Abstract: Aim: To investigate the relationship between factors influencing external environmental microbial exposures (FEMEs), previously identified to be protective or to increase the risk of the development of allergic disease, and cognition and behaviour in infants 2 years of age in an Australian population.

Method: The Barwon Infant Study is a birth cohort (n=1074) in Victoria, Australia. Comprehensive questionnaire, clinical and biological measures were collected at multiple time-points. Multiple linear regression was used to evaluate associations between 56 FEMEs and three outcomes; cognition (Bayley Scales of Infant and Toddler Development (BAYLEY-III)) (n=667, mean (SD) age = 2.45 (0.14) years), internalising and externalising behaviour (Child Behavior Checklist (CBCL)) (n=666, mean(SD) age = 2.45 (0.14)years).

Results: Overall, there were no consistent patterns or dose response found within an outcome nor across all three outcomes, although there was some evidence for individual associations. Breastfeeding and child care were associated with higher cognitive scores (adj. mean diff. [95%CI] = 3.20 [0.23, 6.17] and 0.68 [0.12, 1.24] respectively), and increasing sibling number was associated with lower internalising behaviour (adj. mean diff. [95%CI] = -4.13 [-6.34, -1.91]).

Conclusion: In contrast to allergic disease, there was an absence of epidemiological evidence to support the association between these FEMEs and cognition and behaviour. Direct investigations into the relationship between exposures which influence gut-microbial composition and cognition and behaviour are now needed.

Key words: microbiome; cognition; Bayley-III; internalising behaviour; externalising behaviour; Child Behaviour Checklist

Brief points

What is already known on this topic

- Factors which influence external microbial exposures (FEMEs) such as pet ownership, antibiotic use and caesarean section have been identified as important factors in the development of allergic disease.
- Several of these factors impact the infant gut microbial composition.
- The gut microbiome also influences brain development and behaviour, however, the relationship between these factors and cognition and behaviour has not been well characterised.

What this paper adds

- This is the first epidemiological study to investigate relationship between a comprehensive range of both pre- and postnatal FEMEs and cognition and behaviour.

Introduction

There is intense interest in the role of gut microbiome and early cognition and behaviour. Epidemiological studies have identified a range of early-life factors which influence external microbial exposures (termed FEMEs) that are associated with decreased allergic disease risk, some of which also influence the gut microbial composition. Yet little epidemiological work has been conducted on identifying if these factors also relate to infant cognition and behaviour.

The 'hygiene hypothesis' [1] emerged from the observation that larger household size was associated with decreased allergic disease. Subsequent work has shown that multiple factors which increase external microbial exposure are associated with reduced allergic disease, such as; antenatal exposure to farming environments [2] and antenatal pet ownership. Whereas, factors which reduce microbial exposure have been associated with increased allergic disease such as prenatal antibiotic use[3] and caesarean delivery [4].

There is evidence that some of these FEMEs also influence infant gut microbial composition, at least for; mode of delivery [5], antibiotic exposure [5] and pet ownership [6]. Gut microbes are thought to play a crucial role in allergic disease via immune programming and inflammatory mechanisms [7], although, the precise mechanisms remain unclear.

Evidence from germ-free mouse models suggests that the gut microbiome also modulates brain structure, function and behaviour via the microbiota-gut-brain axis [8]. Bacteria involved in the microbiota-gut-brain-axis are hypothesised to perform a more complex role than immune programming and inflammation alone and are thought to be also integral in metabolic, trophic, gene expression modification, protective and structural functions [8].

However, in contrast to allergic disease, there has been minimal investigation of the relationship between FEMEs and cognition and behaviour in humans. Findings from studies on the allergic disease cannot be extrapolated to cognition and behaviour as the bacteria are involved in different functions. To date, there has been no comprehensive investigation into the relationship between early-life FEMEs, particularly those identified as determinants of the gut microbiome, and cognition and behaviour in humans, although several studies

into single candidate FEMEs have been undertaken, predominately investigating the role of mode of delivery on neurodevelopment [9].

Here we undertake an extensive investigation of over 50 FEMEs across the antenatal and postnatal period and their association with infant cognition and internalising and externalising behaviour at 2 years of age. These factors were selected based on past work indicating variation in allergic disease risk. Some factors have been shown to also impact on the gut microbiome. By using this systematic approach, we aim to; investigate a particular factor's influence on an outcome, identify any patterns across the three outcomes, identify any patterns relating to time of exposure and to be able to compare and contrast these findings with those from allergic disease.

Materials and Methods

The Cohort

The Barwon Infant Study (BIS) is an unselected antenatal sampling frame birth cohort study (n=1,074) with antenatal recruitment south-west of Melbourne, Victoria [10].

The Cohort sample

Women were recruited by 28 weeks gestation between June 2010 and June 2013 using an unselected sampling frame. Population characteristics and exclusion criteria have been previously described [10]. This report is based on infants who completed the BAYLEY-III or Child Behaviour Check List (CBCL) at 2 years of age (Figure 1). Multiple births were excluded.

Measures

Exposures

Comprehensive questionnaire data (including sociodemographic profile, life environment, perinatal associated measures and child care attendance) and an extensive range of clinical measures were collected antenatally and at birth, 4 weeks, 3, 6, 9, 12 and 18 months and 2 years [10]. When the same variable was collected at different time points, each time point was considered a separate factor (e.g. maternal antibiotic use in trimesters 1, 2 and 3 was considered as 3 separate factors).

Outcome measures

Cognitive measure

Bayley Scales of Infant and Toddler Development 3rd edition (BAYLEY-III)

The BAYLEY-III[11] cognitive domain was used to measure cognition. The BAYLEY-III was administered at the 2-year review and scored by trained personnel.

Age-standardised cognitive composite scores have a mean score of 100 (SD = 15) [11]. The standardised sample included children with developmental delay (approximately 10%), which had the effect of lowering the overall mean score. Indeed, healthy term Australian children were found have a significantly higher mean score [12]. As we similarly observed a residual age at assessment for the composite scores, we adjusted for the raw scores for post-conceptional age, the age parameter most related to cognition in this cohort. Note in doing this, we are unable to compare the cognitive ability of our cohort with other age-matched cohorts as they used the cognitive composite scores. This was determined to be unimportant as the objective of this study was to investigate the linear relationship between factors influencing FEMEs and cognition and not the cognitive ability of our cohort. The maximum obtainable score is 91.

Behavioural measures

Child Behaviour Checklist (CBCL)

The CBCL /1.5-5[13] internalising and externalising problem scales were used to measure behaviour[13]. T-scores were used in our analysis and have a mean of 50 (SD = 10). Higher scores indicate greater problems. T-scores 60 ≥ 63 represent borderline scores and T-scores > 63 fall into the clinical range. The CBCL questionnaire was collected at the 2-year interview in conjunction with the BAYLEY-III assessment. The CBCL was completed by the infant's carer.

Statistical analysis

Infant and family characteristics are presented as mean (SD) or percentages for those who completed either the BAYLEY-III or the CBCL or both (Table 1). T-tests were used

to determine mean differences of continuous variables between the inception cohort and BAYLEY-III/CBCL, while Fisher exact test was used to determine differences in proportions of the categorical variables. Non-parametric tests were used where appropriate. Multiple linear regression was used to evaluate associations between the FEMEs and each outcome (cognition, internalising and externalising behaviour) (Table 2; Note that null associations are recorded at the bottom of the table). Significance is reported at the 5% level. Levels of all external microbial exposures (for example, disinfectant use) were ordered so that the reference group was the level that had the least microbial exposure (e.g. '*everyday*') to most microbial exposure (e.g. '*don't use'*) (Table 2). Ordering in this manner was undertaken to investigate possible dose response effects, similar to those undertaken in studies in allergic disease. No adjustments for multiple comparisons were undertaken to reduce the risk of type II errors [14]. Stata 15.0 software (StataCorp, College Station, TX) was used for all analyses.

Confounder selection

As the purpose of this study was to screen for preliminary associations only, fully adjusted models are beyond the scope of this paper. All analyses were adjusted for post-conceptional age and child sex. The cognitive analyses were also adjusted for the processing factors, researcher and researcher experience, which were shown to independently influence the cognitive measure in our cohort.

Ethics

BIS was approved by the Barwon Health Ethics Committee (BHEC 10/24) with informed, written consent obtained from the parents or guardians.

Results

Study populations

The majority of infants in both cohorts were full-term, vaginally-born, and born to Australian-born parents. The mean (SD) age at interview for both the cognitive and CBCL cohort was 2.46 (0.14) years (Table 1). Mothers of infants with BAYLEY-III and CBCL data were marginally older, more likely to have a degree-level education and smoked less than those in the inception cohort. Infants with BAYLEY-III data were breast-fed longer and

155 received more antibiotics than the inception cohort. Less behavioural symptoms were
156 reported on the CBCL than expected from CBCL normative data (Table 1).

157 **Results**

158 Although there were some individual associations (reported below), no consistent
159 patterns of associations or dose response were found within an outcome nor across the
160 three outcomes. Futhermore, no time period appeared more important than any other.

161 ***Antenatal period***

162 Maternal antibiotic use in trimester 1, but not trimester 2 or 3, was associated with
163 reduced externalising behaviour (Table 2).

164 There was an inverse association with the frequency of disinfectant use in T1 and T3
165 and cognitive scores (Table 2).

166 Having any older siblings at birth was inversely associated with cognitive scores at 2
167 years, but positively associated with lower internalising behaviour (Table 2).

168 ***Birth***

169 Exposure to labour expressed was associated with higher internalising behaviour
170 compared to infants born to mothers who underwent scheduled C-section (Table 2).

171 ***Postnatal Period***

172 Infants who received any breastmilk had higher cognitive scores than infants who
173 received no breastmilk and the longer the infant breastfed, the higher the cognitive scores
174 (Table 2).

175 Postnatal antibiotic use only between 3 to 6 months was associated with reduced
176 internalising behaviour (Table 2).

177 Reduced infant hand washing frequency was associated with reduced internalising
178 behaviour, however, evidence was weak (Table 2). A reversal of this relationship was seen at
179 12m, with reduced infant hand washing frequency associated with increased internalising
180 behaviour (Table 2).

Reduced infant bathing frequency at 6 months was associated with lower cognitive scores and higher externalising behaviour when compared to those infants who were bathed once or more times per day (Table 2).

Dummy use at 4 weeks, 6 or 12 months was associated with lower cognitive scores (Table 2). Length of dummy use (hours) at 4 weeks was also associated with lower cognitive scores. Using the mother's mouth to clean the dummy was associated with higher internalising scores at 4 weeks and 6 months (Table 2).

Childcare attendance was associated with higher cognitive performance than children who did not receive any childcare before the age of 1, although the effect size was small (Table 2).

Hygiene scores

We examined composite hygiene patterns based on the ALSPAC score [15] at 4 weeks, 6 and 12 months, but clear patterns were not detected.

Discussion

Factors altering early life external microbial exposure have been shown to be important in allergic disease programming. The intention of this study was to investigate whether environmental factors previously associated with allergic diseases were also associated with cognition and behavioural outcomes at 2 years of age. Factors also previously linked to gut microbiome were of key interest. In particular, we focused on investigating the strength of a particular factor's influence on individual outcomes, identifying any patterns across the three outcomes, identifying any patterns relating to time of exposure and comparing and contrasting these findings with those from allergic disease.

A factor's influence on an outcome

As expected, breastfeeding was the strongest predictor for cognition, followed by disinfectant use in T3, siblings and weak associations for dummy use bathing frequency and child care attendance. Antenatal disinfectant use at different time periods had the strongest association with both internalising and externalising behaviour. Whereas expected factors,

such as siblings and antibiotic use had less influence. No dose response was found with any factor.

Patterns across the three outcomes

In Table 2, although there was perhaps slightly more evidence of associations than expected by chance (20% with p< or =0.05 compared to the expected 5%), there were no clear and consistent patterns of association between factors associated with FEMEs and any outcome. Further, only the association between the number of siblings and the two outcome cognition and internalising behaviour, would have survived Bonferroni correction.

Time of exposure

Multiple time points for multiple variables were investigated. No time point seemed more significant than any other.

Compare and contrast these findings with those from allergic disease.

Unlike studies from asthma and allergy we did not observe an overall pattern that a more sterile environment lead to more adverse outcomes. Indeed, the direction of some associations were opposite to that expected if a more sterile environment is associated with adverse cognitive and behavioural outcomes, such as vaginal delivery and higher internalising behaviour.

Additionally, there were a number of important null findings. Despite dog ownership being associated with reduce allergic disease in both our[16] and a nearby[17] cohorts, dog ownership did not relate to cognition or behaviour. Also, no clear associations were evident with livestock ownership and C-section. Moreover, no consistent associations were found with maternal antibiotic use. Notably, several of these factors have been linked to the gut microbiome.

In contrast to the educative role bacteria play in the immune system where exposure to a greater range of microbes increases the ability of the immune system to be able to distinguish who is friend and who is foe[18] , the role bacteria play in the microbiota-gut-

234 brain axis is more diverse and complex [8]. Different patterns of association have been
235 observed in both animal and human studies. Higher alpha diversity has been associated with
236 lower overall cognitive scores in human 2 year-olds [19]. Additionally, mice who have been
237 treated with antibiotics display reduced microbial diversity, reduced anxiety and increased
238 exploratory behaviour in open spaces [20]. It is likely relationships between FEMEs and
239 cognition and behaviour are non-monotonic. As brain development is multifactorial and
240 intertwined with social influences, future studies teasing out microbial influences from
241 social influences are warranted.

242 Despite the lack of association between FEMEs and cognition and behavioural
243 outcomes found in this study, the gut microbiome may still play a significant role in
244 cognition and behaviour[21]. Firstly, FEMEs are likely to only explain a small portion of the
245 microbiome variation. Secondly, we only studied FEMEs previously shown to alter allergic
246 disease risk. It may be that other environmental and lifestyle factors that are known to
247 influence brain function and structure and microbiome composition, such as exercise [22],
248 stress [23] or diet [24] are more important for cognition and behaviour than those found to
249 be important for immune programming.

250 This study's strength is that it is the first epidemiological study to investigate
251 associations between a comprehensive range of early-life FEMEs and three outcomes:
252 cognition, internalising and externalising behaviour, from a population-derived cohort.

253 There are several limitations to this study. Firstly, as our cohort is an unselected sample, it
254 may not be representative of all mothers and live births. Secondly, as the purpose of this
255 study was to screen for preliminary associations only, models were not fully adjusted and
256 multiple corrections were not applied in order to consider each association on its own [14].
257 Additionally, many of the reference groups have low numbers. Exposures were analysed in
258 this manner, instead of using a reference group with the highest numbers in order to

investigate a dose-response effect and for consistency with past allergy papers. Furthermore, we recognise that the relationships may be non-monotonic, however, non-monotonic investigations are beyond the scope of this paper. Lastly, our cohort reported less behavioural symptoms than expected based on normative data[13]. There may not be sufficient variation in either the levels of exposure or in the levels of cognition or behaviour in our cohort, limiting our ability to detect associations. Further work would ideally assess trajectories rather than outcomes at a single time point.

Conclusion

Overall there was no consistent pattern across the FEMEs and cognition and behaviour at 2 years of age. In contrast to allergic disease, there was no evidence that markers associated with a more sterile early environment increase neurodevelopmental risk. Direct investigations into gut microbiome compositional differences and cognition and behaviour in humans are now necessary to determine the role the gut microbiome plays in brain function and development.

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Figures and Tables

Figure 1: Flow chart of the participating mother-infant dyads in the initial cohort, BIII and CBCL sample (adapted from [10]).

Table 1: Characteristics of the participants who completed the measure(s).

Infant and family characteristics are presented as mean (SD) or n (percentages) for those who completed either the BAYLEY-III (n=667) or the CBCL (n=666).

Table 2: The relationship between the external microbial exposures and cognition and behaviour