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Using difference-in-difference to understand the downside to antibiotic use during infancy

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In this issue of *Pediatric Research*, Lebeaux et al. explore the off-target effects of antibiotics on the infant gut microbiome and resistome.[1] The developing immune system of infants makes antibiotics a key tool in helping infants recover from infection; however, use of antibiotics is not without cost. Use of antibiotics increases antimicrobial resistance, unfortunately moving us into the post-antibiotic era where previously treatable infections are again threats to human health.[2] In infants, antibiotic use is further complicated as it is associated with a range of diseases that develop later in life, including obesity and diabetes.[3] These effects are thought to be mediated by changes in the composition of the gut microbiome and resistome. However, studying the off-target effects of antibiotics in infants is more challenging than studying the off-target effects of antibiotics in adults because the gut microbiome is naturally assembling and changing rapidly during the first 2 years of life.

Lebeaux et al. met the challenge of understanding the off-target impact of antibiotics on the infant gut microbiome by applying the difference-in-difference (DID) approach to microbiome data.[1] The DID approach holds promise for studying the causal effects of interventions on the microbiome when randomization is not feasible.[4] Other potential examples include studying the effects of diet (i.e., human milk feeding versus formula feeding of infants), disease state, and pollution on the gut microbiome. The work presented by Lebeaux et al. demonstrates that this is a viable approach, particularly when longitudinal cohorts with repeated collection and whole metagenomic sequencing of stool samples are available for analysis. Repeated sampling prior to the first exposure permits testing of the common trend assumption for DID models.[4] The common trend assumption is that the changes observed in the control or untreated group are a good proxy for the outcomes in the exposed or treated group if the exposure or treatment had not occurred (Fig. 1).[4] In addition to the rapid rate of change in the assembling gut microbiome making off-target effects of antibiotics more difficult to study, infancy is a time of life when the common trends assumption is more likely to hold as we expect all infants to be experiencing a rapid rate of change in the gut microbiome as they approach a stable, adult-like gut phenotype. The second assumption of DID models that must be considered to understand if the model is valid is strict exogeneity. Strict exogeneity is the assumption that treatment exposures that occur later are unrelated to outcomes measured at earlier periods (full discussion of this is beyond the scope of this commentary, please see Wing et al. for more detail).[4]

The DID approach combined with whole metagenomic sequencing permitted Lebeaux et al. to explore both the changes in gut microbiome taxonomic composition and in antimicrobial resistance gene carriage. The off-target effects of antibiotics on taxonomic composition identified by Lebeaux et al. highlight the importance of selecting the proper sequencing methods. By using whole metagenomic sequencing, Lebeaux et al. were able to explore species levels changes in the infant gut microbiome in response to antibiotics. Of note, some species within the same genus had differing responses to antibiotics. In the genus *Bacteroides*, antibiotics drove an increase in *B. vulgatus* relative abundance but a decrease in *B. fragilis* relative abundance. Within the genus *Bifidobacterium*, antibiotics exposure increased the relative abundance of *B. bifidum* but decreased the relative abundance of *B. longum* and *B. breve*. These species-level differences would not be observable with amplicon sequencing, and serve as a reminder that off-target effects of antibiotics observed in studies that sequence only a small region of the 16S rRNA gene may miss an important nuance. For example, Korpela et al. recently reported that antibiotic use in infancy decreases *Bifidobacterium* levels,[5] but lacks the insight of the Lebeaux et al. paper into which species were affected. In the example of genus *Bifidobacterium*, this nuance is important as members of this genus differ significantly by species in their ability to ferment carbohydrates and to serve as "cross-feeders" for other members of the microbial community.[6]

Infants who both received antibiotics and attended daycare additionally had both an increase in relative abundance of *Escherichia coli* and an increase in antimicrobial resistance genes.[1] The increase in *E. coli* is particularly concerning as *E. coli* serve as a reservoir for transferable antimicrobial resistance genes in the gut microbiome.[7] This suggests that future studies of the resistome of daycares may be warranted, as daycares may represent an environmental reservoir of transferable resistance genes that can readily incorporate into the gut microbiome when infants are exposed to antibiotics.

Among the changes in the resistome, Lebeaux et al. report that among all infants, the *CfxA6* gene experienced the greatest increase in abundance.[1] *CfxA6* is a beta-lactamase, so it is a logical gene to increase given that penicillin and amoxicillin are considered first-line antibiotics for infants. But this result varied

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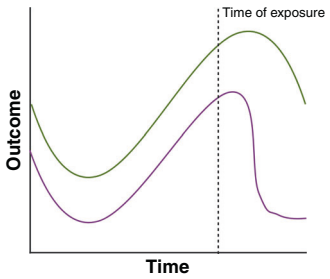

Fig. 1 A generic illustration of the common trend assumption. The common trend assumption means that prior to exposure, changes in the outcome measure occur in parallel between the two groups. Trends do not necessarily have to be linear for this assumption to be met. On this plot, the green line is for the unexposed group and the purple line is for the exposed group. Both groups change in parallel prior to the exposure, after which the exposed group diverges from the unexposed group.

based on whether or not infants attended daycare in the first year of life, as while *CfxA6* abundance was associated with antibiotic use in the full cohort, this association was not detected when considering only infants who attended daycare.[1] Even though *CfxA6* abundance did not change in the daycare attending infants, a number of other antimicrobial resistance genes did increase with antibiotic use in daycare attending infants.[1] This suggests that the lack of difference in *CfxA6* in the daycare attending subset was not due to a decreased sample size and reduced power. Furthermore, another difference between the full cohort and the daycare attending subset was in the overall antimicrobial resistance gene carriage. In the full cohort, there was no association between total resistance gene carriage and antibiotic use, but in the subset of infants who attended daycare, there was a significant increase in total resistance gene abundance with antibiotic use.[1] The different impact of antibiotic use in infants who attended daycare compared to those who did not further supports the need to study daycares as reservoirs of antimicrobial resistance genes, including for the potential of horizontal transmission of microbes from infant to infant serving as a source of bacteria for the developing gut microbiome and resistome. Many major colonizers of the infant gut, such as *B. longum* and *B. breve* tend to be sensitive to antibiotics and are less adept at horizontal gene transfer than some of the more potentially pathogenic species such as *E. coli*. Antibiotic resistance allows these more adaptable species to expand after treatment with antibiotics, driving a dysbiosis of the gut, which might facilitate the transfer of these genes to other infants in a shared environment, reducing some of the differences between exposed and unexposed infants.

This intestinal dysbiosis, including changes in taxonomic composition and increased abundance of antimicrobial resistance genes that result from infant use of antibiotics, serves as an important reminder to us all that antibiotic stewardship is about more than just preserving antibiotics for future use. Antibiotic stewardship is about protecting the future health of the individual. Indeed, antibiotic use in infancy is a double-edged sword, and both improves and harms infant health. As such, there is a need for better metrics to understand both the quantitative and qualitative nature of antibiotic prescribing in pediatric populations.[8] Increased understanding of when antibiotics are being used appropriately in this population will help target interventions to reduce antibiotic use, thus catering to more individualized care while preserving the efficacy of existing antibiotics. But understanding of antibiotic use patterns and reservoirs of antimicrobial resistance genes that infants are exposed to will not do away with the need for antibiotic use entirely. The next challenge is to also learn how to help the microbiome recover after the use of necessary antibiotics. Diet is emerging as a potential option to reduce the carriage of resistance genes,[9] but remains understudied in infants. Korpela et al. suggested *Bifidobacterium* probiotics as a way to help the gut microbiome recover,[5] but the more nuanced, species-level results of Lebeaux et al. highlight the need to better understand the off-target effects of antibiotics and which populations specifically need to recover before probiotics are likely to be an effective intervention. Work in this direction has begun, and includes studies of probiotics to reduce the carriage of antimicrobial resistance genes.[10]

Taken together, the DID approach shows promise for future studies into the impact of external factors on the gut microbiome. In the present work, Lebeaux et al. demonstrate that this approach can identify nuanced differences between populations in both microbial composition and antimicrobial resistome. These differences demonstrate the need for antimicrobial stewardship not only for preserving the effectiveness of antibiotics for the community at large, but also for the long-term health of the individual.

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AUTHOR CONTRIBUTIONS

D.H.T. and A.W.T. co-wrote the initial outline for this paper. D.H.T wrote the initial draft of this manuscript, and both edited the manuscript until it reached its present form. Both authors approved the final draft.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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