Unravelling the Gut Microbiota of Cow's Milk Allergic Infants, their Mothers and Grandmothers

Leticia Mera-Berriatua

CEU San Pablo University: Universidad CEU San Pablo

Elisa Zubeldia-Varela

CEU San Pablo University: Universidad CEU San Pablo

Isabel Adoración Martín-Antoniano

CEU San Pablo University: Universidad CEU San Pablo

Evangelina López de Maturana

Centro Nacional de Investigaciones Oncologicas Carlos III: Centro Nacional de Investigaciones Oncologicas

David Rojo

CEU San Pablo University

Raphaëlle Bazire

Hospital Nino Jesus: Hospital Infantil Universitario Nino Jesus

Paula Cabrera-Freitag

Hospital General Universitario Gregorio Marañón: Hospital General Universitario Gregorio Marañón

Tomás Clive Barker-Tejeda

CEU San Pablo University: Universidad CEU San Pablo

Carles Ubeda

FISABIO: Fundacio per al Foment de la Investigacio Sanitaria i Biomedica

Domingo Barber

CEU San Pablo University: Universidad CEU San Pablo

María del Pilar Francino

FISABIO: Fundacio per al Foment de la Investigacio Sanitaria i Biomedica

María Dolores Ibáñez-Sandín

Hospital Nino Jesus: Hospital Infantil Universitario Nino Jesus

Marina Perez-Gordo (✉ marina.perezgordo@ceu.es)

CEU San Pablo University: Universidad CEU San Pablo <https://orcid.org/0000-0003-2003-4841>

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

L. Mera-Berriatua^{1†}, MSc; E. Zubeldia-Varela^{1,2†}, MSc; IA. Martín-Antoniano^{3,4}, PhD; E. López de Maturana^{1,4}, PhD; D. Rojo, PhD²; R. Bazire⁵, MD; P. Cabrera-Freitag⁶, MD, PhD; TC. Barker-Tejeda^{1,2}, MSc; C. Ubeda^{7,8}, PhD; D. Barber¹, PhD; MP. Francino^{8,9}, PhD; MD. Ibáñez-Sandín^{5‡}, MD, PhD; M. Perez-Gordo^{1‡}, 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 Montepríncipe, 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, FibHNI, 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

33 **Corresponding author:**

34 Marina Perez-Gordo, PhD

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

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

37 Tlf: 91 372 47 00 ext. 14675

38 E-mail: marina.perezgordo@ceu.es

39

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43 Intergenerational cohort

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46	Abbreviations:
47	AG: allergic grandmothers
48	AI: allergic infants
49	AM: allergic mothers
50	ASV: Amplicon Sequence Variant
51	BM: infants fed with breast milk
52	BM_F: infants fed with breast milk together with formula milk
53	BM_H: infants fed with breast milk together with hydrolysate
54	CI: control infants
55	CMA: Cow's Milk Allergy
56	DCs: Dendritic cells
57	F: infants fed with formula milk
58	FA: Food Allergy
59	GIT: Gastrointestinal Tract
60	H: infants fed with hydrolysate
61	L-G: Bray-Curtis distance between infants and their grandmothers
62	L-M: Bray-Curtis distance between infants and their mothers
63	M-G: Bray-Curtis distance between mothers and grandmothers
64	NA-G: non-allergic grandmothers
65	NA-M: non-allergic mothers
66	OR: Odds Ratio
67	TLR: Toll-like receptor
68	

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.¹ 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.^{2,3} 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.⁴⁻⁶

Gut microbiota composition changes throughout life. It is thought that 70% of the gut microbiota primary colonization is of maternal origin,⁷ 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.⁸ 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.⁹ 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.^{10,11}

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.¹² 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.¹³ 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.¹³

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.¹⁴⁻¹⁶ Imbalances of microbial communities, known as dysbiosis, have been related to inadequate modulation of the immune system and the development of

125 pathologies not only related to the GIT. Alterations in gut microbiota have been found in people
126 suffering from rhinitis, atopic eczema, asthma or food allergies such as peanut, egg or cow's milk
127 allergy (CMA).¹⁷⁻²¹ However, whether the imbalance of the gut microbiota is a cause that triggers the
128 disease or, on the contrary, is a consequence of the disease that alters the bacterial populations and
129 their functionality is still unknown. In this sense, previous work from our group has shown that
130 epithelial barriers are compromised in severe respiratory allergic phenotypes regardless of triggering
131 allergen.^{22,23} Moreover, this barrier malfunction is linked to systemic changes.²⁴ Thus, whether this
132 imbalance in the gut microbiota is a cause of allergy or a consequence of a previously altered state of
133 the mucosa is not yet well defined, and this issue needs to be further addressed.

134 Among food allergies, CMA is one of the most prevalent among children. Tolerance is acquired in 80%
135 of CMA patients by the age of 4;^{25,26} despite this, the etiology and pathophysiology of the disease
136 remain unclear. In addition, to what extent alterations of the microbiota of the infant could play a role
137 in this particular allergy is completely unknown.

138 On the other hand, several studies have shown an association between maternal and paternal
139 allergy/asthma and the risk of allergy development in infants. Moreover, most associations are with
140 maternal allergy/asthma.²⁷⁻³⁴ One possible explanation to this link could be that the mother's gut
141 microbiota, transmitted to infants, may predispose infants to allergy. However, to our knowledge this
142 has not been studied yet.

143 The aim of this study was to evaluate the intergenerational effect of the gut microbiota in the allergy
144 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.³⁵ 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.³⁶ 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.³⁷ 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 II³⁸ 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.³⁹ 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 (ANCOMII $p=7.4e-06$ 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%) (ANCOMII $p=1.2e-07$). 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 L-M and L-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).

DISCUSSION

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.⁴⁰ Although the causes of allergy have not been fully determined yet, it is known that genetic predisposition plays an important role.^{41,42} 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.⁴³ Considering the molecular mechanisms underlying the development of allergy, persistent inflammation seems to be a determining factor in its pathophysiology, establishment and severity.⁴⁴ Continuous or repetitive exposure to the allergen causes tissue damage, affecting epithelial barrier function and integrity and therefore increasing the risk of allergen sensitization.^{45,46}

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.⁴⁷ In addition, recent studies have shown that smoking can alter the vaginal microbiota.⁴⁸

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.⁴⁹

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).⁵⁰ In other studies, the increased abundance of *Prevotella* has been associated to rheumatoid arthritis⁵¹ and inflammatory bowel disease.⁵² On the other hand, members of the *Prevotella* genus have also been associated with beneficial effects such as an improved glucose metabolism⁵³ and correlation with a plant-rich diet⁵⁴, 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.^{10,55} However, a longitudinal study including three generations has rarely been performed before.⁵⁶ 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.

378 **Table 1. Study population characteristics**
379

	Control N=16	Case N=34	p.overall
Age (months)	5.00 (1.71)	4.93 (1.47)	0.883
Gender:			0.893
Female	10 (62.5%)	19 (55.9%)	
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%)	8 (23.5%)	

380 **Footnote.** Epidemiological characteristics of the participants in the study. "Ever", within the variable smoking
381 status, refers to the condition of having been a regular smoker at some point in their life. Even if they were not
382 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.

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Author contributions:

M.P-G and MD.I.Š 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. IA.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.

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Figures

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 * $p < 0.05$, ***/ $\Delta\Delta\Delta$ $p < 0.001$.

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

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