamma nei lavori di casa

FARE ATTIVITÀ FISICA COSTANTEMENTE PORTA TANTI BENEFICI!

RIMANI IN FORMA

CONOSCI NUOVI AMICI

DIVENTI FORTE

FA BENE AL CUORE

MIGLIORA IL SONNO

TI FA SENTIRE BENE

AIUTA LA CONCENTRAZIONE

AIUTA LO SVILUPPO E LA COORDINAZIONE

86

ALMENO 60 MINUTI AL GIORNO

Femmine (6-12 anni)	Maschi (6-12 anni)	Adolescenti	Categoria
≥14.000 passi	≥17.500 passi	≥ 13.000 passi	MOLTO ATTIVO
12.000 – 15.000 passi	15.000 – 17.500 passi	11.000 – 13.000 passi	ATTIVO
9.500 – 12.000 passi	12.500 – 15.000 passi	9.500 – 11.000 passi	ABBASTANZA ATTIVO
7.000 – 9.500 passi	10.000 – 12.500 passi	7.000 – 10.000 passi	POCO ATTIVO
<7.000 passi	<10.000 passi	<7.000 passi	SEDENTARIO

TRE VOLTE A SETTIMANA ATTIVITA' PROGRAMMATA

Ginnastica

Danza

Arti marziali

Sport

INFINE, RIDUCI LA SEDENTARIETA'!

3.1.9. Sample Size

The sample size was calculated in a previous study [28] to detect a mean longitudinal variation of 5% or more of HDL cholesterol, based on the baseline mean and standard deviation estimated in children already recruited. Assuming a type I error level of 0.05 with a power of 0.80, and allowing for a drop-out of 5% at least 87 children needed to be recruited [28].

3.1.10. Statistical Analysis

Descriptive data are reported as mean and standard deviation (SD) or 95% confidence interval (CI), or number of observations (percentage). Normality of the distribution of continuous variables was assessed by the Kolmogorov–Smirnov test. Means were adjusted for age, sex, baseline BMI z-score and Tanner stage, as appropriate. Statistical significance of longitudinal variations was tested by the Student's t test for paired data or the Wilcoxon test, and also adjusted by ANOVA for repeated measures. At this analysis non-normally distributed continuous variables entered the model after logarithmic transformation. Association between changes in anthropometric parameters with changes in metabolic parameters was assessed by Spearman's correlation coefficient. All values of $p < 0.05$ were considered to indicate statistical significance (two-tailed test). The statistical package for social sciences (SPSS) package version 20.0 (SPSS Inc., Chicago, IL, USA) for Windows (Microsoft, Redmond, WA, USA) was used, for the statistical analysis.

3.2. RESULTS

One hundred eighteen obese children (94.4%), 54 boys and 64 girls, completed the intervention. At recruitment mean (SD) age and duration of obesity were 9.8 (2.5) years (range 6–15) and 4.2 (2.2) years, respectively. At the end of the intervention reduction of daily energy intake and macronutrient redistribution towards the recommended range were observed (Table 1). Mean (SD) MVPA was 44.4 (32.0) min/day at baseline and 52.8 (34.6) min/day at the end of intervention (p = 0.065). At 6 and 12 months of intervention, compliance with diet and MVPA, evaluated by the 24-h recall (plus FFQ at 12 months) and recall of physical activity, was 87% and 90% (diet) and 84% and 86% (MVPA), respectively.

Table 1. Dietary intake of energy, macronutrients and fiber at baseline and at the end of intervention. Values are mean [SD]†.

Variable	Baseline (n= 118)	End of intervention (n=118)	P-value	Recommended intake [24]
Energy				1380-3330 kcal/d
kcal/d	2492.66 [838.39]	2029.32 [785.11]	<0.001*	depending on age and sex
kcal/kg	46.55 [17.88]	36.99 [16.72]	0.001*	[6-15 y]
Protein				
g/d	97.60 [32.36]	76.08 [26.40]	<0.001*	AR: 16-50 g/die depending on age and sex [6-15 y]
% Energy	15.60 [3.99]	15.00 [2.63]	0.052	-
Carbohydrates				
g/d	342.33 [123.00]	306.66 [119.53]	0.045*	-
% Energy	52.10 [7.71]	57.03 [9.33]	0.001*	45-60% Energy
Sugars				
g/d	113.55 [58.12]	83.78 [46.93]	0.002*	-
% Energy	18.30 [6.09]	15.43 [4.41]	<0.001*	< 15% Energy
Fats				
g/d	90.93 [35.69]	63.97 [21.48]	<0.001*	-
% Energy	31.62 [5.63]	28.72 [4.11]	<0.001*	20-35% Energy
Saturated				
g/d	31.53 [13.33]	19.06 [8.10]	<0.001*	-
% Energy	11.44 [3.39]	8.70 [2.58]	<0.001*	< 10% Energy
Monounsaturated				
g/d	34.65 [15.81]	22.56 [11.84]	<0.001*	-
% Energy	12.72 [4.41]	10.11 [3.40]	0.033*	≤ 15% Energy
Polyunsaturated				
g/d	14.94 [6.63]	9.43 [5.30]	<0.001*	-
% Energy	5.15 [1.73]	4.76 [1.75]	0.072	5-10% Energy
Fiber				
g/d	13.23 [6.82]	17.13 [8.24]	0.002	-
g/1000 kcal	5.50 [2.46]	8.86 [3.70]	<0.001*	8.4 g/1000 kcal

† Mean and related P-value adjusted for age and sex
* Statistically significant

3.2.1. Change in anthropometry and obesity prevalence after 1-year intervention

Throughout the intervention period BMI z-score decreased (mean variation, Δ, -0.52; 95% CI, (-0.59; -0.45)), as well as waist circumference (−0.64; (−1.35; 0.07) cm), triceps skinfold thickness (-2.15; (-3,34; -0,96) mm) and waist to height ratio (-0.02; (-0.03; -.01)). Total lean mass (kg) increased at the end of the intervention (4.43; (3.83; 5.03)). Furthermore, the prevalence of children with waist to height ratio ≥0.60 was reduced by 29.8% (Table 2). The within-subjects longitudinal variation of obesity status was significant (Figure 1-2).

Table 2. Anthropometric measurements at baseline and at the end of intervention. Values are mean [SD]† or number of children (percentage).

Variable	Baseline (n= 118)	End of intervention (n=118)	P-value
BMI Z-score	3.41 [0.95]	2.89 [0.86]	<0.001*
Waist circumference (cm)	84.90 [10.27]	84.26 [10.30]	0.001*
Triceps skinfold thickness (mm)	27.94 [5.35]	25.79 [6.10]	0.036*
Waist/Height	0.60 [0.05]	0.58 [0.05]	0.021*
Total fat mass (kg)	23. 33 [9.20]	24.08 [9.35]	0.139
Total fat mass (%)	38.18 [6.03]	36.56 [6.13]	0.242
Total lean mass (kg)	35.73 [8.73]	40.16 [9.05]	<0.001*
Total lean mass (%)	61.82 [6.03]	63.45 [6.12]	0.243
Waist/Height ≥ 0.60 [Yes]	57 (48.3)	40 (33.9)	0.005*

† Mean and related *P*-value adjusted for age, sex, baseline BMI z-score.
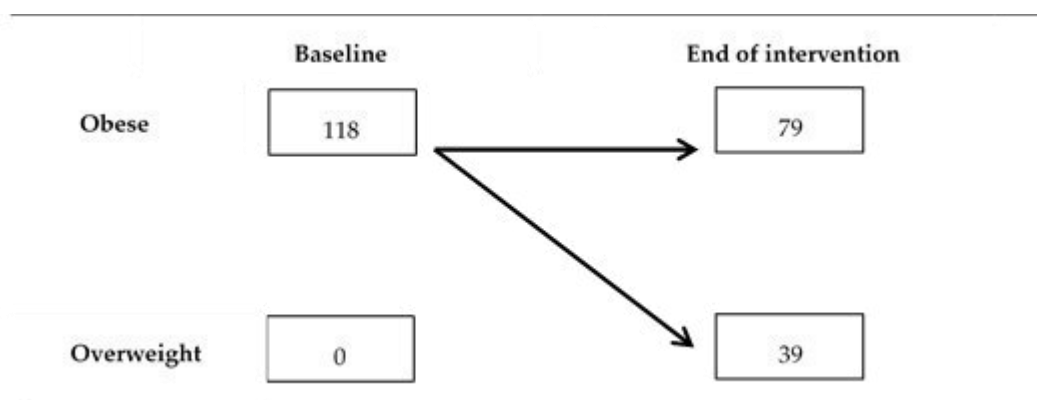* Statistically significant.

Figure 1. Within-subjects longitudinal variation of obesity status (according to Italian BMI charts [1] throughout the intervention period† (figures are number of children).
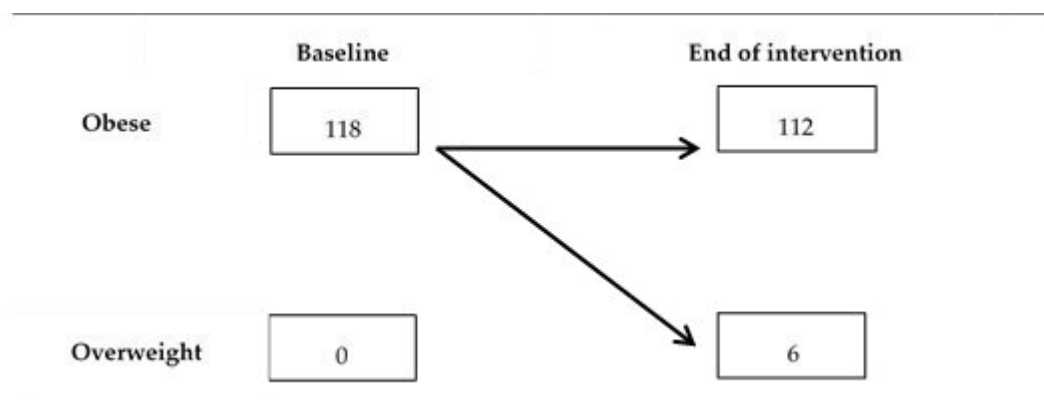† Significance of longitudinal variation was *P*<0.001 (Wilcoxon test).

Figure 2. Within-subjects longitudinal variation of obesity status (according to WHO BMI for age charts [29] throughout the intervention period† (figures are number of children).
† Significance of longitudinal variation was *P*=0.014 (Wilcoxon test).

3.2.2. Change in blood pressure, metabolic profile and liver steatosis after 1-year intervention

At the end of intervention children showed lower mean (SD) systolic blood pressure than at baseline (112.72 (9.61) vs. 114.36 (11.66) mmHg; P = 0.040), while no change was observed for diastolic blood pressure (65.88 (7.99) vs. 66.67 (9.36) mmHg; P = 0.569). Moreover, the prevalence of children with systolic or diastolic blood pressure ≥90th pc for gender, age, and height [6] significantly decreased at the end of the intervention (number of children (percentage): 35 (29.7) vs. 48 (40.7); P = 0.028).

Throughout the intervention period there was a reduction in glucose after OGTT (-7.35; (-10.48; -4.22)) mg/dL), HOMA-β% (-86.90; (-137.60; -36.20), TyG index (-0.25; (-0.32; - 0.18) and triglycerides/HDL cholesterol ratio (-0.69; (-0.94; -0.44)), while QUICK index significantly increased (0.008; (0.004; 0.012). Furthermore, the prevalence of insulin resistance decreased by 30.4% and the prevalence of children with glycated hemoglobin between 39-46 mmol/mol decreased by 36.0% (Table 3). Only one child at baseline had fasting glucose between 100-125 mg/dL (IFG) while only one child at the end of intervention showed post OGGT glycemia between 140-199 mg/dL [13].

Table 3. Glucose metabolism variables and distribution of children according to insulin resistance [8], triglycerides/HDL ≥ 2.2 [12] and pre-diabetes [13], at baseline and at the end of intervention. Values are mean [SD]† or number of children (percentage).

Variable	Baseline (n= 118)	End of intervention (n=118)	*P*-value
Glucose (mg/dL)	84.88 [6.15]	84.82 [5.34]	0.242
Glucose at 120 min (mg/dL)**	98.01 [13.62]	90.66 [12.42]	0.043*
Insulin (μU/mL)	18.12 [13.22]	14.16 [8.51]	0.062
HOMA-IR	3.82 [2.97]	2.98 [1.88]	0.057
HOMA-β%	332.74 [291.60]	245.84 [131.53]	0.043*
QUICK index	0.33 [0.03]	0.33 [0.03]	0.025*
TyG index	8.33 [0.50]	8.08 [0.44]	<0.001*
Triglycerides/HDL	2.49 [1.64]	1.80 [1.12]	<0.001*
Glycated hemoglobin (mmol/mol)	35.26 (4.57)	36.23 (3.54)	0.237
			P-value††
Insulin resistance (yes)	56 (47.5)	39 (33.0)	0.003*
Triglycerides/HDL ≥ 2.2	56 (47.5)	25 (21.2)	<0.001*
Glycated hemoglobin: 39-46 mmol/mol	25 (21.1)	16 (13.6)	0.029*

HOMA-IR, homeostasis model assessment of insulin resistance; QUICK, quantitative insulin sensitivity check; TyG, triglyceride glucose. Insulin resistance (yes): HOMA-IR >3.16 [8]
† Mean and related *P*-value adjusted for age, sex, baseline BMI z-score, Tanner stage.
**Missing value: n=12
† † Wilcoxon test for paired data.
* Statistically significant.

HDL cholesterol increased throughout the intervention period (2.28; (0.77;3.79) mg/dL) while there was a reduction in total cholesterol (-5.60; (-9.65; -1.55) mg/dL), triglycerides (-25.73; (-34.11; -17.35) mg/dL), LDL/HDL cholesterol ratio (-0.17; (-0.25; -0.09)), total/HDL cholesterol ratio (-0.26; (-0.38; -0.14)), triglycerides/HDL cholesterol ratio (-0.69; (-0.94; -0.44)) and atherogenic index (-0.13; (-0.16; -0.10) (Table 4).

Table 4. Blood lipid profile at baseline and at the end of intervention. Values are mean [SD]† or number of children (percentage).

Variable	Baseline (n= 118)	End of intervention (n=118)	P-value
Total cholesterol (mg/dL)	164.14 [29.82]	158.54 [31.66]	0.023*
LDL cholesterol (mg/dL)	97.38 [27.87]	93.56 [29.28]	0.151
HDL cholesterol (mg/dL)	48.02 [9.76]	50.30 [10.33]	<0.001*
Triglycerides (mg/dL)	109.71 [54.87]	83.98 [38.38]	<0.001*
Apo A1 (mg/dL)	132.45 [21.68]	133.96 [20.82]	0.891
Apo B (mg/dL)	74.73 [20.24]	74.23 [19.42]	0.150
ApoB/ApoA	0.57 [0.19]	0.57 [0.19]	0.788
LDL/HDL cholesterol	2.14 [0.80]	1.97 [0.78]	0.007*
Total chol/HDL cholesterol	3.55 [0.95]	3.29 [0.93]	0.001*
Triglycerides/HDL	2.49 [1.64]	1.80 [1.12]	<0.001*
Atherogenic index	0.32 [0.27]	0.19 [0.23]	<0.001*

LDL, low-density-lipoprotein; HDL, high-density-lipoprotein; Apo A1, apolipoprotein A1; Apo B, apolipoprotein B.

† Mean and related *P*-value adjusted for age, sex, baseline BMI z-score, Tanner stage.

* Statistically significant.

The prevalence of children with borderline-high levels of triglycerides decreased significantly during the intervention period by 36.8% and the prevalence of children with triglycerides to HDL cholesterol ratio ≥ 2.2 decreased by 55.4% (Table 5).

Table 5. Distribution of children according to hyperlipidemia cut-offs [18] and triglycerides/HDL ≥ 2.2 [14,16] at baseline and at the end of intervention. Values are number of children (percentage).

Variable	Baseline (n= 118)	End of intervention (n=118)	P-value†
Total cholesterol border/high (≥170 mg/dL)	47 (39.8)	39 (33.0)	0.059
LDL cholesterol border/high (≥110 mg/dL)	35 (29.7)	31 (26.3)	0.285
HDL cholesterol border/low (≤45 mg/DL)	49 (41.5)	41 (34.7)	0.095
Triglycerides border/high (≥75/90 mg/dL) ††	76 (64.4)	48 (40.7)	<0.001*
Triglycerides/HDL ≥ 2.2	56 (47.5)	25 (21.2)	<0.001*

LDL, low-density-lipoprotein; HDL, high-density-lipoprotein.

† Wilcoxon test for paired data.

†† According to age.

* Statistically significant.

With regard to liver function, no significant difference was observed for ALT levels (mean (SD): 28.83 (11.87) vs 32.30 (14.87) U/l; *p*=0.429) while a decrease in AST (29.06 (10.80) vs 29.90 (10.47) U/l; *p*=0.005) and γGT (14.98 (5.62) vs. 18.25 (7.46) U/l; *p*<0.001) was observed.

The rate of increased liver echogenicity declined by 50.0%, from 28.8% to 14.4% (Figure 3).
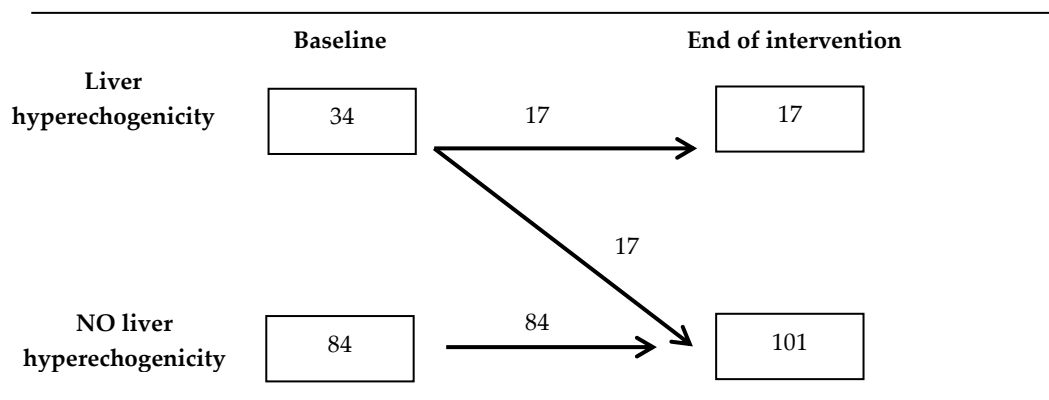

Figure 3. Within-subjects longitudinal variation of liver hyperechogenicity over the intervention period† (figures are number of children).
† Significance of longitudinal variation was *P*=0.001 (Wilcoxon test).

Finally, high sensitivity-CRP decreased at the end of the intervention (n=107; 2.34 (2.41) vs. 4.09 (5.07) mg/L; *p*=0.004) while no difference was observed for erythrocyte sedimentation rate levels (n=101; 26.58 (17.32) vs. 22.74 (20.62) mm/h; *p*=0.460). Furthermore, no significant improvement was observed for vitamin D levels (n=103; 19.61 (5.32) vs 19.77 (7.93) ng/mL; *p*=0.738).

3.2.3. Change in metabolic syndrome prevalence after 1-year intervention

For the evaluation of the effect of the 1-year intervention on metabolic syndrome, a subsample of 61 children of 10 years and older was considered.

At the end of the intervention, prevalence of metabolic syndrome was reduced by 66.7% (Table 6). Considering specific component of metabolic syndrome, the prevalence of children with triglycerides ≥ 150 mg/dL, HDL cholesterol ≤40 mg/dL and high blood pressure was significantly reduced (Table 6). The prevalence of higher liver echogenicity declined by 56.5% (Figure 4). No component worsened for any child. Twenty children recovered one of the metabolic syndrome components and two children recovered 2 components.

All the children with metabolic syndrome had HOMA-IR > 3.16 and triglycerides/ HDL cholesterol ratio ≥2.2. The 44.4% of them had higher liver echogenicity.

Table 6. Distribution of children according to International Diabetes Federation criteria for metabolic syndrome† [19,20] at baseline and at the end of intervention. Values are number of children (percentage).

	Baseline (n= 61)	End of intervention (n= 61)	*P*-value ††
Metabolic syndrome	9 (14.8)	3 (4.9)	0.014*
Component			
Waist circumference ≥ 90th percentile	60 (98.4)	59 (96.7)	0.317
Triglycerides ≥150 mg/dL	13 (21.3)	5 (8.2)	0.021*
HDL cholesterol <40 mg/dL	18 (29.5)	11 (18.0)	0.008*
Blood pressure: Systolic ≥ 130/Diastolic ≥ 85 mmHg	12 (19.7)	5 (8.2)	0.020*
Glucose ≥100 mg/dL	0 (0.0)	0 (0.0)	1.000

HDL, high-density-lipoprotein.

† Evaluated in children aged 10-<16 years (27 boys, 34 girls). Diagnosis of metabolic syndrome requires waist circumference ≥ 90th percentile and two or more of the other components.

†† Wilcoxon test for paired data.
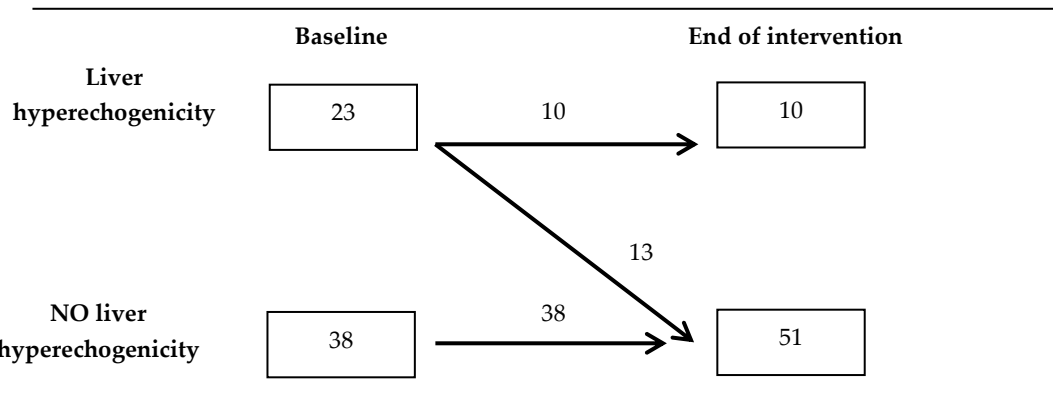
* Statistically significant.

Figure 4. Within-subjects longitudinal variation of liver hyperechogenicity over the intervention period in the subsample of children aged ≥ 10 years (figures are number of children).

† Significance of longitudinal variation was *P*=0.002 (Wilcoxon test).

3.2.4. Association between change in anthropometric parameters and change in metabolic profile variables

Change in BMI z-score was positively associated with change in glucose, insulin, HOMA-IR, HOMA-β% and TyG index and negatively associated with change in QUICK index. Other significant associations have been observed also between changes in waist circumference and triceps skinfold thickness and longitudinal changes of different glucose metabolism variables (Table 7).

Table 7. Correlation coefficient (p-value) † of change (end of treatment-baseline) in anthropometric parameters with change (end of treatment-baseline) of glucose metabolism.

N= 118	Δ BMI Z-score	Δ Waist circumference (cm)	Δ Triceps skinfold thickness (mm)
Δ Glucose (mg/dL)	0.296 (0.004)*	0.171 (0.114)	0.259 (0.045)*
Δ Insulin (μU/mL)	0.289 (0.004)*	0.193 (0.074)	0.340 (0.008)*
Δ HOMA-IR	0.291 (0.004)*	0.181 (0.093)	0.361 (0.005)*
Δ HOMA-β%	0.237 (0.021)*	0.206 (0.055)	0.248 (0.056)
Δ QUICK index	-0.325 (0.001)*	-0.215 (0.045)*	-0.294 (0.022)*
Δ TyG index	0.334 (0.001)*	0.310 (0.003)*	0.055 (0.674)

HOMA-IR, homeostasis model assessment of insulin resistance; QUICK, quantitative insulin sensitivity check; TyG, triglyceride glucose.
† Adjusted for age, sex, baseline BMI z-score, Tanner stage.
*Statistically significant.

Change in BMI z-score was positively associated with change in triglycerides, LDL/HDL cholesterol, triglycerides/HDL cholesterol and atherogenic index. Change in waist circumference was positively associated with change in total cholesterol, LDL cholesterol, triglycerides and related ratios. Similarly, change in triceps skinfold thickness was associated with change in total cholesterol, LDL/HDL cholesterol and total/HDL cholesterol (Table 8).

Table 8. Correlation coefficient (p-value) † of change (end of treatment-baseline) in anthropometric parameters with change (end of treatment-baseline) of lipid variables.

N=118	Δ BMI Z-score	Δ Waist circumference	Δ Triceps skinfold thickness
Δ Total cholesterol (mg/dL)	0.057 (0.582)	0.227 (0.035)*	0.303 (0.019)*
Δ LDL cholesterol (mg/dL)	0.146 (0.157)	0.230 (0.032)*	0.237 (0.069)
Δ HDL cholesterol (mg/dL)	-0.100 (0.337)	-0.057 (0.597)	-0.184 (0.160)
Δ Triglycerides (mg/dL)	0.281 (0.006)*	0.293 (0.006)*	0.017 (0.895)
Δ Apo A1 (g/L)	0.113 (0.277)	0.199 (0.062)	0.188 (0.121)
Δ Apo B (g/L)	0.056 (0.589)	0.066 (0.541)	0.123 (0.350)
Δ LDL/HDL cholesterol	0.204 (0.047)*	0.259 (0.016)*	0.317 (0.014)*
Δ Total chol/HDL cholesterol	0.168 (0.104)	0.283 (0.008)*	0.381 (0.003)*
Δ Triglycerides/HDL cholesterol	0.280 (0.006)*	0.313 (0.003)*	0.076 (0.564)
Δ Atherogenic index	0.298 (0.003)*	0.290 (0.007)*	0.080 (0.542)

LDL, low-density-lipoprotein; HDL, high-density-lipoprotein; Apo A1, apolipoprotein A1; Apo B, apolipoprotein B.
† Adjusted for age, sex, baseline BMI z-score, Tanner stage.
*Statistically significant.

3.3. DISCUSSION

This longitudinal study evaluated whether a 1-year lifestyle intervention, based on a normocaloric Mediterranean balanced diet, promotion of physical activity and behavior changes, may improve obesity and related comorbidities (glucose metabolism alterations, hyperlipidemia, prehypertension/hypertension, high liver echogenicity and metabolic syndrome) in obese children aged ≥6 years. Although beneficial effects on health of Mediterranean diet are widely recognized in adults [30], to our knowledge only a study has evaluated the effect of a Mediterranean-like diet on cardiovascular risk factors in obese children and adolescents [31].

At the end of intervention, children showed a decrease in mean BMI z-score of 15%, and the prevalence of obesity declined from 100% to 67%, with 33% of obese children who became overweight. This result confirms the effectiveness of nutritional/lifestyle interventions in the reduction of BMI z-score [28,31-38] and obesity prevalence, as previously observed [28]. However, differently from our previous results [28], a decrease in waist circumference was observed, confirming results from other studies [32,33,36,38]. Moreover, waist to height ratio and triceps skinfold thickness also decreased at the end of the intervention. Importantly, the prevalence of children with waist/height ratio ≥ 0.6 decreased significantly, thus suggesting also an important reduction of the cardiometabolic risk [4]. Indeed, waist measures may be effective indicator of central adiposity and the waist/height ratio cut-off of 0.6 has been suggested as a useful index of central adiposity and increased risk of abnormal cardiometabolic risk factor [4]. Considering body composition, total lean mass increased significantly, as also observed in obese children after 16 weeks of intervention based on a Mediterranean-like diet [31].

The prevalence of children showing prehypertension or hypertension significantly decreased by 27% at the end of the intervention, similarly to results showed in another study, evaluating the effect of a lifestyle intervention in 83 overweight/obese children [34]. As previously discussed, the prevalence of hypertension among children and adolescents is highly heterogeneous [39]; in this cohort of obese children 41% of them showed, at baseline, systolic or diastolic blood pressure equal or higher than 90th pc for age, gender and height [6]. Specifically, the prevalence of hypertension (systolic or diastolic blood pressure ≥ 95th percentile) was of 24%. Moreover, at the end of the intervention a decrease in mean systolic blood pressure was observed, as also showed by another study evaluating the effect of a 1-year lifestyle intervention on 484 obese children, although they found also decreased diastolic blood pressure [33].

Concerning lipid profile, this study showed a significant reduction of total cholesterol, triglycerides, LDL/HDL cholesterol, total cholesterol/HDL cholesterol, triglycerides/HDL cholesterol ratios and in the atherogenic index of plasma, with a concomitant increase in HDL cholesterol. These results are in agreement with that of other studies evaluating the effect of lifestyle intervention on obese overweight/children and especially showing improvement in triglycerides and HDL cholesterol levels [31,33,34,38]. However, systematic reviews and meta-analysis [40,41] examining the impact of lifestyle interventions, including dietary caloric restrictions and/or nutrition education, on cardio-metabolic risk factors in overweight/obese children, reported that fewer than half of considered studies showed significant improvements in HDL cholesterol or triglycerides levels and that the association of diet plus exercise may determine a greater improvement in HDL cholesterol than diet-only interventions.

An important result of this study is represented by the significant reduction of the atherogenic index of plasma. Indeed, it has been suggested that AIP may predict the risk of atherosclerosis and coronary heart disease in adults, reflecting the relationship between protective and atherogenic lipoprotein and being associated with the size of pre- and anti-atherogenic lipoprotein particle [42]. To our knowledge this study is the first intervention study that evaluated atherogenic index in a pediatric population, and found that it decreased at the end of the intervention.

Interestingly, the prevalence of children with triglycerides to HDL ratio equal or greater than 2.2 declined too, by 55%. The triglycerides/HDL cholesterol ≥2.2 has been confirmed as a marker of atherogenic lipid profile as well as a screening tool able to identify insulin resistance, high blood pressure, metabolic syndrome, liver steatosis, higher carotid intima-media thickness, and concentric left ventricular hypertrophy [14,16]. Finally, it has been suggested that this cut-off may be useful to predict the risk of impaired glucose tolerance in the pediatric population, especially in children [12]. Another important result of this study is represented by the reduction of the prevalence of children with borderline-high triglycerides that are a central element in the definition of atherogenic dislypidemia, characterized by hypertriglyceridemia, increased VLDL, small dense LDL, and reduced HDL cholesterol [43]. To our knowledge no intervention studies on obese children have evaluated the effectiveness of a lifestyle intervention on the prevalence of hyperlipidemia, according to the same criteria [18].

With regard to glucose metabolism, a significant decrease in triglyceride glucose index, an emergent useful indicator, affording an easily and widely available simple laboratory method as a surrogate to estimate the insulin sensitivity [9,10], was observed at the end of the intervention, confirming results from

previously published 1-year nutritional-behavioral intervention study [28]. Moreover, a significant reduction of HOMA-β% and a concomitant increase of QUICK index, indicating increased insulin sensitivity, were observed, while longitudinal variation of insulin levels and HOMA-IR did not reach the statistical significance. This result is not in line with that of other lifestyle-intervention studies, characterized by hypocaloric diet [38] or not [33,35], which reported decreased insulin and/or HOMA-IR after one year of intervention. However, importantly, the prevalence of insulin resistance, evaluated by the HOMA cut-off >3.16 [8], significantly declined by 30%, as previously observed [28], from a baseline prevalence of 47.5%. Although only one child at baseline had fasting glucose higher than 100 mg/dL, a metabolic disorder identified as IFG, 21% of obese children had glycated hemoglobin values in a range of 39-46 mmol/mol, which identifies pre-diabetes, and thus children at high risk for future diabetes [13]. This prevalence declined by 36% at the end of the intervention, thus showing a beneficial effect also on this relevant cardiometabolic risk factor.

Additionally, considering the increasing prevalence of NAFLD in industrialized countries, especially among obese children, the effect of this lifestyle intervention, also based on a Mediterranean diet for children, on liver hyperechogenicity was evaluated. At the end of the intervention, the prevalence of increased liver echogenicity declined by 50%, from a baseline value of 29%, with a concomitant reduction of AST and γGT, thus showing a result comparable to that reported by Verduci et al [44] after a 1-year nutritional intervention on 46 obese children. However, in this study the prevalence of liver hyperechogenicity was lower than that reported by other studies [44,45].

Finally, as obesity is often associated with a chronic low-grade inflammation status, the longitudinal variation of ESR and hs-CRP was evaluated. Indeed, the hepatic production of CRP is stimulated by interleukin-6, one of the several cytokines produced by the adipose tissue [46]. Although no difference was observed in ESR levels, hs-CRP was significantly decreased at the end of the intervention, as also resulted from a 6-month lifestyle intervention study, based also on hypocaloric diet [47].

Considered in the past as a cluster of metabolic abnormalities associated with an increased risk of cardiovascular diseases in adults, metabolic syndrome prevalence is increasing among children and adolescents as a consequence of childhood obesity. In this study the prevalence of metabolic syndrome, evaluated according to IDF criteria [19,20] in a subsample of 61 children aged 10 years of older, was of 15% and declined by about 67% to a prevalence of 5%, thus showing a decline comparable to that observed in other 1-year intervention studies [28,48]. From the study

evaluating the efficacy of a Mediterranean-style diet to decrease cardiovascular risk factors in 24 obese children and adolescents has resulted that at the end of 16 weeks of intervention the prevalence of metabolic syndrome declined from 67% to 21% among children [31]. For what concerns specific components of metabolic syndrome, in this study the number of children with triglycerides ≥ 150 mg/dL, low levels of HDL cholesterol and high blood pressure declined by 61%, 39% and 58%, respectively, while no significant improvement was observed in the prevalence of abdominal obesity, evaluated by waist circumference ≥90th pc. In the study conducted by Reinehr *et al.* [48] variation of IDF components was significant for blood pressure only, with a reduction of about 50%, while in the study by Velázquez-López et al. [31] a significant reduction in the percentage of children with triglycerides ≥ 150 mg/dL and HDL cholesterol ≤40 was observed, from 88% to 8% and from 83% to 29%, respectively.

As previously explained, insulin resistance is highly involved in the pathogenesis of the metabolic syndrome [49]. Therefore, it should be underlined that among the 9 children with metabolic syndrome all of them had insulin resistance and triglycerides/HDL ≥2.2. Furthermore, almost half of them had increased liver echogenicity thus confirming the relationship between metabolic syndrome, insulin resistance and liver steatosis.

In this study we also evaluated if a relationship may exist of longitudinal changes in anthropometric measurements with changes in metabolic profile parameters after a 1-year lifestyle intervention. Indeed, changes in BMI z-score, waist circumference and triceps skinfold thickness were associated with changes of some parameters of both lipid and glucose metabolism profile. This result underlines the importance of the reduction in BMI z-score and the importance of adipose tissue distribution in longitudinal improvement of cardiovascular risk factors and components of metabolic syndrome. In this regard, it has been observed that even a modest reduction in BMI z-score after 1 year of combined hospital/and public health nurse intervention may be associated with improvement in several cardiovascular risk factors, as lower insulin, total cholesterol, LDL and total/HDL cholesterol ratio [35]. Specifically, a BMI z-score reduction ≥0.25 seems to be already associated with improvement in body composition and cardiometabolic risk factors although losing at least 0.5 BMI z-score may determine greater benefits on health [50].

Strength and limitation

A first strength of this study is that the participation rate was high and after 1-year intervention a drop-out of 5.6% only was observed. Moreover, this study was characterized by the use of strict international definitions and cut-off, as that used for blood pressure [6], lipids [18], glucose metabolism alterations [13] and metabolic syndrome [19,20], and accurate anthropometric and biochemistry measurement. Finally, compliance with nutritional intervention was acceptable, with 90% of children who recovered at the end of the intervention towards recommended ranges of macronutrient distribution [24]. A limitation of this study is represented by the use of the FFQ and 24-h recall, instead of a three- or seven-day food diary. However, it should be considered that it was chosen to primary estimate compliance with intervention and not its effect size. Similarly, another limitation of this study is represented by the use of a physical activity recall, instead of portable activity monitors, such as pedometers, heart rate monitors, and accelerometers. Finally, the study did not fully meet the revised CALO-RE taxonomy [26], since only a subset of items was extracted by a consultant psychologist from the original 26-item taxonomy [51]. Anyway, it should be noted that most of the selected items have been recently recognized as providing effective behavior change techniques for childhood obesity [52].

Conclusion

As a conclusion, within the limitations of this study, one may conclude that in obese children, lifestyle intervention, based on a normocaloric Mediterranean balanced diet for pediatric age, promotion of physical activity and behavior changes, may determine a decrease in BMI z-score, waist circumference, triceps skinfold thickness and waist to height ratio, associated with a significant improvement of the metabolic profile. Moreover, this intervention may determine a decrease in the prevalence of obesity and related-comorbidities, reducing the prevalence of insulin resistance, pre-diabetes, prehypertension/hypertension, hypertriglyceridemia, higher liver echogenicity and metabolic syndrome. Although further well-design trials are desirable, results of this study suggest that in children a normocaloric diet, based on a Mediterranean diet pyramid for the pediatric age, associated with regular physical activity, may have an important role in the treatment of obesity and related-comorbidities, with beneficial effects on health.

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3.4. CHANGE IN METABOLIC PROFILE AFTER 1-YEAR NUTRITIONAL-BEHAVIORAL INTERVENTION IN OBESE CHILDREN

The effect of a 1-year intervention, based on normocaloric diet and physical activity, on body mass index, blood lipid profile, glucose metabolism and metabolic syndrome in a cohort of 85 obese children was discussed in a previously published paper.

Change in Metabolic Profile after 1-Year Nutritional-Behavioral Intervention in Obese Children

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Abstract: Research findings are inconsistent about improvement of specific cardio-metabolic variables after lifestyle intervention in obese children. The aim of this trial was to evaluate the effect of a 1-year intervention, based on normocaloric diet and physical activity, on body mass index (BMI), blood lipid profile, glucose metabolism and metabolic syndrome. Eighty-five obese children aged ⩾6 years were analyzed. The BMI z-score was calculated. Fasting blood samples were analyzed for lipids, insulin and glucose. The homeostatic model assessment of insulin resistance (HOMA-IR) was calculated and insulin resistance was defined as HOMA-IR >3.16. HOMA-β%, quantitative insulin sensitivity check index and triglyceride glucose index were calculated. The metabolic syndrome was defined in accordance with the International Diabetes Federation criteria. At the end of intervention children showed a reduction (mean (95% CI)) in BMI z-score (-0.58 (-0.66; -0.50)), triglycerides (-0.35 (-0.45; -0.25) mmol/L) and triglyceride glucose index (-0.29 (-0.37; -0.21)), and an increase in HDL cholesterol (0.06 (0.01; 0.11) mmol/L). Prevalence of insulin resistance declined from 51.8% to 36.5% and prevalence of metabolic syndrome from 17.1% to 4.9%. Nutritional-behavioral interventions can improve the blood lipid profile and insulin sensitivity in obese children, and possibly provide benefits in terms of metabolic syndrome.

Keywords: childhood obesity; nutritional-behavioral intervention; lipid profile; glucose metabolism; metabolic syndrome

1. Introduction

Childhood obesity has become a worldwide concern, affecting children of high-income countries as well as middle-income and low-income countries [1]. Although recent studies suggested that progresses have been made in the control of the obesity epidemic [2,3] the prevalence of childhood obesity remains high [1]. Obese children are exposed to detrimental short and long-term effects on health, thus showing components of metabolic syndrome [4], such as dyslipidemia [5], hypertension [6], insulin resistance and disturbed glucose metabolism [7]. In most cases, obesity is consequence of a chronic imbalance between energy intake and energy expenditure, involving environmental and lifestyle factors, e.g., easy access to energy-dense foods, increased portion sizes, reduced physical activity and increased time spent in sedentary activities [8]. Chronic exposure over time to these factors may potentiate weight gain over many years [8].

Guidelines for treatment of childhood obesity recommend intensive lifestyle interventions, involving diet, behavioral and physical activity for the child and the entire family, in an age-appropriate manner [9]. A Cochrane systematic review stated that in children, family-based lifestyle interventions aimed at changing dietary, behavioral and physical activity patterns can lead to a reduction in overweight, compared to standard care or self-help [10]. A meta-analysis of randomized trials conducted on overweight/obese children showed a small to moderate effect from combined lifestyle interventions on body mass index (BMI) [11]. The largest effects were observed when lifestyle modifications were implemented with parental involvement [11]. In a recent meta-analysis, Ho *et al.* [12] evaluated randomized controlled trials with a follow-up period of at least 2 months from baseline and highlighted that lifestyle interventions, incorporating diet and physical exercise and/or behavioral treatment, can lead to improvement in weight and cardio-metabolic outcomes, compared to no treatment/wait-list control, usual care, or written education materials. These authors also reported that although both diet-only and combined interventions (diet plus exercise) may result in weight loss and metabolic improvement in the overweight/obese pediatric population, combined interventions can determine larger improvement in levels of high-density lipoprotein (HDL) cholesterol, fasting glucose and insulin over 6 months [13]. Other authors suggested that improved weight status may be achieved by a reduced-energy diet, but the need for an adequate content of macronutrients has to be considered when aiming at specific cardio-metabolic risk factors [14]. Studies evaluated the effect of a lifestyle intervention, in overweight/obese children, characterized by nutritional counseling and education, within an intervention period ranging from 20 weeks to 12 months [15–19]. While a decrease in BMI *z*-score has been observed [15–19], research findings are inconsistent about improvement of specific cardio-metabolic variables.

The primary aim of this study was to evaluate whether a 1-year intervention based on normocaloric diet and physical activity may impact the BMI status, blood lipid profile and glucose metabolism indicators in obese children. Additionally, metabolic syndrome was assessed.

2. Experimental Section

A cohort of 90 obese children (44 boys and 46 girls) was consecutively recruited among those admitted with diagnosis of obesity by primary care pediatricians to the Department of Pediatrics, San Paolo Hospital, Milan, Italy, between 1 January 2012 and 31 December 2014, according to the following eligibility criteria: age ⩾6 years, weight at birth ⩾2500 g and <4000 g, gestational age 37–42 weeks, single birth, children having white parents and residing in Milan or neighborhood (<30 km). Children having syndromic, organic and hormonal conditions besides obesity were excluded.

A child was defined obese in accordance with the International Obesity Task Force, *i.e.*, if her/his BMI was above the age- and sex-adjusted BMI Cole's curve passing through the cut-off of 30 kg/m^2 at age 18 years [20]. The parents of eligible children or their legal guardian received detailed explanation about the aim of the study, and signed a consent form. The Hospital Ethics Committee approved the study protocol and gave ethical clearance.

2.1. Anthropometry and Blood Pressure

A medical history was collected at recruitment from parents by a standardized questionnaire during a personal interview conducted by the same pediatrician that saw children for a general examination and evaluated the Tanner stage of puberty [21]. The pediatrician also took anthropometric measurements and blood pressure of children both at recruitment and at the end of intervention, assisted by an experienced operator. Body weight and height were measured using a mechanical column scale (seca 711; seca GmbH & KG, Hamburg, Germany) with integrated measuring rod (seca 220; seca GmbH & KG). BMI was calculated from the ratio of weight to height squared (kg/m^2). BMI *z*-scores were calculated and adjusted for age and sex by using Cole's LMS method [22] and Italian reference data [23]. Waist circumference (WC) was measured using the

measuring tape seca 203 (seca GmbH & KG) to the nearest 0.1 cm at the mid-point between the iliac crest and the lower edge of the ribs at the end of a normal expiration. Triceps skinfold thickness was measured on the left side of the body, using the Harpenden Skinfold Caliper (Chasmors Ltd, London, UK) halfway between the acromion process and the olecranon process [24]. Blood pressure was measured according to recommendations of the National High Blood Pressure Education Program Working Group [25].

2.2. Biochemistry

Biochemical measurements were performed within 3 ± 1 day (baseline) of recruitment and one year (± 5 day) after starting intervention (end of intervention). Fasting blood samples were taken at $8\,h \pm 30$ min a.m. and immediately analyzed at the hospital laboratory of biochemistry for total cholesterol, HDL cholesterol, low-density-lipoprotein (LDL) cholesterol, triglycerides, apolipoprotein A1, apolipoprotein B, insulin and glucose on the cobas$^{®}$ 6000 analyzer series, c501 and e601 modules (Roche Diagnostics GmbH, Hoffmann-La Roche ltd, Mannheim, Germany), which has been recognized as providing robust chemistry and immunochemistry [26]. The homeostatic model assessment of insulin resistance (HOMA-IR) was calculated as the product of fasting glucose (mmol/L) and fasting insulin (µU/mL) divided by 22.5 [27], and insulin resistance was defined as HOMA-IR >3.16 [28]. The quantitative insulin sensitivity check (QUICK) index was calculated as $1/(\log_{10}$ fasting plasma insulin in µU/mL + $\log_{10}$ glucose in mg/dL) [29]. Pancreatic β-cell function was evaluated by HOMA-β% as (20 × fasting insulin in µU/mL)/(fasting glucose in mmol/L $-$ 3.5) [27]. The triglyceride glucose index (TyG index) was calculated as ln [fasting triglycerides (mg/dL) × fasting glucose (mg/dL)/2] [30,31].

2.3. Dietary Habits

Dietary habits of children were assessed at baseline and at the end of intervention by a food frequency questionnaire (FFQ) originally developed at our Department in 1990's on the original Block FFQ [32] and then revised and updated in 2008 on the basis of the full-length Block 2005 FFQ © (NutritionQuest, Berkeley, CA, USA) and the 2007 national food composition tables [33], to appropriately adjust for cultural food/beverage items of the Italian pediatric population. Parents completed the FFQ about their children's habits during an interview of approximately 50 min, conducted at hospital by the same experienced dietitian unaware of the obesity status of children. Each meal was analyzed to find out which food was eaten and how often. Usual portion sizes were estimated using household measures and the weight (e.g., pasta) or unit (e.g., fruit juice) of the purchase. A 24-h recall was additionally recorded at the end of the interview to standardize the usual serving size. Quantification and analysis of the energy intake and nutrient composition were performed with an *ad hoc* PC software program developed by a consultant.

2.4. Metabolic Syndrome

Metabolic syndrome was defined in accordance with the International Diabetes Federation (IDF) criteria for children and adolescents [34,35]. As IDF suggests that below the age of 10 years metabolic syndrome cannot be diagnosed [34,35], in this study it was evaluated only in children of 10 years or older.

2.5. Intervention

The intervention was based on promotion of a normocaloric diet, balanced for the macronutrient distribution, in accordance with the national guidelines for treatment of childhood obesity [36]. Specifically, it was recommended that children follow, for a 1-year period, a normocaloric diet (daily caloric intake by age and sex [37]) consisting of protein (12%–15%), carbohydrates (55%–60%), fat (25%–30%; <10% saturated fatty acids, polyunsaturated up to 10%, monounsaturated up to 15%) and fiber (range: age (year) plus 5 g–age (year) plus 10 g) [36,37]. Additionally, it was recommended

that children engage in at least 60 min of moderate- to vigorous-intensity physical activity (MVPA) daily [38], based on walking, and tailored to individual preferences. MVPA was estimated using 3-day physical activity recall (3DPAR). During a first round 1-h educational session, held at hospital on the day of recruitment, a pediatrician and an experienced dietitian provided illustration and instructed parents and children about the intervention to be performed and actions to maintain through a 1-year period. Education was based and focused on regulation of energy expenditure, body composition, physical activity, consequences of sedentary lifestyle, principles of nutrition, food sources, glycemic index and glucose metabolism, to continuously enhance and maintain parental and self-efficacy for dietary change. This education managing also took into account a range of behavior change techniques from the revised CALO-RE taxonomy (items 1, 2, 5, 6, 8, 16, 21, and 26) [39]. In particular, written guidelines were given to the parents, including general nutritional advice, food choice lists, selected week menu, and recommended average servings for principal food categories, according to age and sex. General nutritional advice included increasing fruit and vegetable intake, increasing legume and fish intake while decreasing meat consumption, using more whole grain food, avoiding sugary beverages and limiting sweets. Educational and incentive documentation (friendly, illustrated brochures) about potential benefits of a routinely normocaloric diet and physical activity for the child and family were also given to parents, together with a diary for recording the physical activity of their child, in terms of type, frequency, duration and intensity. A second explanatory session tailored for parents requests was held at the hospital on the day of blood sampling (*i.e.*, within 3 ± 1 day of recruitment) to resolve any doubts parents had about intervention, providing them with an instructive point-by-point reply. Lastly, the study design scheduled a dietitian to contact the parents by phone on a midweek day at 3-month intervals to fill out a 24-h recall and ask about the physical activity of the child as recorded in the diary. Parents were also invited to actively contact a pediatrician by phone (8–20 h) at any time of the intervention, when necessary.

2.6. Outcomes

The primary outcome measures were the change in BMI *z*-score and HDL cholesterol at the end of intervention. Secondary measures were the change in the other blood lipid variables and insulin resistance, and in the prevalence of metabolic syndrome.

2.7. Sample Size

The sample size was calculated iteratively to detect a mean longitudinal variation of 5% or more of HDL cholesterol, based on the baseline mean and standard deviation estimated in children already recruited. Assuming a type I error level of 0.05 with a power of 0.80, and allowing for a drop-out of 5% at least 87 children needed to be recruited.

2.8. Statistical Analysis

Descriptive data are reported as mean and standard deviation (SD) or 95% confidence interval (CI), or number of observations (percentage). Normality of the distribution of continuous variables was assessed by the Kolmogorov–Smirnov test. Means were adjusted for age, sex and baseline BMI *z*-score, as appropriate. Statistical significance of longitudinal variations was tested by the Student's *t* test for paired data or the Wilcoxon test, and also adjusted by ANOVA for repeated measures. At this analysis non-normally distributed continuous variables entered the model after logarithmic transformation. All values of $p < 0.05$ were considered to indicate statistical significance (two-tailed test). The statistical package for social sciences (SPSS) package version 20.0 (SPSS Inc., Chicago, IL, USA) for Windows (Microsoft, Redmond, WA, USA) was used, for the statistical analysis.

3. Results

Eighty-five children (94.4%), 42 boys and 43 girls, completed the intervention. At recruitment, mean (SD) age and duration of obesity were 9.7 (2.6) years (range 6–15) and 4.0 (2.1) years, respectively. At the end of intervention there was a reduction of daily energy intake and macronutrient redistribution towards the recommended range (Table 1). Mean (SD) MVPA was 45.4 (33.2) min/day at baseline and 54.7 (35.0) min/day at the end of intervention ($p = 0.089$). No change was observed for systolic (113.33 (11.2) *vs.* 112.8 (9.5) mmHg; $p = 0.524$) and diastolic (68.47 (9.25) *vs.* 67.83 (7.52) mmHg; $p = 0.321$) blood pressure.

Table 1. Daily dietary intake of energy, macronutrients and fiber, and overall glycemic index and glycemic load at baseline and at the end of intervention. Values are mean (SD) [†].

Variable	Baseline (*n* = 85)	End of Intervention (*n* = 85)	*p*-Value	Recommended Intake [36,37]
		Energy		
kcal/day	2460.46 (795.84)	1855.62 (614.31)	<0.001 *	1380–3330 kcal/day depending on age and sex (6–15 year)
kcal/kg/day	45.43 (18.86)	34.39 (16.94)	0.001 *	
		Protein		
g/day	96.32 (29.50)	71.12 (23.74)	<0.001 *	12%–15% Energy
% Energy	15.82 (3.02)	15.54 (3.16)	0.006 *	
		Carbohydrates		
g/day	332.69 (113.51)	268.04 (106.59)	<0.001 *	55%–60% Energy
% Energy	54.20 (7.19)	58.07 (9.62)	0.001 *	
		Fats		
g/day	84.54 (37.94)	57.27 (21.04)	<0.001 *	25%–30% Energy
% Energy	31.44 (5.48)	27.97 (4.16)	<0.001 *	
		Saturated		
g/day	31.64 (14.40)	19.48 (8.36)	<0.001 *	<10% Energy
% Energy	11.69 (3.07)	9.44 (3.15)	<0.001 *	
		Monounsaturated		
g/day	34.53 (15.49)	22.31 (9.28)	<0.001 *	≤15% Energy
% Energy	12.64 (2.87)	10.80 (3.52)	0.022 *	
		Polyunsaturated		
g/day	14.92 (7.20)	9.83 (3.93)	<0.001 *	≤10% Energy
% Energy	5.40 (1.57)	4.72 (1.74)	0.016 *	
Fiber g/day	11.30 (5.15)	17.11 (8.02)	<0.001 *	age (year) plus 5 g–age (year) plus 10 g
Overall Glycemic Index	43.58 (22.42)	41.57 (22.32)	0.146	
Glycemic Load	418.73 (565.56)	307.56 (377.64)	0.124	

[†] Mean and *p*-value adjusted for age and sex; * Statistically significant.

At the end of intervention children showed lower BMI *z*-score than at recruitment (2.96 (0.96) *vs.* 3.54 (1.04); $p < 0.0001$) and lower triceps skinfold thickness (24.05 (5.74) *vs.* 27.18 (5.42) mm; $p < 0.038$), while no difference was found for waist circumference (81.76 (9.88) *vs.* 83.69 (10.68) cm; $p = 0.150$). The within-subject longitudinal variation of obesity status was significant (Figure 1).
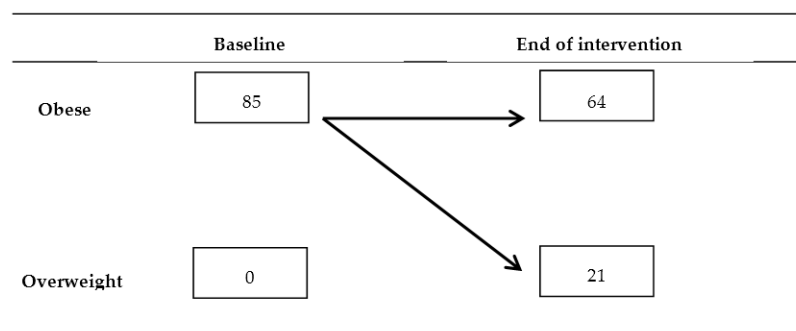
Figure 1. Within-subject longitudinal variation of obesity status through the intervention period (figures are number of children). Significance of longitudinal variation was $p < 0.0001$ (Wilcoxon test).

HDL cholesterol increased through the intervention period (mean variation, Δ, 0.06; 95% CI, (0.01; 0.11) mmol/L) while there was a reduction in triglycerides (-0.35; (-0.45; -0.25) mmol/L) and triglycerides/HDL cholesterol ratio ($\Delta = -0.36$; (-0.46; -0.26)) (Table 2). Reduction in triglyceride glucose index (-0.29; (-0.37; -0.21)) and prevalence of insulin resistance were observed (Table 3).

Table 2. Blood lipid profile at baseline and at the end of intervention. Values are mean (SD) [†].

Variable	Baseline (*n* = 85)	End of Intervention (*n* = 85)	*p*-Value
Total cholesterol (mmol/L)	4.40 (0.62)	4.21 (0.70)	0.072
LDL cholesterol (mmol/L)	2.58 (0.58)	2.50 (0.65)	0.116
HDL cholesterol (mmol/L)	1.26 (0.21)	1.32 (0.26)	0.034 *
Triglycerides (mmol/L)	1.29 (0.66)	0.94 (0.40)	0.024 *
Apo A1 (g/L)	1.33 (0.23)	1.34 (0.21)	0.772
Apo B (g/L)	0.76 (0.19)	0.70 (0.17)	0.164
ApoB/ApoA	0.58 (0.18)	0.54 (0.16)	0.546
Triglycerides/HDL cholesterol	1.13 (0.77)	0.77 (0.45)	0.018 *
LDL/HDL cholesterol	2.14 (0.80)	1.99 (0.76)	0.181
Total cholesterol/HDL cholesterol	3.62 (0.95)	3.34 (0.95)	0.078

LDL, low-density-lipoprotein; HDL, high-density-lipoprotein; Apo A1, apolipoprotein A1; Apo B, apolipoprotein B. SI conversion factors: to convert cholesterol, divide values by 0.0259; to convert triglycerides, divide values by 0.0113; to convert Apo A1 and Apo B divide values by 0.01. [†] Mean and *p*-value adjusted for age, sex, baseline BMI *z*-score. * Statistically significant.

Table 3. Glucose metabolism variables at baseline and at the end of intervention. Values are mean (SD) [†] or number of children (percentage).

Variable	Baseline (*n* = 85)	End of Intervention (*n* = 85)	*p*-Value
Glucose (mmol/L)	4.78 (0.33)	4.74 (0.29)	0.341
Insulin (pmol/L)	133.28 (102.22)	98.64 (59.51)	0.399
HOMA-IR	4.10 (3.30)	3.01 (1.92)	0.281
HOMA-β%	317.10 (221.23)	237.61 (123.26)	0.368
QUICK index	0.32 (0.03)	0.33 (0.03)	0.250
TyG index	8.38 (0.51)	8.09 (0.43)	0.030 *
Insulin resistance (yes)	44 (51.8)	31 (36.5)	0.008 *,[††]

HOMA-IR, homeostasis model assessment of insulin resistance; QUICK, quantitative insulin sensitivity check; TyG, triglyceride glucose. SI conversion factors: to convert glucose, divide values by 0.0555; to convert insulin, divide values by 6.945. Insulin resistance (yes): HOMA-IR >3.16. [†] Mean and *p*-value adjusted for age, sex, baseline BMI *z*-score. [††] Wilcoxon test. * Statistically significant.

At the end of intervention, prevalence of metabolic syndrome was reduced by 71.4% (Table 4). No component worsened for any child. The only child who had waist circumference decreased below the 90th percentile was not syndromic at baseline. Fourteen children recovered one of the other metabolic components and two children recovered 2 components.

At 3, 6, 9 and 12 months of intervention, compliance with diet and MVPA, evaluated by the 24-h recall (plus FFQ at 12 months) and diary of physical activity, was 84%, 86%, 88% and 92% (diet) and 83%, 86%, 85% and 87% (MVPA), respectively.

Table 4. Distribution of children according to International Diabetes Federation criteria for metabolic syndrome [†] [34,35] at baseline and at the end of intervention. Values are number of children (percentage).

	Baseline (*n* = 41)	End of Intervention (*n* = 41)	*p*-Value [††]
Metabolic syndrome	7 (17.1)	2 (4.9)	0.025 *
Component			
Waist circumference ⩾90th percentile	40 (97.6)	39 (95.1)	0.317
Triglycerides ⩾1.7 mmol/L	10 (24.4)	3 (7.3)	0.008 *
HDL cholesterol <1.03 mmol/L	12 (29.3)	7 (17.1)	0.025 *
Blood pressure: Systolic ⩾130/Diastolic ⩾85 mmHg	9 (22)	3 (7.3)	0.034 *
Glucose ⩾5.6 mmol/L	0 (0)	0 (0)	1.000

HDL, high-density-lipoprotein. SI conversion factors: to convert cholesterol, divide values by 0.0259; to convert triglycerides, divide values by 0.0113. [†] Evaluated in children aged ⩾10 years (20 boys, 21 girls). Diagnosis of metabolic syndrome requires waist circumference ⩾90th percentile and two or more of the other components. [††] Wilcoxon test. * Statistically significant.

4. Discussion

This longitudinal study evaluated whether a 1-year nutritional-behavioral intervention, based on normocaloric balanced diet and physical activity, may impact the BMI status and metabolic profile of obese children aged ⩾6 years. The participation rate was high, ranging from 100% at baseline to 94.4% at the end of intervention. Strict international definitions and accurate anthropometric and biochemistry measurement procedures were used. Compliance with treatment, as based on national recommended dietary energy and macronutrient intakes [36,37], was acceptable, with more than 90% of children who recovered at the end of intervention towards the recommended range. However, owing to the study design, which did not include a control group of obese children on a free diet, and based on the dietary assessment on the Food Frequency Questionnaire, caution should be exercised in drawing definitive conclusions. Indeed, it should be pointed out that while the absence of a control group on a free diet is a limitation, the recruitment of such a group was discouraged by the Hospital Ethics Committee due to the opinion that all obese children and their families should have the same opportunity to be instructed about dietary recommendations, while also taking into account the current international guidelines [9]. The use of the FFQ and 24-h recall, instead of a three- or seven-day food diary, was chosen as it provides for immediate collection of data and because the dietary assessment was primarily planned to estimate compliance with intervention and not its effect size. Another limitation is that the study did not fully meet the revised CALO-RE taxonomy [39]. Indeed, while the revised CALO-RE taxonomy was not available before starting this study, only a subset of items was extracted by a consultant psychologist from the original 26-item taxonomy [40] due to financial constraints. Regardless, it should be noted that most of the selected items have been recently recognized as providing effective behavior change techniques for childhood obesity [41].

At the end of intervention, children showed a decrease in mean BMI *z*-score of 16%, and 25% of them recovered from obesity to overweight. Other studies [15–19,42,43] conducted on overweight/obese children found, at the end of nutritional/lifestyle interventions, a decrease in mean BMI *z*-score, ranging from about 5% [18,19] to 20% [15]. In this study no overall significant change was detected for waist circumference while a decrease in triceps skinfold thickness was observed. It should be pointed out that skinfold thickness is *de facto* a measure of subcutaneous fat unable to quantify visceral adiposity, while waist circumference is a useful indicator for identifying children at increased risk of cardiovascular disease and metabolic syndrome [44]. The authors observed a waist circumference decrease in obese/overweight children after lifestyle interventions based on hypocaloric diet [43] or not [15,16,19] while no change of waist circumference was

detected in obese/overweight children aged 7–9 years after a 1-year lifestyle intervention based on a recommended dietary allowance of about 1800 kcal or nutrition education program [17]. A study conducted on 484 children who underwent a 1-year intervention based on physical activity, nutrition education, and behavior therapy found decreased blood pressure [16]. In this study no significant change was observed in mean blood pressure, as was also found in a trial that evaluated the effects of a 20-week exercise and diet guidance intervention on 19 overweight school-aged children [15].

HDL cholesterol and triglycerides have a key role in cardiovascular disease. HDL cholesterol protects against vascular disease by removing the "bad" cholesterol from the walls of arteries while high triglycerides increase the risk of atherosclerotic cardiovascular disease [45]. In our study, increased HDL cholesterol and decreased triglycerides levels were found. While these findings agree with other studies [16,17,43], it should be noted that a recent systematic review and meta-analysis examining the impact of lifestyle interventions, including dietary caloric restrictions and/or nutrition education, on cardio-metabolic risk factors in overweight/obese children reported that fewer than half of evaluated studies demonstrated significant improvements in HDL cholesterol or triglycerides levels [12]. The same authors [13] suggested that diet plus exercise interventions may produce greater improvement in HDL cholesterol than diet-only interventions. Concerning the other lipid variables, no improvement in total cholesterol, LDL cholesterol, Apolipoprotein A1 and Apolipoprotein B was observed while other authors reported a significant change of at least one of these lipid variables [16–18,43].

Lifestyle interventions based on a hypocaloric diet [43] or not [16,18] reported decreased insulin and/or HOMA-IR after one year of intervention. In this study, the change in insulin, HOMA-IR, HOMA-β% and QUICK index was not statistically significant. However it should be noted that the prevalence of insulin resistance decreased by 30%, from a baseline value of 51.8%, which is comparable with estimates reported in the literature, ranging from 32% [46] to 52% [47]. Triglyceride glucose index is an emergent useful indicator, affording an easily and widely available simple laboratory method as a surrogate to estimate the insulin sensitivity [30,31]. Only one study examined its usefulness in pediatric age, suggesting that it could be used in the metabolic evaluation of obese adolescents [31]. To our knowledge this study is the first intervention study that evaluated triglyceride glucose index in a pediatric population, and found that it decreased at the end of the intervention. This result also suggests that assessment of triglyceride glucose index might be included in future research investigating on glucose-metabolism alterations in obese children.

Metabolic syndrome was firstly defined in adult population as "a link between insulin resistance, hypertension, dyslipidemia, impaired glucose tolerance and other metabolic abnormalities associated with an increased risk of athero-sclerotic cardiovascular diseases" [48] and it has also been successively defined in the pediatric population. Recently, the International Diabetes Federation has suggested a unified definition that can be profitably used in children [35]. In this study, the prevalence of metabolic syndrome, defined according to IDF criteria [34,35], was 17% and 5% at, respectively, the baseline and the end of intervention. Reinehr *et al.* [49], when using the IDF definition, found in obese children a comparable decline of prevalence, going from 19% to 9% after 1-year lifestyle intervention. Other authors found a variation of prevalence from 17% to 10%, after 1-year lifestyle intervention characterized by a diet with a caloric intake 250–500 kcal less per day than the daily requirement, but variation was not statistically significant [43].

Concerning specific components of metabolic syndrome, the percentage of children with blood level of triglycerides ⩾1.7 mmol/L, lower levels of HDL cholesterol and high systolic or diastolic blood pressure significantly declined by 70%, 42% and 67%, respectively. In the study conducted by Reinehr *et al.* [49] variation of IDF components was significant for blood pressure only, with a reduction of about 50%. Direct comparison with findings reported in other studies is not here possible due to the different adopted definition of the metabolic syndrome (e.g., [17]).

On the whole, within the limitations of this study, one may conclude that in obese children, interventions based on normocaloric diet and physical activity could result in a decrease of BMI

z-score and also benefit blood lipid profile and insulin sensitivity. Additionally, it might play a positive role in terms of metabolic syndrome. Large longitudinal trials with adequate power, and hopefully meeting the revised CALO-RE taxonomy, are desirable to better evaluate the clinical relevance and long-term effectiveness of nutritional-behavioral interventions based on normocaloric diet and physical activity.

Author Contributions: E.V. conceived and designed the experiments; C.L., R.G. and G.B. performed the experiments; C.L. and G.R. analyzed the data; E.V., C.L., V.L.M. and G.R. wrote the paper; E.V. and G.R. supervised the trial and the final version of the manuscript.

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4. GUT MICROBIOTA BIODIVERSITY IN OBESE AND NORMAL-WEIGHT CHILDREN

The aims of this study were to evaluate qualitatively and quantitatively gut microbiota biodiversity in obese and normal-weight children and to compare gut microbiota profiles with SCFAs and BMI z-scores to gain insights into the structure and activity of the microbiota in pediatric obesity.

Methods and results have been largely discussed in the following published papers.

Relative Abundance in Bacterial and Fungal Gut Microbes in Obese Children: A Case Control Study

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Abstract

Background: Differences in relative proportions of gut microbial communities in adults have been correlated with intestinal diseases and obesity. In this study we evaluated the gut microbiota biodiversity, both bacterial and fungal, in obese and normal-weight school-aged children.

Methods: We studied 28 obese (mean age 10.03 ± 0.68) and 33 age- and sex-matched normal-weight children. BMI z-scores were calculated, and the obesity condition was defined according to the WHO criteria. Fecal samples were analyzed by 16S rRNA amplification followed by denaturing gradient gel electrophoresis (DGGE) analysis and sequencing. Real-time polymerase chain reaction (PCR) was performed to quantify the most representative microbial species and genera.

Results: DGGE profiles showed high bacterial biodiversity without significant correlations with BMI z-score groups. Compared to bacterial profiles, we observed lower richness in yeast species. Sequence of the most representative bands gave back *Eubacterium rectale*, *Saccharomyces cerevisiae*, *Candida albicans,* and *C. glabrata* as present in all samples. *Debaryomyces hansenii* was present only in two obese children. Obese children revealed a significantly lower abundance in *Akkermansia muciniphyla, Faecalibacterium prausnitzii*, *Bacteroides/Prevotella* group, *Candida* spp., and *Saccharomyces* spp. ($P = 0.031$, $P = 0.044$, $P = 0.003$, $P = 0.047$, and $P = 0.034$, respectively).

Conclusion: Taking into account the complexity of obesity, our data suggest that differences in relative abundance of some core microbial species, preexisting or diet driven, could actively be part of its etiology. This study improved our knowledge about the fungal population in the pediatric school-age population and highlighted the need to consider the influence of cross-kingdom relationships.

Introduction

Over the last decade, the microbial composition of the gut has been widely investigated, and recognized as having an impact in various physiological and pathological conditions.[1] The Human Microbiome Project[2] and Metagenomics of the Human Intestinal Tract (MetaHIT) (www.metahit.eu/) project clearly demonstrated that gut microorganisms are not just passive residents, carrying out a range of biological functions that are important in nutrition and well-being of the individual.

Molecular analyses revealed the presence of more than 1000 microbial species and highlighted the deep diversity within human gastrointestinal (GI) tracts.[3] However, these species belong to only 8 of the 55 known bacterial phyla,

with the *Firmicutes* (low-GCC gram-positives), *Bacteroidetes,* and *Actinobacteria* (high-GCC gram-positives) being the most widely represented.[4]

Moreover, the human microbiota is more complex than a bacterial community. It also involves Archeabacteria and fungi.[5] Very few studies encompass the fungal gut population, and even fewer in the pediatric population. Thus, the exact role of colonizing fungi has not been fully explored. Coexistence of cross-kingdom communities within the human gut could affect the final relationship with the host. The most representative genera are *Candida* and *Saccharomyces.*[6]

The composition and activity of the gut microbiota co-develop with the host from birth and is subjected to a complex interplay that depends on the host genome,

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nutrition, and lifestyle. The gut microbiota in relation to pediatric metabolic disorders has been poorly studied, although in recent years has emerged as a significant factor involved in obesity, even if no causal relationship has been established.[7]

Turnbaugh et al.[8] reported that adult obesity is associated with microbial compositional changes at the phylum level. They found that individuals with high BMIs had a lower proportion of *Bacteroidetes* and a higher proportion of *Actinobacteria* when compared to leaner individuals. However, a number of studies have failed to confirm this association or reported the opposite association.[9,10] Karlsson et al.[11] investigated the gut microbial biodiversity in preschool children with normal and excessive body weight; they found in the overweight group a significant reduction of *Akkermansia muciniphila* and *Desulfovibrio* spp., together with an increase of *Enterobacteriaceae*.

In our study we aimed at evaluating, both qualitatively and quantitatively, gut microbiota biodiversity in obese and normal-weight children aged 8–12 years. Prevention and treatment of childhood obesity involve mainly school-aged children, and additional research needs to be done on such a cohort in order to understand the critical window of a child's life in which environmental factors such as diet and microbiota can be shaped to promote health.

Methods

Subjects and Sample Collection

This observational case control study included 28 obese children (15 females and 13 males), mean age 10.03 (standard deviation [SD] 0.68), among patients consecutively admitted to the pediatric department of the San Paolo Hospital, Milan, Italy, between December 2013 and September 2014 in order to evaluate obesity-related metabolic profile, and 33 sex- and age-matched normal-weight children. Inclusion criteria were children living in north Italy born from Caucasian parents with birthweight ≥2500 grams, gestational age 37–42 weeks, singleton birth, no neonatal disease or congenital malformation. Exclusion criteria were having chronic or acute intestinal diseases and treatments with antibiotic and probiotic/prebiotic in the previous month, obesity-related comorbidity conditions (*e.g.,* insulin resistance, nonalcoholic fatty liver disease).

Data were collected for all subjects concerning mode of delivery and exclusively breastfeeding or formula feeding. Weight (kg), height (cm), and BMI (kg/m^2) were transformed to age- (in days) and sex-specific z scores according to WHO growth standards.[12,13] Obesity was defined by using WHO criteria.[14] Fasting blood samples were analyzed for insulin and glucose. Insulin resistance was estimated by homeostatic model assessment (HOMA) and defined as HOMA >3.16 according to the most recent cut-off for the pediatric population.[15] Abdominal ultrasonography (US) was performed according to a randomized sequence to evaluate liver echogenicity.

A fresh fecal sample was self-collected at home by each enrolled subject and stored immediately at −20°C. The collection took place in the same week as the dietary record. Subsequently, the fecal samples were transported to the laboratory and stored at −20°C until further analysis. The dietary habits of the children were assessed at recruitment by means of an age-adjusted food frequency questionnaire made up of 116 items.[16]

DNA Extraction and 16S Ribosomal (rRNA Bacteria) and ITS Ribosomal (Fungi) DNA Amplification

Total microbial DNA extraction was performed with the Spin Stool DNA Plus Kit (Stratec Molecular, Berlin, Germany) according to manufacturer instruction, using 200 mg aliquot of wet feces. The V2-V3 region of the gene that encodes for 16S rRNA was amplified using the following primers: HDA1-GC (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCACGG GGG G-ACT CCT ACG GGA GGC AGCAGT-′3) and HDA2 (5′-GTA TTA CCG CGG CTG CTG GCA C-′3). Primers were used in a reaction mix (Thermo Scientific Dream Taq Master Mix) at a final concentration of 0.5 μM. The amplification cycles were as follow: initial denaturation at 94°C for 5 minutes, followed by 35 cycles at 94°C for 1 minute, 56°C for 1 minute, 68°C for 1 minute, and a final extension at 68°C for 7 minutes.[17] Polymerase chain reaction (PCR) products (220 bp) were visualized on a 1.5% agarose gel and subsequently subjected to denaturing gradient gel electrophoresis (DGGE) analysis. For fungi, the 5.8S ITS rDNA region was amplified by means of a nested-PCR approach using the primers ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′), ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′), NL1 with a GC clamp (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA TAT CAA TAA GCG GAG GAA AAG-3′), and LS2 (5′-ATT CCC AAA CAA CTC GAC TC-3′). Amplification cycles were as follows: initial denaturation at 94°C for 3 minutes and 35 cycles of 94°C for 30 seconds, 52°C for 30 seconds, and 74°C for 2 minutes followed by 74°C for 10 minutes.[18] PCR products were separated by gel electrophoresis on a 1.0% (wt/vol) agarose gel, detected by ethidium bromide staining, and subsequently subjected to DGGE analysis.

Denaturing Gradient Gel Electrophoresis Analysis

DGGE was performed using a PhorU system (Ingeny International, Goes, The Netherlands) in 1X tris-acetate-EDTA (TAE) buffer at 60°C. PCR products were loaded onto 8% polyacrylamide gels in 1X TAE. The electrophoretic conditions were the following: 18 hours at 90 V in a 40%–60% denaturing agent gradient. The gels were stained in 1X TAE buffer with SYBR Green I nucleic acid stain (Roche Products Ltd., Welwyn Garden City, UK) for 30 minutes and visualized by UV radiation. Banding patterns of DGGE profiles were analyzed with Fingerprinting II software (Bio-Rad Laboratories, Hercules, CA), using the Pearson product moment correlation coefficient and the unweighted-pair group method with averages (UPGMA) for

the generation of dendrograms. Pearson coefficient is a measure of the degree of similarity. Two identical profiles create a similarity value of 100%, whereas completely different profiles result in a similarity value of 0%.[8]

Excision and Sequence Analysis of Products

Individual bands were cut out from the gel, placed in 50 μl sterile distilled water, and incubated overnight at 4°C. Two microliters of the eluate were amplified with the original primer pairs and amplification products checked by agarose gel electrophoresis. PCR products were purified using the NucleoSpin Extract II kit (Macherey-Nagel GmbH, Düren, Germany) and subjected to Sanger sequencing (Eurofins Biolab S.r.l., Milan, Italy). The sequences were compared with those available in the National Center for Biotechnology Information (NCBI) GenBank (www.ncbi.nlm.nih.gov/) and those in the Ribosomal Database Project (RDP) using the Sequence Map tool (rdp.cme.msu.edu/).

Quantification by Real-Time Polymerase Chain Reaction

Absolute quantification by real-time PCR was performed using the following control strains: *Escherichia coli* American Type Culture Collection (ATCC) 25922[T], *Akkermansia muciniphila* DSM 22959[T], *Candida albicans* ATCC 90028[T], Deutsche Sammlung von Mikroorganismen und Zellkulturen; *Bacteroides uniformis*, *Lactobacillus reuteri*, *Bifidobacterium animalis*, *Faecalibacterium prausnitzii*, and *Saccharomyces cerevisiae* (from the Clinical Microbiology Laboratory collection, Health Sciences Department, Università degli Studi di Milano). Firstly, the microbial DNA was extracted for each control strain using Prepman Ultra (Applied Biosystems, Foster City, CA). Real-time PCR was carried out using the StepOne instrument (Applied Biosystems) and SYBR® Green chemistry (Thermo Scientific, Waltham, MA). The analysis was performed in a total volume of 15 μl and each sample analyzed in triplicate. Standard curve was carried out for each qPCR run using five serial dilutions of control DNA. The specific 16S rRNA primers and qPCR conditions used for *Bacteroides*/*Prevotella* group, *Bifidobacterium* spp., *Enterobacteriaceae* group, and *F. prausnitzii* are reported by Bartosch et al.;[16] for *Lactobacillus* spp. and *A. muciniphila* we used the conditions designed by Delroisse et al.[17] and Collado et al.[20] The specific yeast quantification was carried out for *Candida* spp. and *Saccharomyces* spp. as described by Hierro et al.[21] and Zott et al.,[22] respectively.

Data Analysis

Statistical analysis has been performed using Graph Pad Prism (Graph Pad Software, La Jolla, CA) statistical software. Variables have been expressed as mean and standard deviation. Mean values were compared among subjects using the Whitney U test, and ANOVA was used to compare variables between all groups. A probability value (P value) less than 0.05 was considered statistically significant.

Results

Cohort Composition

Twenty-eight children were included in the obese (O) group and 33 in the control normal-weight (N) group. BMI z-scores were 2.9 (SD 0.66) and 0.29 (SD 0.79), respectively ($P < 0.001$). Eighty percent of normal-weight and 54% of obese children were born by vaginal delivery; furthermore, 50% of N and 53% of O were exclusively breastfed. Compared to normal-weight children, obese children showed higher dietary intakes of energy—1906 (SD 474) Kcal vs. 2593 (SD 989) Kcal, $P = 0.0007$; proteins, 64 (SD 13) grams vs. 97 (SD 31) grams, $P < 0.0001$; carbohydrates 271 (SD 55) grams vs. 368 (SD 137) grams, $P = 0.0004$; total fats 58 (SD 15) grams vs. 92 (SD 37) grams, $P < 0.0001$; saturated fats 19 (SD 6) grams vs. 31.5 (SD 11) grams, $P < 0.0001$; monounsaturated fats 20 (SD 6) grams vs. 31 (SD 11) grams, $P < 0.0001$; and polyunsaturated fats 7 (SD 3.5) grams vs. 13 (SD 7) grams, $P < 0.0001$.

Gut Microbial Ecosystem

The DGGE analysis of the gut bacterial population indicated a high degree of individual variation in the intestinal microbial community profiles obtained by using universal 16S rRNA primers (Figure 1a), while the DGGE patterns obtained for the yeast population contained a relatively low number of bands and had low interindividual variability (Figure 1b).

Figure 1a shows an example of the unweighted pair group method with arithmetic mean (UPGMA) dendrogram obtained for the children's microbiota. Two main clusters (50% similarity) are reported; one is represented by a sole severely obese child and the other group included four subclusters without significant differences among BMI z-score groups. The most representative bands—13 individual bands, identified from A to P (Figure 1a)—were excised from DGGE gel and subjected to sequencing. The respective bacterial species are indicated in Table 1. A similarity rate ≥90% was considered significant. In particular, sequencing of excised bands revealed that band F/G (*Eubacterium rectale*) was present in all children, while band H (*F. prausnitzii*) were absent in two severely obese children. The UPGMA dendrogram obtained for the fungal gut population revealed two main clusters (37% similarity), one represented by the sole severely obese child and the second composed by two subclusters without significant differences among BMI z-score groups (Figure 1b). The sequencing of fungal gut population DGGE excised bands, reported in Table 1, revealed that the bands relative to *Saccharomyces cerevisiae*, *Candida albicans*, and *Candida glabrata* were present in all samples, whereas bands 7 and 11 (*Debaryomyces hansenii*) were present only in two obese children.

Microbial Genome Quantification by Real-Time Polymerase Chain Reaction

Quantification of the bacterial and yeast groups using real-time PCR was performed on N and O distinct populations
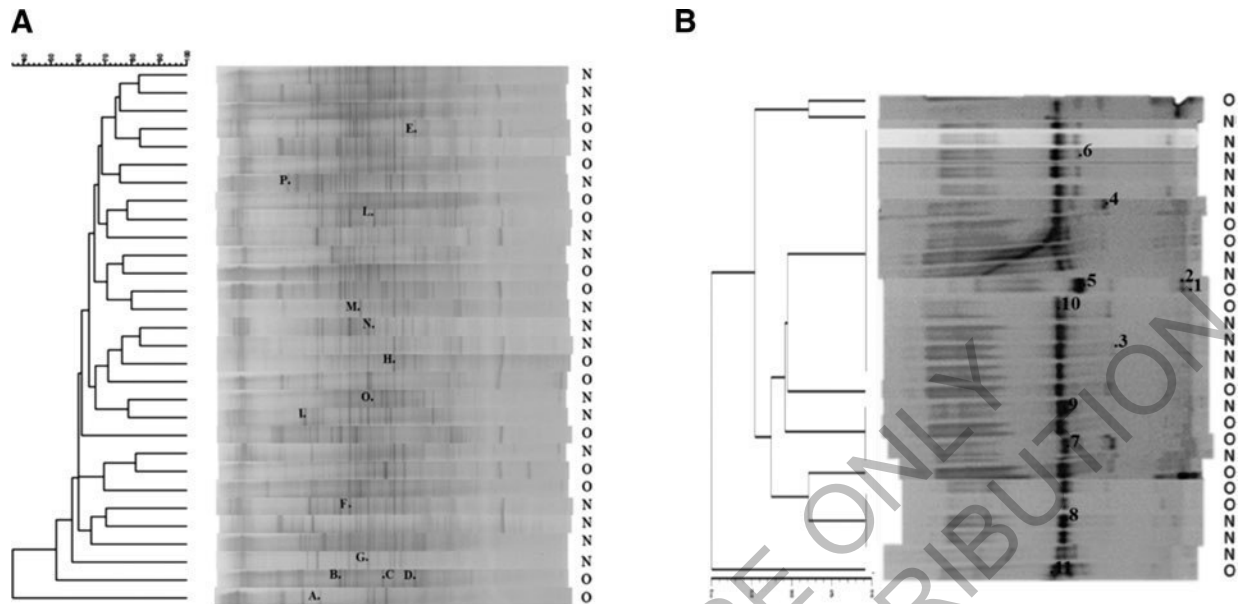
A

B

Figure 1.   Representative cluster analysis of DGGE profiles in obese and normal-weight children (panels A and B). Panel A: bacterial population; panel B: fungal population. O = obese; N = normal weight.

(Figure 2). We did not observe differences in the *Bifido-bacterium* spp. ($P = 0.606$), *Lactobacillus* spp. ($P = 0.420$), and *Enterobacteriaceae* ($P = 0.168$), whereas *A. mucini-phyla*, *F. prausnitzii*, and *Bacteroides*/*Prevotella* group were significantly less abundant in obese children ($P = 0.031$, $P = 0.044$, and $P = 0.003$, respectively). The average copy number of the yeasts was higher in the normal-weight pop-ulation compared to the obese population, with a significant P value for *Candida* spp. ($P = 0.047$) and for *Saccharomyces* spp. ($P = 0.034$). No significant differences in microbiota

Table 1. Sequenced DGGE bands and relative species identification

|	Band letters	Nearest species	Similarity	Accession number
Bacterial species	A	*Parabacteroides distasonis*	92%	NR 074376
	B	*Alistipes putredinis.*	98%	NR 113152
	C	*Clostridium parabutiricum*	98%	NR 11903
	D	*Paraprevotella clara*	96%	NR 113073
	E	*Flavonifractor plautii*	94%	NR 452852
	F/G	*Eubacterium rectale*	100%	NR 074634
	H	*Faecalibacterium prausnitzii*	97%	NR 043680
	L	*Clostridium aldenense*	100%	NR 043680
	M	*Clostridium* spp.	97%	NR 029355
	N/O	*Gemmiger formicilis*	93%	NR 104846
	P	*Flavobacterium* spp.	96%	NR 108535
Fungal species	1/2/4/5/6/13	*Saccharomyces cerevisiae*	100%	KM103045
	10	*Candida albicans*	99%	AM998790
	3	*Candida parapsilosis*	99%	EF568035
	7/11	*Debaryomyces hansenii*	98%	KF214434.1
	8/9/11	*Candida glabrata*	99%	AF336837
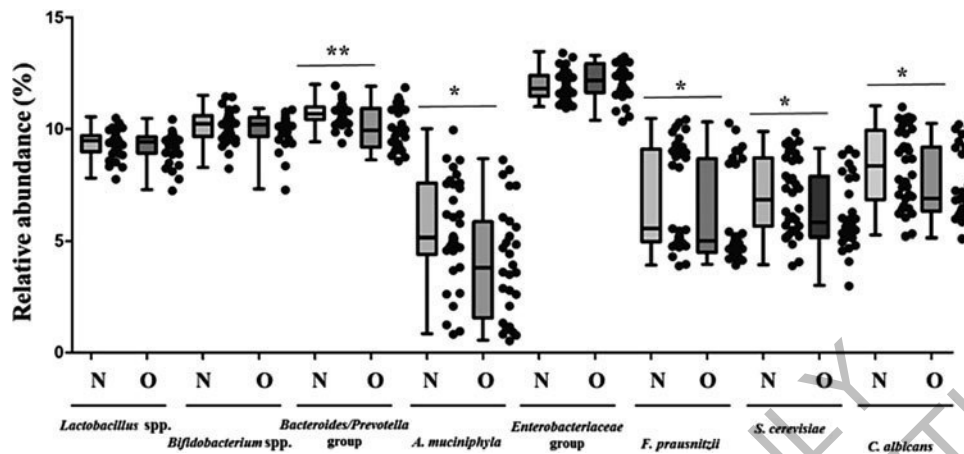
Figure 2. Box and whisker plots to compare relative abundance of microbial species in obese and normal-weight children. The box plot representation shows the median (designated by a line) and the 25th and 75th percentiles. Significant values (*P*-values*)* between the two groups are displayed on the plots. *P < 0.05; **P < 0.01. O = obese; N = normal weight.

composition were observed comparing different types of delivery: *Bifidobacterium* spp. (*P* = 0.868), *Lactobacillus* spp. (*P* = 0.908), *Enterobacteriaceae* (*P* = 0.959), *A. muciniphyla* (*P* = 0.800)*, F. prausnitzii,* (*P* = 0.389), *Bacteroides/Prevotella* group (*P* = 0.070), *Candida* spp. (*P* = 1), and *Saccharomyces* spp. (*P* = 0.959).

The lack of significant differences was also observed comparing breast and formula feeding: *Bifidobacterium* spp. (*P* = 0.228), *Lactobacillus* spp. (*P* = 0.354) and *Enterobacteriaceae* (*P* = 0.102), *A. muciniphyla* (*P* = 0.384), *F. prausnitzii* (*P* = 0.612), *Bacteroides/Prevotella* group (*P* = 0.236), *Candida* spp. (*P* = 0.658), and *Saccharomyces* spp. (*P* = 0.684).

Discussion

Key activities of the gut microbiota are the efficient extraction of calories from ingested food and the regulation of fat storage by modulation of lipoprotein lipase activity and subsequent triglyceride storage. The diet could induce strong modifications of the gut microbiota composition, and indeed, obese people have been reported to have lower bacterial diversity in the gastrointestinal tract compared to lean subjects.[23] Modifications in the proportions of microorganisms in the gut and, consequently, in the concentrations of metabolites produced and released by them in the lumen, have been suggested to play a role in the development of several pathological conditions[24] such as metabolic disorders.

In this study we evaluated, both qualitatively and quantitatively, the gut microbiota biodiversity in obese and normal-weight children. The combined use of two methods—DGGE (qualitative) and real-time (quantitative)—allows for a good evaluation of microbial ecology profile in the two studied populations. The DGGE profiles of total bacterial population demonstrated high interindividual heterogeneity in the microbial species. We failed to find common pattern strongly associated to high BMI z-score,

even though a lower microbial biodiversity was highlighted in subjects with severe obesity.

The presence of interindividual differences is well reported in the literature, although the presence has been demonstrated of a core of microbiota, relatively stable and resilient,[25] that depends on the age, health, diet, and even geographical location of the individuals. To rule out differences related to the above conditions, our cohort was selected to minimize differences related to age and geographical location. Both groups of children had a ''Western-style'' diet, high in fat and refined sugars. The quantification of the common genera that are part of the core microbiota showed no significant differences in the number of genomes of *Lactobacillus* spp., *Bifidobacterium* spp., and *Enterobacteriaceae*.

It is well known from the literature that the significant less abundance of *A. muciniphyla* is related with an excessive weight in adult and preschool children.[11,26] In this study we corroborate this finding in school-aged children. *A. muciniphyla* is a mucin-degrading bacterium (phylum *Verrucomicrobia*) and the dominant colonizer of the intestinal mucus layer.[27] In the mouse, a high-fat diet induced increased body mass, endotoxemia, adiposity, fasting hyperglycemia, and insulin resistance, while all of the above were reversed with the administration of *A. muciniphyla*.[26] In addition, *A. muciniphyla* increases the expression of acylglycerols, important compounds in gut barrier integrity, and reverses the thinning of the mucus layer caused by a high-fat diet.

In addition, we found in obese children a reduction of *F. prausnitzii*. Different authors suggested that a lower presence of *F. prausnitzii* could result from a long-standing inflammation.[28] *F. prausnitzii* appears to have significant anti-inflammatory activity with decreased production of proinflammatory cytokines (IL-8, IL-12, and IFN-γ) and increased production of anti-inflammatory IL-10 in cell cultures.[29] Studies in germ-free mice suggest that *F. prausnitzii* in conjunction with another common commensal, *Bacteroides thetaiotaomicron*, plays a role in goblet

cells differentiation and in the production of the mucus layer.[30] An intact mucus layer is an important component of the intestinal barrier that limits exposure of the epithelial monolayer to proinflammatory bacteria in the gut lumen. Concerning the *Bacteroides*/*Prevotella* group, we found a lower abundance of this microbial group in obese subjects. This data is in agreement with other authors reporting in the obese adult population a variation of the *Firmicutes/Bacteroides* ratio, with an increase in *Firmicutes*.[7–8,31]

In order to obtain a complete picture of the studied cohort, the fungal microbiota has been investigated. DGGE profiles of fungal population showed a lower biodiversity, expressed by a lower bands number, compared with bacteria profiles, without significant differences between normal-weight and obese children. However, we have to consider that the overall fungal population is underrepresented compared to the bacterial one, and the DGGE technique may in turn underestimate some species that are quantitatively poorly present. At this time, our knowledge on commensal fungi inhabiting the gut of children is incomplete. Nonetheless, the few literature data on adult population are in agreement with our results on Ascomycota as the most detected phylum. Indeed, *C. albicans*, *C. glabrata,* and *S. cerevisiae* were the most representative species in both groups. *Debaryomyces hansenii,* sequenced from two excised bands, was found only in two obese children; this species in closely related to food, in particular to cheeses and dry-cured meat products. Future studies are needed to better understand if the presence of *D. hansenii* is due to the ingestion of yeast-containing foods or if this species might contribute to obesity pathology.

However, we found significant differences in the two groups in terms of abundance. Normal-weight group was characterized by a higher number of genomes of *Candida* spp. and of *Saccharomyces* spp. Hoffmann and coworkers[32] analyzed the influence of diet on fungal and bacterial levels in the gastrointestinal tract. *Candida* spp. was positively correlated with carbohydrate consumption and negatively correlated with total saturated fatty acids, but no correlation was observed for *Saccharomyces* spp. with both diets. Moreover, *Candida* species are well-known human commensals, whereas for the *Saccharomyces* species we cannot rule out if they are transiently present because of diet (bread, pizza) or true commensals.

The influence of trans-kingdom relationships and diet on the coexistence of the microbial communities within the human gut has not yet been defined. One possible scenario could be the role of *Candida* spp. in breaking down starch in carbohydrate foods, leading to the release of simple sugars, which are in turn fermented by bacteria (for example, *Prevotella* and *Ruminococcu*s).

One limitation of the present study is that Archaebacteria are not included in the analysis of the gut microbial ecology, although their contribution to obesity is still debated. Animal studies suggested a potential role for methanogens Archaea, mainly *Methanobrevibacter smithii*, in promoting obesity.[33,34] Literature data on humans, however, are inconsistent,

with some authors reporting a negative association with BMI[35,36] and others reporting a positive association.[37,38] Further studies, especially on obese children, would be useful in elucidating their role.

In conclusion, taking into account the complexity of obesity, our data suggest that changes of some core microbial species, preexisting or diet induced, could actively be part of the syndrome's etiology. Obese children are highly prone to become obese adults, and in order to fight obesity-related complications, prevention and prompt treatment are crucial. The most important strategies to manage childhood obesity are therapeutic lifestyle changes, but the failure rate of such interventions is still high. The microbiota analysis of obese children could provide new elements of the puzzle to pave the way for designing customized diets and improve the current strategies.

Ethical Considerations

The medical ethical committee of our institution approved this study (protocol number 2015/ST/135). Written informed consent was signed by a parent of all the enrolled subjects.

Author Disclosure Statement

The authors declare that no competing financial interests exist.

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Pediatric obesity is associated with an altered gut microbiota and discordant shifts in *Firmicutes* populations

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Summary

An altered gut microbiota has been linked to obesity in adulthood, although little is known about childhood obesity. The aim of this study was to characterize the composition of the gut microbiota in obese ($n = 42$) and normal-weight ($n = 36$) children aged 6 to 16. Using 16S rRNA gene-targeted sequencing, we evaluated taxa with differential abundance according to age- and sex-normalized body mass index (BMI *z*-score). Obesity was associated with an altered gut microbiota characterized by elevated levels of *Firmicutes* and depleted levels of *Bacteroidetes*. Correlation network analysis revealed that the gut microbiota of obese children also had increased correlation density and clustering of operational taxonomic units (OTUs). Members of the *Bacteroidetes* were generally better predictors of BMI *z*-score and obesity than *Firmicutes*, which was likely due to discordant responses of *Firmicutes* OTUs. In accordance with these observations, the main metabolites produced by gut bacteria, short chain fatty acids (SCFAs), were higher in obese children, suggesting elevated substrate utilisation. Multiple taxa were correlated with SCFA levels, reinforcing the tight link between the microbiota, SCFAs and obesity. Our results

suggest that gut microbiota dysbiosis and elevated fermentation activity may be involved in the etiology of childhood obesity.

Introduction

The gut microbiota is involved in the regulation of multiple host pathways and participates in metabolic and immune-inflammatory axes connecting the gut with the liver, muscle and brain. The gut microbiota co-develops with its host from birth and is subjected to a complex interplay that is influenced by host genome, nutrition and lifestyle (Nicholson *et al.*, 2012). Diet can have a particularly marked impact on the gut environment, affecting factors such as gut transit time and pH. In particular, alterations in the intake of carbohydrates, proteins and fats can significantly affect the composition of the microbiota (Scott *et al.*, 2013). One of the main activities of the gut microbiota is to break down substrates such as resistant starch and dietary fiber, which are incompletely hydrolysed by host enzymes in the small intestine. The main fermentation products resulting from fiber breakdown are the short chain fatty acids (SCFAs) acetate, propionate and butyrate, which play different roles in energy salvage (Schwiertz *et al.*, 2009). Microbially-derived SCFAs provide an additional source of energy for the body: propionate is taken up by the liver and used as a precursor for liponeogenesis, gluconeogenesis and protein synthesis; acetate is used as a substrate for cholesterol synthesis; and butyrate is the main energy supply for colonic epithelial cells (Kallus *et al.*, 2012).

The adult human gastrointestinal tract microbiota has been extensively studied in relation to its role in gut homeostasis and various diseases (Schwiertz *et al.*, 2009). Notably, alterations in the gut microbiome and metabolome have been associated with the development of obesity (Choquet *et al.*, 2010; Vinolo *et al.*, 2011). Obesity is a multifactorial disease that predisposes to several comorbidities (Ang *et al.*, 2013) and is considered to be a global epidemic by the World Health Organisation (Schwiertz *et al.*, 2009). In recent years, the prevalence of childhood obesity has increased substantially worldwide,

and currently 23% of children and adolescents in developed countries can be classified as overweight or obese (Ng *et al.*, 2014).

Information regarding the structure and function of the gut microbiota during childhood is limited. Although it has been suggested that the microbiota reaches a relatively stable adult-like state in the first three years of life, other evidence indicates that it continues to develop through adolescence (Hollister *et al.*, 2015). As such, childhood may provide unique opportunities for microbiota interventions to promote health or prevent disease. It is, therefore, vital to establish a baseline understanding of pediatric gut microbiota structure and function, as during this period the gastrointestinal tract undergoes a transition from an immature to a mature state (Hollister *et al.*, 2015).

The goal of the present study was to characterize the composition of the gut microbiota in obese and normal-weight children using 16S rRNA gene-targeted sequencing. We recruited a large cohort of children from the same geographic area to reduce variation unrelated to obesity. We compared gut microbiota profiles with SCFAs and BMI *z*-scores to gain insights into the structure and activity of the microbiota in pediatric obesity.

Results

Pediatric cohort characteristics

A total of 78 children were enrolled at the Pediatric Department of San Paolo Hospital, Milan, Italy. Fecal samples were collected from 36 normal-weight (N) and 42 obese (O) children (N, BMI *z*-score: -2.12 to 1.56; O, BMI *z*-score: 2.14–5; $p < 0.0001$). Cohort characteristics, including age, sex, BMI *z*-score, mode of delivery in childbirth and history of breastfeeding or formula feeding as an infant were considered (Supporting Information Table S1). There was no significant relationship between history of breastfeeding or formula feeding as an infant with obese and normal-weight classification (Chi-square test; $p = 0.610$). Children born by Caesarean section tended to be obese, although this trend did not reach statistical significance at the $p = 0.05$ level (Chi-square test; $p = 0.068$). Dietary habits were also collected and obese children showed higher dietary intakes of energy and macronutrients (proteins, carbohydrates, sugars and fats) compared to normal-weight subjects (Supporting Information Table S3).

SCFAs are increased in the stool of obese children

We observed significantly higher concentrations of acetate, propionate and butyrate, as well as total SCFAs, in the stool of obese compared to normal-weight subjects ($p < 0.05$ for all comparisons; Supporting Information Table S2). Moreover, we found that the concentration of total SCFAs was significantly associated with obesity ($p = 0.0317$) and was positively correlated with BMI *z*-score ($p = 0.001$).

The intestinal microbiota is altered in obese children

At the phylum level, the predominant bacterial taxa in feces of both obese and normal-weight subjects were *Bacteroidetes* and *Firmicutes*, followed by *Actinobacteria*, *Verrucomicrobia* and *Proteobacteria* (Fig. 1A, Supporting Information Table S4). The most abundant families were *Ruminococcaceae*, *Lachnospiraceae*, *Bacteroidaceae*, *Veillonellaceae*, *Bifidobacteriaceae*, *Prevotellaceae*, *Verrucomicrobiaceae*, *Rikenellaceae* and *Christensenellaceae* (Fig. 1B, Supporting Information Table S4). The most abundant genera were *Bacteroides*, *Subdoligranulum*, *Faecalibacterium*, *Dialister*, *Bifidobacterium*, *Pseudobutyrivibrio* and *Blautia* (Supporting Information Fig. S1, Supporting Information Table S4).

The overall composition of the intestinal microbiota, considered at OTU level as well as taxonomic levels ranging from genus to phylum, was significantly affected by obesity, as determined by non-parametric multivariate analysis of variance testing (perMANOVA; $p < 0.05$ for all levels). Ordination showed that samples from normal-weight and obese children were distinctly grouped (Fig. 1C). This grouping was confirmed for every taxonomic level by the analysis of similarity (ANOSIM) test, which evaluates significance of sample grouping ($p < 0.01$ for all levels). The intestinal microbiota of obese children was enriched in *Firmicutes* (N: 60.9 ± 14.1, O: 72.1 ± 12.1; mean $\pm$ sd) and depleted in *Bacteroidetes* (N: 30 ± 12.6, O: 16.6 ± 11.8) (Supporting Information Table S5). Accordingly, the *Firmicutes/Bacteroidetes* ratio was significantly elevated in obese children ($p < 0.0001$; N: 2.6 ± 1.83, O: 7.7 ± 7.1) (Fig. 1D). In agreement with previous observations, the *Firmicutes/Bacteroidetes* ratio for obese children displayed a much larger range than for normal-weight children, which may be partially attributable to the wide range of BMI *z*-score in the obese group. Consistent with the shifts observed at phylum level, at the family level *Ruminococcaceae* (N: 33.3 ± 11.5, O: 42.5 ± 12.7) was enriched and *Bacteroidaceae* (N: 21.4 ± 12.2, O: 10 ± 7.1) was depleted. At the genus and OTU levels, we observed significant depletion of *Bacteroides* (N: 21.4 ± 12.2, O: 10.5 ± 7.1) as well as *Bacteroides* OTU 7 (best BLAST hit: *Bacteroides vulgatus* with 100% sequence similarity of 422 bp). There were, however, no significant shifts in members of the *Ruminococcaceae* (Supporting Information Table S5).

Gut microbiota richness estimates were not significantly different between samples from obese and normal-weight children (Observed species: $p = 0.59$; Chao1 estimated richness: $p = 0.98$). Likewise, alpha diversity metrics, which take into account both community richness and evenness, were not significantly different between groups
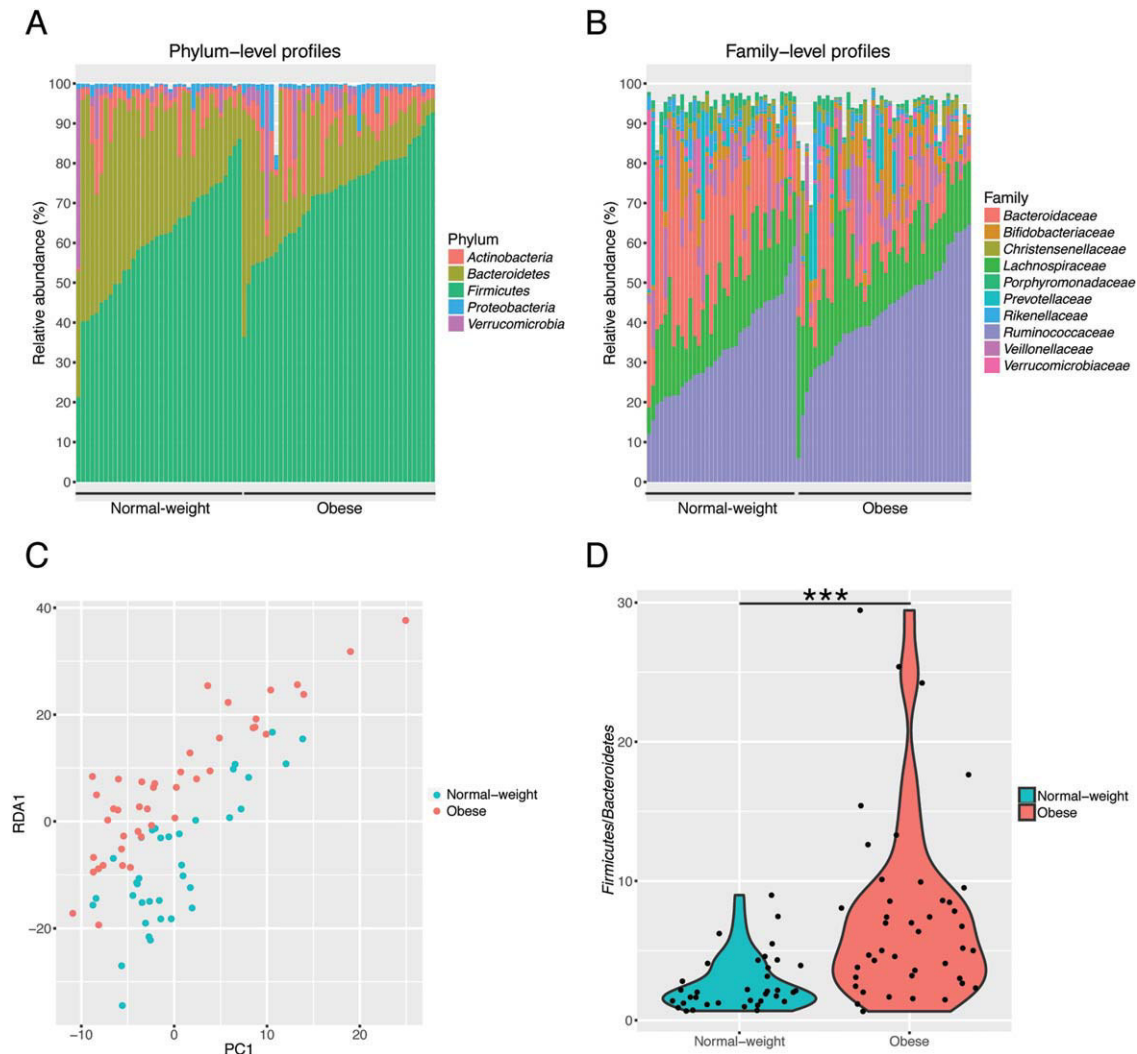
Fig. 1. Abundant bacterial taxa in stool samples of normal-weight (*n* = 36) and obese (*n* = 42) children.
Phylum-level (A) and family-level (B) taxon profiles are shown. Abundant taxa, defined as having a mean relative abundance of >1%, are shown.
C. Redundancy analysis ordination of the gut microbiota according to normal-weight (blue) and obese (red) groups.
D. *Firmicutes/Bacteroidetes* ratio for normal-weight (N) and obese (O) children. The ratio is significantly higher in obese compared to normal-weight children ($p < 0.0001$).

(Shannon: $p = 0.065$; inverse Simpson $p = 0.34$) (Supporting Information Fig. S2). We also found that mode of delivery and infant feeding were not significantly associated with microbiota composition at any taxonomic level (perMANOVA, $p > 0.05$ for all levels). In order to determine whether the higher proportion of Caesarean deliveries among the obese group could impact the difference in microbiota profiles, we grouped the samples according to delivery mode (vaginal or Caesarean section) and calculated if there was a difference in the abundance of taxonomic groups. No significant differences were found at any taxonomic level. Therefore, we conclude that delivery mode did not significantly influence the composition of the microbiota.

BMI z-score and SCFAs are associated with intestinal microbiota composition

Childhood obesity is typically defined using age and sex normalized BMI (BMI *z*-score), to classify subjects into normal-weight and obese. In order to gain a more fine-grained understanding of the relationship between the intestinal microbiota and obesity, we evaluated how BMI *z*-score was associated with microbiota composition. BMI *z*-score and the SCFAs acetate and propionate were significantly associated with microbiota composition at every taxonomic level (OTU to phylum; $p < 0.05$ at all levels). Additionally, alpha diversity metrics were largely negatively correlated with BMI *z*-score and SCFA levels (Supporting
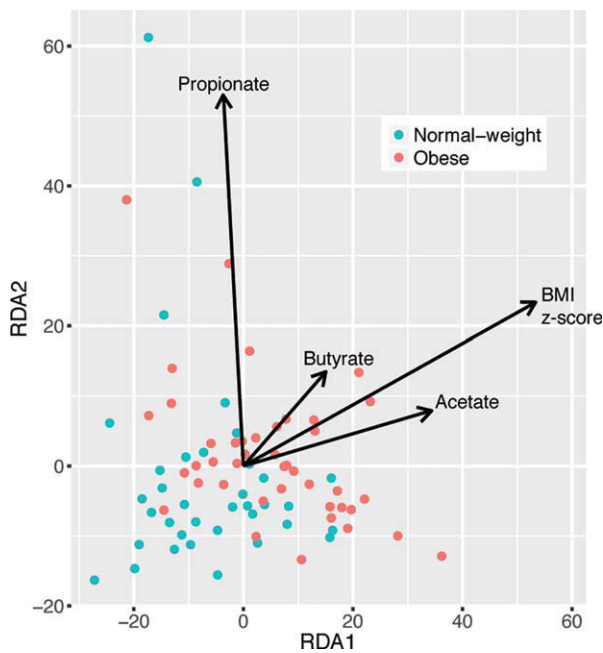
Fig. 2. Redundancy analysis of gut microbiota composition with respect to BMI *z*-score, acetate, propionate and butyrate. The direction of the arrows shows the correlation between variables. Normal-weight samples are represented by blue dots and obese with red dots.

Information Table S6). We performed redundancy analysis to visualize the relationship between microbiota composition, BMI *z*-score and SCFAs (Fig. 2). This analysis revealed a strong relationship between BMI *z*-score and acetate, and to a lesser extent, butyrate levels.

BMI *z*-score was positively correlated with the abundance of *Firmicutes* (as well as *Ruminococcaceae*) and negatively correlated with *Bacteroidetes* (as well as *Bacteroidaceae* and *Bacteroides*) (Table 1). At the OTU level, *Faecalibacterium* OTU 3 (Best BLAST hit: *Faecalibacterium prausnitzii* with 100% seq. similarity over 402 bp) was positively correlated with BMI *z*-score, and *Bacteroides* OTUs 7 and 49 (Best BLAST hit: *Bacteroides stercoris* spp. with 99% seq. similarity over 422 bp) were negatively correlated with BMI *z*-score.

The abundances of multiple taxa were also correlated with levels of major SCFAs. Several genera within the *Bacteroidetes* were negatively correlated with acetate levels, and multiple genera within the *Firmicutes* were either positively or negatively correlated with acetate (Supporting Information Table S7). At the OTU level, *Faecalibacterium* OTU 3 was positively correlated with acetate. Compared to acetate, the number of correlations was much more limited for propionate and butyrate. The family *Prevotellaceae*, the genus *Prevotella* as well as *Prevotella* OTU 26 (Best BLAST hit: *Prevotella copri* with 99% seq. similarity over 422 bp) were positively correlated with propionate levels.

The genus *Faecalibacterium* as well as *Faecalibacterium* OTU 3 were positively correlated with butyrate levels.

Comparing models to predict BMI z*-score based on microbiota composition*

We next determined the best microbial predictors of BMI *z*-score by comparing generalized linear regression models at different taxonomic levels. This revealed that the total explanatory power of the models increased at more refined taxonomic levels (Supporting Information Fig. S3). *Bacteroides* was the main contributor to the genus-level model (relative importance: 0.172), followed by two genera of the *Ruminococcaceae*, *Faecalibacterium* and *Subdoligranulum* (rel. imp.: 0.08 and 0.03 respectively). At the OTU-level, the main contributors to the model were *Bacteroides* OTU 7 (rel. imp.: 0.12), *Faecalibacterium* OTU 3 (rel. imp.: 0.08) and *Bacteroides* OTU 49 (rel. imp.: 0.07) (Supporting Information Fig. S3).

The obese gut microbiota has an altered correlation network structure

We performed a correlation network analysis to evaluate if obesity was associated with changes in the correlation structure and putative interaction structure of the gut microbiota. We found that networks constructed from samples of normal-weight children had fewer edges, a lower mean degree and lower transitivity, indicating that there were fewer significant correlations and less clustering of OTUs compared to samples from obese children (Fig. 3A and B; Supporting Information Table S8). The betweenness centrality was higher in normal-weight sample networks, which indicates that only a few OTUs are highly connected in the network.

We next evaluated whether there were differences in intra-taxon correlations within the families *Bacteroidaceae* and *Ruminococcaceae*. Interestingly, in both networks *Bacteroidaceae* OTUs with intra-family correlations were positively correlated with one another, whereas *Ruminococcaceae* OTUs had both positive and negative intra-family correlations (Supporting Information Table S8). To further explore the difference in intra-taxon correlations between these groups we extracted clusters of correlating *Bacteroidaceae* and *Ruminococcaceae* OTUs (Supporting Information Table S9). We found that *Bacteroidaceae* OTUs form two communities based on co-abundance patterns, with the most abundant (*Bacteroidaceae* CB1: 8 OTUs, including OTUs 7 and 49) negatively correlated with BMI *z*-score and the less abundant not significantly correlated (*Bacteroidaceae* CB2: 2 OTUs). *Ruminococcaceae* was composed of three communities based on co-abundance patterns, and while the most abundant (*Ruminococcaceae* CR1: 11 OTUs, including OTU 3) was

Table 1. Bacterial taxa correlated with BMI *z*-score.

Taxonomic level	Taxon	*R*	*p*-value
Phylum	*Firmicutes*	0.4145	0.0001
	Bacteroidetes	−0.4538	<0.0001
Class	*Clostridia*	0.3688	0.0008
	Bacteroidia	−0.4538	<0.0001
Order	*Clostridiales*	0.3687	0.0008
	Bacteroidales	−0.4538	<0.0001
Family	*Ruminococcaceae*	0.3778	0.0006
	Bacteroidaceae	−0.4930	<0.0001
Genus	*Bacteroides*	−0.4930	<0.0001
OTU	OTU 7: *Bacteroides vulgatus*	−0.4321	<0.0001
	OTU 3: *Faecalibacterium prausnitzii*	0.3058	0.0064
	OTU 49: *Bacteroides stercoris*	−0.3252	0.003

Pearson correlation coefficient (*r*) and *p*-value are shown for significantly correlating taxa and operational taxonomic units (OTUs).

positively correlated with BMI *z*-score, the second was negatively correlated (*Ruminococcaceae* CR2: 8 OTUs), and the third (CR3: 20 OTUs) was not significantly correlated (Supporting Information Figs. S4 A, B).

Discussion

The gut microbiota is affected by many factors, such as diet, genetics, health status, environment and lifestyle (Rodríguez *et al.*, 2015). Childhood and adult obesity are accompanied by changes in the composition of the gut microbiota (Karlsson *et al.*, 2012; Bervoets *et al.*, 2013; Borgo *et al.*, 2016). In the present study we found alterations in gut microbiota composition and SCFA levels in a cohort of 42 obese and 36 normal-weight Italian children. We observed that children born by Caesarean section tended to be obese, although this result did not reach statistical significance. Past studies have found that Caesarean section delivery increases the risk of obesity (Goldani *et al.*, 2011; Mueller *et al.*, 2015; Portela *et al.*, 2015) and impacts the infant gut microbiota (Grönlund *et al.*, 1999). In our study, delivery mode and infant feeding history (breast-fed vs. formula-fed) were not significantly associated with obesity or the gut microbiota composition of children (mean age = 11). The impact of delivery mode and infant feeding history on the gut microbiota may, therefore, be lost after the first years of life, although it is still unclear exactly when (Penders *et al.*, 2006; Biasucci *et al.*, 2010). Gut microbiota composition has been reported to begin to converge toward an adult-like microbiota by the end of the first year of life and fully resemble the adult microbiota by 2.5 years of age (Clemente *et al.*, 2012), although other studies have shown that the microbiota of children up to 4 years of age differs from that of adults (Kulka *et al.*, 2013; Hollister *et al.*, 2015), suggesting that conversion to an 'adult-like' microbiota may be a long and gradual process.

Recent scientific advances implicate the gut microbiota as a contributor to over-nutrition. The gut microbiota enables hydrolysis of indigestible polysaccharides to easily-absorbable monosaccharides and activation of lipoprotein lipase by direct action of the villous epithelium. Consequently, glucose is rapidly adsorbed and fatty acids are stored in excess (Kalliomäki *et al.*, 2008), providing an additional source of energy for the body (Turnbaugh *et al.*, 2006). The significantly higher concentration of SCFAs in obese participants in our study may indicate that in obese children colonic fermentation is elevated, or alternatively that there is decreased SCFA absorption due to low-grade inflammation or more rapid gut transit. This has previously been observed in cohorts of both children (Payne *et al.*, 2011) and adults (Schwiertz *et al.*, 2009; Fernandes *et al.*, 2014). Elevated fecal concentrations of total or individual SCFAs might result from increased microbial production, shifts in microbial cross-feeding patterns or low mucosal absorption (Schwiertz *et al.*, 2009).

We observed a clear alteration in the gut microbiota in obese children at every taxonomic level. This was characterized at the phylum level by an increased abundance of *Firmicutes* and a decreased abundance of *Bacteroidetes* in obese children. It has been hypothesized that an increased ratio of *Firmicutes* to *Bacteroidetes* may contribute to the pathophysiology of obesity and is associated with increased production of SCFAs and energy harvest from colonic fermentation (Turnbaugh *et al.*, 2006; Fernandes *et al.*, 2014). Although an elevated *Firmicutes/Bacteroidetes* ratio in obese subjects has been reported in multiple studies (Turnbaugh *et al.*, 2009; Xu *et al.*, 2012; Bervoets *et al.*, 2013), a reduced *Firmicutes/Bacteroidetes* ratio in obese adults has also been found (Schwiertz *et al.*, 2009). A recent meta-analysis concluded that there were no statistically significant differences across multiple studies in the *Firmicutes/Bacteroidetes* ratio between obese and normal-weight adults (Walters *et al.*, 2014). In agreement with this meta-analysis, some pediatric studies have
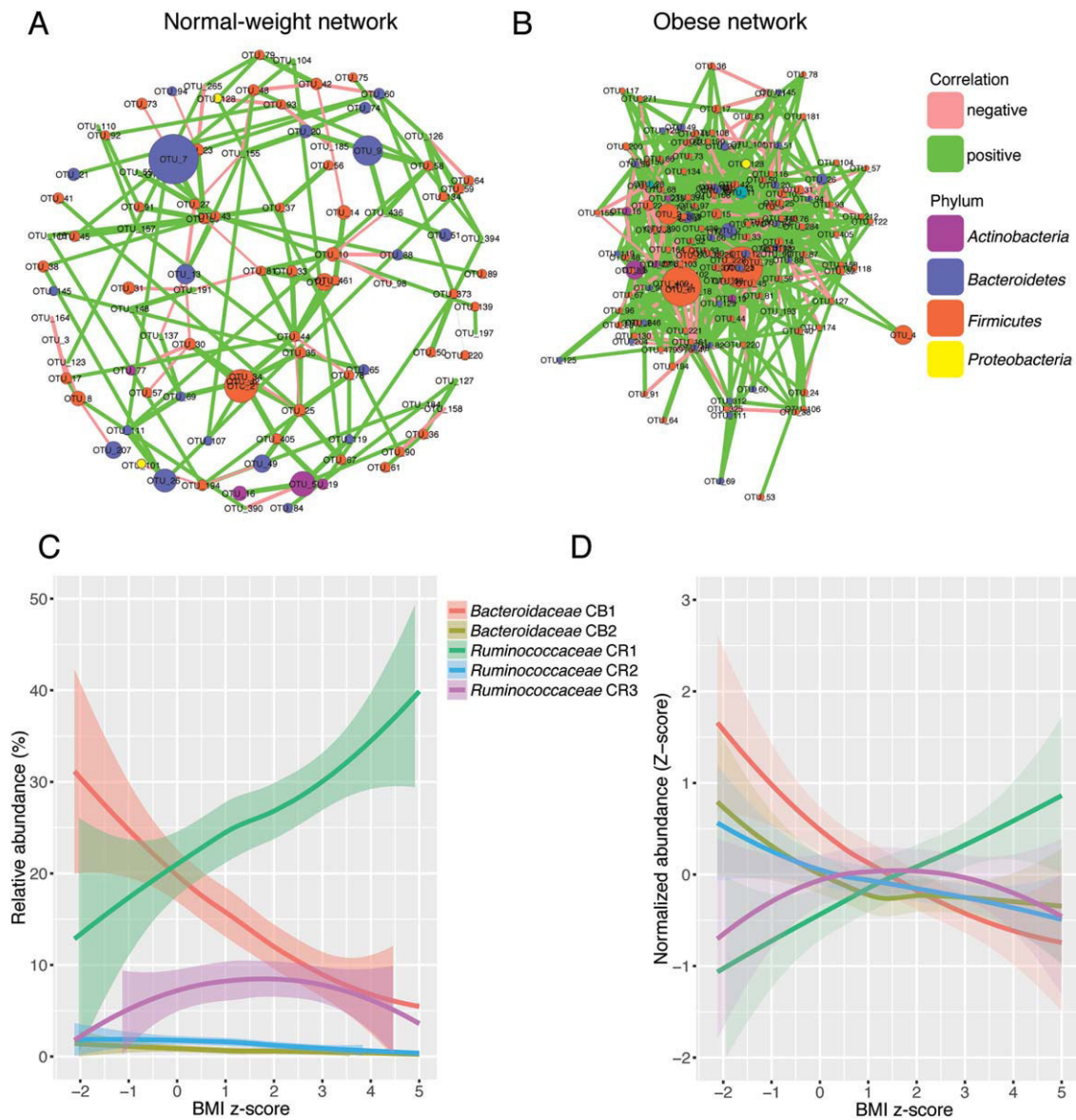
Fig. 3. Correlation networks of samples from normal-weight and obese children.
A, B. Networks show significant positive (green) and negative (pink) pairwise correlations between operational taxonomic units (OTUs). OTUs are coloured by phylum affiliation and sized by mean relative abundance.
C, D. Correlating communities of *Bacteroidaceae* (CB) and *Ruminococcaceae* (CR) and their abundances with respect to BMI *z*-score.
Relative abundances (C) and *z*-score transformed abundances (D) are shown. Data points were processed using Lowess smoothing and 95% confidence intervals are shown.

found an increase in *Firmicutes* and a decrease in *Bacteroidetes* (Bervoets *et al.*, 2013; Ferrer *et al.*, 2013) while others have not (Abdallah Ismail *et al.*, 2011; Payne *et al.*, 2011). Although in our study the *Firmicutes*/*Bacteroidetes* ratio was significantly elevated in obese individuals, we observed large variation in the ratio, particularly within the obese group. 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.

We reasoned that the classification of individuals into normal-weight and obese groups might be too coarse of a description for the physiological differences present at different BMI *z*-scores. We found that the alpha diversity of the gut microbiota was negatively correlated with BMI *z*-score and we recovered the same broad trends as we observed with obesity classification such as a positive correlation with the *Firmicutes*/*Bacteroidetes* ratio, but with additional insights such as positive correlation of *Faecalibacterium* OTU 3 (*F. prausnitzii*) with BMI *z*-score and a

negative correlation of *Bacteroides* OTU 7 and 49 (*B. vulgatus* and *B. stercoris* respectively) with BMI *z*-score. *Faecalibacterium*, a group of major butyrate producers in the colon (Louis *et al.*, 2009), was also positively correlated with acetate and butyrate, reinforcing the tight link between SCFAs and obesity. Literature data are conflicting about the level of *F. prausnitzii* in obesity, with studies showing positive (Balamarugan *et al.*, 2010), negative (Borgo *et al.*, 2016) or no association (Feng *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 *et al.*, 2009; Hippe *et al.*, 2016). Indeed, Hippe and colleagues (2016) suggested that the two identified phylotypes display different physiological properties and seem to produce different amounts of butyrate in the gut. Propionate levels were positively correlated with members of the *Prevotellaceae*, which are known propionate producers (Schwiertz *et al.*, 2009), although this was not related to BMI *z*-score. Interestingly, increase of colonic propionate has been shown to prevent weight gain in overweight adults by stimulating the release of PYY and GLP-1 from human colonic cells and thereby reducing energy intake (Chambers *et al.*, 2014).

In order to determine if the structure of the gut microbiota is also altered in obesity, we performed a correlation network analysis and found that there were fewer correlations and less clustering of OTUs in normal-weight compared to obese children. The betweenness centrality was higher in the normal-weight network, which indicates that in the obese microbiota there are more OTUs that are highly connected to other OTUs. It is tempting to speculate that the altered network structure in obese children may be involved in the increased fermentation capacity of the gut microbiota.

Interestingly, intra-taxon correlations within the families *Bacteroidaceae* and *Ruminococcaceae* demonstrated that in both networks *Bacteroidaceae* OTUs were positively correlated with one another, whereas *Ruminococcaceae* OTUs had both positive and negative intra-family correlations. This indicates a lack of intra-family ecological cohesion for *Ruminococcaceae* across these samples and may explain why *Bacteroidetes* taxa were generally better predictors of BMI and obesity than *Firmicutes* taxa. To further investigate the difference between *Bacteroidaceae* and *Ruminococcaceae* responses, we extracted from the complete network the communities of co-abundant OTUs from these two groups. We identified five distinct correlating communities (2 *Bacteroidaceae* [CB1 and CB2] and 3 *Ruminococcaceae* [CR1-CR3]). Interestingly, while *Ruminococcaceae* CR1 was positively correlated with BMI *z*-score, *Ruminococcaceae* CR2 was negatively correlated. The divergent response of members of the *Ruminococcaceae* with respect to BMI *z*-score may indicate different

niche preferences within this group and may also help to explain why the increased *Firmicutes/Bacteroidetes* ratio is not found in all studies, as it groups together *Firmicutes* populations with discordant shifts in obesity. Divergent responses of members of the clostridia have previously been observed in other conditions such as inflammation (Berry *et al.*, 2012). It is likely that the extensive physiological and metabolic diversity in members of the clostridia is responsible for these contrasting responses, and additional studies are needed to better characterize and functionally categorize the members of this abundant group.

Although it is recognized that the gut microbiota has the potential to change along with the development of its host, information regarding the structure and function of the microbiome in children remains limited (Hollister *et al.*, 2015). We hypothesized that an aberrant gut microbiota composition and activity might contribute to the development of childhood obesity. We found that members of the *Bacteroidetes* and certain populations of *Firmicutes* were associated with childhood obesity, although members of the *Firmicutes* exhibited contrasting shifts. Additional studies are needed to better characterize the members of *Firmicutes* and their roles in obesity. Obesity is often associated with altered dietary habits, and in the present study obese children had higher caloric intake. It is therefore not possible to determine if an altered microbiota is a causative factor in pediatric obesity or a consequence of diet, and this must be tested with future research that takes into account diet and physiology and which includes detailed functional analyses of the metabolic activity of the gut microbiota. Together, this will advance our understanding of the role of the gut microbiota in obesity and provide opportunities to improve health and prevent disease.

Experimental procedures

Subjects and sample collection

Seventy-eight children (36 males/42 females, 9–16 years) were enrolled in the study at the Pediatric Department of San Paolo Hospital in Milan from December 2013 to February 2015. The enrollment conditions were performed as previously described (Borgo *et al.*, 2016). Briefly, children's BMI was calculated by reported weight/height^2 (kg m^{-2}), and classification of obese (O) and normal weight (N) was made according to Cole (Cole *et al.*, 2000). Weight (kg), height (cm) and BMI (kg m^{-2}) were transformed to age and sex-specific *z*-scores (Cole *et al.*, 1995). Inclusion criteria were: children living in Northern Italy born from Caucasian parents with birth weight $\geq$ 2500 g, gestational age 37–42 weeks and singleton birth. Children with neonatal disease, congenital malformation, antibiotic or probiotic/prebiotic usage in the previous six months, chronic or acute intestinal and obesity-related co-morbidity conditions were excluded. Data concerning mode of delivery and type of feeding were collected for all subjects and the dietary habits were assessed at recruitment by means of an age-adjusted food frequency questionnaire made up of 116

items (Verduci *et al.*, 2007). Fecal samples were collected 24 h before medical examination and stored at −20°C until processing.

The study was conducted in accordance with the local medical ethical committee (protocol number 2015/ST/135). Written informed consent was given by a parent for all enrolled subjects.

DNA extraction and preparation of 16S rRNA gene amplicon libraries

The total bacterial DNA extraction was performed using the Spin stool DNA kit (Stratec Molecular, Berlin, Germany), according to the manufacturer's instructions and amplified by PCR. Amplification was performed with a two-step barcoding approach according to Herbold and colleagues, 2015. In the first-step PCR, 16S rRNA genes of all Bacteria were amplified with forward primer S-D-bact-0341-b-S-17 (5-CCTACGGGNGGCWGCAG-3′) and reverse primer S-D-bact-0785-a-A-21 (5-GACTACHVGGGTATCTAATCC-3′), which also contained head adaptors (5′-GCTATGCGCGAGCTGC-3′). In the second-step PCR, PCR products from the first step were amplified with primers consisting of the 16 bp head sequence and a sample-specific 8 bp barcode from a previously published list at the 5′ end (Hamady *et al.*, 2008). Each PCR reaction (20 μL in first step, 50 μL in second step) consisted of 10× Taq buffer (Fermentas, USA), 2 mM dNTPmix (Fermentas), 25 mM MgCl$_2$ (Fermentas), 5 U μl^{-1} Taq DNA polymerase (Fermentas), 20 mg ml^{-1} bovine serum albumin (Fermentas), 50 μM of each of the forward and reverse primers and 5 μl of sample. Thermal cycle conditions were: 95°C for 3 min; 95°C for 30 s, a primer-specific annealing temperature of 55°C for 30 s, 72°C for 1 min for 25 cycles and an elongation time of 72°C for 7 min (step1); 52°C for 30 s, 72°C for 1 min for 5 cycles (step 2) and an elongation step of 72°C for 7 min. The first PCR reaction was performed in triplicate, pooled for use as a template in the second step and evaluated qualitatively by gel electrophoresis. The barcoded amplicons were purified between the first step and the second step and after the second step with ZR-96 DNA Clean-up Kit (Zymo Research, USA) and quantified using the Quant-iT PicoGreen dsDNA Assay (Invitrogen, USA). An equimolar library was constructed by pooling samples, and the resulting library was sent sequenced on the Illumina MiSeq platform at Microsynth AG (Balgach, Switzerland). Sequence data have been deposited in the NCBI Short Read Archive under SRP073251.

Short chain fatty acids (SCFAs) measurement

Stool samples were analysed for acetic acid, propionic acid and butyric acid using capillary electrophoresis. For determination of SCFAs concentration one aliquot of frozen fecal sample (50 mg) was used and 200 μl of Milli-Q filtered water was added. The solution was mixed by vortexing for 10 min and then centrifuged 30 min at 21,000 × g. A standard mix composed of acetic acid, propionic acid, butyric acid, lactic acid, formic acid and succinic acid with consecutive concentration of 50 μM, 100 μM, 250 μm and 350 μM, were run as external standards and calibrated. Caproic acid (100 μM final concentration) was used as internal control. A buffer with 0.01M NaOH, 500 μM CaCl$_2$ and 100 μM caproic acid was prepared to run samples. Because we detected interference between phosphates and propionic acid peaks, a final concentration of 500 μM of CaCl$_2$ was added in order to precipitate phosphates usually present in human fecal matter. Ceofix Anions 5 kit (Beckman Coulter, USA) was utilized to prepare anion buffers for the machine. SCFAs concentration was determined in 100μl supernatant using P/ACE MDQ Molecular Characterisation System Beckam Coulter (USA) with a fused silica capillary of 75 μm internal diameter × 363 μm outer diameter (Polymicro Tecnologies, USA). Thirty-two karat software (Beckman Coulter, USA) was used for data processing. SCFAs concentration in fecal samples was expressed in micromoles per gram (μmol g^{-1}) of feces.

Sequence pre-processing and data analysis

Sequence data were sorted into libraries using the 8 nt sample-specific barcode and primer using a custom-made in-house script, quality-filtered according to the Earth Microbiome Project guidelines and paired end reads were concatenated (Bokulich *et al.*, 2013). Reads were then clustered into species-level operational taxonomic units (OTUs) of 97% sequence identity, checked for chimeras using USEARCH, and taxonomically classified using the Ribosomal Database Project näive Bayesian classifier (Wang *et al.*, 2007). Statistical analysis was performed using the statistical software R (https://www.r-project.org/). To avoid biases related to uneven library depth, sequencing libraries were subsampled to a number of reads smaller than the smallest library (2000 reads). The statistical significance of factors affecting microbiota composition was evaluated using nonparametric permutational multivariate analysis of variance (perMANOVA), significant clustering of groups was evaluated with analysis of similarities (ANOSIM), ordination was performed using redundancy analysis (RDA) in the vegan package (Oksanen *et al.*, 2010). Alpha and beta diversity metrics were also calculated with the vegan package. Indicator species analysis was performed using the indicspecies package (De Caceres *et al.*, 2009). Network analysis was performed for all OTUs present in at least 30% of samples as recommended in (Berry and Widder, 2014) using graphical lasso technique cclasso to mitigate biases associated with compositional data (Danaher *et al.*, 2014). Network topological and node-level properties were determined using the igraph package (Csardi, 2015) and networks were visualized using Cytoscape (Shannon *et al.*, 2003). Statistical analysis of cohort-related data was performed using Student's *t*-test, chi-square test, correlation analysis (Pearson correlation coefficient) and linear regression modeling. Variables were expressed as mean ± standard deviation (sd), and for multiple comparisons *p*-values were adjusted with the False Discovery Rate method. A *p*-value less than or equal to 0.05 was considered statistically significant.

Acknowledgement

Conflict of interest

All authors declare no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Fig. S1. Abundant bacterial taxa in stool samples of normal-weight (n=36) and obese (n=42) children. Genus level taxon profiles are shown. Abundant taxa, defined as having a mean relative abundance of >1%, are shown.

Fig. S2. Intestinal microbiota richness and diversity in normal-weight and obese children. Observed species, Chao1 estimated richness, Shannon diversity, and inverse Simpson diversity estimators show no significant difference between the two groups (Observed species: p=0.59; Chao1: p=0.98; Shannon: p=0.065; Inverse Simpson p=0.34).

Fig. S3. Generalized linear regression models at different taxonomic levels. (A) The coefficient of determination (R^2), which indicates the proportion of the variance in the dependent variable that is predictable from the independent variable, increases at genus and OTU levels. (B) The Akaike information criterion (AIC), a measure of the relative quality of statistical models for a given set of data, is lowest at genus and OTU levels.

Fig. S4. Correlating communities of *Bacteroidaceae* (CB) and *Ruminococcaceae* (CR) and their abundances with respect to BMI z-score. Relative abundances (A) and z-score transformed abundances (B) are shown. Data points were processed using Lowess smoothing and 95% confidence intervals are shown.

Table S1. Characteristics of the study cohort. The cohort was composed of normal-weight (N) and obese (O) children. Body mass index (BMI) was calculated as weight/height^2 (kg/m^2), and was transformed to age- and sex-adjusted z-scores. Values are expressed as mean ± sd. [a]Information not available for two subjects. [b]Information not available for three subjects.

Table S2. Short chain fatty acid (SCFA) levels in the stool of normal-weight (N) and obese (O) subjects. Concentrations are calculated as μmol/g wet weight and are expressed as mean ± sd. Total SCFA is calculated as the sum of acetate, propionate, and butyrate concentrations.

Table S3. Daily caloric and dietary intake in obese and normal-weight children. Values are expressed as mean ± sd.

Table S4. The relative abundance of abundant bacteria taxa in the study. Abundant taxa are defined as having a mean abundance greater than 1%. *Taxa significantly increased or decreased in obese children (complete details are presented in Table S4).

Table S5. Taxa that were increased (+) or decreased (−) in abundance in obese children (O).

Table S6. Correlation of alpha diversity metrics with BMI z-score and SCFAs. Observed OTUs, Chao1 estimated richness, Shannon and inverse Simpson diversity indexes were correlated and the Pearson correlation coefficients (r) and respective p-values are shown. *indicates p<=0.05 and **indicates p <=0.01.

Table S7. Taxa correlated with acetate concentration. The Pearson correlation coefficients (r) and respective p-values are shown.

Table S8. Properties of correlation networks generated from samples from normal-weight (N) or obese children (O). Nodes are OTUs and edges are significant correlations between OTUs. Other parameters are metrics related to the topology of the network.

Table S9. Clusters of correlating *Bacteroidaceae* and *Ruminococcaceae* OTUs extracted from the correlation network. The closest cultured species and its similarity to each OTU (% sequence similarity) are shown.

5. SERUM SALICYLIC ACID AND FRUIT AND VEGETABLE CONSUMPTION IN OBESE AND NORMAL-WEIGHT CHILDREN

The aim of this study was to evaluate the concentrations of serum salicylic acid in a group of obese children, compared to normal-weight children, and to evaluate if an association may exist between serum salicylic acid and fruit and vegetable consumption.

Methods and results have been largely discussed in the following published paper.

Serum salicylic acid and fruit and vegetable consumption in obese and normal-weight children: a pilot-study

Carlotta Lassandro, Giuseppe Banderali, Benedetta Mariani, Alberto Battezzati, Lucia Diaferio, Vito Leonardo Miniello, Giovanni Radaelli & Elvira Verduci

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RESEARCH ARTICLE

Serum salicylic acid and fruit and vegetable consumption in obese and normal-weight children: a pilot-study

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ABSTRACT

Salicylic acid (SA), a phenolic compound produced by plants, may play a beneficial role on health. This pilot study evaluated whether there might be an association between serum SA and fruit and vegetable (FV) consumption in obese and normal-weight children. Thirty-four obese children (17 boys and 17 girls) and 34 normal-weight children were recruited. Dietary intake was evaluated by the 7-day dietary record. Serum SA was measured using gas chromatography-mass spectrometry method. FV intake in obese and normal-weight children was not different between groups (175.00 (97.66) g versus 192.29 (90.54) g, $p = .455$). Obese children had lower serum SA than normal-weight children [mean difference, -0.025; 95% CI (-0.044; -0.006) μmol/L]. Serum SA was not associated with daily intake of FV in obese ($p = .111$) and normal-weight ($p = .092$) children. Further studies are needed to evaluate the role of FV on serum SA, taking into account also the quantity and the type.

Introduction

Fruits and vegetables (FV) are known for their health-promoting properties in the prevention of chronic diseases, as dietary sources of fibre, vitamins, minerals and phytochemicals (Slavin & Lloyd 2012). Among these phytochemicals salicylic acid (SA), a phenolic compound produced by plants as a defence system against pathogens and stress, may play a beneficial role on health (Paterson & Lawrence 2001; Paterson et al. 2006). It has been suggested that beneficial effects associated with diets rich in FV could be due, at least in part, to salicylate intake (Paterson & Lawrence 2001; Paterson et al. 2006), although it is not yet fully clear what component or combination of components in FV is protective (Duthie & Wood 2011). Moreover, accurately estimating the dietary intake of SA might be difficult, since salicylate content varies considerably among foods (Swain et al. 1985; Wood et al. 2011) and is additionally affected by factors as seasonality, storage and cooking (Duthie & Wood 2011). To date, a systematic review of the literature (Wood et al. 2011) shows the most definitive estimate of salicylate content of foods. By using this database, the total salicylate intake in a

Scottish population was estimated of 4.42 and 3.16 mg/day, for males and females respectively, with FV as major sources (Wood et al. 2011).

It has been reported that vegetarian adults may exhibit higher serum concentration of SA than non-vegetarians (Blacklock et al. 2001) and that in healthy adults not in therapy with aspirin, the most common anti-inflammatory drug, serum SA can be related to FV consumption (Spadafranca et al. 2007). Although in the future SA may become a nutritional biomarker of FV intake, nowadays no study has been performed to define cut-offs distinguishing between "normal", "excess" or "deficiency" levels of serum SA nor to evaluate the response of this compound to therapeutic interventions, such as nutritional–behavioural intervention.

SA has anti-inflammatory properties and possibly the ability to modulate activity and/or expression of components involved in oxidative stress processes (Duthie & Wood 2011). Both inflammation and oxidative stress are related to the pathogenesis of many chronic diseases, including cardiovascular diseases, diabetes and cancer (Camps & García-Heredia 2014). The prevalence of childhood overweight and obesity has increased dramatically worldwide in the last

decades, becoming a public health issue (Lobstein et al. 2015). Obesity is often associated with chronic low-grade systemic inflammation (Makki et al. 2013) and in children, not only it may be associated with several acute health problems, but it can also result in later adult obesity and its related comorbidities (Lakshman et al. 2012).

Studies on this issue are lacking in the paediatric age. This pilot study evaluated whether an association may exist between serum SA and FV consumption in obese and normal-weight children.

Materials and methods

This observational case–control pilot study included a series of 34 obese children (17 boys and 17 girls), consecutively recruited among those admitted with diagnosis of obesity at Day Hospital of the Department of Paediatrics, San Paolo Hospital, Milan, Italy, between 1 January and 30 June 2015, and 34 healthy children (control group). For each obese child, an age (±1 year) and sex-matched control was recruited within 2 weeks among those undergoing minor surgery at the Day Surgery Clinic of San Paolo Hospital. Inclusion criteria were age ≥6 years; weight at birth ≥2500 g and <4000 g; gestational age 37–42 weeks; single birth; having Caucasian parents. Exclusion criteria were any syndromic, organic and hormonal conditions besides obesity; use of anti-inflammatory drugs, including aspirin, in the last month; any allergy, food intolerance or adoption of special diets (gluten-free, vegetarian, vegan diet).

At recruitment, a medical history was collected from parents by a standardised questionnaire during a personal interview, conducted by the same paediatrician that was in charge of the children's general examination. Body weight and height were measured using a mechanical column scale (seca 711; seca GmbH & KG, Hamburg, Germany) with integrated measuring rod (seca 220; seca GmbH & KG). Body mass index (BMI) was calculated from the ratio of weight to height squared (kg/m^2). BMI z-scores were calculated and adjusted for age and sex by using Cole's LMS method (Cole 1990) and Italian reference data (Cacciari et al. 2002). A child was defined obese in accordance with the International Obesity Task Force (Cole et al. 2000). The parents or legal guardian of eligible children received a detailed explanation about the aim of the study, and signed a consent form. The Hospital Ethics Committee approved the study protocol and gave ethical clearance.

Daily dietary record

Dietary intake of children was assessed after recruitment using a 7-day dietary record. Parents received complete oral and written instructions about how to weigh food and the recording of such data. They were trained by a dietician to weigh each food offered to the child before consumption and the leftovers, and to record these weights each time. Vegetable intake was quantified, excluded potato and legumes. Quantification and analysis of the energy intake and nutrient composition were performed with an ad hoc PC software programme (Mètadieta®, 2013). Individual salicylate intakes, derived from FV, of the last day before the blood sample were estimated by using a dietary database containing the median salicylate content of 27 types of fruits and 21 vegetables (Wood et al. 2011) and, for those items missing, a database developed in 1985 by Swain et al. (1985) (Table 1).

Serum salicylic acid determination

Fasting blood samples were taken on the day after the dietary record was completed, at 8 h ± 30 min a.m., since it has been observed that circulating SA is significantly related both to daily FV intake of the entire previous week and of the last day (Spadafranca et al. 2007). Measurement of SA serum concentration was performed using a sensitive stable isotope dilution and gas chromatography-mass spectrometry method, as previously described (Battezzati et al. 2006). This procedure provides high sensitivity and is adequate for population studies as small serum quantity is required (~100 μl) (Battezzati et al. 2006).

Statistical analysis

Descriptive data are reported as mean and SD, median and 25th–75th percentile. Normality of the distribution of continuous variables was assessed by the Kolmogorov–Smirnov test. Comparison between obese

Table 1. Salicylate content of fruit and vegetables adapted from Wood et al. (2011) and Swain et al. (1985)[a].

Food item	Salicylates/mg kg^{-1}	Food item	Salicylates/mg kg^{-1}
Fruits		*Vegetables*	
Grapes red	4.71	Peppers-green	6.01
Cherry	4.43	Broccoli	3.25
Lemon	2.50	Peppers	2.07
Pear	1.46	Asparagus	1.35
Strawberry	0.63	Onion	1.20
Apple	0.55	Cauliflower	0.80
Plum	0.50	Green beans	0.59
Banana	0.40	Carrots	0.50
Kiwi fruit	0.31	Tomato	0.36
Melon honeydew	0.11	Cucumber	0.24
Orange	0.11	Peppers-red	0.10
Apricot	0.10	Peppers-yellow	0.10
Tangerine	0.06	Spinach	0.06
Grapes white	0.04	Lettuce	0.02
Lychees	0.04	Courgette	0.00

[a]Only fruit and vegetables consumed by the studied children are reported here.

and normal-weight children for continuous variables was performed by the Student's t-test for unpaired data or the Mann–Whitney test, as appropriate. A multivariate analysis using binary logistic regression analysis was performed to evaluate whether daily dietary intake of energy, macronutrients, fibre and FV were influenced by age and sex and whether serum SA concentration was influenced by age, sex, FV intake and salicylate intake from FV. The association of SA with FV consumption was assessed by Spearman's correlation coefficient.

All values of $p < .05$ were considered to indicate statistical significance (two-tailed test). The Statistical Package for Social Sciences (SPSS), version 20.0 (SPSS Inc., Chicago, IL) for Windows (Microsoft, Redmond, WA), was used for the statistical analysis.

Results

Mean (SD) age of obese and normal-weight children was 10.2 (2.2) years and 10.3 (2.2) years, respectively, ($p = .882$). BMI z-score was 3.17 (0.83) in obese children and 0.14 (0.89) in normal-weight children ($p < .001$).

Daily dietary intake of energy, protein, carbohydrates, fats and fibre is shown in Table 2. Obese children had higher energy intake than normal-weight children. In both groups protein intake (as % of total energy) was close to the upper recommended

threshold, while carbohydrates and lipids were within the third quartile of the recommended range (SINU 2014). In obese children, fibre intake (g/1000 kcal) was lower ($p < .001$) than recommended (SINU 2014). No significant difference was observed for FV intake between obese and normal-weight children (Table 2). Estimated dietary salicylate intake was lower in obese children than normal-weight children [0.15 (0.12) versus 0.21 (0.10) mg/day, $p = .030$].

Figure 1 compares serum SA between obese and normal-weight children. Obese children had lower
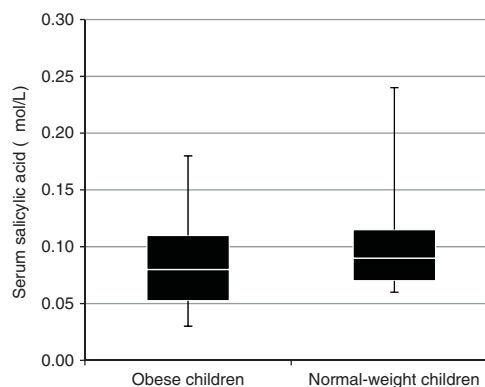

Figure 1. Box-Whisker plot of salicylic acid in obese and normal-weight children. Significance of difference between groups was $p = .013$ (crude) and $p = .608$ (adjusted for age, sex, FV intake and estimated salicylate intake from FV).

Table 2. Daily dietary intake of energy, macronutrients, fibre and fruit and vegetables in obese and normal-weight children.

Variable	Obese children ($n = 34$)	Normal-weight children ($n = 34$)	p Value[a]	Recommended intake[b]
Average of the 7-day record				
Energy				
kcal	2316.44 (473.17)	1723.61 (389.02)	<.001*	1380–3170 kcal/day depending on age and sex
kJ	9691.98 (1979.75)	7211.60 (1627.67)		
Protein				
g	90.07 (26.92)	64.71 (20.64)	<.001*	
% Energy	15 (3)	15 (3)	.576	<15% Energy
Carbohydrates				
g	309.70 (79.65)	239.22 (53.95)	<.001*	
% Energy	54 (5)	56 (7)	.206	45–60% Energy
Fats				
g	79.13 (21.82)	57.44 (20.90)	<.001*	
% Energy	31 (4)	30 (5)	.562	20–35% Energy
Fibre				
g	16.44 (5.69)	16.02 (6.43)	.784	
g/1000 kcal	7.10 (1.93)	9.44 (2.56)	<.001*	8.4 g/1000 kcal
Fruit and vegetables				
Average of the 7-day record				
Amount (g)	175.00 (97.66)	192.29 (90.54)	.455	≥400 g/day
Median (25th–75th percentile)	125.00 (76.75–275.00)	170.00 (100.00–255.00)		
Last day record				
Amount (g)	180.00 (93.07)	205.00 (91.18)	.268	≥400 g/day
Median (25th–75th percentile)	143.00 (65.33–293.12)	181.00 (90.80–315.71)		

Values are mean (SD).
[a]Adjusted for age and sex.
[b]Energy, macronutrients and fibre (SINU 2014); amount of fruit and vegetables (WHO/FAO 2003).
*Statistically significant.

mean (SD) SA serum concentration [0.08 (0.04) μmol/L versus 0.11 (0.04) μmol/L, $p = .013$ (crude), $p = .608$ (adjusted for age, sex, FV intake and estimated salicylate intake from FV)]. Median (25th–75th percentile) serum SA was 0.08 μmol/L (0.05–0.11) in obese children and 0.09 μmol/L (0.07–0.12) in normal-weight children. Correlation coefficient of serum SA with daily intake (g) of FV (average of the 7-day record) was 0.286 ($p = .111$) in obese children and 0.301 ($p = .092$) in normal-weight children.

Discussion

This is the first study evaluating if there might be an association between serum SA and FV consumption in obese and normal-weight children. While serum SA was detected in all children, ranging within an interval (0.03–0.24 μmol/L) partially overlapping that reported in non-vegetarian adults (Blacklock et al. 2001; Spadafranca et al. 2007), the mean concentration was around 30% lower in obese than normal-weight children, with 38% of the obese children having serum SA below the 25th percentile of normal-weight children.

Consistent with the results from an active national surveillance system, showing that out of 46,307 children aged 8–9 years, 48.8% did not eat vegetables and 28.7% did not eat fruit daily (Lauria et al. 2015), in this study the mean amount of FV consumed by children was not compliant with the World Health Organisation guidelines (WHO/FAO 2003), i.e. it was about 50% lower of the minimum recommended value of 400 g daily. This result is also in agreement with data from the PRO GREENS cross-sectional survey (Lynch et al. 2014), performed in a sample of 8158 children aged 11 years in 10 European countries, showing that total mean consumption of FV was 263 g/day and therefore none of the participating countries met the WHO population goal. Considering the well-known beneficial effects associated with FV consumption, these data on the paediatric population are alarming. In this regard, recently, data from IDEFICS study (González-Gil et al. 2015), a large multicentre study involving 16.228 children aged 2–9 years from eight European countries, found that high-frequency intake of vegetables was negatively associated with inflammatory status in children, evaluated measuring high sensitivity-c reactive protein levels.

Dietary macronutrient distribution did not differ between obese and normal-weight children, as also previously observed (Verduci et al. 2007). In obese children mean intake of fibre was 15% lower than the defined "adequate intake" (SINU 2014). In this regard, NHANES 2003–2006 data (Brauchla et al. 2012) report

a lower risk for childhood obesity with increasing dietary fibre intake. Moreover, it should be noted that fibre intake (g/1000 kcal) in obese children was significantly lower than observed in normal-weight children, although no difference in FV intake was observed. This result could be due to a lower consumption of legumes and whole grain in obese than normal-weight children.

In this study, the relationship of serum SA with FV consumption did not reach statistical significance both in obese and normal-weight children. Spadafranca et al. (2007) evaluated 36 healthy adults and found an association of serum SA with FV consumption. However, it should be noted that the subjects taken in consideration (Spadafranca et al. 2007) consumed a mean daily amount of FV about three times higher than children examined in this study. While studies in paediatric age are lacking, other studies on adults have suggested that SA concentration may be influenced by FV consumption (Blacklock et al. 2001; Rinelli et al. 2012).

Differently from other studies that did not determine salicylate intake through the diet (Blacklock et al. 2001; Spadafranca et al. 2007), results from this study showed that, although FV consumption did not differ between obese and normal-weight children, estimated salicylate intake was lower in obese children. This result may be due to different choices in the type of FV consumed, as well as to a greater monotony in food habits of obese children.

Salicylates have anti-inflammatory properties, in part by modifying the binding of transcription factors to the promoter region of genes involved in pro-inflammatory processes, and are involved in the regulation of activity/expression of transcription factors of oxidative stress processes (Duthie & Wood 2011). Moreover treatment with salicylates at high doses may have a role in glucose metabolism. Indeed, a review (Rumore & Kim 2010) suggests that potential underlying mechanisms of salicylates on glucose metabolism may include the inhibition of NF-κB, a transcription factor that stimulates inflammatory responses associated with insulin resistance, and the increased expression of peroxisomal proliferator-activated receptor-γ and adiponectin, an adipokine with insulin-sensitizing effect that is often decreased in obesity (Lara-Castro et al. 2007). However, it should be considered that salicylates doses employed in trials exceed the quantity which can be obtained from diet alone (Duthie & Wood 2011) and that besides salicylates, other different phenolic compounds with recognised anti-inflammatory and redox-related bioactivity are widely distributed in the plant kingdom (Duthie & Wood 2011).

As a conclusion, results obtained in this pilot study indicate that obese children have lower levels of serum SA than normal-weight children as well as lower estimated salicylate intake from FV. FV consumption was not different between groups, and was lower in all children compared to WHO recommendation. Therefore, it can be speculated that the absence of an association of serum SA with FV may be due to the lower intake of FV, also compared to that observed in Spadafranca et al. (2007), as well as to specific FV choices. Moreover this result could also be due to the small sample size, which is one of the main limitations of the study. Another limitation that should be considered is that dietary intake was assessed by a 7-day dietary record. This methodology cannot exclude the "Hawthorne effect", especially in obese children and their parents, thus probably lowering the inter-individual variability of FV intake.

Nutrition education of both children and parents towards an adequate FV consumption should be stressed in children. Additionally, the promotion of FV with higher content of SA might be considered as part of the nutrition counselling for obese children, taking into account individual dietary habits and health conditions, such as food allergy or intolerance (Skypala et al. 2015). Further larger studies with appropriate power calculation of sample size are needed in the paediatric age to confirm these results and to better evaluate the role of FV on serum SA levels, taking into account both the quantity and the type of FV intake.

Disclosure statement

The authors report no conflicts of interest.

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6. CONCLUSION

Unhealthy dietary patterns and sedentary behaviors are major determinants of childhood obesity, one of the most serious global public health challenges. The great problem of childhood obesity epidemic is that it may be associated with adverse health complications and an increased risk of premature morbidity and mortality later in life. Therefore, addressing the alarming rates of obesity and noncommunicable diseases is a major priority.

Our results confirm that obesity is associated with detrimental effects on health already during pediatric age, thus children may show prehypertension/hypertension, insulin resistance, pre-diabetes, hyperlipidemia, liver steatosis and metabolic syndrome.

Moreover, our findings suggest that childhood obesity may be associated with changes of some core microbial species, preexisting or diet-induced, and these changes may be involved in the etiology of obesity. Indeed, an alteration of the gut microbiota composition of obese children, characterized by an increased abundance of Firmicutes and a decreased abundance of Bacteroidetes, has been observed. It could be hypothesized that an aberrant gut microbiota composition and activity might contribute to the development of childhood obesity, although to date it is not possible to determine if it is a causal factor or a consequence of unbalanced dietary patterns.

It is well-known from the literature that the adherence to a Mediterranean pattern has beneficial effects on health in adults. Fruit and vegetables, widely recommended for their health-promoting properties as important sources of dietary fiber, vitamins, minerals and phytochemicals, are at the base of the Mediterranean diet pyramid. However, results from our study show that fruit and vegetables consumption in children is very low, about 50% lower of the minimum recommended value by WHO (400 g daily). Furthermore, obese children have lower levels of serum salicylic acid, a phenolic compound produced by plants and thus found also in fruit and vegetables, than normal-weight children. This result may have a relevant role since, although further studies are needed, it has been suggested that beneficial effects associated with fruit and vegetables could be due, at least in part, to salicylate intake. Therefore, results from our study suggest that nutrition education towards an adequate fruit and vegetables consumption should be stressed in children. Moreover, the promotion of fruit and vegetables with higher content of salicylic acid might be considered as part of the nutrition counseling for obese children.

Finally, findings from our longitudinal study clearly highlight the importance of a lifestyle intervention, based on a normocaloric Mediterranean balanced diet for pediatric age, promotion of physical activity and behavior changes, in the improvement of cardio-metabolic risk factors and in the reduction of prevalence of some obesity-related comorbidities, as insulin resistance, pre-diabetes, prehypertension/hypertension, hypertriglyceridemia, higher liver echogenicity and metabolic syndrome. Although further well-design trials are desirable, we can suppose that the developed Mediterranean diet pyramid for the pediatric age provides a useful instrument for a food-based approach in the treatment of childhood obesity and related comorbidities.

ANNEX - LIST OF PAPERS AND ABSTRACTS

International publications

- Borgo F, Verduci E, Riva A, **Lassandro C**, Riva E, Morace G, Borghi E. Relative Abundance in Bacterial and Fungal Gut Microbes in Obese Children: A Case Control Study. Child Obes. 2017;13:78-84.

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populations. "XXIII Congresso Nazionale SIGENP". 29 September - 1 October 2016, Milan (accepted as poster).

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➢ Verduci E, **Lassandro C**, Mariani B, Brambilla M, Radaelli G, Banderali G. Circulating salicylic acid in obese children: association with fruit and vegetable consumption. 56TH ESPR/ESN Annual Meeting - 5th International Congress of UENPS, September 15-20 2015, Budapest.

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