Initial Dietary and Microbiological Environments Deviate in Normal-weight Compared to Overweight Children at 10 Years of Age

Raakel Luoto, *Marko Kalliomäki, †Kirsi Laitinen, ‡Nathalie M. Delzenne, §Patrice D. Cani, ¶Seppo Salminen, and §Erika Isolauri

ABSTRACT

Objective: The aim of the study was to characterize early nutritional and microbiological environments (maternal colostrum adiponectin concentration and early gut microbiota composition) in children subsequently becoming normal weight versus overweight.

Patients and Methods: Fifteen overweight children at 10 years of age were identified from an ongoing prospective nutrition, allergy, mucosal immunity and intestinal microbiota project. Normal-weight children (n = 15), matched for sex, gestational age and body mass index at birth, mode of delivery, probiotic intervention, and duration of breast-feeding, were identified from the same cohort as controls. To characterize the early dietary environment we analyzed the adiponectin concentration in the maternal colostrum. With an aim to assess the initial microbiological environment, we analyzed the gut microbiota composition by fluorescent in situ hybridization in these children at the age of 3 months. Additionally, putative early markers of low-grade inflammation, such as serum-soluble innate microbial receptor sCD14, were analyzed at the age of 3 months.

Results: The colostrum adiponectin concentration was significantly higher in mothers whose children were normal weight than in those whose children were overweight at the age of 10 years (P = 0.001). In parallel, the normal-weight children had significantly higher sCD14 concentrations in the serum (P = 0.049) and tended to have higher bifidobacterial numbers in the gut microbiota (P = 0.087) at the age of 3 months.

Conclusions: The results of the present study suggest that early dietary and gut microbiological environments have a more complex effect on the metabolic programming of a child than previously anticipated.

Key Words: adiponectin, bifidobacteria, gut microbiota, obesity, overweight, sCD14

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An obesity epidemic has swept across Western societies, which now face a growing threat of obesity-related metabolic disorders (1). Children are the most relevant target for primary prevention in that there is an outstanding likelihood of overweight children entering adulthood with overweight and consequently carrying a risk of metabolic abnormalities later in life (2). The key cause of overweight is an imbalance between energy intake and expenditure. This conception is, however, challenged by evidence accumulating from epidemiological studies: the foundation of overweight and obesity is already laid during the fetal period or the first months of life (3,4). In fact, the diet during this period, that is, breast milk with few solid foods, is markedly homogeneous worldwide and is thus unlikely to be the explanation for later overweight development.

We therefore hypothesized that the obscure bridge between early nutrition and weight development may lie in compositional differences in mother’s breast milk, this being dependent on the mother’s nutritional and immunological state and gut microbiota composition. The maternal gut microbiota provides the first inoculum to the development of the offspring’s microbiota (5), the composition of which recently has been linked to overweight development (6). The gut microbiota, a massive immunomodulatory and metabolic regulator, has namely been shown to contribute not only to the control of energy harvest and metabolism (7) but also to the inflammatory state characterizing obesity (8) and insulin resistance (9). The early colonization process appears to be dependent on both prenatal and postnatal factors such as mothers’ prepregnancy body mass index (BMI) and weight gain during pregnancy (10), the vaginal and intestinal microbiota of the mother and the microbiota of the initial environment (5), and the biological and microbiological composition of breast milk (11).

For the purpose of testing the hypothesis that compositional differences in mother’s breast milk are related to the development of overweight, we sought, in a case-control manner, to assess the concentration of the adipocytokine adiponectin in the maternal colostrum in 15 children of normal weight and 15 children overweight at age 10 years. Adiponectin, with its antiatherogenic and anti-inflammatory properties (12) and participation in glucose and lipid metabolism (13,14), represents a key molecule in protection against metabolic syndrome (15). Additionally, to assess the potential relation of the early compositional development of the gut microbiota with overweight development, the gut microbiota composition at the age of 3 months was determined in these 30 children by fluorescent in situ hybridization.
situ hybridization (FISH). For putative early markers of low-grade inflammation, we also analyzed serum-soluble innate microbial receptor (sCD14), lipopolysaccharide (LPS), lipopolysaccharide-binding protein (LBP), and high-sensitivity C-reactive protein (hsCRP) concentrations in the serum in these 30 children at age 3 months. The age point of 3 months was chosen on the grounds of detected timing of deviation in growth patterns between children of normal weight and those with overweight at age 10 years.

SUBJECTS AND METHODS

Study Design
Subjects were selected from a prospective follow-up study on probiotics supplementation (http://www.clinicaltrials.gov/ct/show/NCT00167700), as described in greater detail elsewhere (16). In brief, the original study population comprised 159 infants who had at least 1 close relative (mother, father, sibling) with atopic dermatitis, allergic rhinitis, or asthma. The mothers of these children were recruited in antenatal clinics in the city of Turku and randomizes in a double-blind, placebo-controlled manner to receive 1 × 1010 colony-forming units of Lactobacillus rhamnosus GG (ATCC 53103) or placebo (microcrystalline cellulose) capsules once daily or mechanical expression as soon as lactation had commenced, or at age 10 years.

Gut Microbiota Analysis by FISH

Fecal specimens were collected from diapers after defecation. The specimens were immediately cooled to 6°C to 8°C and transported to the study clinic within 24 hours to be frozen at −75°C. All together, 20 fecal samples were available for FISH analysis at 3 months old and 5 at 3 weeks old. The main groups of fecal bacteria were analyzed. The samples were suspended in phosphate-buffered saline (PBS) and homogenized. Bacteria were fixed with 4% paraformaldehyde, washed with PBS, and stored in 50% ethanol-PBS at −20°C until analysis. Probes included Bac303 (5′-CAAATGTTGGGGACCTT) for the Bacteroides-Prevotella group, Bif616 (5′-CATCCGGCATATCACC) for the Bifidobacterium genus, CH150 (5′-TTATGCGGTATTAATCTTC) for the Clostridium histolyticum group, and Lab158 (5′-GGTAGTATTACGTGTTTCCA) for the Lactobacillus-Lactococcus-Enterococcus group. Total bacterial counts were determined by staining with 4′,6-diamino-2-phenylindole. The bacteria were washed and filtered on 0.2-μm polycarbonate filters. These were then mounted on slides and counted visually under an epifluorescence microscope (BX51; Olympus, Hamburg, Germany) using Cy3-labeled probes and 4′,6-diamino-2-phenylindole–specific filters. At least 15 random fields were counted on each slide, and the average count was used for analysis.

Serum sCD14, LBP and LPS, Analyses

Serum sCD14, LBP, and LPS analyses were performed in the laboratory of the Metabolism and Nutrition Research Group, Université Catholique de Louvain, Louvain Drug Research Institute. Human sCD14 and LBP concentrations were assayed using a solid-phase enzyme-linked immunosorbent assay based on the sandwich principle (Hycult Biotechnology, Uden, the Netherlands) and adapted as follows: sera were diluted 1/10 with the appropriate buffer and subjected to an ultrasonic bath for 3 minutes and homogenized by vortex for 30 seconds before a high dilution rate: 1/500 (sCD14) and 1/1000 (LBP). The LPS concentration was measured using Endosafe-MCS (Charles River laboratories, Lyon, France) based on the Limulus amebocyte lysate kinetic chromogenic methodology, which measures the color intensity directly related to the endotoxin concentration in a sample. Sera were diluted 1/10 with endotoxin-free buffer to minimize interferences in the reaction (inhibition or enhancement) and heated for 15 minutes at 70°C. Each sample was diluted 1/46 with endotoxin-free Limulus amebocyte lysate reagent water (Charles River Laboratories, Wilmington, MA) and treated in duplicate, and 2 spikes per sample were included in the determination. All of the samples have been validated for recovery and coefficient of variation determination. The lower limit of detection was 0.01 EU/mL.

Serum hsCRP Analysis

The National Institute for Health Research Cambridge Biomedical Research Centre, Core Biochemical Assay Laboratory (Abingdon, Oxfordshire, UK). All of the samples were analyzed in duplicate. Samples in which the coefficient of variation of the duplicates was >10% were repeated. Quality control samples with concentrations spanning the working range of the assay were run at the beginning and end of each assay. According to the manufacturer, the assay measures all multimeric forms of adiponectin together, that is, the total adiponectin concentration. The lower limit of detection was 0.8 ng/mL (in-house data), and the between-batch imprecision was 5.4% at 3.6 μg/mL, 5.2% at 9.2 μg/mL, and 5.8% at 15.5 μg/mL (in-house data).
analyzed the samples. Serum hsCRP levels were determined using an automated colorimetric immunoassay on the Dade Behring Dimension RXL autoanalyzer (Siemens Healthcare, Camberly, Surrey, UK). The lower limit of detection was 0.1 mg/L. The imprecision between batches was 5.1% at 1.8 mg/L and 2.4% at 6.4 mg/L. Any hsCRP measurements >8 mg/L were excluded from the analysis because such a level is suggestive of active inflammation or infection. The final number of subjects in this analysis was 11 in children of normal weight and 12 in children overweight at age 10 years.

Statistical Analysis

The clinical characteristics of the study subjects are given as mean values (standard deviations [SDs]) for continuous variables and as numbers and proportions for categorical variables. Differences in clinical characteristics and main outcome measures between the study groups were assessed using the $\chi^2$ test for categorical variables, Student $t$ test for normally distributed continuous variables, and Mann-Whitney $U$ test for skewed continuous variables (ie, concentration of adiponectin in colostrum and LPS and sCD14 in serum). The analysis of variance for repeated measurements was used to compare BMIs at different age points between the study groups. The Breslow-Day interaction test was used to analyze possible interactions between fecal microbial counts and ages when the fecal samples were taken. Because no interaction was detected, all of the samples were combined in further analyses. Fecal microbial counts are expressed as means with 95% confidence intervals, and by reason of non-normal distribution, the unpaired $t$ test was applied to compare the counts between the groups after logarithmic (log) transformation. To examine the relation between continuous variables, linear regression models were constructed. Data were analyzed using SPSS version 15.0 (SPSS Inc, Chicago, IL). A $P$ value < 0.05 was considered statistically significant.

RESULTS

Clinical Characteristics and Growth of the Children

The clinical characteristics of the study subjects are presented in Table 1. None of the children had received antibiotics during the first 3 months of life. The mean prepregnancy BMI (range) of the women was 23.08 (18.40–28.37) kg/m$^2$, and all of the mothers were metabolically healthy. The mean (SD) prepregnancy BMI of the mothers whose children were normal weight than in those whose children were overweight at age 10 years: 15.2 (23.4) vs 5.9

<table>
<thead>
<tr>
<th>TABLE 1. Clinical characteristics of the study subjects</th>
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<tbody>
<tr>
<td>Normal-weight children, n = 15</td>
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<tr>
<td>Probiotic supplementation$^*$</td>
</tr>
<tr>
<td>Probiotic supplementation directly to the child$^*$</td>
</tr>
<tr>
<td>Sex (male)$^*$</td>
</tr>
<tr>
<td>Cesarean delivery$^*$</td>
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<tr>
<td>Maternal peripartal antibiotic treatment$^*$</td>
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<tr>
<td>Gestational age at birth, wk$^1$</td>
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<tr>
<td>Birth weight, g$^1$</td>
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<tr>
<td>Birth length, cm$^1$</td>
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<tr>
<td>Exclusively breast-fed, mo$^1$</td>
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<tr>
<td>Total duration of breast-feeding, mo$^1$</td>
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</tbody>
</table>

Results are given as mean (SD) or as numbers (%) of subjects. None of the differences between the normal-weight and overweight children was statistically significant.

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(2.1) ng/mL, respectively ($P = 0.001$) (Fig. 2). In the regression model the colostrum adiponectin concentration did not correlate with the prepregnancy BMI of the mothers, but an inverse correlation was detected with the BMI of the children at age 10 years ($r = -0.198$, $P = 0.009$).

**Fecal Microbiota Analysis**

There were no statistically significant differences in fecal bacterial counts at age 3 months, as assessed by FISH with microscopic detection, between the children of normal weight and the children overweight at age 10 years. However, a tendency toward higher bifidobacterial numbers was detected in children remaining normal weight in comparison with those becoming overweight by age 10 years (Table 2).

**Concentrations of sCD14, LPS, LBP, and hsCRP in Serum**

The normal-weight children at age 10 years had statistically significantly higher mean (SD) sCD14 concentrations in the serum at age 3 months than those who were overweight by age 10 years: 45.15 (5.82) vs 38.71 (10.04) μg/mL ($P = 0.049$) (Fig. 3). The mean (SD) LBP did not differ between the groups: 27.66 (15.84) μg/mL in normal-weight vs 26.12 (15.09) μg/mL in overweight children ($P = 0.712$). Nor did the mean (SD) LPS concentration (2.24 [3.22] vs 2.45 [3.61] EU/mL) or mean (SD) hsCRP concentration (1.50 [1.93] vs 1.23 [1.77] mg/L) in serum at age 3 months differ between normal-weight and overweight children at age 10 years ($P = 0.836$ and $P = 0.487$, respectively). However, one-third (5/15) of the children of normal weight and 2/15 (13.3%) of those overweight at age 10 years had hsCRP levels at age 3 months below the detectable limit (<0.10 mg/L); $P = 0.134$.

**DISCUSSION**

The present article reports differences in adiponectin concentrations in the maternal colostrum and in fecal bifidobacterial counts at age 3 months between children of normal weight and children overweight at age 10 years, both pointing to the importance of the first few months of life as a window of opportunity to influence subsequent weight development. Interestingly, although birth size did not differ between the children studied due to the matched BMI at birth, the excessive weight gain was observed to set in immediately after 3 weeks of age, the difference already being

![FIGURE 2. Maternal adiponectin concentration in colostrum (ng/mL) in children of normal weight and those overweight at age 10 years; $P = 0.001$, Mann-Whitney U test. The box represents the interquartile range, the horizontal line represents the median, and the whiskers represent the 95th and 5th percentiles of distribution. The singular high value (98.7 ng/mL) in 1 mother of a normal-weight child is not represented.](image1)

![FIGURE 3. Serum sCD14 concentration at age 3 months (μg/mL) in children of normal weight and those overweight at age 10 years; $P = 0.049$, Mann-Whitney U test. The box represents the interquartile range, the horizontal line represents the median, and the whiskers represent the 95th and 5th percentiles of distribution. sCD14 = soluble innate microbial receptor.](image2)

**TABLE 2. Bacterial counts in fecal samples analyzed by FISH at age 3 months**

<table>
<thead>
<tr>
<th>Bacterial Count</th>
<th>Normal-weight children, n = 12</th>
<th>Overweight children, n = 13</th>
<th>$P^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bifidobacteria ($\times 10^9$)</td>
<td>10.0 (3.7–17.1)</td>
<td>3.9 (2.4–5.3)</td>
<td>0.087</td>
</tr>
<tr>
<td>Bacteroides ($\times 10^7$)</td>
<td>4.4 (2.4–6.3)</td>
<td>7.9 (2.3–18.2)</td>
<td>0.873</td>
</tr>
<tr>
<td>Lactobacilli/enterococci ($\times 10^9$)</td>
<td>5.6 (1.1–12.3)</td>
<td>4.7 (1.5–8.0)</td>
<td>0.651</td>
</tr>
<tr>
<td>Clostridia ($\times 10^7$)</td>
<td>9.4 (0.2–18.8)</td>
<td>12.0 (1.5–22.8)</td>
<td>0.581</td>
</tr>
<tr>
<td>Total cell count ($\times 10^9$)</td>
<td>14.0 (5.9–22.2)</td>
<td>6.9 (4.4–9.5)</td>
<td>0.170</td>
</tr>
</tbody>
</table>

The bacterial counts are represented as mean (95% confidence interval) of number of bacteria per gram feces.

* Unpaired $t$ test was performed after logarithmic transformation of bacterial counts.
significant at age 3 months. Notwithstanding the preliminary nature of the present study and the limited number of subjects studied from early infancy up to 10 years of age, the results reinforce the conception of early nutritional and microbial environments as potential initial requisites for later overweight development.

Thus far, only scarce evidence has demonstrated an association between breast milk adiponectin concentration and childhood weight gain. Weyermann et al. (19) found in a 674 mother–infant cohort that higher levels of adiponectin in breast milk 6 months postpartum were associated with overweight at 2 years of age in infants, particularly among those who were breast-fed for at least 6 months. Contrary to this finding, Woo et al. (20) demonstrated in 2 independent cohorts (n = 45 and 277 mother–infant cohorts) that higher breast milk adiponectin was associated with lower infant weight-for-age z score and weight-for-length z score during a 6-month follow-up. On the basis of pleiotropic biological properties of adiponectin, especially its propensity to modulate metabolic and inflammatory processes and constrain the pathophysiology of obesity-linked disease and its presence in breast milk, it has been hypothesized that human milk adiponectin can biologically relevant and presumably involved in infant metabolic programming (21). Although the exact mechanisms of adiponectin action have yet to be deciphered, mounting evidence suggests that its effects are transmitted through innate immune system regulation (22). It must be acknowledged, however, that it is possible that the colostrum adiponectin levels could be only a proxy for maternal factors that themselves influence newborn nutritional programming. Long-term follow-up studies with larger populations and more accurate data of the mothers are needed to confirm our preliminary results.

An interesting finding in the present study was that the normal-weight children at age 10 years had significantly higher sCD14 concentrations in the serum at age 3 months than those who were overweight by age 10 years. Because the sCD14 levels are strongly associated with LPS levels, current orthodoxy holds that sCD14 is a valuable marker of low-grade inflammation (23). An explanation for the result presented here may be that adiponectin has dual opposing actions in macrophages and adipocytes (24). Adiponectin is fundamental in establishing systematic suppression of the proinflammatory responses mediated via the LPS-LBP ligand and the CD14/Toll-like receptor 4 (TLR-4) (25), the same signaling channel of innate immunity that intestinal microbes (26) and fatty acids in nutrition (27) also engage. During the initiation of inflammation, adiponectin can, however, have locally an opposing, proinflammatory effect (24). Because a tolerogenic response has been shown to accomplish sustained exposure to LPS or adiponectin itself, the higher concentrations of sCD14 in the normal-weight children at age 10 years can be hypothesized to be a consequence of a sufficient adiponectin exposure and to be further involved in downregulation of the deleterious immune responses mediated through TLR-4 (28), thus preventing excessive weight gain later in life. Our results call certainly for further multidisciplinary trials with an aim to better understand the mechanism by which colostrum adiponectin could be involved with neonatal metabolic programming via the LPS-LBP ligand and the CD14/TLR-4.

Concomitant with the commencement of lactation, bacterial colonization of the infant’s gut takes place. It is feasible that the “core microbiome,” with its metabolic effects on the host, is more important than the mere presence of a particular bacterial species (29), even though the Bifidobacterium microbiota has been shown to protect against gut barrier dysfunction (30), metabolic endotoxemia (8), insulin resistance (31), and obesity (6). The importance of a balanced host–microbiome interrelation is further supported by the contribution of the early gut microbiota in the maturation of gut-related immunity (32), and thus in counterbalancing between immunoregulatory and proinflammatory responses (33). We suggest that the mechanism linking the trend of lower numbers of fecal Bifidobacteria seen during the first months of life to later overweight development, as shown in the present study, is mediated via both increased energy harvest and unfavorable stimuli of the immature immunomodulatory pathways, the latter being additionally reinforced by insufficient adiponectin exposure from the colostrum.

The nutritional environment, especially the augmentative amount of energy and fat in the diet, shapes the gut microbiota composition by altering the gut barrier function (8) and thus metabolic endotoxemia via increasing concentrations of LPS (9), this acting as a triggering factor for insulin resistance and metabolic diseases. Our study is the first to show that despite the trend of lower numbers of Bifidobacteria in stools in those children whose excessive weight gain was initiated during the first months of life, no signs of endotoxemia were yet detectable at the age of 3 months. It is feasible that despite the differences in fecal Bifidobacterium microbiota among the children, the amount and composition of fat in the diet, that is, breast milk or formula, were still too similar during the first months of life to have elicited endotoxemia. Furthermore, the hsCRP synthesized solely by the hepatocytes is, on the contrary, mainly regulated by the proinflammatory cytokines produced by the adipocytes of visceral fat (34), the concentration of which does rise until central obesity and age increase.

CONCLUSIONS

In revealing the contribution of the early nutritional and microbial environment to the subsequent development of overweight, the results of the present study may improve the understanding of the mechanisms that program and regulate the individual body weight development and also call for further intensive research into novel therapeutic interventions with the aim of reversing the obesity trend of recent decades. We here further hypothesize that the size and longevity of an immune response originates not only from the amount or quality of antigens provided by the initial dietary and microbiological environment but also from the volume of individual signals from damage and stress induced by these molecular patterns (35). Hence, this excitability evolving during early postnatal life is likely to determine lifelong reactivity and responsiveness to medical and lifestyle interventions.

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