Development of Synbiotics for Repairing the Microbiota of Children with Acute Malnutrition and Restoring Healthy Growth

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Jeffrey Gordon *is Dr. Robert J. Glaser Distinguished University Professor and Director of the Edison Family Center for Genome Sciences and Systems Biology at the Washington University School of Medicine in St. Louis, Missouri. He is Principal Investigator of this three-year project investigating the role of the microbiota in children's health. Michael Barratt, Associate Professor of Pathology and Immunology and Director of the Washington University School of Medicine's Center for Gut Microbiome and Nutrition Research has been named Deputy Supervisor, and Tahmeed Ahmed, Senior Director of the Nutrition and Clinical Services Division and Executive Director of the International Centre for Diarrhoeal Disease Research in Bangladesh (icddr,b) is Co- Principal Investigator. Administrative responsibility rests with the Washington University School of Medicine. For nearly ten years, the Washington University-icddr,b collaboration has leveraged the deep clinical and nutritional sciences expertise and long-standing community engagement of Dr. Ahmed and his colleagues at icddr,b, and the experimental and computational approaches (including gnotobiotic animal models) for characterizing the dynamic operations and host effects of the human gut microbiota/microbiome developed in the Gordon lab at Washington University in St. Louis.*

Context

Childhood undernutrition is the leading cause of death in those <5-years-old worldwide. It is not due to food insecurity alone; many aspects of its pathogenesis remain uncharacterized and current treatments are inadequate. Gordon's lab's birth-cohort studies in low-/middle-income countries, including Bangladesh, identified 'age-discriminatory' gut bacterial strains whose changes in representation define a program of normal gut microbiota development that is largely executed during the first two postnatal years. Together with Tahmeed Ahmed, Executive Director of the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b), they discovered that moderate and

severe acute malnutrition (MAM/SAM) are associated with impaired postnatal gut microbiota development ('immaturity') that is not repaired with current nutritional interventions. By transplanting microbiomes from healthy and undernourished children into germ-free mice, Gordon's lab identified bacterial strains associated with key facets of postnatal growth/development; these strains, a number of which are inappropriately represented in the immature microbiomes of undernourished children, became their therapeutic targets.

To develop culturally acceptable, affordable and scalable treatments, combinations of Bangladeshi complementary foods (i.e., those given as children are being weaned) were screened in gnotobiotic mice and gnotobiotic piglets harboring gut communities from undernourished Bangladeshi children, yielding microbiome-directed complementary food (MDCF) prototypes that increased the fitness and beneficial metabolic activities of the targeted bacterial strains. Randomized controlled clinical trials of their lead MDCF showed that it produced significantly greater rate of ponderal compared to an existing ready-to-use supplementary food (RUSF), even though the MDCF had 15% lower caloric density. Linear growth also became significantly better, a remarkable finding considering how ineffective current therapies are for stunting.

MDCF's superior effect on growth was accompanied by superior microbiota repair and greater effects on levels of plasma protein mediators of musculoskeletal development, neurodevelopment, metabolism, and inflammation (NEJM 384,1517 (2021); Sci.Transl.Med.16, adn2366 (2024)).

As described below, the project has proceeded to; (i) characterize the genomes of bacterial strains whose abundances were positively-associated with growth of these children, (ii) define changes in expression of these strains' metabolic pathways as a function of treatment type and host responses, (iii) identify the key bioactive carbohydrate structures in MDCF; (iv) demonstrate that *Prevotella copri* strains positively-correlated with growth were major contributors to the metabolism of these bioactive glycans and key effectors of changes in gut epithelial metabolism; and (v) identify novel mediators of host physiology produced by a novel enzyme expressed by a growth-promoting MDCF microbial target (Nature 625, 237 (2024); Nature Microbiology 9, 922 (2024); Sci.Transl.Med.16, adn2366 (2024); Science 386, eado6828 (2024)). Together with studies of Bangladeshi infants with severe acute malnutrition (Sci.Transl.Med.14, abk1107(2022)) and the small intestinal microbiota of stunted Bangladeshi children with environmental enteric dysfunction (EED) (NEJM 383, 321(2020); biorxiv.org/content/10.1101/2024.11.01.621574v1), Gordon's team's work is revealing a causal link between gut microbiome development, systems physiology and healthy growth, and a new approach for treating malnutrition.

Progress on the four specific aims of Gordon's Balzan Foundation Research Project is summarized below.

Aim 1 – Compare the genome sequences of *P. copri* strains cultured from the fecal microbiota of healthy and undernourished Bangladeshi children, and their mothers, living in several demographically distinct areas of Bangladesh.

Gordon's research group has performed a pan-genome analysis using the microbial community (mc)SEED database (Overbeek et al., Nucleic Acids Res. 33:5691; 2005). This analysis encompassed 339 members of *Provetella* including 89 *P. copri* strains spanning four phylogenetically distinct clades (A-D) and involved *in silico* metabolic reconstructions with mcSEED. mcSEED represents over 100 subsystems that

include pathways for biosynthesis of essential nutrients (vitamins, amino acids, nucleotides), respiration and utilization of carbohydrates. The latter (largest) category includes pathways for degradation and utilization of 16 polysaccharides, 15 oligosaccharides and 42 mono- and disaccharides.

By comparing the distribution of the reconstructed metabolic pathways across 40 Bangladeshi isolates and 50 related reference genomes from the same species-level groups, they identified conserved and variable pathways within and between each species-level group. This information proved to be vital in identifying bacterial isolates, cultured from the fecal microbiota of children representing the MDCF study population, that correspond to the *P. copri* metagenome assembled genomes (MAGs) Bg0018 and Bg0019 that they had identified in their randomized clinical trials of Bangladeshi children with moderate acute malnutrition (MAM) as (i) positively correlated with participant growth responses and (ii) the principal source of transcriptional responses involving metabolism of the bioactive polysaccharides present in our MDCF (Hibberd et al., 2024; Chang et al., 2024).

Aim 2 – Conduct in vitro tests of the capacity of the different cultured *P. copri* strains to utilize different glycan components contained in their lead MDCF.

To contextualize their observations regarding the conserved polysaccharide degradation features of *P. copri* strains (metagenome associated genomes) identified in their randomized controlled clinical trials of Bangladeshi children with moderate acute malnutrition (MAM), representatives of these MAGs from fecal samples obtained from trial participants were cultured and characterized. They focused on five *P. copri* isolates that represented diverse repertoires of polysaccharide utilization loci (PULs) as well as a range of phylogenetic distances from the growth-associated MAGs Bg0018 and Bg0019 (**Fig. 1a,b**).

Cultured *P. copri* strain BgF5_2 is highly related phylogenetically to Bg0018 and Bg0019. Notably, it possesses nine of the ten conserved PULs in these MAGs (Fig. 1a). Based on (i) the predicted glycan targets of each conserved PUL, (ii) the bioactive glycan components of MDCF that we had identified by mass spectrometry and (iii) the pattern of expression of PULs in these *P. copri* MAGs in trial participants as a function of MDCF treatment, selected eight glycan preparations (sugar beet arabinan, wheat arabinoxylan, barley β-glucan, potato galactan, carob galactomannan, soybean rhamnogalacturonan, tamarind xyloglucan, and beechwood xylan) were selected for in vitro screening of their effects on growth and PUL gene expression in *P. copri* strain BgF5_2 plus four other Bangladeshi *P. copri* isolates with overlapping but distinct PUL repertoires. Chondroitin sulfate was included in the panel as a negative control given its resistance to degradation by *P. copri*. Each cultured isolate was grown in a defined medium containing 1% (w/v) of each glycan as the sole carbon source.

The results underscored the broad glycan utilization capabilities of the *P. copri* isolates but also highlighted their distinct preferences for individual glycans. Importantly, growth phenotypes aligned with PUL repertoires, the known and predicted substrate specificities of the carbohydrate-active enzymes (CAZymes) encoded by PULs, and the results of mass spectrometry-based quantification of their consumption of monosaccharide components of the tested glycans (**Fig. 1c,d**). Isolates whose PUL profiles matched the two growth-associated *P. copri* MAGs most closely (strains BgF5_2, Bg2C6, Bg2H3) displayed the strongest preference for glycan substrates that were enriched in or unique to MDCF relative to RUSF, including arabinans (arabinan, arabinoxylan) and

galactans/mannans (galactan, galactomannan). Notably, strain BgF5_2 displayed growth preferences for arabinoxylan and galactan whereas all other strains favored arabinan over arabinoxylan. Together, these results support predictions of the capacities of the two growth-associated *P. copri* MAGs to utilize MDCF glycans. In addition, they also revealed that *P. copri* strain BgF5_2 could be considered to be a cultured representative of MAGs Bg0018 and Bg0019.

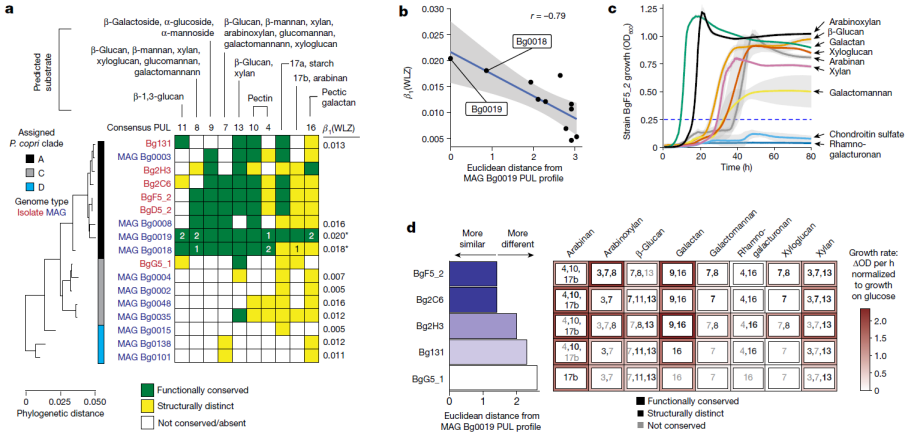

Fig. 1 Relationship between PULs in *P. copri* MAGs and cultured isolates and growth responses of isolates to different MDCF glycans. a, PUL conservation in *P. copri* MAGs identified in clinical trial participants (blue font) and in *P. copri* isolates cultured from Bangladeshi children (red font). The marker-gene-based phylogenetic tree (left) indicates the relatedness of *P. copri* MAGs and isolates. The β1(WLZ) coefficient in a linear mixed effects model relating MAG abundance to ponderal growth response (anthropometrically defined as weight-for-length Z score, WLZ)) is plotted on the y-axis; significant associations ($q < 0.05$) are indicated by asterisks. The matrix in the center depicts PUL conservation among *P. copri* MAGs and cultured isolates relative to the growth-associated MAG Bg0019. **b**, Relationship between PUL conservation in the 11 P. copri MAGs identified in study participants and the association of each MAG abundance with ponderal growth (WLZ). The gray ribbon indicates the 95% confidence interval. **c,d**, *In vitro* growth assays for five *P. copri* isolates in defined medium supplemented with individual purified glycans representative of those in MDCF. $n = 3$ replicates per condition; two independent experiments were performed; representative results from one are shown. c, Results obtained with *P. copri* BgF5_2, the isolate whose PUL profile is most similar to MAGs Bg0019/Bg0018. Mean values ± s.d. (gray ribbons) for optical density at 600 nm (OD600). d, Summary of PUL conservation and growth rates for the five *P. copri* strains tested. Each colored box lists PULs in each strain (rows) that are predicted to metabolize the indicated carbohydrates. PULs are denoted as functionally conserved (black, bold), structurally distinct but functionally similar (black, not bold) or not conserved (gray) according to the scheme shown in a. The color intensity surrounding each box indicates the mean maximum growth rate for each isolate in the presence of each glycan.

Aim 3 – Advance lead glycans and lead *P. copri* strains from Aim 2 to preclinical studies involving gnotobiotic mouse models of mother-to-child microbiota transmission.

The goal of the project is to leverage the knowledge being gained from preclinical and translational (randomized controlled clinical studies) of MDCF and microbiome structure/function relationships to guide development of mixtures of prebiotics, comprised of bioactive polysaccharide structures that are present in MDCF but

obtained from the byproduct streams of food manufacture. These prebiotic mixtures are envisioned as means for supplementing locally available foods to treat undernutrition and for creating healthy snacks that help ensure normal development of the microbiota during and following weaning. The availability of these mixtures could help overcome the many challenges of manufacturing and distributing shelf-stable therapeutic foods that are acceptable to individuals representing different cultural traditions.

As noted above, metagenomic and meta-transcriptomic analyses of fecal samples from children enrolled in Gordon's clinical translational studies of MDCF in Bangladesh identified MAGs whose abundances and/or expressed functions, especially those related to carbohydrate utilization, are linked to improvements in growth. These include MAGs assigned to *Prevotella copri, Faecalibacterium prausnitizii* and members of *Blautia*. Their culturing initiative has focused on recovering Bangladeshi strains representative of these key WLZ-associated MAGs based on their whole-genome average nucleotide identity and functional characteristics (i.e., encoded mcSEED metabolic pathways, PUL and CAZyme repertoires). This effort has successfully yielded isolates belonging to *P. copri* (see above) as well as other WLZ-associated taxa, collectively referred to as 'therapeutic targets', for our ongoing prebiotic discovery efforts.

Using gnotobiotic mice that had been colonized during the pre-weaning and weaning periods by dam-to-pup transfer of serially administered consortia of cultured, genome-sequenced Bangladeshi age- and growth-discriminatory bacteria, it was determined that *P. copri* strain BgF5_2 mediates weight gain, plays a key role in degradation of MDCF bioactive glycans, and modulates energy metabolism in intestinal epithelial cells (Chang et al., 2024). Importantly, the establishment of this model has enabled direct tests of the effects of prebiotics designed to promote the representation/expressed beneficial functions of these bacterial therapeutic targets as well as mechanistic characterization of their effects on host physiology (gut, brain, bone, etc.).

Mass spectrometry-based analyses have been applied to the carbohydrate containing components of MDCF (peanut, chickpea, soy and plantain, sugar) and RUSF (rice, lentil, plus milk powder and sugar). As noted above, these studies have revealed that MDCF contains a distinct composition of complex polysaccharides compared to RUSF, with greater representation of various galactans, mannans and pectic glycans (including rhamnogalacturonans, oligo-galacturonans) (Hibberd et al., 2024). It has also been shown that (i) isolates representing WLZ-associated taxa (e.g., *P. copri*) preferentially metabolize a number of these polysaccharide species, and (ii) their breakdown products (e.g. rhamnose, galactose, arabinose, fucose, etc.) are utilized by other growth-associated taxa (*F. prausnitzii, Blautia* spp.) *in vitro* (Hibberd et al., 2024; Lynn et al., *in preparation*). Initial studies of individual or simple combinations of representative glycans (glucomannan, rhamnogalacturonan I) in this gnotobiotic model revealed transcriptional responses in growth-associated taxa consistent with their utilization of these glycans.

Two parallel strategies are being employed to assemble prebiotic mixtures for testing in their gnotobiotic mouse model:

> *Strategy 1* - Use a mixture of ***purified*** 'research grade' polysaccharides (with analytical QC) selected based on (i) knowledge of bioactive MDCF polysaccharide structures, (ii) the predicted and/or known substrate specificities of their CAZyme targets (the latter knowledge gained in part from our direct biochemical tests of these CAZymes expressed in and purified from *E. coli*), and (iii) the *in vitro* growth and transcriptional responses of growth-associated taxa to

these glycans. This approach is limited by the cost of these purified glycans, the potential for their production to be scaled to levels needed for clinical translational studies, and their cost.

Strategy 2 - Prioritize prebiotics that are already available at scale (e.g., food supplements, products obtained from the waste streams of food manufacture). The advantage of this approach is speed with a focus on existing scalable ingredients over purity/specificity. The risks of this strategy relate to efficacy and ease of interpretation of results. Not all glycan components in MDCF have existing large scale commercial sources. The purity of material obtained from commercial sources is likely lower and potentially more variable. Additional structural characterization of commercial glycan preparations may be needed (together with an assessment of their cost).

The effects of adding these prebiotic mixtures to a background Bangladeshi diet is being assessed in gnotobiotic mice that have been colonized by dam-to-pup transmission of consortia of age- and growth-discriminatory Bangladeshi bacteria strains (**Fig. 2**). Microbial RNA-Seq of fecal/cecal samples are initially being used to identify glycan utilization pathways that are most strongly upregulated in age- and growth-discriminatory consortia members to guide follow-on studies of refined prebiotic mixtures. Results of these experiments will allow prioritization of leads and establishment of MTAs with commercial suppliers of food grade materials.

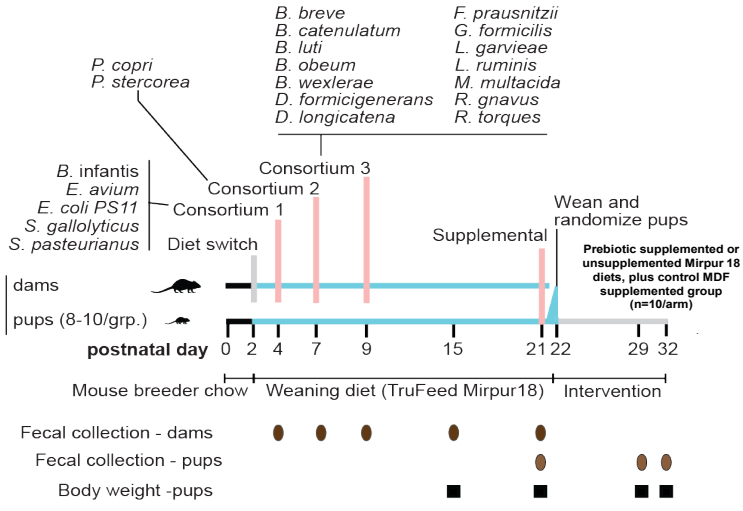

Fig. 2 –Design of the dam-pup model of transmission of a defined community of age- and growth-associated bacterial taxa that is being used to assess the efficacy of prebiotic candidates.

In addition to these experiments involving mice colonized with defined consortia of cultured, genome-sequenced, age- and growth-discriminatory bacterial strains, parallel experiments have involved mice colonized with mixtures of intact uncultured fecal

samples obtained from children with MAM or SAM. Donors initially are from Bangladesh but this type of preclinical model containing 'metacommunities' can be extended to involve fecal microbiota obtained from children with MAM or SAM (and control healthy counterparts) living in other low- and middle-income countries. This approach will make it possible to test lead prebiotic mixtures added to diets corresponding to those consumed by these different groups of undernourished children and use the results to inform design of future clinical translational studies involving the very population whose microbiota have been incorporated into our preclinical model.

Analyses of these gnotobiotic mice include assessments of (i) microbiota reconfiguration (abundances of bacterial strains, microbial RNA-Seq of expression of carbohydrate utilization and other metabolic pathways in age- and growth-discriminatory strains plus exclusion of potentially pathogenic strains, (ii) lean body mass gain and bone growth, (iii) levels of translatable plasma biomarkers of various facets of systems physiology (bone, CNS, musculoskeletal development), (iv) targeted mass spectrometry of microbial community and host metabolism, (v) immune profiling including assessments of gut mucosal barrier function, and (vi) bulk- and single nucleus RNA-Seq of intestinal gene expression.

Aim 4 – Support the creation of a 'Clinical Microbiome Laboratory Medicine Program' at icddr,b through training programs and reciprocal secondments between the project's two institutions.

icddr,b has recently established a Genomics Center led by Dr. Mustafizur Rahman. This Center is integral to the capacity building efforts supported by the Balzan Award. Members of our Washington University team have provided a series of Zoom training sessions on the project's bioinformatics pipelines for analyzing metagenomic datasets. These training sessions have facilitated analyses by icddr,b colleagues of datasets comprised of sequenced bacterial V4-16S rRNA amplicons generated by icddr,b team members from fecal specimens collected from Bangladeshi infants, children and mothers of childbearing age with various clinical phenotypes. Manuscripts describing the results are currently in preparation.

In 2023 and 2024 two icddr,b 'Balzan Foundation scholars' were hosted for 6 months each in Gordon's lab at Washington University. These individuals were selected from a shortlist handpicked by the Executive Director of icddr,b, Dr. Tahmeed and members of his senior leadership team. Selection was based on their scientific and leadership potential. The specific learning objectives for the 6-month training secondments included mastery of pipelines for (i) characterizing bacterial strains by 16S rDNA amplicon sequencing of DNA isolated from biospecimens obtained from participants in the project's clinical translational studies, (ii) characterizing MAGs (metagenome-assembled genomes) present in these biospecimens including annotation of their CAZymes and mcSEED metabolic pathway components, (iii) characterizing MAG gene expression (from microbial RNA-Seq analyses), (iv) sequencing, assembly and annotation of the genomes of cultured gut bacterial strains corresponding to growth-associated MAGs and (v) qPCR-based quantification of specific bacterial strains within the complex matrix of human fecal samples.

Their training was subsequently applied to datasets generated from a set of fecal samples serially collected by icddr,b scientists from a Nutritional Counselling study of women at risk for undernutrition who lived in a rural site in Bangladesh. Both scholars have returned to icddr,b and are now in the process of writing a manuscript about the results of their analyses (with guidance from senior scientists in the Gordon Lab).

Members of Gordon's lab visited iccdr,b in February 2025 to participate in project planning meetings and to discuss progress with development of the Genome Center. This visit also provided them with an opportunity to reconnect with their first two scholars and meet the two 2025 Awardees. At the time of writing this progress report, these Awardees have arrived in St. Louis and are undergoing training in the lab. Their goal is to become familiar with the mass spectrometry-based metabolomic and anaerobic microbiology pipelines there, with a view to bolstering this capability at icddr,b upon their return. Their specific projects are focused on characterizing a novel class of bacterial fatty acid amide hydrolase enzymes recently identified in Gordon's lab (Cheng et al., 2024). These enzymes, present in a number of growth-associated bacterial strains found in the fecal microbiota of Bangladeshi children, exhibit different specificities for (i) hydrolyzing a variety of *N*-acylamides, including oleoylethanolamide (OEA), neurotransmitters and quorum-sensing *N*-acyl homoserine lactones, and (ii) synthesizing a range of *N*-acylamides, notably *N*-acyl amino acids. The project has shown that some of these metabolites have pharmacologic activity *in vitro* and immunomodulatory effects in gnotobiotic mice, and anticipates that the contributions of the icddr,b Balzan scholars to this project will not only provide them training in Gordon's experimental and analytic pipelines but also lead to impactful publications in this emerging and potentially clinically important area of microbiome research.

These capacity building efforts are allowing the icddr,b to establish a *human microbial observatory program* of the Washington University-icddr,b type of team described in Gordon et al. (2024). The goal of this program is several fold: (i) define normal microbiome development and expressed functions in healthy infants and children through the first decade of life as well as the effects of pregnancy on the structural and functional configuration of the maternal gut microbiome (in different regions of Bangladesh where populations with different cultural/socioeconomic features reside and where climate change is already and/or is forecast to have marked effects on the environment, agriculture, and lifestyles); (ii) conduct long-term follow-up studies of the effects of treatment with MDCF (and later, prebiotic mixtures) on systems physiology including assessments of CNS development, vaccine responses, the impact of age of initiation of microbiome repair on efficacy and whether post-treatment 'booster doses' of microbiome-directed therapeutics allow for greater and more sustained physiologic benefit; (iii) study the effects of gut microbiome repair in undernourished young women on pregnancy outcomes; (iv) discover and develop complementary foods and healthy snacks that support healthy microbiome development; (v) advance initiatives related to the ethical, policy and educational issues raised by microbiome-related research in mothers, infants and children, and (vi) build the infrastructure required for a group of young talented researchers to advance work at the intersection between climate change, agriculture, microbiome science, food science and nutritional science.

Recent publications from the icddr,b – Wash U collaboration related to this report.

M. C. Hibberd, et al., Bioactive glycans in a microbiome-directed food for children with malnutrition. *Nature* **625**, 157–165 (2024).

H.-W. Chang, et al., *Prevotella copri* and microbiota members mediate the beneficial effects of a therapeutic food for malnutrition. *Nat. Microbiol.* **9**, 922–937 (2024).

Mostafa, et al., A microbiota-directed complementary food intervention in 12–18-month-old Bangladeshi children improves linear growth. *eBioMedicine* **104**, 105166 (2024).

Hartman, S.J. et al. A microbiome -directed therapeutic food for children recovering from severe acute malnutrition. *Science Translational Medicine,* **16,** eadn2366 (2024).

J. Cheng, et al., A human gut *Faecalibacterium prausnitzii* fatty acid amide hydrolase. *Science* **386,** eado6828. (2024).

J.I. Gordon et al., Establishing human microbial observatory programs in low-and middle-income countries. *Annals New York Acad Sci.* **1540,** 13-20 (2024).