Unravelling the gut microbiota of cow’s milk allergic infants, their mothers and grandmothers

L. Mera-Berriatua¹, MSc; E. Zubeldia-Varela¹,² MSc; IA. Martín-Antoniano³,⁴ PhD; E. López de Maturana¹,⁴ PhD; D. Rojo, PhD; R. Bazire⁵, MD; P. Cabrera-Freitag⁶, MD, PhD; TC. Barker-Tejeda¹,², MSc; C. Ubeda⁷,⁸, PhD; D. Barber¹, PhD; MP. Francino⁸,⁹, PhD; MD. Ibáñez-Sandín⁵, MD, PhD; M. Perez-Gordo¹⁰, PhD.

¹Institute of Applied Molecular Medicine (IMMA), Department of Basic Medical Sciences. Facultad de Medicina. Universidad San Pablo-CEU, CEU Universities, ARADyAL, Madrid, España.
²Centre for Metabolomics and Bioanalysis (CEMBIO), Department of Chemistry and Biochemistry, Facultad de Farmacia. Universidad San Pablo-CEU, CEU Universities, Urbanización Montepriíncepe, 28660 Boadilla del Monte. Madrid Spain.
³Institute of Applied Molecular Medicine (IMMA), Department of Clinical Medical Sciences. Facultad de Medicina. Universidad San Pablo-CEU, CEU Universities, Madrid, España.
⁴Genetic and Molecular Epidemiology Group, Spanish National Cancer Research Centre (CNIO), Madrid, Spain.
⁵Department of Allergy, H. Infantil Universitario Niño Jesús, FibHNJ, ARADyAL- RETICs Instituto de Salud Carlos III, IIS-P, Madrid, Spain.
⁶Allergy Paediatric Unit. Allergy Service, Hospital General Universitario Gregorio Marañón, Gregorio Marañón Health Research Institute (IiSGM), Madrid, Spain.
⁷Fundació per al Foment de la Investigació Sanitària i Biomèdica de la Comunitat Valenciana (FISABIO), Valencia, España.
⁸CIBER en Epidemiología y Salud Pública, Madrid, España.
⁹Joint Research Unit in Genomics and Health, Fundació per al Foment de la Investigació Sanitària i Biomèdica de la Comunitat Valenciana (FISABIO) and Institut de Biologia Integrativa de Sistemes (Universitat de València / Consejo Superior de Investigaciones Científicas), Avda. Catalunya 21, València 46020, Spain.

†: These Authors have equally contributed
‡: These Authors have equally contributed
Corresponding author:

Marina Perez-Gordo, PhD

IMMA, Instituto de Medicina Molecular Aplicada, Facultad de Medicina, San Pablo CEU University.

Avda. Monteprincipe s/n, 28668 Boadilla del Monte, Madrid, España.

Tlf: 91 372 47 00 ext. 14675

E-mail: marina.perezgordo@ceu.es

Short title: Intergenerational gut microbiota study of CMA

Word count: 3528 (including headers).

Key words: Gut microbiota, Food Allergy, Cow’s milk allergy, 16S rRNA Gene Sequencing, Intergenerational cohort
Abbreviations:

AG: allergic grandmothers

AI: allergic infants

AM: allergic mothers

ASV: Amplicon Sequence Variant

BM: infants fed with breast milk

BM_F: infants fed with breast milk together with formula milk

BM_H: infants fed with breast milk together with hydrolysate

CI: control infants

CMA: Cow’s Milk Allergy

DCs: Dendritic cells

F: infants fed with formula milk

FA: Food Allergy

GIT: Gastrointestinal Tract

H: infants fed with hydrolysate

L-G: Bray-Curtis distance between infants and their grandmothers

L-M: Bray-Curtis distance between infants and their mothers

M-G: Bray-Curtis distance between mothers and grandmothers

NA-G: non-allergic grandmothers

NA-M: non-allergic mothers

OR: Odds Ratio

TLR: Toll-like receptor
ABSTRACT

Background: Cow’s Milk Allergy (CMA) is one of the most prevalent food allergies (FA) among infants. Gut microbiota dysbiosis has been related to the development of FA. The primary colonization of the gut microbiota occurs via maternal route. We hypothesized that a longitudinal influence in the composition of the gut microbiota, transmitted from mothers to offspring, could be directly related to CMA development.

Methods: 148 faecal samples of 34 CMA and 16 control 0-8 month-old infants and their respective mothers and grandmothers were studied. Gut microbiota was profiled by 16S rRNA gene sequencing using the Illumina MiSeq platform. 16S rRNA sequencing analysis was performed using the DADA2 pipeline. Descriptive statistics of the epidemiological variables of the three generations were analysed. Statistical analyses were performed with R 3.4.0 software.

Results: Mothers allergy status and smoking habits of mothers and grandmothers were associated to infant CMA. We found that adult gut microbiota is richer and more diverse than that of infants. Relative abundance of the Prevotellaceae family was significantly different between infant groups, and between hydrolysate-fed and formula-fed infants. Finally, the Bray-Curtis distance between members of the same family was independent of the allergy status.

Conclusions: Microbiota from allergic children do not differ from non-allergic at the onset of allergy. Moreover, microbiota inheritance was similar in healthy and allergic infants. Maternal smoking and allergy status were the most significant epidemiological risk factors associated with CMA. Finally, microbiota composition of infants was influenced by diet and allergy status; however, these were confounded variables.
INTRODUCTION

The human microbiota is a complex ecosystem made up of bacteria, fungi, viruses, archaea and parasites that cohabit on or inside the human body, where bacteria are the most abundant. These microorganisms and their entire set of genes are referred to as the microbiome. Among the microbial ecosystems of the human organism, the most complex and diverse is the one associated with the digestive system, particularly in the gastrointestinal tract (GIT), where the density of microorganisms is the highest of the entire body. The number of bacteria in the GIT, particularly in the section between the colon and the appendix, is around $10^9$ per gram of luminal content, and the number of genera swings between 1000 and 3000. Faeces are representative of the microbiota composition of the colon segment. This fact and their noninvasive collection method make them the biological sample of choice for the study of the gut microbiota.

Gut microbiota composition changes throughout life. It is thought that 70% of the gut microbiota primary colonization is of maternal origin, and that the first 1,000 days of life, when the body is faced for the first time with external factors, are very important for the development of the intestinal microbiota. Moreover, the development of the gut microbiota in the first years of life correlates with the development and maturation of the intestine and the immune system. From birth, a symbiotic relationship is established between the microbiota and our cells, which evolves over time, adapting to changes. After the first 2-3 years of life, the gut microbiota becomes similar to what it will be for the rest of our life. However, the composition of the gut microbiota is dynamic and dependent on host-associated confounding factors such as age, diet, use of antibiotics, lifestyle, and environmental conditions.

The greatest source of stimulation of the immune system is found in the mucous surfaces of the body, in contact with the external environment. About 70-80% of immune system cells are found in the small and large intestines. The gut microbiota stimulates and modulates the immune system by a DCs-mediated immune regulation. Microbes promote the differentiation of regulatory T cells by the activation of DCs present in the mucous surface of the intestine through the Toll-Like Receptor (TLR) pathway. These activated cells produce cytokines that in turn activate naïve T cells or Th0 cells so that they mature towards the corresponding T cell subtype, T helper cells (Th1, Th2, Th17) and regulatory T cells.

In healthy individuals, all Th cell subpopulations are present in dynamic balance with regulatory T cells. Several studies have shown that the onset of tolerogenic responses to antigens is mediated by the presence of certain bacteria in our GIT. Imbalances of microbial communities, known as dysbiosis, have been related to inadequate modulation of the immune system and the development of
pathologies not only related to the GIT. Alterations in gut microbiota have been found in people suffering from rhinitis, atopic eczema, asthma or food allergies such as peanut, egg or cow’s milk allergy (CMA). However, whether the imbalance of the gut microbiota is a cause that triggers the disease or, on the contrary, is a consequence of the disease that alters the bacterial populations and their functionality is still unknown. In this sense, previous work from our group has shown that epithelial barriers are compromised in severe respiratory allergic phenotypes regardless of triggering allergen. Moreover, this barrier malfunction is linked to systemic changes. Thus, whether this imbalance in the gut microbiota is a cause of allergy or a consequence of a previously altered state of the mucosa is not yet well defined, and this issue needs to be further addressed.

Among food allergies, CMA is one of the most prevalent among children. Tolerance is acquired in 80% of CMA patients by the age of 4; despite this, the etiology and pathophysiology of the disease remain unclear. In addition, to what extent alterations of the microbiota of the infant could play a role in this particular allergy is completely unknown.

On the other hand, several studies have shown an association between maternal and paternal allergy/asthma and the risk of allergy development in infants. Moreover, most associations are with maternal allergy/asthma. One possible explanation to this link could be that the mother’s gut microbiota, transmitted to infants, may predispose infants to allergy. However, to our knowledge this has not been studied yet.

The aim of this study was to evaluate the intergenerational effect of the gut microbiota in the allergy status of infants over the maternal route within three generations.
MATERIALS AND METHODS

Study design

We performed an intergenerational and observational case-control study approved by the Regional Ethics Committee for Clinical Research of Hospital Universitario Infantil Niño Jesús in Madrid according to the ethical guidelines outlined in the Declaration of Helsinki and its amendments. All participants provided informed consent.

Study population

The final study population consisted of 148 participants: 50 infants up to eight months of age (16 healthy control subjects and 34 with CMA), their 50 mothers and their 48 maternal grandmothers. Recruitment of the study population was highly complex and took two years. Study participants were classified into six groups according to their allergy status: allergic infants (AI); control infants (CI); allergic mothers (AM); non-allergic mothers (NA-M); allergic grandmothers (AG) and non-allergic grandmothers (NA-G). Inclusion criteria and sample collection and processing are detailed in Supplementary Information.

Epidemiological variables and analysis

Demographic, clinical and lifestyle variables from all subjects involved in the study were analysed. Descriptive statistics (mean and standard deviations for continuous variables, percentages for categorical variables, and p-values) were used to describe the epidemiological variables and are detailed in Supplementary Information. The two groups were compared their proportions using the chi-squared test.

16S rRNA Gene Sequencing

DNA extraction

DNA was extracted from 200 mg of faeces using the QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer’s instructions plus an additional membrane disruption step using glass beads. The extracted DNA was quantified by Qubit® 2.0 Fluorometer following the dsDNA HS Assay Kit protocol. Those samples with a concentration higher than 0.2 µg/µL were selected for sequencing (n=115).

16S rRNA gene amplification and sequencing
The V3-V4 regions of the 16S rRNA gene were amplified and sequenced using the MiSeq platform from Illumina, as described in the manual for “16S Metagenomic Sequencing Library Preparation” of the MiSeq platform (Illumina, San Diego, California, EEUU).

**Bioinformatics and statistical analyses**

16S rRNA sequences were denoised and processed with DADA2 v1.11 in order to define ASVs. In addition, DADA2 and the command removeBimeraDenovo were used. Shannon’s α-diversity index was estimated using the package vegan v2.5-3 and the R 3.4.0 software. Richness was defined as the number of ASVs identified in a given sample. The Bray-Curtis distance between pairs of samples was calculated using the vegan package. The permutational multivariate analysis of variance (PERMANOVA) test was applied using the Bray-Curtis distance matrix. ANCOM II was applied to identify specific taxonomic differences between groups of samples. To adjust for multiple hypothesis testing, we used the FDR approach by Benjamini and Hochberg implemented in the fdr.R package. Detailed information is described in Supplementary section.
RESULTS

Epidemiological and intergenerational risk factors for allergy in infants

The characteristics of the 50 infants including variables of their mothers and grandmothers are shown in Table 1. Infant cases and controls had similar age (4.93 vs 5.00 months, respectively) and gender distribution, and no significant differences (p>0.05) were found regarding their delivery mode and the use of antibiotics at birth. Having older siblings or pets was not significantly associated (p>0.05) with an increased risk of allergy in infants. A highly significant association was found between type of feeding at the moment of sample collection of infants (p<0.01). Mothers and grandmothers of cases and controls were of similar ages (p>0.05). In addition, it was observed a trend between allergy status of infants and the counterpart of their mothers (p=0.06), but not with that of their grandmothers (p=1.00). Interestingly, smoking status of both mothers (p=0.019) and grandmothers (p=0.077) might be associated with the allergy status of the infant.

Figure 1 shows the forest plot including the Odds Ratio (OR) estimates and confidence intervals for the association between the risk of allergy in infants and allergy and smoking status of their (grand)mothers, obtained in the univariate logistic regressions. Allergic mothers were five times more likely to have an allergic child than non-allergic ones (p=0.03). Regarding smoking status, non-smoker mothers showed 90% lower odds of having allergic infants than smokers. Likewise, non-smoker grandmothers were associated with a decreased risk of having an allergic infant (OR = 0.28, p-value = 0.05).

We also performed a multivariate analysis including the smoking status of mothers, grandmothers and allergy status of the mothers. In this case, only the association between the smoking status of the mother and the risk of having an allergic child remained significant (OR = 0.10, p-value = 0.04) (Table 1S).

Gut microbiome profile differences over three generations

The 16S rRNA gene sequencing was performed in samples from infants between 4 and 6 months which had sufficient quantity of DNA, including 11 AG; 32 NA_G; 18 AM; 27 NA_M; 19 AI and 7 CI (n=114 out of 148, 77%). This selection allowed to standardize even more the AI and CI groups.

Overall, a total of 19,523,010 sequences were generated, out of which 12,955,391 remained after quality and length filtering and chimera removal, resulting in 9,641 ASVs for the gut microbiome.
Relative abundance of the bacterial phyla and families found in individuals of the three generations according to their allergic status are shown in Figure 2A and 2B.

The faecal microbiome composition was different between adults and infants (PERMANOVA $p=0.017$). At the phylum level, the adult microbiota was mainly constituted by Firmicutes (60%), Bacteroidetes (15%), Actinobacteria (7%), Verrucomicrobia (4%) and Proteobacteria (3%), whereas in infants, the relative abundance of both Actinobacteria and Proteobacteria phyla was higher (ANCOM $p=7.4e^{-06}$ for Actinobacteria and $p=9.8e^{-14}$ for Proteobacteria), representing 30% and 15% of the infant gut microbiota composition. On the contrary, the relative abundance of Firmicutes was lower in infants (30% vs. 60%) (ANCOM $p=1.2e^{-07}$). At the family level, the predominant families in adult microbiota were Ruminococcaceae (25%), Lachnospiraceae (23%), Bacteroidaceae (10%), Coriobacteriaceae (5%), Bifidobacteriaceae (2-4%), Verrucomicrobiaceae (2-4%) and Rikenellaceae (2-4%), while in the case of infants the most abundant families were Bifidobacteriaceae (25-30%), Enterobacteriaceae (15-20%) and Lachnospiraceae (5%) (Figure 2B).

Alpha diversity (Shannon’s diversity index) and richness were analysed showing a statistically significant increase in adults compared to infants ($p<0.001$) (Figure 2C-D).

**Gut microbiota differences associated with CMA and type of feeding in infants**

Aiming to assess if the allergic state of infants was associated with a different microbiota composition, the 15 most abundant families (relative abundance > 0.1%) identified using 16S rRNA gene sequencing were compared between AI and CI. The AI phenotype showed an increased relative abundance of Bifidobacteriaceae, Clostridiaceae, Verrucomicrobiaceae, Lactobacillaceae and Streptococaceae families, while Ruminococcaceae, Coriobacteriaceae, Bacteroidaceae and Veillonellaceae families were decreased compared to CI. However, these differences were not significant (Figure 3S_A). On the other hand, AI had decreased within-sample bacterial diversity compared to CI ($p<0.05$) (Figure 2C). Moreover, multivariate analysis using PERMANOVA showed that there were statistical compositional differences at bacterial family level in the gut microbiota between AI and CI (Adonis $p=0.025$) (Figure 3B). However, the overall microbiome composition of their mothers did not significantly differ (Figure 2S). Differences regarding abundant bacterial families and ASVs between infant groups were identified using the ANCOM-II method. Interestingly, the relative abundance of Prevotellaceae and Acidaminococcaceae, two of the families with the lowest relative abundances, was significantly different between AI and CI (Figure 3C). At ASV level, significant differences between the
relative abundances for *Veillonella parvula*, *Veillonella dispar*, *Streptococcus lutetiensis* and *Enterococcus casseliflavus* were detected between AI and CI (Figure 3S_B & 3S_C).

We identified 5 groups according to the feeding regime of infants: breast milk (BM), breast milk together with hydrolysate (BM_H), breast milk together with formula milk (BM_F), hydrolysate (H) and formula milk (F). In relation to the sequenced samples, AI were fed with H, BM or BM_H while CI were fed with F or BM_F. As can be observed in Figure 4A, the relative abundance of the bacterial families changes according to the feeding regime. The most remarkable compositional changes were observed between infants fed with H (all belonging to the AI group) and those with F feeding (all belonging to the CI group) (PERMANOVA p=0.005), finding an increase of *Prevotellaceae* bacterial family in F group (Figure 4B-C). At the ASV level, we identified an increase of the relative abundance of *Veillonella parvula* and *Enterococcus casseliflavus* in BM_H and BM_F groups compared to F group, respectively, while *Streptococcus lutetiensis* was decreased in H group compared to F (Figure 4S).

**Intra-family β-diversity is not associated with CMA**

In order to investigate the dissimilarity between infants, mothers and grandmothers of the same family we computed the Bray-Curtis distance between infants and their mothers (L-M), mothers and grandmothers (M-G) and infants and their grandmothers (L-G). As can be observed, M-G distance was lower than I-M and I-G distances, which correlates with the fact that adult microbiotas are more similar to each other and more different to the infant gut microbiota. On the other hand, we also aimed to see if there was a dysbiotic pattern in allergic families compared to non-allergic. We found that the distance among subjects of the same family was similar regardless of allergy status (Figure 5).
To our knowledge, this is the first study to investigate the longitudinal influence, transmitted from grandmothers to mothers and from mothers to offspring, on the composition of the gut microbiota and its relationship with the development of cow’s milk allergy in infants. Grandmothers, mothers, and infants were recruited in this study to understand the perceptible effect of allergy on the microbiome from a novel point of view. The strengths of this study include the heterogeneity of samples including three different generations of patients from two Hospitals and five health centres in Madrid, rigorous determination of CMA and the use of high throughput culture-independent techniques for microbiota identification. On the other hand, samples were collected only at one timepoint during the study so changes in the composition of the microbiota over time could not be assessed.

We found evidence that maternal history of allergy is associated with a 5-fold increased risk of infant allergy. This result is in line with the findings reported by Pali-Schöll et al, who found that the degree of risk for allergy appears to be directly related to the family history of allergy and especially to maternal atopy. Although the causes of allergy have not been fully determined yet, it is known that genetic predisposition plays an important role. In addition to the genetic component, other factors such as maternal diet during pregnancy, alcohol consumption and exposure to tobacco smoke may contribute to allergy in the offspring. Considering the molecular mechanisms underlying the development of allergy, persistent inflammation seems to be a determining factor in its pathophysiology, establishment and severity. Continuous or repetitive exposure to the allergen causes tissue damage, affecting epithelial barrier function and integrity and therefore increasing the risk of allergen sensitization.

Among all the variables considered in this study (see Table 1) smoking was also found to be associated with risk of allergy in infants. Maternal tobacco consumption was significantly associated with a higher prevalence of infant allergy. This association remained significant even after adjusting for the maternal allergy status and the maternal grandmother smoking status, confirming that maternal smoking is an independent risk factor for allergy status in infants. The loss of significance of the maternal allergy status and maternal grandmother smoking status in the multivariate model maybe due to the limited sample size of our study.

Maternal smoking during pregnancy is a modifiable environmental risk factor for many diseases like atopic eczema, dermatitis syndrome and bronchial asthma, and has intergenerational and organ-specific effects for the lungs as well as inducing epigenetic changes in the foetal allergen-specific
immune responses. In addition, recent studies have shown that smoking can alter the vaginal microbiota.

In our infant multi-factor models, maternal smoking and allergy were identified as significant factors associated with infant allergy, suggesting that tobacco smoke in general is an important determinant.

In this work, we have identified that Prevotellaceae family was significantly increased in CI and in F fed infants compared to AI and H fed infants, respectively. However, at genus level there was no significant increase within this family. The significant association between the feeding regime and allergy status in infants may be explained by the fact that for most of the infants diagnosed with CMA, the feeding was changed to H either alone or combined with BM (~70%) as a regular clinical practice. In contrast, most of the controls were fed with F. Therefore, each infant’s diet was conditioned by their allergy status and it is highly difficult to separate both variables as they are usually linked. To minimize this bias as much as possible, infants who ingested H for more than two weeks were excluded from the study. Likewise, since samples were collected only once, we cannot state that those changes are a consequence of either the allergy status of the infant or their feeding regime. Prevotella together with Bacteroides are the most prevalent genera of the Bacteroidetes phylum within the gut. Prevotella is a large genus with high species diversity and high levels of genome diversity between strains. Previous studies have shown that a decrease of this genus in the lung microbiota is associated to asthma and chronic obstructive pulmonary disease (COPD). In other studies, the increased abundance of Prevotella has been associated to rheumatoid arthritis and inflammatory bowel disease. On the other hand, members of the Prevotella genus have also been associated with beneficial effects such as an improved glucose metabolism and correlation with a plant-rich diet, so the role of this genus is still poorly understood and is probably highly dependent on the specific strains involved.

Other potential risk factors, including delivery mode (caesarean or vaginal delivery) and antibiotics consumption were analysed although no significant results were obtained as most of the participants recruited in the study had a vaginal delivery and very few participants received antibiotics at birth. In addition, no significant association was observed between CMA risk and having pets or older siblings, probably due to the limited sample size of our study.

Several authors have reported the association between age and the human microbiome. However, a longitudinal study including three generations has rarely been performed before. The structure of the gut microbiome community showed differences in diversity and richness when age groups were compared according to their allergic status, showing a statistically significant decrease in infants.
compared to adults, understood as both mothers and grandmothers (Figure 2). The Actinobacteria
and Proteobacteria phyla were highly abundant in infants compared to adults. The Actinobacteria
phylum is mainly represented by the *Bifidobacteriaceae* family and in the case of infants it is 25-30% 
of the total gut microbiota.\(^{57}\)

In addition, we found that microbiota Bray-Curtis distances between infants and their respective 
mothers were similar regardless of allergy status, suggesting a similar microbiota inheritance and 
development in healthy and allergic infants. Moreover, gut microbiota from allergic children does not 
differ significantly from that of non-allergic at these stages of life. These results suggest that dysbiosis 
does not precede the onset of CMA.

Allergy involves changes in underlying molecular processes, such as lipid metabolism, that can lead to 
epithelial barrier dysfunction and dysbiosis.\(^{58}\) These changes and the immune responses taking place 
at mucous membranes are linked to a specific and systemic release of metabolites. Previous work of 
our group showed that epithelial barrier integrity is compromised in severe allergic phenotypes 
regardless of triggering allergen and that this has systemic consequences.\(^{22,24}\) In our study, epithelial 
barrier integrity of allergic infants could be compromised beforehand due to metabolic alterations 
helping to establish the allergic phenotype. Dysbiosis could be, therefore, a consequence of the 
disease. However, prospective studies in this sense would be necessary to confirm this hypothesis. 
Moreover, the identification by -omic techniques of altered metabolic pathways and the metabolites 
implicated, such as lipid mediators, would complement our findings and help to discover additional 
mechanisms whereby the early-life intestinal microenvironment influences food allergy risk.

**CONCLUSIONS**

To sum up, there are no differences between allergic and non-allergic families in terms of Bray-Curtis 
distance, suggesting that there are no differences in the longitudinal microbial pattern related to 
allergy at these stages of life. Maternal smoking and allergy were identified as the most significant 
epidemiological factors associated with infant allergy. However, a larger intergenerational study 
should be conducted to confirm them as independent risk factors. Regarding gut microbiota 
composition, we have confirmed that the microbiota of infants is less diverse than that of adults. 
Finally, it was not possible to assign the differences we have found to diet or allergy. Taking this into 
account, prospective studies with longitudinal follow-up in close intervals during the first six months 
will be optimal to shed light into the causal effect of microbiome differences between infant cases and 
controls.
Table 1. Study population characteristics

|                               | Control N=16 | Case N=34 | p.overall |
|-------------------------------|--------------|-----------|-----------|
| **Age (months)**              | 5.00 (1.71)  | 4.93 (1.47)| 0.883     |
| **Gender:**                   |              |           |           |
| **Female**                    | 10 (62.5%)   | 19 (55.9%)| 0.893     |
| **Male**                      | 6 (37.5%)    | 15 (44.1%)|           |
| **Type of birth:**            |              |           | 1.000     |
| **Vaginal**                   | 13 (81.2%)   | 28 (82.4%)|           |
| **Cesarea**                   | 3 (18.8%)    | 6 (17.6%) |           |
| **Antibiotics:**              |              |           | 1.000     |
| **No**                        | 14 (87.5%)   | 28 (82.4%)|           |
| **Yes**                       | 2 (12.5%)    | 6 (17.6%) |           |
| **Detailed Feeding:**         |              |           | <0.001    |
| **Formula**                   | 7 (43.8%)    | 0 (0.00%) |           |
| **Breast milk**               | 2 (12.5%)    | 7 (20.6%) |           |
| **Hydrolysate**               | 0 (0.00%)    | 12 (35.3%)|           |
| **Breast milk + Formula**     | 7 (43.8%)    | 3 (8.82%) |           |
| **Breast milk + Hydrolysate** | 0 (0.00%)    | 12 (35.3%)|           |
| **Older sibling:**            |              |           | 1.000     |
| **No**                        | 9 (56.2%)    | 20 (58.8%)|           |
| **Yes**                       | 7 (43.8%)    | 14 (41.2%)|           |
| **Pet:**                      |              |           | 0.508     |
| **No**                        | 13 (81.2%)   | 24 (70.6%)|           |
| **Yes**                       | 3 (18.8%)    | 10 (29.4%)|           |
| **Mother’s age**              | 33.8 (4.45)  | 34.3 (3.98)| 0.715    |
| **Allergy status (mother):** |              |           | 0.060     |
| **No**                        | 14 (87.5%)   | 19 (55.9%)|           |
| **Yes**                       | 2 (12.5%)    | 15 (44.1%)|           |
| **Smoking status (mother):**  |              |           | 0.019     |
| **Never**                     | 15 (93.8%)   | 19 (55.9%)|           |
| **Ever**                      | 1 (6.25%)    | 15 (44.1%)|           |
| **Grandmother’s age**         | 65.4 (5.28)  | 63.4 (6.08)| 0.263   |
| **Allergy status (grandmother):** |          |           | 1.000     |
| **No**                        | 12 (75.0%)   | 24 (70.6%)|           |
| **Yes**                       | 4 (25.0%)    | 8 (23.5%) |           |
| **‘Missing’**                 | 0 (0.00%)    | 2 (5.88%) |           |
| **Smoking status (grandmother):** |         |           | 0.077     |
| **Never**                     | 11 (68.8%)   | 12 (35.3%)|           |
| **Ever**                      | 5 (31.2%)    | 20 (58.8%)|           |
| **‘Missing’**                 | 0 (0.00%)    | 2 (5.88%) |           |
| **Smoking & Allergy status (mother):** |    |           | 0.011     |
| **Smoker non-allergic mother**| 1 (6.25%)    | 8 (23.5%) |           |
| **Smoker allergic mother**    | 0 (0.00%)    | 7 (20.6%) |           |
| **Non-smoker non-allergic mother** | 13 (81.2%) | 11 (32.4%)|           |
| **Non-smoker allergic mother** | 2 (12.5%)    | 6 (23.5%) |           |

**Footnote.** Epidemiological characteristics of the participants in the study. "Ever", within the variable smoking status, refers to the condition of having been a regular smoker at some point in their life. Even if they were not smokers at the time of collection.
**Figure Legends**

**Figure 1.** Forest plot for the odds ratio estimates of the associations between allergy status in infants and allergy and smoking status of their mothers and grandmothers obtained in the univariate logistic regressions.

**Figure 2.** Gut microbiota composition over three generations: A, Bacterial phyla composition B, Bacterial family composition (top 35 most abundant families). C, Alpha diversity differences among groups (Shannon’s diversity index). D, Richness (N) differences among groups. T-test was performed for statistical analyses using GraphPad Prism. * indicates statistical differences between AI and the rest of groups, Δ indicates statistical differences between CI and the rest of groups * \( p < 0.05 \), **/**ΔΔΔ \( p < 0.001 \).

**Figure 3.** Distinct gut microbiota composition associated to CMA in infants. A, Bacterial family composition in AI and CI. B, Principal coordinate analysis showing clustering of individual gut microbiota composition. C, Bacterial families with a significantly different abundance between AI and CI using ANCOMII test (q<0.1). * \( p < 0.05 \), ****p \( < 0.001 \).

**Figure 4.** Distinct gut microbiota composition associated to infants’ diet. A, Bacterial family composition in BM_H, BM_F, BM, H and F groups. B, Principal coordinate analysis showing clustering of individual gut microbiota composition as well as PERMANOVA multivariate analysis (PERMANOVA \( p \)-value). C, Bacterial families with a significantly different abundance between groups using ANCOMII test (q<0.1). * \( p < 0.05 \), ** \( p < 0.01 \), **###p \( < 0.001 \).

**Figure 5.** Beta diversity analysis based on Bray-Curtis distance between members of the same family. T-test was performed for statistical analyses using GraphPad Prism.
Declarations

Ethics approval and consent to participate
The study was approved by the Regional Ethics Committee for Clinical Research of Hospital Universitario Infantil Niño Jesús in Madrid (R-0004/17) according to the ethical guidelines outlined in the Declaration of Helsinki and its amendments. All participants provided informed consent.

Consent for publication
Not applicable

Availability of data and materials
Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

Competing interests
Domingo Barber has received consultancy fees from ALK and Aimmune therapeutics companies. María Dolores Ibañez has received personal fees from Faes Farma, Merck, LETI, ROXALL and CIRCASSIA. Dr. Bazire reports personal fees from LETI Pharma and grants from FAES and Aimmune Therapeutics, outside the submitted work. The rest of the Authors have no conflict of interest. All authors have read and approved the manuscript.

Sources of funding for the research reported in the article
This work was supported by Instituto de Salud Carlos III (PI17/01087) and Fundación Sociedad Española de Alergia e Inmunología Clínica (FSEAIC_2016). It was co-funded by the European Regional Development Fund “Investing in your future” for the thematic network and co-operative research centers ARADyAL RD16/0006/0015 and RD16/0006/0026. D.R and E.Z-V acknowledge funding from the Spanish Ministry of Science, Innovation and Universities (RTI2018-095166-B-I00). CU acknowledges funding from the Spanish Ministry of Economy (SAF2017-90083-R).

Author contributions:
M.P-G and MD.I.S were the PI. D.B participated in the concept design of the study and contributed to the analysis. R.B., P.C-F. and MD.I.S included all study patients and the clinical data. L.M-B and E.Z-V performed the sample processing and treatment, DNA isolation and data treatment. TC.B-T participated in sample processing, figures editing and language revision. C.U and MP.F supervised the
16S rRNA gene sequencing analysis and the statistical analysis. M.P-G and D.R supervised genomic data treatment and interpretation. I.A.M-A and E.LM performed the epidemiological part of the study. All authors contributed to the writing of the manuscript and have given approval to the final version of the manuscript.

Acknowledgments

We would like to thank all institutions and hospitals involved: Institute of Applied Molecular Medicine (IMMA, San Pablo CEU University, Madrid), Sequencing and Bioinformatics Service (FISABIO, Valencia), Hospital Universitario Infantil Niño Jesús (Madrid, España) and Hospital Universitario Gregorio Marañón, Centro de Salud (C.S) Eloy Gonzalo (Feliciano López, Ana María López Madrazo, Sonia Luna Ramírez), C.S Baviera (Mercedes Velez García-Nieto), C.S Ibiza (Alberto Morlan Sala), C.S Justicia (Mª Rosario Antón Jiménez), C. S Valleaguado (Paloma Ortiz Ramos) y C.S. Goya (Yolanda Martín Peinador). All authors would also like to thank the help received from all IMMA researchers that have participated in sample processing.
REFERENCES

1. Power SE, O’Toole PW, Stanton C, Ross RP, Fitzgerald GF. Intestinal microbiota, diet and health. *Br J Nutr.* 2014;111:387-402.

2. Sartor RB. Microbial influences in inflammatory bowel diseases. *Gastroenterology.* 2008;134:577-594.

3. Falony G, Joossens M, Vieira-Silva S, et al. Population-level analysis of gut microbiome variation. *Science.* 2016;352:560-564.

4. Theriot CM, Bowman AA, Young VB. Antibiotic-Induced Alterations of the Gut Microbiota Alter Secondary Bile Acid Production and Allow for Clostridium difficile Spore Germination and Outgrowth in the Large Intestine. *mSphere.* 2016;1.

5. Quiñones-Rada P, Choy YY, Calvert CC, Waterhouse AL, Lamuela-Raventos RM. Use of metabolomics and lipidomics to evaluate the hypocholesterolemic effect of Proanthocyanidins from grape seed in a pig model. *Mol Nutr Food Res.* 2016;60:2219-2227.

6. Zubeldia-Varela E, Raczkowska BA, Ferrer M, Perez-Gordo M, Rojo D. Chapter 4 - Techniques for Phenotyping the Gut Microbiota Metabolome. In: Faintuch J, Faintuch S, eds. *Microbiome and Metabolome in Diagnosis, Therapy, and Other Strategic Applications.* Academic Press; 2019:33-41.

7. Gomez de Agüero M, Ganal-Vonarburg SC, Fuhrer T, et al. The maternal microbiota drives early postnatal innate immune development. *Science.* 2016;351:1296-1302.

8. Wopereis H, Oozeer R, Knipping K, Belzer C, Knol J. The first thousand days - intestinal microbiology of early life: establishing a symbiosis. *Pediatr Allergy Immunol Off Publ Eur Soc Pediatr Allergy Immunol.* 2014;25:428-438.

9. Moles L, Gómez M, Heilig H, et al. Bacterial diversity in meconium of preterm neonates and evolution of their fecal microbiota during the first month of life. *PloS One.* 2013;8:e66986.

10. Yatsunenko T, Rey FE, Manary MJ, et al. Human gut microbiome viewed across age and geography. *Nature.* 2012;486:222-227.

11. Rojo D, Méndez-García C, Raczkowska BA, et al. Exploring the human microbiome from multiple perspectives: factors altering its composition and function. *FEMS Microbiol Rev.* 2017;41:453-478.

12. Furness JB, Kunze WA, Clerc N. Nutrient tasting and signaling mechanisms in the gut. II. The intestine as a sensory organ: neural, endocrine, and immune responses. *Am J Physiol.* 1999;277:G922-928.

13. Pascal M, Perez-Gordo M, Caballero T, et al. Microbiome and Allergic Diseases. *Front Immunol.* 2018;9:1584.

14. Ivanov II, Frutos R de L, Manel N, et al. Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell Host Microbe.* 2008;4:337-349.

15. Ostman S, Rask C, Wold AE, Hultkrantz S, Telemo E. Impaired regulatory T cell function in germ-free mice. *Eur J Immunol.* 2006;36:2336-2346.
16. O’Mahony C, Scully P, O’Mahony D, et al. Commensal-induced regulatory T cells mediate protection against pathogen-stimulated NF-kappaB activation. *PLoS Pathog.* 2008;4:e1000112.

17. Muir AB, Benitez AJ, Dods K, Spergel JM, Fillion SA. Microbiome and its impact on gastrointestinal atopy. *Allergy.* 2016;71:1256-1263.

18. Aitoro R, Paparo L, Amoroso A, et al. Gut Microbiota as a Target for Preventive and Therapeutic Intervention against Food Allergy. *Nutrients.* 2017;9:672.

19. Abrahamsson TR, Jakobsson HE, Andersson AF, Björkstén B, Engstrand L, Jenmalm MC. Low diversity of the gut microbiota in infants with atopic eczema. *J Allergy Clin Immunol.* 2012;129:434-440, 440.e1-2.

20. Bisgaard H, Li N, Bonnelykke K, et al. Reduced diversity of the intestinal microbiota during infancy is associated with increased risk of allergic disease at school age. *J Allergy Clin Immunol.* 2011;128:646-652.e1-5.

21. Dong P, Feng J, Yan D, Lyu Y, Xu X. Early-life gut microbiome and cow’s milk allergy- a prospective case - control 6-month follow-up study. *Saudi J Biol Sci.* 2018;25:875-880.

22. Rosace D, Gomez-Casado C, Fernandez P, et al. Profilin-mediated food-induced allergic reactions are associated with oral epithelial remodeling. *J Allergy Clin Immunol.* 2019;143:681-690.e1.

23. Sanchez-Solares J, Delgado-Dolset MI, Mera-Berriatua L, et al. Respiratory allergies with no associated food allergy disrupt oral mucosa integrity. *Allergy.* 2019;74:2261-2265.

24. Obeso D, Mera-Berriatua L, Rodriguez-Coira J, et al. Multi-omics analysis points to altered platelet functions in severe food-associated respiratory allergy. *Allergy.* 2018;73:2137-2149.

25. Zeiger RS, Heller S, Mellon MH, Halsey JF, Hamburger RN, Sampson HA. Genetic and environmental factors affecting the development of atopy through age 4 in children of atopic parents: a prospective randomized study of food allergen avoidance. *Pediatr Allergy Immunol.* 1992;3:110-127.

26. Zeiger RS, Heller S. The development and prediction of atopy in high-risk children: Follow-up at age seven years in a prospective randomized study of combined maternal and infant food allergen avoidance. *J Allergy Clin Immunol.* 1995;95:1179-1190.

27. Lim RH, Kobzik L. Maternal transmission of asthma risk. *Am J Reprod Immunol N Y N.* 1989;61:1-10.

28. Kurukulaaratchy RJ, Waterhouse L, Matthews SM, Arshad SH. Are influences during pregnancy associated with wheezing phenotypes during the first decade of life? *Acta Paediatr Oslo Nor.* 2005;94:553-558.

29. Celedón JC, Litonjua AA, Ryan L, Platts-Mills T, Weiss ST, Gold DR. Exposure to cat allergen, maternal history of asthma, and wheezing in first 5 years of life. *Lancet Lond Engl.* 2002;360:781-782.

30. Kurukulaaratchy RJ, Matthews S, Waterhouse L, Arshad SH. Factors influencing symptom expression in children with bronchial hyperresponsiveness at 10 years of age. *J Allergy Clin Immunol.* 2003;112:311-316.
31. Latzin P, Frey U, Roiha HL, et al. Prospectively assessed incidence, severity, and determinants of respiratory symptoms in the first year of life. *Pediatr Pulmonol*. 2007;42:41-50.

32. Martinez FD, Wright AL, Taussig LM, Holberg CJ, Halonen M, Morgan WJ. Asthma and wheezing in the first six years of life. The Group Health Medical Associates. *N Engl J Med*. 1995;332:133-138.

33. Litonjua AA, Carey VJ, Burge HA, Weiss ST, Gold DR. Parental history and the risk for childhood asthma. Does mother confer more risk than father? *Am J Respir Crit Care Med*. 1998;158:176-181.

34. Lim RH, Kobzik L, Dahl M. Risk for asthma in offspring of asthmatic mothers versus fathers: a meta-analysis. *PloS One*. 2010;5:e10134.

35. World Medical Association Declaration of Helsinki: Ethical Principles for Medical Research Involving Human Subjects. *JAMA*. 2013;310:2191-2194.

36. Thompson JA, Oliveira RA, Djukovic A, Ubeda C, Xavier KB. Manipulation of the quorum sensing signal AI-2 affects the antibiotic-treated gut microbiota. *Cell Rep*. 2015;10:1861-1871.

37. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods*. 2016;13:581-583.

38. Kaul A, Mandal S, Davidov O, Peddada SD. Analysis of Microbiome Data in the Presence of Excess Zeros. *Front Microbiol*. 2017;8.

39. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *J R Stat Soc Ser B Methodol*. 1995;57:289-300.

40. Pali-Schöll I, Namazy J, Jensen-Jarolim E. Allergic diseases and asthma in pregnancy, a secondary publication. *World Allergy Organ J*. 2017;10:10.

41. Sicherer SH, Furlong TJ, Maes HH, Desnick RJ, Sampson HA, Gelb BD. Genetics of peanut allergy: a twin study. *J Allergy Clin Immunol*. 2000;106:53-56.

42. Ullemar V, Magnusson PKE, Lundholm C, et al. Heritability and confirmation of genetic association studies for childhood asthma in twins. *Allergy*. 2016;71:230-238.

43. Pali-Schöll I, Renz H, Jensen-Jarolim E. Update on allergies in pregnancy, lactation, and early childhood. *J Allergy Clin Immunol*. 2009;123:1012-1021.

44. Kraft S, Kinet J-P. New developments in FcepsilonRI regulation, function and inhibition. *Nat Rev Immunol*. 2007;7:365-378.

45. Galli SJ, Tsai M, Piliponsky AM. The development of allergic inflammation. *Nature*. 2008;454:445-454.

46. Presland RB, Dale BA. Epithelial structural proteins of the skin and oral cavity: function in health and disease. *Crit Rev Oral Biol Med Off Publ Am Assoc Oral Biol*. 2000;11:383-408.

47. Shinohara M, Matsumoto K. Fetal Tobacco Smoke Exposure in the Third Trimester of Pregnancy Is Associated with Atopic Eczema/Dermatitis Syndrome in Infancy. *Pediatr Allergy Immunol Pulmonol*. 2017;30:155-162.
48. Nelson TM, Borgogna JC, Michalek RD, et al. Cigarette smoking is associated with an altered vaginal tract metabolomic profile. *Sci Rep.* 2018;8:852.

49. Levin AM, Sitarik AR, Havstad SL, et al. Joint effects of pregnancy, sociocultural, and environmental factors on early life gut microbiome structure and diversity. *Sci Rep.* 2016;6:31775.

50. Larsen JM. The immune response to Prevotella bacteria in chronic inflammatory disease. *Immunology.* 2017;151:363-374.

51. Aquino SG de, Abdollahi-Roodsaz S, Koenders MI, et al. Periodontal Pathogens Directly Promote Autoimmune Experimental Arthritis by Inducing a TLR2- and IL-1–Driven Th17 Response. *J Immunol.* 2014;192:4103-4111.

52. Kleessen B, Kroesen AJ, Buhr HJ, Blaut M. Mucosal and Invading Bacteria in Patients with Inflammatory Bowel Disease Compared with Controls. *Scand J Gastroenterol.* 2002;37:1034-1041.

53. Kovatcheva-Datchary P, Nilsson A, Akrami R, et al. Dietary Fiber-Induced Improvement in Glucose Metabolism Is Associated with Increased Abundance of Prevotella. *Cell Metab.* 2015;22:971-982.

54. Clemente JC, Pehrsson EC, Blaser MJ, et al. The microbiome of uncontacted Amerindians. *Sci Adv.* 2015;1.

55. Ruiz-Ruiz S, Sanchez-Carrillo S, Ciordia S, et al. Functional microbiome deficits associated with ageing: Chronological age threshold. *Aging Cell.* 2020;19:e13063.

56. Chaudhari DS, Dhotre DP, Agarwal DM, et al. Gut, oral and skin microbiome of Indian patrilineal families reveal perceptible association with age. *Sci Rep.* 2020;10:5685.

57. Rinninella E, Raoul P, Cintoni M, et al. What is the Healthy Gut Microbiota Composition? A Changing Ecosystem across Age, Environment, Diet, and Diseases. *Microorganisms.* 2019;7:14.

58. Murakami M. Lipid mediators in life science. *Exp Anim.* 2011;60:7-20.