Fecal microbiota signatures of insulin resistance, inflammation and metabolic syndrome in youth with obesity. A pilot study.

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Abstract

Background: Growing evidences show a relation between gut microbiota, metabolic syndrome (MS) and other risk factors of cardiovascular disease. We investigated fecal microbiota profiles associated with metabolic abnormalities belonging to the MS, high count of white blood cells (WBCs) and insulin resistance (IR).

Methods: Sixty-eight young patients with obesity were stratified for percentile distribution of MS abnormalities. A MS risk score was defined as low, medium and high MS risk. High WBCs were defined as a count ≥7.0 10^3/μL; severe obesity as body mass index Z-score ≥2 standard deviations; IR as homeostatic assessment model algorithm of IR (HOMA) ≥3.7. Stool samples were analyzed by 16S rRNA-based metagenomics.

Results: We found reduced bacterial richness of fecal microbiota in patients with IR and high diastolic blood pressure (BP). Distinct microbial markers were associated to high BP (*Clostridium* and Clostridiaceae), low high-density lipoprotein cholesterol (Lachnospiraceae, Gemellaceae, *Turicibacter*), and high MS risk (Coriobacteriaceae), WBCs (*Bacteroides caccae*, Gemellaceae), severe obesity (Lachnospiraceae), and impaired glucose tolerance (*Bacteroides ovatus* and Enterobacteriaceae). Conversely, taxa such as *Faecalibacterium prausnitzii*, *Parabacterodes*, *Bacteroides caccae*, *Oscillospira*, *Parabacterodes distasonis*, *Coprococcus* and *Haemophilus parainfluenzae* were associated to low MS risk score, triglycerides, fasting glucose and HOMA-IR, respectively.

Conclusions: This was a proof-of-concept study opening the way at the identification of fecal microbiota signatures, precisely associated with cardio-metabolic risk factors in young patients with obesity. These evidences led us to infer that while some gut bacteria have a detrimental role in exacerbating metabolic risk factors some others are beneficial ameliorating cardiovascular host health.

Background

Prevalence of overweight and obesity in childhood has substantially increased worldwide in recent decades with children becoming obese at progressively younger ages [1].

Obesity in children carries a wide range of serious complications, such as high blood pressure (BP) and triglycerides, low high-density lipoprotein cholesterol (HDL-C) and impaired glucose metabolism. All these metabolic abnormalities stem from the enhanced insulin resistance (IR) [2], that is deemed as a condition of systemic low-grade inflammation and is associated with all the metabolic alterations that belong to the metabolic syndrome (MS) [3]. IR as well as these abnormalities contribute to early atherosclerosis, anticipated incidence of cardiovascular disease (CVD) and type 2 diabetes (T2D) in young adulthood [4]. Identification and timely treatment of youths with increased risk of CVD and T2D is thus becoming a priority for the health care systems worldwide [3].

Robust epidemiological and molecular investigations demonstrate that gut dysbiosis contributes significantly to increase the risk of these disease conditions throughout different mechanisms (*i.e.* release of byproducts that trigger innate immunity, enhanced inflammation and IR, and impaired energy metabolism) [5, 6]. The gut microbiota is virtually an endocrine organ, arguably the largest, capable of contributing and reacting to circulating signaling molecules within the host [7]. Gut microbiota produces a large number of small metabolites through primary or secondary metabolic pathways that can have either pro- or anti-inflammatory properties in their own, *i.e.* bile acids [8], short chain fatty acids [5], trimethylamine oxide [9], endotoxins [5] and bacterial peptidoglycan [10].

The purpose of the present study was to identify fecal microbiota signatures that are specifically associated with IR, low-grade inflammation and CVD risk factors belonging to the MS. To accomplish the aim, we scored the CVD risk based on the distribution of metabolic variables belonging to the syndrome, WBCs and values of homeostatic assessment model algorithm of insulin resistance (HOMA-IR) in the studied population. Hence, we investigated the fecal microbiota profiles in relation to these features.

Methods

Subject recruitment

One hundred and twenty children and adolescents were consecutively enrolled among those referred for overweight or obesity by general paediatricians to the Unit for Multifactorial Diseases and Complex Phenotypes at the "Bambino Gesù" Hospital (OPBG) between October 2013 and December 2015. Patients were invited to participate to the MD-PAEDIGREE study (Model-Driven European Paediatric Digital Repository; http://www.md-paedigree.eu; 7th Framework Programme EU GA no 600932). Primary aim of the study was deep phenotyping of young patients with obesity in order to provide medical professionals decision support wherever they treat young patients with obesity. As ancillary analysis, a cross-sectional association between fecal microbiota profiles and CVD risk factors was investigated in 68 patients from the MD-PAEDIGREE study population.

Inclusion criteria were age ranging from 9 to 18 years; overweight or obesity; no previous treatment for obesity; no systemic and endocrine disease; no use of medication, alcohol or recreational drug.

The study was approved by the OPBG ethical committee (protocol #615/2013) and was conducted in accordance with the Principles of Good Clinical Practice and the Declaration of Helsinki. Written informed consent was obtained from all participants.

Body weight and height, were measured according to standardized procedures. The BMI was calculated as weight (kilograms) divided by height (meters) squared. Classifications of normal weight, overweight, and obesity were defined according to the International Obesity Task Force criteria [11]. Systolic (SBP) and diastolic blood pressure (DBP) were measured on the right arm with the participant seated using an automated oscillatory system and appropriately sized arm cuffs (Dinamap; Criticon Inc) [12]. The mean of 3 BP measurements was used.

A standard oral glucose tolerance test (1.75 g of glucose per kilogram of body weight up to a maximum of 75 g) was performed with measurements of fasting and 2 hour plasma glucose (2HPG).

Additionally, 38 healthy children and adolescents (CTRLs) were recruited between July and December 2013 among the research staff offspring who had participated in the six month before (January to June 2013) to the “Bambino Gesù study: Profiling the genetic risk of complex diseases in the Italian population”. The primary aim of the study was to dissect the genetic architecture of glucose homeostasis in the Italian children and adolescents. At the time of the Bambino study, BP, lipid profile, liver function tests, WBCs, high sensitivity reactive C protein, fasting glucose and insulin were all tested in the study participants [13]. Those participants, who were offspring of the research staff, were recalled having normal BMI, BP values and laboratory parameters, and asked to provide stool samples between July and December. Blood tests were not repeated since normal at the previous visit while anthropometrics and blood pressure values were annotated.

Laboratory Evaluation

Fasting plasma and serum were collected by centrifugation at 3000 rpm for 15 min. Glucose was measured by the glucose oxidase technique (Cobas Integra; Roche); insulin and C-peptide by chemiluminometric immunoassays (DiaSorin Liaison Analyzer, DiaSorin, Saluggia, Italy); triglycerides, total and HDL-cholesterol, levels using colorimetric kits (modular systems P/S Can 433; Roche/Hitachi); alanine aminotransferase (ALT), aspartate aminotransferase (ASP), γ-glutamyltransferase (γ-GT) using a radioimmunoassay method (ADVIA 1650; Bayer Diagnostics). Hemoglobin A1c (HbA1c) was measured by high-performance liquid chromatography with the use of a fully automated glycated hemoglobin analyzer system (Hitachi L-9100, Hitachi-Merck, Rahway, NJ). WBCs were counted by an automated blood cell counter (Cell-Dyn 3500; Abbott Core Laboratories, Abbott Park, IL).

Metabolic syndrome risk score

For the computation of the MS risk score, we considered as low HDL-C cholesterol an HDL value ≤ 10th percentile, and as high fasting glucose, triglycerides, and blood pressure (either systolic or diastolic) values ≥ 90th percentile for age and sex in the studied population. A MS score was computed as described elsewhere [14, 15] as the sum of the above metabolic abnormalities (0 if absent, 1 present). Therefore, the total metabolic score could range from 0 to 5. The study population was then stratified according to the MS score; patients with score 0 were considered at low risk, those having 1 or 2 risk factors at medium risk and those with ≥ 3 risk factors as at high risk.

The HOMA-IR was calculated using the mean of 3 fasting glucose and insulin values using the HOMA calculator provided by the University of Oxford [16] (https://www.dtu.ox.ac.uk/homacalculator/). IR was defined based on the median distribution of the HOMA-IR index in the population. IR patients were those with HOMA-IR ≥ 3.7.

Severe obesity was defined as a BMI Z-score ≥ 2 SDS.

Patients were defined at risk of low-grade inflammation when the WBC count was ≥ 7.0 10^3/μL, the median value in the population, and at low risk when the WBC count was below [17]. Impaired glucose tolerance (IGT) was defined as 2HPG ≥ 140 mg/dl.

Stool collection and DNA extraction

Fresh stool samples were collected by the participants at home and were immediately frozen in their home freezer (approx. −20 °C). Frozen samples were transported in a refrigerated box to the Biobanking and BioMolecular Resources Research Infrastructure_Microbiome Biobank at the OPBG Human Microbiome Unit and stored at -80 °C, until DNA extraction.

Total fecal genomic DNA was extracted from 200 mg using QIAamp Fast DNA stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The V1-V3 regions of the 16S ribosomal RNA (rRNA) gene were amplified by PCR. The amplicons (520 bp) were sequenced on a 454-Junior Genome Sequencer (Roche 454 Life Sciences, Branford, USA), according to the pipeline described in Del Chierico et al., 2017 [18]. Primers (FW: 5'-GAGTTTGATCNTGGCTCAG-3'; RV 5'-GTNTTACNGCGGCKGCTG-3') were barcoded by 8 unique nucleotide sequences (Roche 454 Life Sciences, Branford, USA). The polymerase chain reactions were performed using a Hi-Fi PCR Taq polymerase (FastStart™ High Fidelity PCR System, dNTPack, Roche Diagnostics, Mannheim, Germany) [18].

Sequencing reads and the associated metadata are available at BioProject database of NCBI (PRJNA356507 and PRJNA280490) (https://www.ncbi.nlm.nih.gov/bioproject/).

Microbiota Biocomputational and statistical analyses

Quantitative Insights Into Microbial Ecology (QIIME) suite of tools, v1.8 [19] was used for analyzing raw reads and quality filtering, read length and chimera presence were included. High-quality sequences were clustered into Operational Taxonomic Units (OTUs) at 97% threshold. Representative OTU sequences were aligned using the PyNAST algorithm v.0.1 [20] against the Greengenes 13_08 database with a 97% similarity for bacterial sequences, and a taxonomic tree was constructed via the NAST (Nearest Alignment Space Termination) algorithm [21]. The relative abundance of taxa was computed using QIIME pipeline, v1.8. The α- and β-diversity, β-diversity PERMANOVA, α-diversity Monte Carlo analysis were carried out by QIIME software.

Shapiro-Wilk test was applied to test distribution of the clinical features. Statistical descriptive tests, Mann-Whitney U test and Spearman's coefficient analysis were performed by SPSS software v. 20 (IBM).

Linear discriminant analysis effect size (LEfSe) analysis was used to identify microbiota biomarkers. This analysis couples Kruskall-Wallis test for all metagenomic variables; Wilcoxon test and Linear Discriminant Analysis (LDA) model. The analysis was performed with α value equal to 0.05 and logarithmic LDA score threshold of 2.0 [22].

Results

This study included 68 children and adolescents with obesity aged 9 to 18 years-old (44 males, 64.7%) and 38 normal-weight healthy subjects aged 9 to 16 years-old (15 males, 39.5%).

The median values of the clinical and anthropometric features are reported in Table 1.

Table 1
Median values and interquartile range (IQR) of anthropometric and clinical characteristics of the population[1].

CTRL features	Median	25th	75th	IQR
Age (years)	11.0	10.0	13.0	3.0
Weight (kg)	38.5	33.5	42.8	9.2
Height (m)	142.3	138.1	146.5	8.4
BMI (kg/m^2)	18.7	17.6	19.6	2
BMI Z score (SDS)	0.4	0.1	0.5	0.4
obese features	**Median**	**25th**	**75th**	**IQR**
Age (years)	14.0	13.0	15.3	2.3
Weight (kg)	81.5	70.1	89.8	19.6
Height (m)	162.0	156.0	167.9	11.9
BMI (kg/m^2)	30.4	27.7	33.4	5.7
BMI Z score (SDS)	2.0	1.8	2.3	0.5
SBP (mmHg)	113.5	108.0	123.0	15.0
DBP (mmHg)	66.0	60.8	70.0	9.3
Fasting glucose (mg/dl)	84.0	79.0	90.0	11.0
2HPG (mg/dl)	103.0	91.0	115.0	24.0
Fasting insulin (mIU/mL)	18.0	13.6	24.8	11.2
HbA1c (mmol/mol)	34.0	32.0	35.3	3.3
Peptide C (ng/ml)	2.0	1.5	2.4	0.9
Total Cholesterol (mg/dl)	147.5	132.8	169.3	36.5
HDL-C (mg/dl)	43.0	37.0	47.3	10.3
Triglycerides (mg/dl)	89.0	59.3	120.3	61.0
ALT (IU/L)	24.5	18.0	29.0	11.0
AST (IU/L)	23.5	19.8	31.3	11.5

[1]Abbreviations: BMI, body mass index; SDS, standard deviation score; SBP, systolic blood pressures; DBP, diastolic blood pressures; 2HPG, 2 hour plasma glucose; HbA1c, Hemoglobin A1c; ALT, alanine aminotransferase; AST, aspartate aminotransferase, γ-GT, γ-glutamyl transferase; WBC count, white blood cell count; HOMA-IR, homeostasis model assessment; HDL-C, low high density lipoprotein-cholesterol.

CTRL features	Median	25th	75th	IQR
γ-GT (IU/L)	15.0	12.0	21.3	9.3
WBCs (10^3/μL)	7.0	6.0	7.9	1.9
HOMA-IR	3.7	2.8	4.9	2.1

[1]Abbreviations: BMI, body mass index; SDS, standard deviation score; SBP, systolic blood pressures; DBP, diastolic blood pressures; 2HPG, 2 hour plasma glucose; HbA1c, Hemoglobin A1c; ALT, alanine aminotransferase; AST, aspartate aminotransferase, γ-GT, γ-glutamyl transferase; WBC count, white blood cell count; HOMA-IR, homeostasis model assessment; HDL-C, low high density lipoprotein-cholesterol.

Table 2 shows the distribution of parameters used for computing the MS risk score; Table 3 reports combination of metabolic abnormalities included in the MS score and prevalence of other CVD risk factors.

Table 2

Distribution of metabolic abnormalities within the metabolic syndrome and number of cases[1].

Clinical parameters	Median value	Min value	Max value	Percentiles		Cases N (%)	
				10th	90th	≤ 10th	≥ 90th
Triglycerides (mg/dl)	89.0	23	224	45.0	155.4	-	7 (10.3%)
Fasting glucose (mg/dl)	84.	62	102	69.9	96.1	-	7 (10.3%)
HDL-C (mg/dl)	43.0	27	74	34.0	55.1	8 (11.8%)	-
SBP (mmHg)	113.5	96	152	102	130.1	-	8 (11.8%)
DBP (mmHg)	66.0	36	88	52.7	76.1	-	8 (11.8%)

[1]Abbreviations: SBP, systolic blood pressures; DBP, diastolic blood pressures; HDL-C, low high density lipoprotein-cholesterol.

Table 3

Metabolic syndrome risk scores and other risk factor threshold registered in the study group[1]

CVD risk factors		Cases N (%)
MS score	Low (0 abnormality)	15 (22)
	Medium (1–2 abnormalities)	39 (57.3%)
	High (3–5 abnormalities)	14 (20.6%)
Insulin resistance	HOMA-IR ≥ 3.7	34 (50.0%)
High WBCs	WBCs ≥ 7.0×10^3/μL	34 (50.0%)
Severe obesity	BMI z-score ≥ 2	35 (51.4%)
IGT	2HPG ≥ 140 mg/dl	5 (7.3%)

[1]**CVD, cardio-vascular disease; HOMA-IR, homeostatic assessment model algorithm–insulin resistance (median value); 2HPG, 2 hour plasma glucose; WBCs, white blood cells (median value); BMI, body mass index (median value); IGT, impaired glucose tolerance** [45].

Fecal microbiota profiles of obese and CTRL subjects

To exclude gender and age-related differences in fecal microbiota between CTRL subjects and participants with obesity, we compared fecal microbiota features of male and female participants aged below and above the median population age (13 years). Microbiota profiled were not different, proving that microbiota profiles were independent of age and gender (**Table S1**). Then, we performed an ecological analysis (Chao1 and observed species indexes) to assess the overall differences in the microbial community structures between patients with obesity and CTRLs. The Chao1 index gives more weight to the less abundant species (rare species), while the observed species index is the count of the unique OTUs found in each sample (**Figure S1**). For both, we found no statistically signifcant significance between CTRLs and participants with obesity.

Principal coordinate analysis (PCoA) based on weighted and unweighted UniFrac distances was performed to uncover differences in the structure of fecal microbiota across all the samples. This method is based on the phylogenetic distance of OTUs weighted or unweighted for OTUs relative abundance.

The analysis revealed a high distance between patients with obesity and CTRLs. Findings were confirmed by the succeeding PERMANOVA analysis (weighted $p = 0.002$; unweighted $p = 0.0001$) (**Figure S2**).

To identify the specific bacterial taxa associated with obesity, we compared the colonic microbiota of patients with obesity and CTRLs using LEfSe analysis **(Figure S3**). We found 6 OTUs abundant in patients with obesity, Clostridiaceae, *Dorea*, *Streptococcus*, *Blautia*, Erysipelotrichaceae and

Lachnospiraceae; and 7 OTUs in the CTRLs, Rikenellaceae, *Bacteroides*, Barnesiellaceae, *Oscillospira*, *Bacteroides ovatus*, *Parabacteroides* and Bacteroidaceae.

Metabolic abnormalities and fecal microbiota profiles in patients with obesity

The ecological analysis showed statistically significant differences in fecal microbiota richness of patients with IR (patients with HOMA-IR ≥ 3.7 *versus* those with HOMA-IR < 3.7; p = 0.015; Fig. 1A; p = 0.014 Fig. 1B) and high diastolic blood pressure (DBP) (patients with DBP ≥ 90th *versus* those with DBP < 90th percentile; p = 0.007, Fig. 1C; p = 0.042, Fig. 1D). No difference was found in association with other metabolic abnormalities.

The β-diversity analysis discriminated patients with severe obesity from those with non-severe obesity (unweighted p = 0.003; weighted p = 0.029); patients with high versus low HDL-C (weighted p = 0.013) and with MS risk score from low to high (unweighted p = 0.049) (Fig. 2, **Panels A and B**).

The patients' bacterial communities were analyzed in relation to any metabolic abnormality (Fig. 3). As reported in Fig. 3, *Clostridium* was significantly higher in patients with values of DBP > 90th. Bacteroidaceae was enriched in patients with SBP values < 90th and Clostridiaceae in patients with SBP values ≥ 90th. Lachnospiraceae, Gemellaceae and *Turicibacter* were associated to low HDL-C values, while *Bacteroides uniformis* to high HDL-C values. *Parabacteroides* and *Bacteroides caccae* were high in patients with low fasting glucose. *Faecalibacterium prausnitzii* was associated to low values of triglycerides.

Moreover, we evaluated the impact of MS risk scores and other risk factors on the fecal microbiota profiles of patients with obesity. Patients with high MS score were associated to high abundance of Coriobacteriaceae, while low MS score with high abundance of *Oscillospira* and *Parabacterodes distasonis. Coprococcus* and *Haemophilus parainfluenzae* were abundant in patients with HOMA-IR < 3.7. High WBC count was related to the increased presence of *Bacteroides caccae* and *Gemellaceae*, while low WBCs to that of Mogibacteriaceae.

Having a BMI Z-score ≥ 2 was associated to high levels of Lachnospiraceae while BMI Z-score < 2 to overrepresentation of *Bacteroides* and Bacteroidaceae. In patients with IGT, there were increased levels of *Bacteroides ovatus* and Enterobacteriaceae, while in patients with normal glucose tolerance (NGT) of *Clostridium* and Ruminococcaceae.

Correlation analysis between metabolic abnormalities and microbiota OTUs

We found significant correlations between BMI Z-score and OTUs, i.e. Actinobacteria (ρ = 0.39; p value = 0.001), Lachnospiraceae (ρ = 0.30; p value = 0.013), *Granulicatella* (ρ = 0.33; p value = 0.006), Bacteroidetes (ρ=-0.38; p value = 0.001), Bacteroidaceae (ρ=-0.30; p value = 0.011), and *Odoribacter* (ρ=-0.32; p value = 0.007). Significant correlations were also found between WBCs and Gemellaceae (ρ = 0.34; p value = 0.001); DBP and *Dialister* (ρ = 0.30; p value = 0.013); HDL-C and *Clostridium* (ρ = 0.34; p value = 0.024), *Turicibacter* (ρ=-0.30; p value = 0.012) and Gemellaceae (ρ=-0.40; p value = 0.001); HOMA-

IR and Clostridiaceae (ρ=-0.30; p value = 0.011), *Coprococcus* (ρ=-0.33; p value = 0.006) and *Haemophilus parainfluenzae* (ρ=-0.37; p value = 0.002); IGT and *Clostridium* (ρ=-0.35; p value = 0.001) (**Figure S4**).

Discussion

This is the first investigation on fecal microbiota profiles that are specifically associated with cardio-metabolic abnormalities belonging to MS, IR and low-grade inflammation in patients with obesity. On the other hand, we confirmed differences in fecal bacterial richness and composition between young individuals with obesity and healthy age-matched normal-weight subjects. Indeed, we found increased representation of some microbial markers, i.e. *Streptococcus* and Lachnospiraceae, and reduced one of some others, i.e. *Bacteroides* spp., Barnesiellaceae and *Oscillospira* in the group of individuals with obesity as compared to controls [23].

With regard to signatures that were specific of each metabolic abnormality, we found a reduced bacterial richness, that is characteristic of many inflammatory conditions [24, 25], prevalently in patients with more severe IR but also higher DBP suggesting that the close pathogenic link between IR and hypertension can be mediated by the gut microbiota dysbiosis and passes through a condition of enhanced inflammation [5, 26, 27].

We found some microbial taxa that inhabit the gut ecosystem with low abundance (*i.e.* the Gemellaceae) to be associated with metabolic abnormalities. In general, few microbial taxa dominate almost all types of human-associated samples, while the majority of them are present in low abundance and represent a kind of “rare biosphere” [28]. Nevertheless, in our sample rare taxa seemed to contribute significantly to the diversity of the host’s microbiome and hence to the health to disease balance.

We found significant associations of microbial taxa with metabolic abnormalities. In brief, we found an increase of Clostridiaceae and *Clostridium* in patients with high blood pressure as already seen in the CARDIA (Coronary Artery Risk Development in Young Adults) study [26]. Nonetheless, in our series, *Clostridium* was more abundant also in NGT patients as compared with those with IGT, suggesting a not clear role in the obesity context.

We found also an over representation of Lachnospiraceae, Gemellaceae and *Turicibacter* in patients with low-levels of HDL-C. A previous study in individuals either with normal or high cholesterol found a statistically significant correlation between *Turicibacter* and low levels of HDL-C [29]. Gemellaceae were also associated with high count of WBCs. The Gemellaceae family is present in the gut microbiota with a low relative abundance in different disease conditions characterized by an inflammatory status [30, 31].

The increased representation of Lachnospiraceae in patients with low-levels of HDL-C and severe obesity confirmed the close connection of this taxa with severe obesity and altered lipid metabolism [32].

Parabacteroides and *Bacteroides caccae* were highly represented in patients with low levels of fasting glucose. The treatment of obese high-fat diet (HFD)-fed mice with *P. distasonis* was effective to reduce

weight gain, hyperglycemia, and hepatic steatosis in the animals owing likely to the dramatically altered bile acid profile and increased gut levels of succinate [33]. As to the *B. caccae*, it seems playing a dualistic role in the gut being a beneficial inhabitant or an opportunistic pathogen. Indeed, it was found in the gut microbiota of normal weight adolescents [23] as well as in patients with inflammation as appraised by high WBC count [34, 35].

Furthermore, the *P. distasonis* together with the *Oscillospira* was associated with a low MS risk score, suggesting the former may serve as marker of a relatively healthy metabolic profile of obesity. *Oscillospira* has also been related to a healthy metabolic profile consisting of high HDL-cholesterol and low systolic and diastolic blood pressure, fasting glucose and triglycerides in large cohort study [36].

On the contrary, the Coriobacteriaceae were associated with a high risk score for the metabolic syndrome. In a murine model of streptozocin-induced diabetes, a study demonstrated a beneficial effect of Coriobacteriaceae in the amelioration of the glucose metabolism following the Roux-en-Y gastric bypass. [37].

Coprococcus and *H. parainfluenzae* were more abundant in patients with low IR. In literature, it has been reported that representation of both bacteria is associated with the amelioration of the IR in HFD-high sucrose mice [38] and the glucose tolerance status in humans [39].

There was also an abundance of *Bacteroides ovatus* and Enterobacteriaceae in children with IGT. The Enterobacteriaceae is a family belonging to the phylum Proteobacteria that has been found over represented, with an undoubted causative role, in patients T2D [40, 41]. Its abundance seems depending on the diet. Indeed, Proteobacteria were found increased in European children who consumed a calorie-dense, high-fat, low-fiber diet as compared with children from Burkina Faso who were low-fat, high-fiber consumers [42]. Furthermore, the representation of a specific Proteobacteria, the Betaproteobacteria, was found positively correlated with plasma glucose levels in adults with different degree of glucose tolerance [40].

In our series, low levels of triglycerides were associated to the presence of *F. prausnitzii* that is regarded as marker of a healthy gut. A study on HFD fed mice demonstrated the significant reduction of triglycerides in mice following the oral administration of *F. prausnitzii* [43].

We are aware of bias in the present investigation: small-size and metabolic heterogeneous sample of patients with obesity and normal-weight controls; cross-sectional design; no use of gold standard techniques to estimate IR; no estimation of microbiome metabolites that are known to cause inflammation, IR and CVD.

Nonetheless, the present study can be deemed as a pilot methodological investigation to open the way at the identification of specific fecal microbiota signatures of metabolic abnormalities even in the population of adolescents in which the diagnosis of MS is not univocally recognized. Indeed, in keeping with the recommendations of the European Childhood Obesity working group on MS [44], we categorized

our patients on percentile distributions of metabolic abnormalities. Drawbacks of this study could be overcome by a multi-center cohort investigation with characterization of host inflammation and host and microbiome metabolites.

In conclusion, in young individuals with obesity, we observed reduced heterogeneity in groups with higher propensity to the metabolic syndrome and pro-inflammatory conditions. We also identified single fecal taxa, in some cases rare taxa, that were relatively over abundant in relation to a specific metabolic abnormality, *i.e.* impaired glucose tolerance or high white blood cell count. On top, findings hint that microbiota signatures can be informative on the risk of incident metabolic syndrome.

Finally, our study provided insight to dissect phenotype heterogeneity of a complex disease and this methodological approach might be replicated in other multifactorial heterogeneous conditions, i.e. the autism spectrum disorder, in which microbiota plays a pivotal role.

Abbreviations

2HPG, 2 hour plasma glucose;

ALT, alanine aminotransferase;

AST, aspartate aminotransferase;

BMI, body mass index;

CVD, cardiovascular disease;

DBP, diastolic blood pressures;

HbA1c, Hemoglobin A1c;

HDL-C, high-density lipoprotein cholesterol;

HFD, high-fat diet

HOMA-IR, homeostatic assessment model algorithm of insulin resistance;

IGT, impaired glucose tolerance;

IQR, interquartile range;

IR, insulin resistance;

LDA, Linear Discriminant Analysis;

LEfSe, Linear discriminant analysis effect size;

MS, metabolic syndrome;

NAST, Nearest Alignment Space Termination;

NGT, normal glucose tolerance;

OPBG, "Bambino Gesù" Children's Hospital;

OTUs, Operational Taxonomic Units;

PCoA, Principal coordinate analysis;

QIIME, Quantitative Insights Into Microbial Ecology;

SBP, systolic blood pressures;

SDS, standard deviation score;

T2D, type 2 diabetes;

WBCs, white blood cells;

γ-GT, γ-glutamyl transferase;

Declarations

Ethics approval and consent to participate: The study was approved by the OPBG ethical committee (protocol #615/2013) and was conducted in accordance with the Principles of Good Clinical Practice and the Declaration of Helsinki. Written informed consent was obtained from all participants.

Consent for publication: Written informed consent was obtained from all participants.

Availability of data and materials: Sequencing reads and the associated metadata are available at BioProject database of NCBI (PRJNA356507 and PRJNA280490) (https://www.ncbi.nlm.nih.gov/bioproject/).

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Russo; Project administration, Marzia Bianchi; Resources, Danilo Fintini and Lorenza Putignani; Software, Valentina Tortosa and Andrea Quagliariello; Supervision, Lorenza Putignani; Writing – original draft, Federica Del Chierico and Melania Manco; Writing – review & editing, Federica Del Chierico, Melania Manco, Alessandra Russo, Marzia Bianchi, Valentina Tortosa, Andrea Quagliariello, Blegina Shashaj, Danilo Fintini and Lorenza Putignani. All authors had final approval of the submitted and published versions.

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Figures

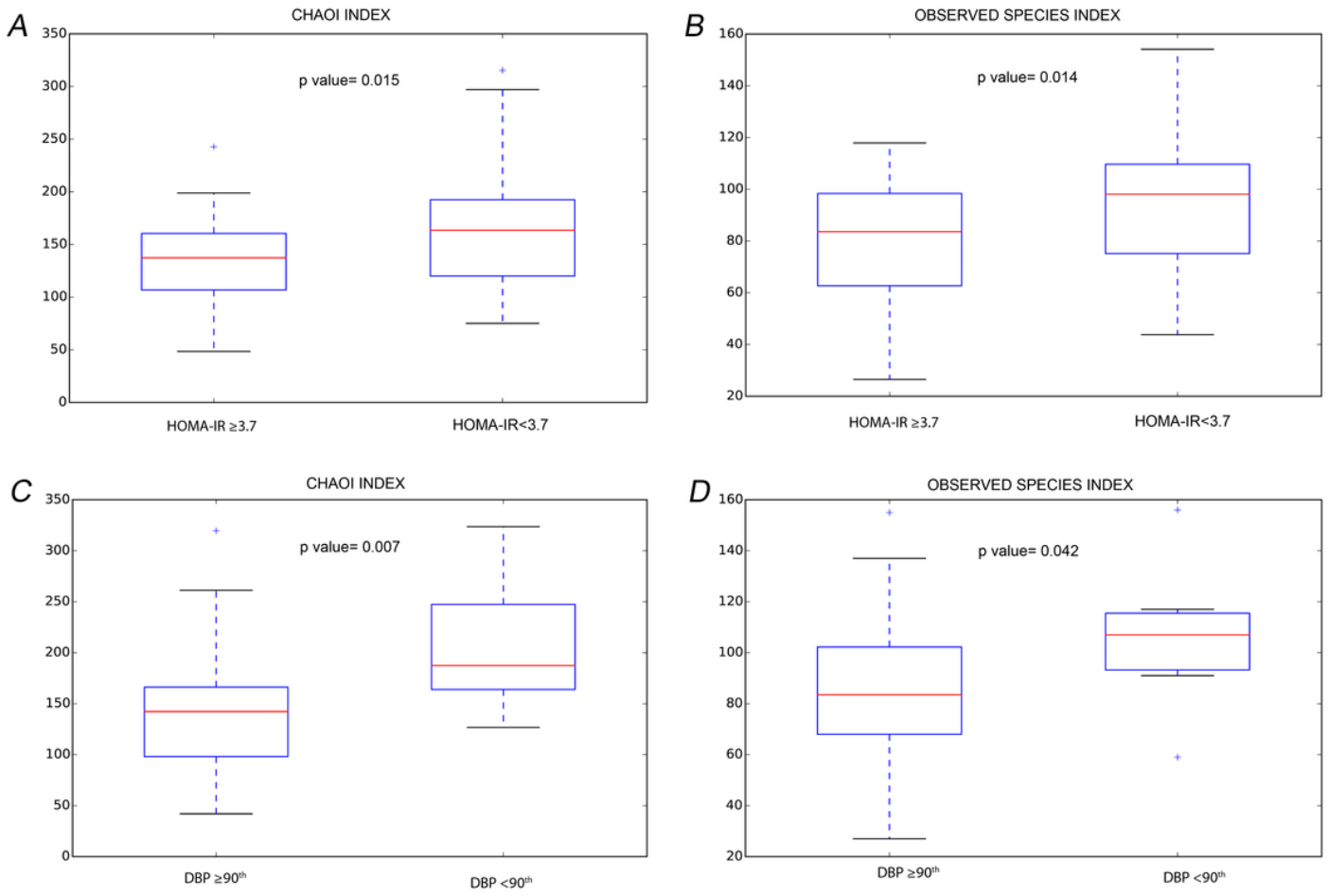

Figure 1

Boxplots representing α-diversity indices. Panel A and B represent the Chao1 and Observed species indexes for samples stratified for Homeostasis Model Assessment Algorithm of Insulin Resistance (HOMA-IR) ≥3.7 and HOMA-IR<3.7. Panel C and D represent the Chao1 and Observed species indexes for samples stratified for Diastolic Blood Pressure (DBP) values ≥90th and DPB<90th. The interquartile range is represented by the box and the line in the box is the median. The whiskers indicate the largest and the lowest data points, respectively, while the dots symbolize outliers.

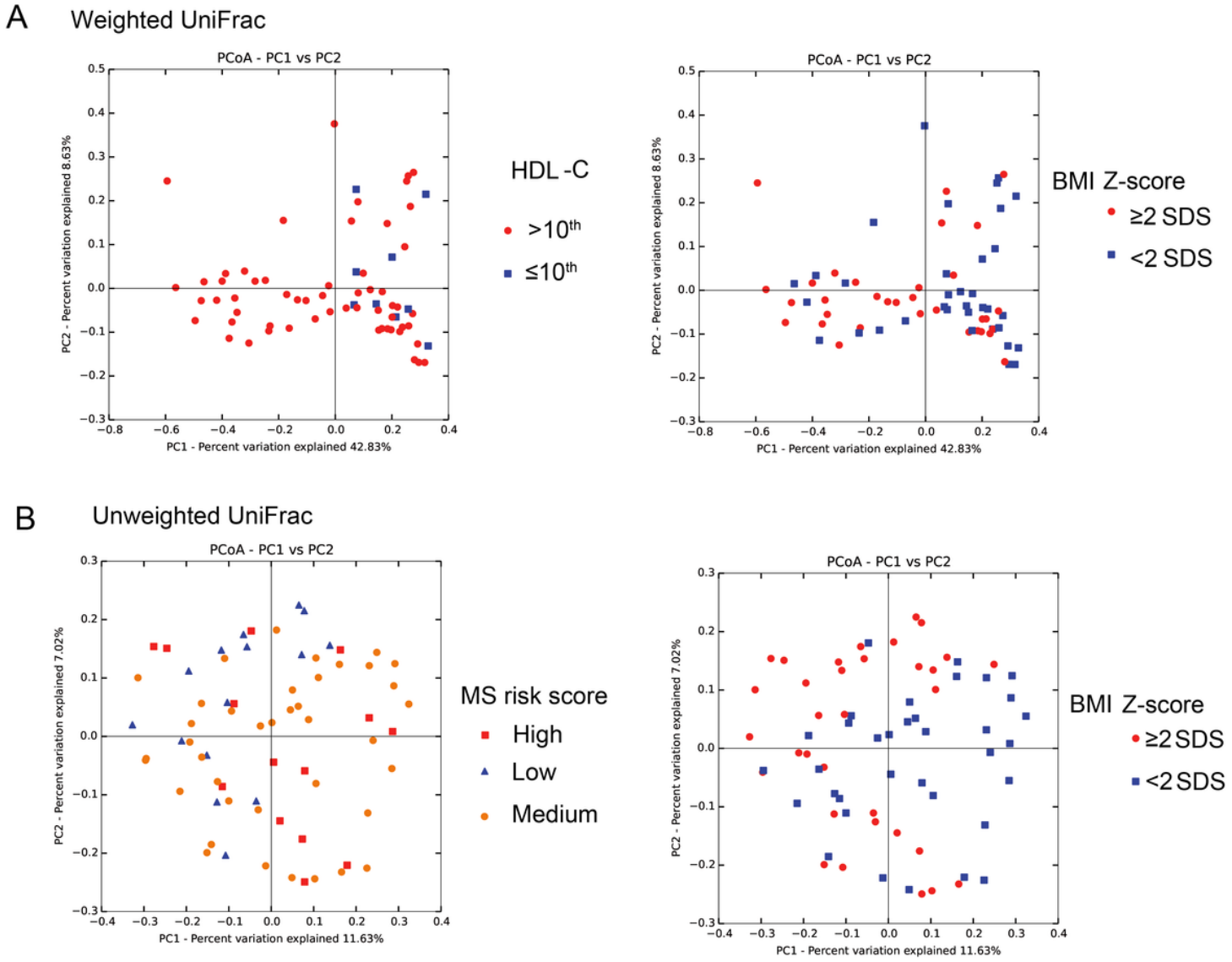

Figure 2

Principal component analysis plot (PCA) of the bacterial communities using UniFrac algorithm. Axes represent the first 2 components from principal coordinate (PCo) analysis based on the phylogenetic distance between Operational Taxonomic Units (OTU) representative sequences. Panel A, UniFrac weighted PCoA plots of High Density Lipoprotein (HDL-C) and Body Mass Index (BMI) Z-score groups. SDS, standard deviation score. Panel B, unweighted UniFrac PCoA plots of Metabolic Syndrome (MS) score and BMI Z-score groups.

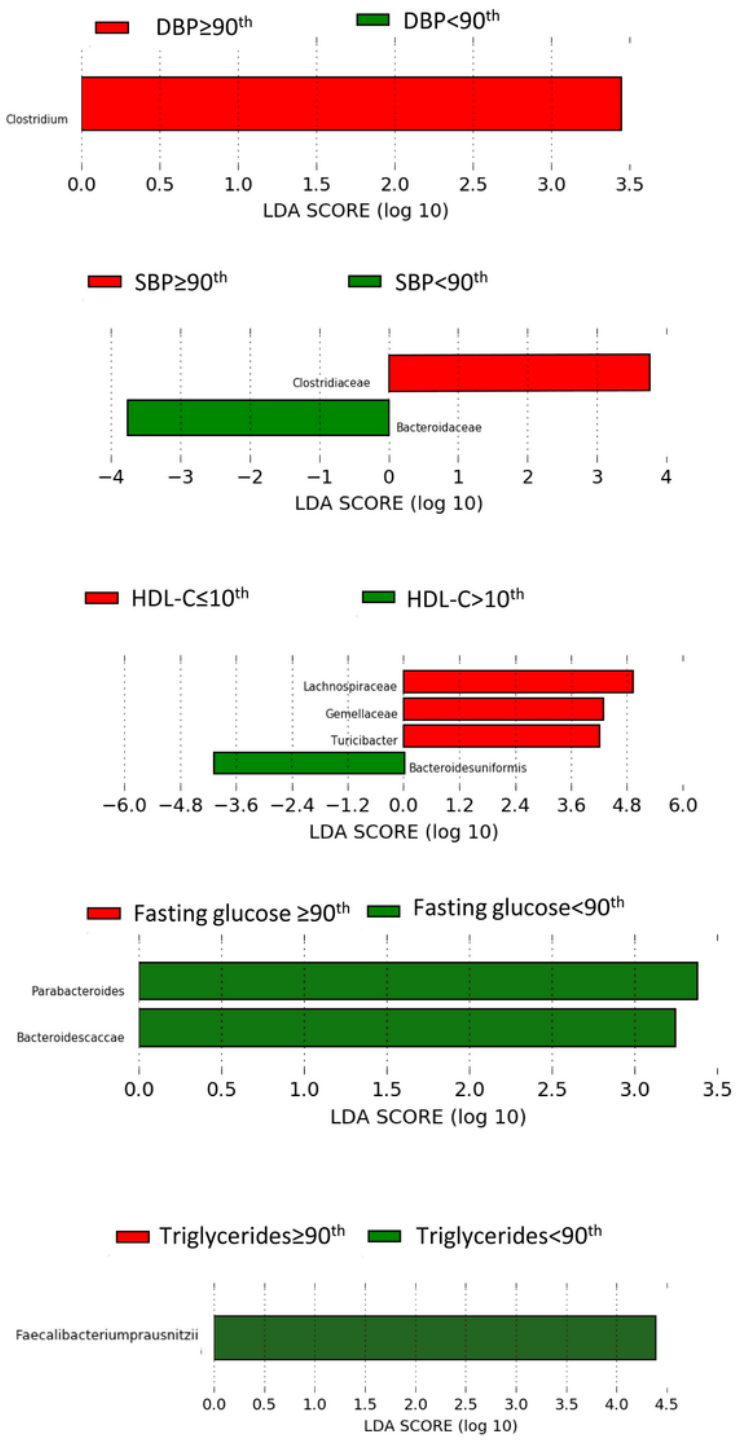

Figure 3

Linear discriminative analysis (LDA) effect size (LEfSe) analysis in relation to each metabolic abnormality. Analysis was performed grouping patients on the bases of 90th percentile distribution for diastolic blood pressure (DBP), systolic blood pressure (DBP), fasting glucose, and triglycerides and of 10th for high density lipoprotein cholesterol (HDL-C).

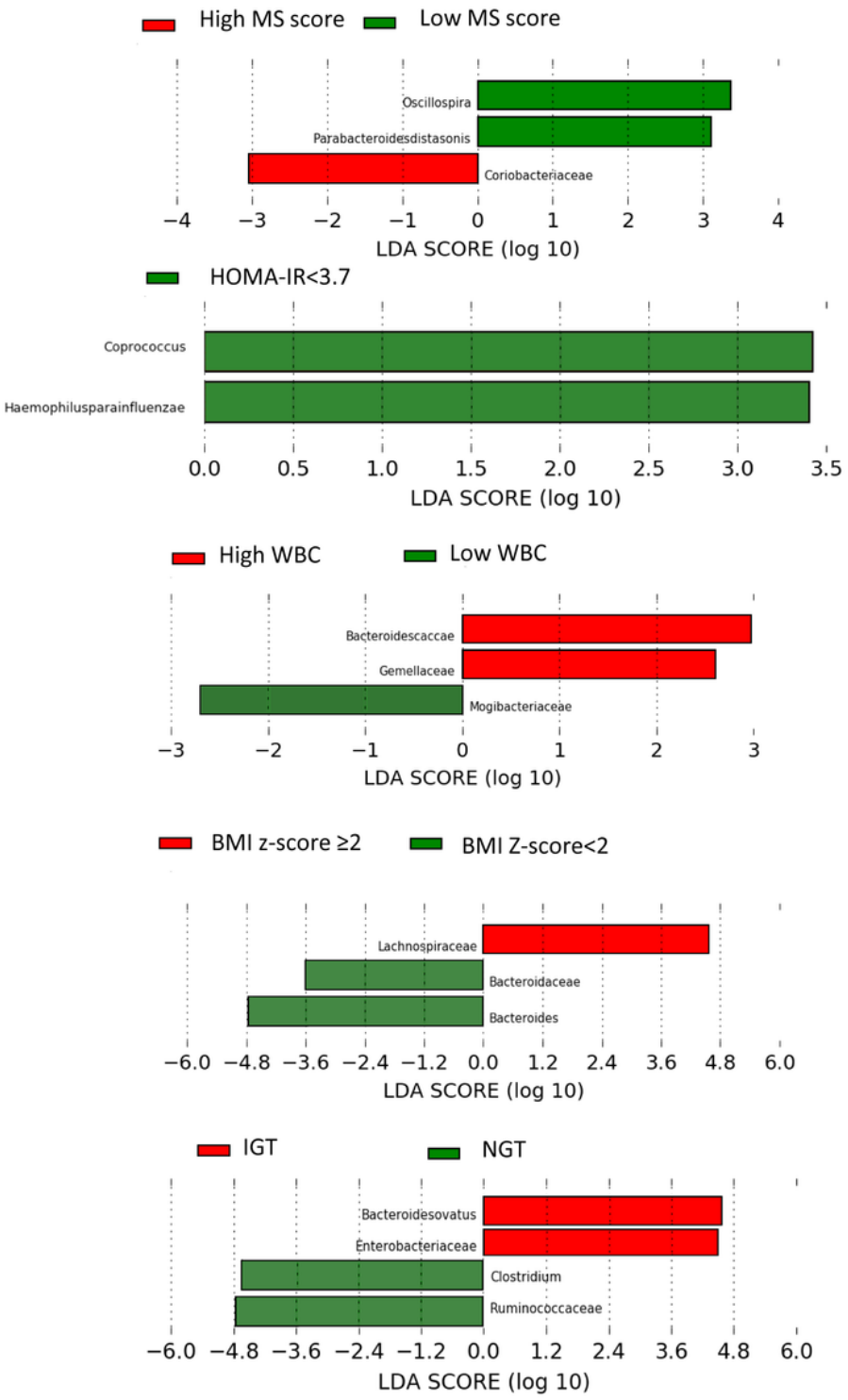

Figure 4

Linear discriminative analysis (LDA) effect size (LEfSe) analysis in relation to metabolic syndrome risk scores and other risk factors. Analysis was performed grouping patients as reported in Table 3.

Supplementary Files

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