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Intestinal Microbiota and Serum LPS Level and Correlation to Fatty Liver in Obese Egyptian Children.

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ABSTRACT

Changes of gut microbiota and inflammation and its role in the pathogenesis of obesity-related disorders are increasingly recognized. This study investigated the changes in gut bacteria and serum LPS Level associated with obesity. Also, is serum LPS level correlate to presence of fatty liver in obese children? Twenty five obese children randomly selected, and 25 children with normal weight serving as controls. Clinical and anthropometric evaluations were done. The Fatty liver quantifies visceral fat and subcutaneous fat were measured by ultrasonography. Quantification of Bacteroides, Enterobacteria, Bifidobacterium, and Lactobacillus in feces was done by real-time PCR. Serum LPS level was measured using ELISA. Insulin resistance was calculated by the homeostasis model. There were significant higher differences in obese children in HOMA-IR, serum LPS. Obesity in children was associated with a significant reduction in gut Bifidobacteria. Serum LPS, HOMA-IR. Frequency of junk food had significant positive correlation with BMI. Bifidobacterium had highly significant negative correlations with BMI. A significant negative correlation was detected between fatty liver and Bifidobacterium. Conclusion: Obesity causes significant alterations of the gut microbiota.

Keywords: LPS, Insulin Resistance, Obesity, Visceral Fat, Fatty liver

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INTRODUCTION

The human Intestinal microbiota is estimated to be composed of 10^{13} to 10^{14} microorganisms per gram of content [1]. The 1.5 kilograms of bacteria that we each carry in our intestines have an enormous impact on our health and well-being. The bacteria normally live in a sensitive equilibrium but if this equilibrium is disrupted our health could suffer [2].

The number of people suffering from obesity world-wide has risen rapidly in recent years [3]. Animal and human data demonstrate that phylogenic changes occur in the micro biota composition in obese individuals [4-6]. Furthermore, evidence from animal models suggests that the alterations of the gut microbiota with obesity results in increased intestinal permeability and metabolic endotoxemia. The relation between gut microbiota and inflammation and its role in the pathogenesis of obesity-related disorders are increasingly recognized. Animals' models of obesity detected a connection between an altered gut microbiota composition and the development of obesity, insulin resistance, and type 2 diabetes in the host. This connection is through several mechanisms: increased energy harvest from the diet, altered fatty acid metabolism and composition in adipose tissue and liver, modulation of gut peptide YY and glucagon-like peptide (GLP)-1 secretion, activation of the lipopolysaccharide toll-like receptor-4 axis, and modulation of intestinal barrier integrity by GLP-2[7,8].

Recent work has shown that gut bacteria can initiate the inflammatory state of obesity through the activity of lipopolysaccharide (LPS) [9]. The endogenous bacterial lipopolysaccharide (LPS) from the Gram-negative intestinal microbiota is continuously produced in the gut by the death of Gram-negative bacteria and physiologically translocate into intestinal capillaries through a TLR4-dependent mechanism [10] .Then it is transported from the intestine toward target tissues by a mechanism facilitated by lipoproteins [11]. It triggers the secretion of proinflammatory cytokines when it binds to the complex of mCD14 and the TLR4 at the surface of innate immune cells [12, 13].

Our aim is to detect the changes in gut bacteria and serum LPS Level associated with obesity. Also, is serum LPS level correlate to presence of fatty liver in obese children?

MATERIALS AND METHODS

Subjects

A total number of 50 children were recruited from NRC Pediatrics Clinic during the period between September 2012 and December 2013. They were 25 normal weight and 25 obese children, with age range from 10 years to 18 years old.

Children who had secondary obesity such as Cushing syndrome, children with obesity because of corticosteroid therapy or hypothyroidism or those with dysmorphic features suggestive of syndromes (e.g. Laurence–Moon–Biedl or Prader Willi), or received antibiotic therapy within the last two months were excluded. Informed consents were obtained from the parents of our study groups according to the guidelines of the ethical committee of the National Research Centre, Egypt.

Clinical Examination

- All the children in the study were subjected to the following: a full history taking including the complete present history, with particular emphasis on history of medication and antibiotic intake and clinical examinations, both general and systemic. Frequency of fast food intake/ week was evaluated in all children.
- Blood pressure measured according to American Heart Association guidelines.
- Anthropometric indices: Body weight measured to the nearest 0.1 kg with a balance scale and height measured to the nearest 0.1 cm. Body mass index was calculated as weight divided by height squared

(kg/m2). Waist circumference (WC) was measured at the level midway between the lowest rib margin and the iliac crest. Hip circumference (HIP C) was measured at the widest level over the greater trochanters in a standing position by the same examiner; then waist to hip ratio (WHR) and Waist to height ratio (WHTR) were calculated [14]. Percentage body fat was calculated by the equation:

Child Body Fat % = (1.51 × BMI) − (0.70 × Age) − (3.6 × gender) + 1.4 where (female = 0 and male = 1) [15].

Abdominal Ultrasonography

In addition to the routine abdominal ultrasound examination based on the clinical indication, ultrasonography (US) distinctively quantifies visceral fat and subcutaneous fat [16].

Laboratory Measurements

Blood samples

Five milliliters of blood was withdrawn from the anticubital vein after a fast of 12–14 h under aseptic conditions. Samples were labeled and left to clot at room temperature for 15 min then centrifuged, sera were collected and aliquated for evaluation of the following parameters and inflammatory markers:

- FBS and Lipid profile were assessed by an OLYMPUS AU 400 Chemistry Analyzer.
- Insulin and hsCRP levels were estimated by Enzyme immunoassay (ELISA).
- Insulin resistance was calculated by the homeostasis model (HOMA-IR) using the following formula: HOMA- IR = fasting insulin (mU/L) × fasting glucose (mmoL/L)/ 22.5 [17].
- Lipopolysaccharide (LPS) was performed using Enzyme immunoassay (ELISA) Glory Science Co., td TX78840, USA .

Bacteriology

Collection of human fecal samples.

Fecal samples were collected from all subjects. The samples are kept in in 10 ml of phosphate buffer (pH 7) and immediately frozen at – 80ºC.

Analysis of stool

Fecal sample preparation, bacterial cultivation and counting.

Fecal samples were homogenized and serially diluted in sterile anaerobic solution. Appropriate dilutions were incubated aerobically or anaerobically at 37°C in duplicate using selective media.

Bacterial strains and growth conditions

The bacterial strains used for standard curve: *Bifidobacterium longum* subsp. *infantis* (ATCC 15697), *Enterobacter cloacae* subsp. *cloacae* (ATCC 29005)**,** *Bacteroides fragilis* BAA (ATCC 2283), and *Lactobacillus gasseri* (ATCC 33323) were purchased from the American Type Collection Culture (ATCC). Bacteria were cultured aerobically or anaerobically on selective broth as recommended by ATCC. The cultivation in anaerobic conditions was done using the anaerobic jar with AnaeroGen (Oxoid). For each culture, the total number of bacteria, in terms of CFU, was determined by plating.

DNA extraction

Genomic DNA was extracted from pure cultures by the use of MiniPrepDNA extraction kit (Fermentas Life Sciences, Vilnius, Lithuania) according to the manufacturer's instructions with addition of lysozyme for gram positive bacteria. Total DNA was extracted from stool by the use of a Qiagen MiniPrep Stool DNA extraction kit according to the manufacturer's instructions with slight modification. The samples were resuspended in ASL buffer and subjected to 95°C for 10 min. The DNA was then quantified using a spectrophotometer and stored at -20°C.

Primers and probes for quantification bacterial groups

TaqMan probe quantitative PCR was used to quantify Bifidobacterium species. Quantitative PCR using SYBR-Greens was performed for the Bacteroides/Prevotella group Enterococcus group, and for the Lactobacillus/Leuconostoc/Pediococcus group. Primers and probe used in this study (Table 1) were designed based on 16S rRNA gene sequences (EMBL database) aligned with the program CLUSTAL W [18].

Table 1: Group and species-specific 16S rRNA gene-targeted primers and probe. (Probe sequences are in bold).

Target microorganism	Primer / probe	Reference
Bifidobacterium	F_Bifid 09: CGG GTG AGT AAT GCG TGA CC	Furet et al. (2009) [19]
	R_Bifid 06: TGA TAG GAC GCG ACC CCA	
	P_Bifid: 6FAM-CTC CTG GAA ACG GGT G	
Bacteroides/ Prevotella	F_Bacter 11: CCT WCG ATG GAT AGG GGT T	Furet et al. (2009) [19]
	R_Bacter 08: CAC GCT ACT TGG CTG GTT CAG	
Lactobacillus/ Leuconostoc/ Pediococcus	F_Lacto 05: AGC AGT AGG GAA TCT TCC A	Furet et al. (2009) [19]
	R_Lacto 04: CGC CAC TGG TGT TCY TCC ATA TA	
Enterococcus	F_Entero: CCC TTA TTG TTA GTT GCC ATC ATT	Rinttila¨ et al. (2004) [20]
	R_Entero: ACT CGT TGT ACT TCC CAT TGT	

Standard curves and quantification

Seven 10-fold dilutions with known cell numbers (ranging from 10^2 to 10^9 CFU/ml) of each microorganism were used to extract DNA. These DNAs were then used as templates in real-time PCR to generate standard curves for quantification of the target groups: *Bacteroides*, *Enterococcus*, *Bfidobacteria*, and *Lactobacillus*. Standard curves were generated by plotting threshold cycles (Cq) vs. bacterial quantity (CFU). The total number of bacteria (CFU) was interpolated from the averaged standard curves. When PCR was performed on unknown faecal samples, we used these standard curves to quantify each bacterial population.

Real-time PCR.

Real-time qPCR was performed using Thermo Scientific PikoReal real-time PCR machine 24-well block format. Each reaction was carried out in duplicate in a volume of 25 µl with 0.2 mM final concentration of each primer, 0.25 mM final concentration of each probe and 10 mL of appropriate dilutions of DNA samples. The detection was carried out using DyNAmo™ Flash Probe qPCR Kit or DyNAmo™ ColorFlash SYBR® Green qPCR 2X master mix (Thermo scientific). Amplifications were carried out using the following ramping profile: one cycle at 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, and 60 °C for 1 min. SYBR-Greens reactions ended with melting step 60-98 °C. The *Cq* (cycle at which the signal crosses a threshold) values were plotted as a linear function of the base 10 logarithm of the number of respective bacterial cells in the culture as determined by plate counts. The standard curves were then used to quantify the fecal samples with unknown cell concentrations collected during the study.

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Statistical Analysis

Each variable was assessed for a normal distribution. The standard computer program SPSS for Windows, release 12.0 (SPSS Inc., USA) was used for data entry and analysis. All numeric variables were expressed as mean±SEM. Comparison of different variables in various groups were carried out using the Student t-test and the Mann–Whitney U-test for normal and nonparametric variables, respectively. Pearson's and Spearman's correlation tests (r=correlation coefficient) were used for correlating normal and nonparametric variables, respectively. For all tests, a probability (P) less than 0.05 (<0.05) is considered significant.

RESULTS

Demographic Data of Patients and Controls

The study included 50 Egyptian children (25 were obese, and 25 were of normal weight), 55.6 % females and 44.4 % males. Their mean age was 5.57 ± 2.9 years.

Comparison between anthropometric measurements and clinical data in obese children and normal weight-matched controls

The clinical characteristics and comparison between obese and normal weight children are provided in Table 2. A significant higher difference was observed in BMI, WHTR, Percentage of body fat and Frequency of junk food (P = 000). However, there were insignificant sex differences in the age, waist/hip ratio, systolic and diastolic blood pressures.

Table 2: Anthropometric measurements and clinical data of obese children and controls

	1=non obese 2=obese	N	Mean	Std. Deviation	Sig. (2-tailed)
AGE (years)	1	25	12.46	3.86	NS
	2	25	13.88	2.76	
BMI	1	25	22.32	2.45	.000**
	2	25	33.71	4.16	
WHTR	1	25	.50	.074	.000**
	2	25	.61	.04	
WHR	1	25	.87	.08	NS
	2	25	.87	.045	
Percentage of body fat	1	25	27.70	3.98	.000**
	2	25	41.46	5.71	
Systolic BP mmHg	1	25	102.75	10.32	NS
	2	25	106.66	13.96	
Diastolic BP mmHg	1	25	65.75	5.19	NS
	2	25	68.54	8.78	
Frequency of junk food	1	25	2.09	1.79	.000 **
	2	25	5.84	1.65	
Visceral Fat [cm]	1	25	4.02	2.21	.014**
	2	25	5.54	1.87	
subcutaneous fat [cm]	1	25	1.51	.92	.004**
	2	25	2.17	.57	

. . ** P value is highly significant

Comparison between ultrasonography data in obese and normal weight children

Visceral and subcutaneous were significantly higher in obese (P were.o14and 0.014 respectively). Using the Mann–Whitney U-test compare between the presence of fatty liver in obese and normal weight children , it was significantly higher in obese group (P=.003).

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Comparative results of the laboratory data and the stool bacteriological analysis of both obese and normal weight children.

Table 3 shows the comparative results of the laboratory data such (HOMA-IR and serum LPS) and the stool bacteriological analysis of both obese and normal weight children. There were significant higher differences in obese children in HOMA-IR, serum LPS and Bifidobacterium.

Table 3: Comparison between obese children and controls regarding Gut microbiota and some laboratory data

Bacteria	1=non obese 2=obese	N	Mean	Std. Deviation	Sig. (2-tailed)
Lactobacillus (log 10 CFU/ml)	1	25	3.19	1.90	NS
	2	25	2.72	1.62	
Bacteroides (log 10 CFU/ml)	1	25	5.20	1.39	NS
	2	25	5.62	1.92	
Bifidobacterium (log 10 CFU/ml)	1	25	4.60	1.53	.019*
	2	25	3.48	1.74	
Enterobacteria (log 10 CFU/ml)	1	25	5.37	1.56	NS
	2	25	5.86	1.48	
LPS [ng/mL]	1	25	7.66	4.77	.033*
	2	25	11.32	6.82	
HOMA-IR	1	25	1.47	.94	.000**
	2	25	3.44	1.51	

* P value is significant. ** P value is highly significant. Lipopolysaccharide (LPS), HOMA-IR=the homeostasis model assessment

Correlation between anthropometric measurements and some variables in obese children

Correlations between BMI, Lactobacillus, Bacteroides, Bifidobacterium, Enterobacteria, LPS,HOMA-IR and Frequency of junk food for the total sample are presented in Table 4. Serum LPS, HOMA-IR and Frequency of junk food had significant positive correlation with BMI. Bifidobacterium had highly significant negative correlations with BMI. However, insignificant correlation was detected between Lactobacillus, Bacteroides and Enterobacteria and BMI.Table4 shows the correlation between fatty liver and gut microbiota. A significant positive correlation was detected between fatty liver and Bifidobacterium.

Table 4: Correlation between anthropometric measurements and some variables in obese children

		BMI	Bifidobacterium	Enterobacteria	LPS	HOMA-IR	fatty liver	Frequency of junk food
BMI	r	1.000	-.398(**)	.065	.115	.715(**)	.727(**)	.616(**)
	Sig. (2-tailed)	.	.004	.655	.425	.000	.000	.000
	N	50	50	50	50	50	50	50
Bifidobacterium	r	-.398(**)	1.000	-.430(**)	.214	-.133	-.474(**)	-.306(*)
	Sig. (2-tailed)	.004	.	.002	.136	.357	.008	.036
	N	50	50	50	50	50	50	50
Enterobacteria	r	.065	-.430(**)	1.000	-.133	-.070	.109	.093
	Sig. (2-tailed)	.655	.002	.	.357	.627	.566	.533
	N	50	50	50	50	50	50	50
LPS	r	.115	.214	-.133	1.000	.157	-.156	.302(*)
	Sig. (2-tailed)	.425	.136	.357	.	.277	.412	.039
	N	50	50	50	50	50	50	50
HOMA-IR	r	.715(**)	-.133	-.070	.157	1.000	.610(**)	.425(**)
	Sig. (2-tailed)	.000	.357	.627	.277	.	.000	.003
	N	50	50	50	50	50	50	50
fatty liver	r	.727(**)	-.474(**)	.109	-.156	.610(**)	1.000	.256
	Sig. (2-tailed)	.000	.008	.566	.412	.000	.	.181
	N	50	50	50	50	50	50	50
Frequency of junk food	r	.616(**)	-.306(*)	.093	.302(*)	.425(**)	.256	1.000
	Sig. (2-tailed)	.000	.036	.533	.039	.003	.181	.
	N	50	50	50	50	50	50	50

** Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed).

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DISCUSSION

To the best of our knowledge, this is the first study analyzing the cross-sectional relationships between serum LPS, insulin resistance, fatty liver in childhood obesity. There were significant higher differences in obese children in HOMA-IR, serum LPS and frequency of fast food intake. Many researchers reported insulin resistance in obese children [16, 21,22]. Cani et al. demonstrated that mice fed a high-fat diet for 2 to 4 weeks exhibited a significant increase in circulating LPS levels (described as ''metabolic endotoxemia'') and that these mice became obese and had obesity associated metabolic disorders. Similarly, mice infused with LPS (to reach levels observed in mice that were fed a high-fat diet) also had obesity and obesity-associated metabolic disorders [23,24]. Insulin resistance was detected in LPS-infused mice. CD14 mutant mice resisted most of the LPS and high-fat diet–induced features of metabolic diseases. This new finding demonstrates that metabolic endotoxemia dysregulates the inflammatory tone and triggers body weight gain. Neal et al. reported that endogenous LPS is continuously produced in the gut by the death of Gram-negative bacteria and physiologically translocated into intestinal capillaries through a TLR4-dependent mechanism [25].

Fast food consumption was more prevalent in obese children in our study than in controls. This result is consistent with the findings of Omuemu and Omuemu [26], Ismail et al. [27] and Briefel et al. [28]. Vreugdenhil et al. found that LPS is transported from the intestine toward target tissues by a mechanism facilitated by lipoproteins, notably chylomicrons freshly synthesized from epithelial intestinal cells in response to a high-fat diet [11].This could explain the positive correlation between frequency of fast food intake and LPS.

This work has shown that obesity in children was associated with a significant reduction in gut Bifidobacteria . Bifidobacteria can synthesize bioactive isomers of conjugated linoleic acid from free linoleic acid, which have antidiabetic, anti- atherosclerotic, immunomodulatory,and anti-obesity properties [29]. This was in agreement with the results of a prospective study [30], which found a children becoming overweight by 7years of age had lower levels of gut Bifidobacteria.

While no significant difference was observed in Bacteroidetes between the two groups. Our results showed a non-significant decrease in Lactobacillus and a non-significant increase in enterobacteria. Our results are however, in agreement with those reported by Turnbaugh et al. indicating that the state of obesity shapes the gut microbiota [31]. They reported a marked difference in the abundance of Lactobacillus, which had higher abundances in lean children while enterobacteria had higher abundances in obese group.

A significant negative correlation were detected between fatty liver and Bifidobacterium.This could be explained by Terpstra et al who reported that mammalian intestinal Bifidobacteria can synthesize from free linoleic acid bioactive isomers of conjugated linoleic acid, which have antidiabetic, anti- atherosclerotic, immunomodulatory, and anti-obesity properties [29]. Wall et al found that the supplementation of Bifidobacterium and linoleic acid to different mammalian species resulted in a two- to threefold higher intestinal, hepatic, and adipose tissue content of cis-9, trans-11 conjugated linoleic acid, eicosapentaenoic acid, and docosahexaenoic acid, concomitantly with a reduced proinflammatory cytokines TNF-, IL-6, and interferon-ex-pression, than the linoleic acid-alone supplemented diet [32].

CONCLUSION

These preliminary results are encouraging to conduct more extensive studies to evaluate the effect of avoiding junk food to develop novel approaches to obesity and its comorbidity therapeutics.

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