Frequency of Firmicutes and Bacteroidetes in gut microbiota in obese and normal weight Egyptian children and adults

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Abstract

Introduction: Obesity and associated metabolic disorders are a worldwide epidemic. Recent evidence suggests that the microbial community in the human intestine may play an important role in the pathogenesis of obesity. The aim of this study was to assess the differences in the composition of the intestinal microbiota between obese and normal weight Egyptian children and adults.
Material and methods: The study included 79 subjects among whom 51 were obese (23 children and 28 adults), and 28 were subjects of normal weight (17 children and 11 adults). Faecal samples were collected from all subjects. Total DNA was extracted from collected stool samples and submitted to conventional PCR for detection of Firmicutes and Bacteroidetes. All the studied group was subjected to clinical and anthropometric evaluation. Laboratory assessment of fasting glucose, high-sensitivity C-reactive protein (hsCRP) and lipid profile was performed.
Results: The proportions of the phyla Firmicutes and Bacteroidetes were statistically significantly increased in the obese group compared to the normal weight group ($p < 0.001$, $p = 0.003$ respectively). The study also found a statistically significant positive trend for higher hsCRP in subjects with positive Firmicutes ($p = 0.004$). However, no associations were found between positive Bacteroidetes and hsCRP.
Conclusions: The results of this study indicate that obesity in Egyptian children and adults is associated with compositional changes in faecal microbiota with increase in the phyla Firmicutes and Bacteroidetes. This could be considered when developing strategies to control obesity and its associated diseases by modifying the gut microbiota.

Key words: obesity, gut microbiota, Firmicutes, Bacteroidetes, high-sensitivity C-reactive protein.

Introduction

Obesity represents one of the five major health risks in modern societies with a frequency of more than 20% of the population in developed countries [1]. As a consequence of the expected dramatic increase in obesity prevalence rates, life expectancy may start to decrease for the first time in recent history due to numerous co-morbid disorders [2, 3].

The intestinal flora has been recently proposed as an environmental factor involved in the control of body weight and energy homeostasis

[4-8]. The human intestine contains a large variety of microorganisms, consisting of at least 1014 bacterial cells and up to 500-1000 different species [9]. As a whole this represents overall more than 100 times the human genome [9, 10], and is called the "metagenome". Thus, the intestinal flora can be considered as an "exteriorized organ" which contributes to our homeostasis with multiple, highly diversified functions. Studies have shown that the gut microbiota is dominated by 2 bacterial phyla, the gram-negative Bacteroidetes and the gram-positive, low GC% Firmicutes [11, 12], with other phyla represented at subdominant levels including the Actinobacteria, Fusobacteria and Verrucomicrobia phyla [11]. Within these phyla the dominant bacterial groups include the *Clostridium coccoides-Eubacterium rectale* group, *Clostridium leptum* group (Firmicutes), *Bacteroides-Prevotella* species (Bacteroidetes), *Bifidobacterium* and *Atopobium* genera (both Actinobacteria) [13]. Firmicutes are in the class Bacillus [14], which is rare in the human or mouse gut microbiota; none is a member of the Clostridium XIV class, which contains the most abundant representatives of this division in the distal guts of mice and humans. Hence, the physiological contributions of Firmicutes to the intestinal ecosystem, and to fuel partitioning, are unclear [14].

Diet-induced and genetically obese mice (*ob/ob* or *db/db*) showed a significant upregulation of expression of Toll-like receptors 1 to 9 in adipocytes and pre-adipocytes along with higher cytokine production upon stimulation [15]. In particular, it is known that Toll-like receptor 4 (TLR-4) can be activated by both lipopolysaccharide (LPS) and dietary saturated fatty acids inducing upregulation of common intracellular inflammatory pathways, such as JNK and NF-κB in adipocytes and macrophages, related to the induction of insulin resistance and increased adiposity [16]. Recently, metabolic endotoxaemia, characterized by an increase in serum LPS levels, has been demonstrated to be an inflammatory factor, causing body weight gain, insulin resistance, and diabetes in high-fat fed animal models [17-19].

C-reactive protein (CRP) is an acute-phase protein that is a sensitive marker for systemic inflammation. Several inflammation-related diseases such as respiratory disease, rheumatoid arthritis, diabetes mellitus, and cardiovascular disease have been associated with elevated CRP concentrations. Moreover, recent studies have reported a positive relation between body mass index and CRP concentrations [20-22]. In obesity, an elevated CRP level concentration is explained by increased interleukin-6 expression in adipose tissue and its release into the peripheral circulation. The role of the gut microbiota in modulating plasma LPS levels, which triggers the inflammatory tone and the onset of obesity and type 2 diabetes, is under evaluation [23]. More studies are needed to unravel how changing gut microbiota impacts on the development of obesity and related metabolic alterations. The idea that gut microbial diversity is linked to obesity deserves exploration in humans, because it may yield new treatment strategies for this growing worldwide threat to our health.

The aim of this study was to assess the differences in the composition of the intestinal microbiota between obese and normal weight Egyptian children and adults by comparing the Firmicutes and Bacteroidetes frequency in the stool and its correlation with high-sensitivity C-reactive protein (hsCRP), as a marker of inflammation, and also the correlation with the amount of dietary fat and carbohydrate intake.

Material and methods

All subjects were recruited between September 2008 and December 2009 from outpatient clinics at the National Research Centre (NRC). Out of 120 subjects randomly chosen, only 79 were included in this study, among whom 51 were obese (23 children and 28 adults), and 28 were subjects of normal weight (17 children and 11 adults). Cases with hepatic disease, hypertension, asthma, infection, tumour, febrile conditions (or had febrile conditions within the previous 2 months), congestive heart failure, diseases affecting the gut microbiota, recent antibiotic intake and other acute inflammatory diseases were excluded from this study. Obesity in children and adults was considered as body mass index (BMI) $\geq$ 30 kg/m^2 and overweight as BMI $\geq$ 25 kg/m^2 and < 30 kg/m^2 [24]. Corresponding normative data for Egyptian children and adolescents were used. Overweight was defined as between the 90th and 95th BMI percentiles and obesity as > 95th percentile in both children and adolescents.

A detailed medical and family history was obtained from all subjects and a complete physical examination was performed, including anthropometric parameters (height, weight), fatness indices (BMI, standard deviation score-BMI – SDS-BMI, and basal blood pressure).

Nutritional evaluation

Twenty-four-hour dietary recalls for three successive days were done for all cases and were evaluated according to the food pyramid guidelines. Food frequency questionnaires (FFQs) ask about the usual frequency of consumption of a list of foods mainly to evaluate fat and carbohydrate intake.

Stool samples were collected from all subjects and were diluted as 20% using sterile EDTA (50 mM),

then all samples were purified from stool deprise by centrifugation at 5000 rpm for 10 min [25].

DNA extraction

DNA was extracted from diluted stool samples using Wizard Genomic DNA Purification Kit (Promega, Cat. No. A1120), according to the manufacturer's instructions, and extracted DNA was stored at –20°C for detection of Bacteroidetes and Firmicutes by PCR [25, 26].

Polymerase chain reaction

Oligonucleotide primers

For amplification of selected DNA segments of Bacteroidetes and Firmicutes, two pairs of oligonucleotide primers were used as detected in stool samples using primers reported by Dick and Field, 2004 and Scupham *et al*., 2006, respectively [25, 27]. The primer sequences were: BactFA ACGCTAGCTACAGGCTTAACA (Forward) and BactR ACGCTACTTGGCTGGTTCA (Reverse), for Bacteroidetes, and FirmF GCGTGAGTGAAGAAGT (Forward) and FirmR CTACGCTCCCTTTACAC (Reverse) for Firmicutes.

PCR amplification

Polymerase chain reaction amplification was carried out in 50 µl reaction mixture containing 10 µl of DNA, 5 µl of 10 × PCR buffer, 4 µl of $MgCl_2$ (25 mM), 0.5 µl (5 U/µl) of Go Taq Flexi DNA Polymerase (Promega Cat. No. M8301) [27], 1 µl of BactF upstream primer (50 pmol/µl), 1 µl of BactR downstream primer (50 pmol/µl), for Bacteroidetes detection or 1 µl of FirmF upstream primer (50 pmol/µl), and 1 µl of FirmR downstream primer (50 pmol/µl), for Firmicutes detection, and 4 µl of dNTPs (10 mM each), and 24.5 µl of DEPC-treated water. The PCR amplification profile was an initial denaturation step of 5 min at 95°C followed by 40 cycles of denaturation at 94°C for 45 s, annealing at 62°C for 45 s (for Bacteroidetes amplification), while the annealing was 58°C at 45 s (for Firmicutes amplification), and extension at 72°C for 1.5 min, with a final extension step of 10 min at 72°C. The PCR thermal cycling was done using a Biometra PCR system and the expected PCR product characteristic for Bacteroidetes was 396 bp and that characteristic for Firmicutes was 161 bp.

Gel electrophoresis

The amplified PCR products were electrophoresed through 1.5% agarose gel in Tris acetate/EDTA (TAE) buffer by using 100 V for 1 h, and the PCR product bands were visualized by staining with 0.01% ethidium bromide (0.5 µg/ml). Polymerase chain reaction product bands were visualized under UV light using a UV transilluminator and photos were taken using a gel documentation system [28].

Blood samples

Five ml of blood were drawn from the antecubital vein under aseptic precautions from fasting (for 12-14 h) subjects. After centrifugation serum was collected from each subject to evaluate inflammatory markers [hs-CRP, lipid profile (total cholesterol, HDL cholesterol, LDL cholesterol, triglyceride)]. 1 – lipid analysis: Lipid assays in sera were determined by chemistry analyzer Olympus AU 400. 2 – the serum hs-CRP assay was based on the chemiluminescence technique. Determination of hs-CRP concentrations was performed with the Diagnostic Products Immulite 2000 analyzer (Block Scientific, Nutley, NJ); assay limits ranged from below 0.1 mg/l to above 10.0 mg/l.

Statistical analysis

Statistical analysis was performed using the software SPSS 12.0 (SPSS: Chicago, IL). Variables were expressed as mean ± standard deviation (SD). Mean values were compared among subjects using Student's *t* test and ANOVA was used to compare variables between all groups. For comparing categorical data, χ^2 test was performed. The exact test was used instead when the expected frequency was less than 5. Spearman's correlation coefficient rho was used to correlate between non-normally distributed continuous variables. A probability value (p value) less than 0.05 was considered statistically significant.

Results

The results of PCR product bands for Bacteroidetes and Firmicutes by the gel documentation system of some cases are shown in Figure 1. Distribution of Bacteroidetes and Firmicutes was significantly increased in the obese group compared to the normal weight group ($p = 0.003$, $p < 0.001$ respectively) (Table I, II). Only 21 subjects had one positive bacteria (13 were positive for Firmicutes and 8 for Bacteroidetes). Table III shows the means and SDs of different variables and their statistical significance in both groups. Both cholesterol and hsCRP were significantly higher in obese individuals ($p = 0.03$, $p = 0.05$ respectively). Table IV shows the cross-tabulation between Firmicutes and Bacteroidetes in children and adults. Firmicutes showed a statistically significant increase in the adult group ($p = 0.031$). As regards the results of dietary analysis, subjects were divided into low and high fat intake. According to carbohydrate intake they were divided into four groups. The fourth group had the highest carbohydrate intake. We could not correlate changes in gut microbiota with fat or

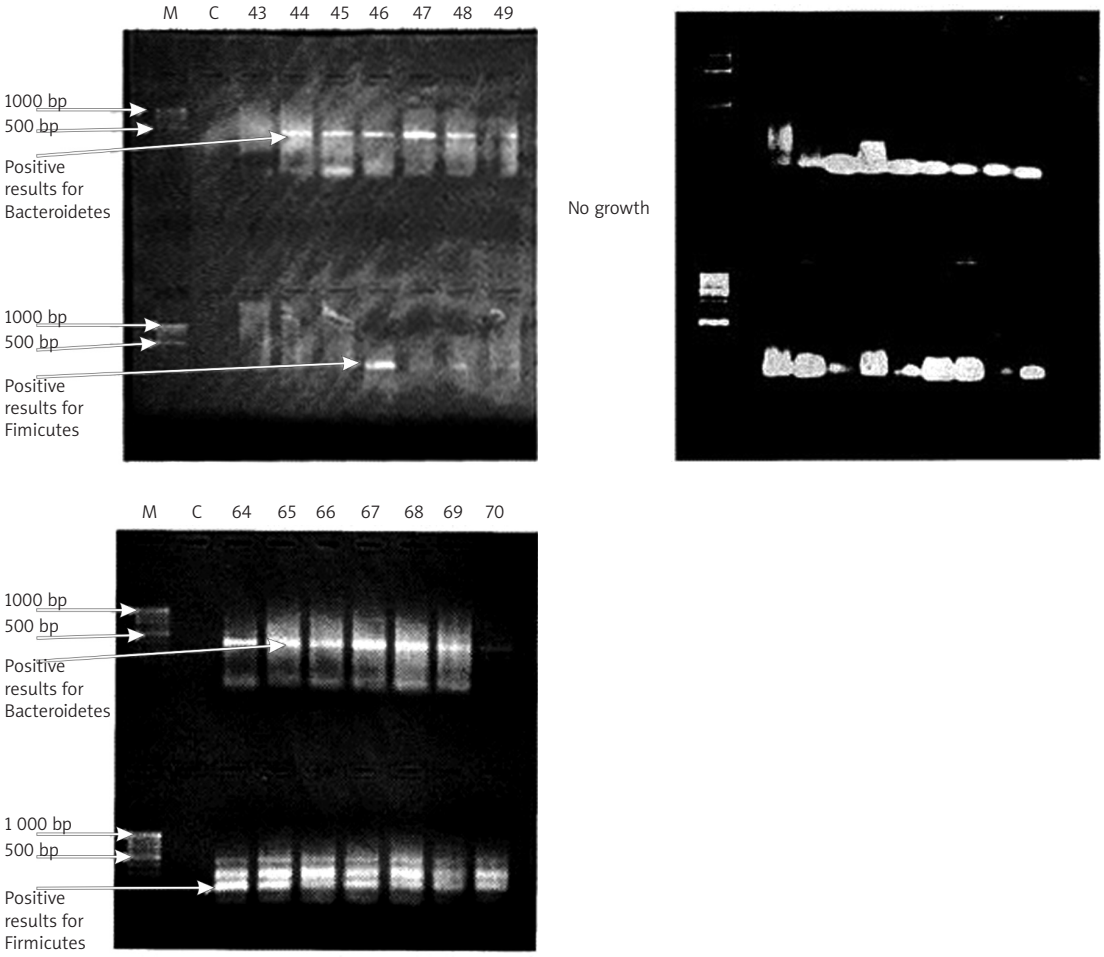

Figure 1. Results of the PCR product bands for Bacteroidetes and Firmicutes by gel documentation system of some cases

Table I. Distribution of Bacteroidetes and Firmicutes in obese and normal weight subjects

		Bacteroidetes		Total	Firmicutes		Total
		1.00	2.00		1.00	2.oo	
Obesity	Count	43	9	52	45	7	52
	% Within obesity	82.7%	17.3%	100.0%	86.5%	13.5%	100.0%
Normal weight subjects	Count	13	14	27	12	15	27
	% Within normal weight	48.1%	51.9%	100.0%	44.4%	55.6%	100.0%

Table II. χ^2 Tests for Bacteroidetes and Firmicutes in obese and normal weight subjects

	Asymp. sig. (2-sided)	Exact sig. (2-sided)	Exact sig. (1-sided)	Asymp. sig. (2-sided)	Exact sig. (2-sided)	Exact sig. (1-sided)
	Bacteroidetes			Firmicutes		
Pearson χ^2	0.001			< 0.001		
Continuity correction	0.003			< 0.001		
Likelihood ratio	0.002			< 0.001		
Fisher's exact test		0.003	0.002		< 0.001	< 0.001
Linear-by-linear association	0.001			< 0.001		
N of valid cases	< 0.001			< 0.001		

carbohydrate intake. Interestingly, the study found a trend for subjects with high fat intake to have positive Firmicutes, and those with the highest carbohydrate intake to have positive Bacteroidetes and Firmicutes (Figure 2). The study also showed a significant positive trend for higher hsCRP in subjects with positive Firmicutes ($p = 0.004$), as shown in Table V.

Discussion

Obesity is now characterized by a cluster of several metabolic disorders, and by low grade inflammation. The evidence that the gut microbiota composition can be different between healthy and obese and type 2 diabetic patients has led to the study of this environmental factor as a key link between the pathophysiology of metabolic diseases and the gut microbiota. Techniques based on the PCR are commonly used and provide rapid qualitative information on the composition of the intestinal microbiota [29]. Qualitative changes of the gut microbiota according to gender and species are well known and there are wide interpersonal variations in gut microbiota as a result of host genetic and environmental factors (e.g. mother's microbiota, birth place, diet, and living conditions). Our study revealed that the proportions of the phyla Firmicutes and Bacteroidetes were statistically significantly increased in the obese group compared to the normal weight group ($p < 0.001$, $p = 0.003$ respectively). Results obtained in both rodents and humans suggest that obesity is associated with an altered composition of gut microbiota. Turnbaugh *et al.* (2009) observed wide shifts of the two major phyla: obese are characterized by more Firmicutes versus lean individuals; some authors show a drop in Bacteroidetes upon obesity [30], whereas other authors show no change or even an increase of Bacteroidetes in overweight [30-33]. This highlights the danger of generalizing observations of changes in microbiota composition from one population to another.

To date, 73 genomes of members of Firmicutes have been fully or partially sequenced. Interestingly, hydrogen-producing members of the family Prevotellaceae within the Bacteroidetes phylum, as well as certain Firmicutes, were more prominent in microbiota of obese individuals. This is believed to accelerate fermentation of otherwise indigestible carbohydrates, thereby leading to increased acetate production and subsequently higher energy absorption [34]. A question remains open: are the changes in gut microbiota in overweight or obese individuals due to the obesity per se, or due to changes in nutritional habits in obese people?

To explore the roles of diet on intestinal microbial ecology, dietary records were analysed for carbohydrate and fat contents. We could not correlate changes in gut microbiota with fat or carbohydrate intake. Interestingly, the study showed a trend for subjects with high fat intake to have positive Firmicutes, and those with the highest carbohydrate intake to have positive Bacteroidetes and Firmicutes. Other studies have correlated changes in gut microbiota with carbohydrate intake [35]. Data obtained in animals suggest that a high-fat diet – and not the obese state per se – can modulate microbiota composition towards an increase in Firmicutes and a proportional decrease in Bacteroidetes [36]. This highlights the likelihood

Table III. Comparison between the obese and normal weight groups

	Obesity	Mean	Standard deviation	Sig. (2-tailed)
FBS	1	93.07	21.82	0.426
	2	89.83	11.04	0.419
Cholesterol	1	197.93	37.04	0.038
	2	180.91	34.65	0.037
Triglyceride	1	112.31	38.68	0.127
	2	96.45	52.97	0.137
HDL	1	41.11	8.16	0.624
	2	42.10	9.83	0.629
LDL	1	131.76	37.81	0.143
	2	119.72	33.34	0.139
HsCRP	1	6.43	10.31	0.053
	2	2.35	3.31	0.036
BMI	1	34.82	5.84	0.000
	2	21.85	2.66	0.000

1 – obese group, 2 – normal weight group
*FBS – fasting blood glucose, HDL – high density lipoprotein, LDL – low density lipoprotein, HsCRP – high-sensitivity C-reactive protein, BMI – body mass index, *p is significant < 0.05*

Table IV. Firmicutes and Bacteroidetes in children and adults

			Firmicutes		Total	Bacteroidetes		Total
			1 (+ve)	2 (–ve)		1 (+ve)	2 (–ve)	
Age group	1 – children	Count	22	18	40	27	13	40
		% Within age	55.0%	45.0%	100.0%	67.5%	32.5%	100.0%
	2 – adults	Count	31	8	39	29	10	39
	Adult	% Within age	79.5%	20.5%	100.0%	74.4%	25.6%	100.0%

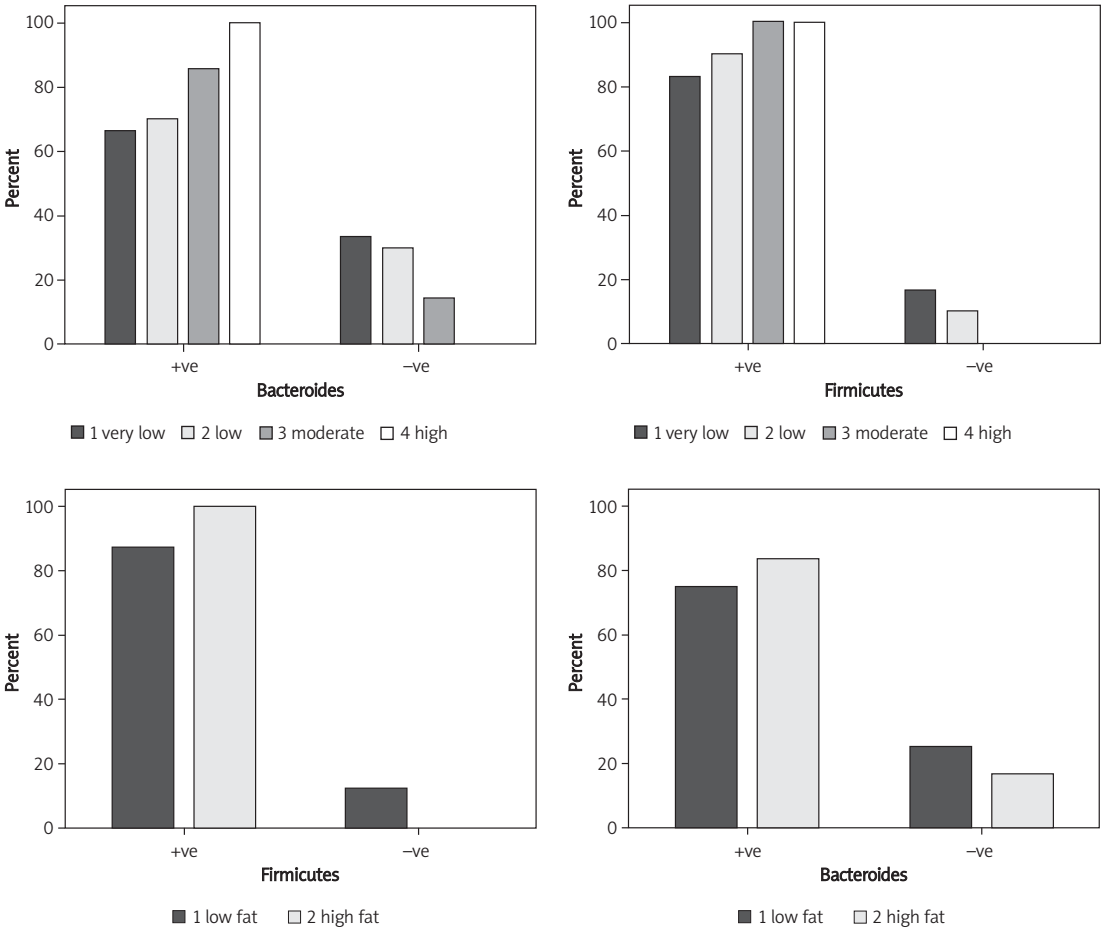

Figure 2. Relation between Firmicutes and Bacteroides and fat or carbohydrates intake

Table V. HsCRP in positive and negative Firmicutes and Bacteroidetes subjects

	HsCRP	*N*	Mean [mg/l]	Standard deviation	Sig. (2-tailed)
Firmicutes	–ve	11	1.3745	1.12117	0.004
	+ve	50	5.3426	4.91056	
Bacteroidetes	–ve	14	4.0264	8.02824	0.759
	+ve	47	4.8060	8.28610	

of external factors such as diet, and potentially host factors, in modulating the microbiota.

Another point remains to be elucidated: could we attribute the low grade inflammatory process observed in obese subjects to changes in the gut bacteria? Our study showed that hsCRP was significantly higher in obese individuals ($p = 0.05$). The study also showed a significant positive trend for higher hsCRP in subjects with positive Firmicutes ($p = 0.004$). The role of the gut microbiota in modulation of plasma lipopolysaccharide (LPS) levels, which triggers the inflammatory tone and the onset of obesity, might be possible. Cani *et al.* (2007) have shown that subcutaneous infusion of LPS can cause weight gain and insulin resistance in mice without altering energy intake [37]. In accordance with this, mice lacking Toll-like receptor 4 (TLR4), which recognizes LPS, are resistant to diet-induced obesity and insulin resistance [38].

In conclusion, our data support the key idea that the gut microbiota can contribute to the pathophysiology of obesity. This could be considered when developing strategies to control obesity and its associated diseases by modifying the gut microbiota.

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