ORIGINAL ARTICLE

Autism and Folate-dependent One-carbon Metabolism: Serendipity and Critical Branch-point Decisions in Science

自闭症和叶酸依赖一碳代谢：科学的意外发现和关键的分支点决定

El autismo y el ciclo de un carbono dependiente de folato: los hallazgos casuales en la ciencia y las decisiones fundamentales en los puntos de ramificación

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Folate-dependent one-carbon metabolism is present in every cell of the body. It represents a central systems biology hub that reverberates into countless other pathways with more specialized roles in specialized cell types throughout the body. I have spent 25 years of research on this core biochemical pathway with several unanticipated iterations that led me from Down syndrome to congenital heart defects to leukemia and finally to autism about 12 years ago.

Figure 1 provides an overview of the three interdependent pathways involved in folate-dependent methionine "transmethylation" and "transsulfuration." Methionine is necessary for the synthesis of S-adenosylmethionine (SAM), the major methyl donor for all cellular methylation reactions. It is also the major precursor for cysteine, the rate-limiting amino acid for glutathione synthesis linking transmethylation and transsulfuration pathways. Methionine levels can be negatively affected by genetic and environmental factors that reduce folate availability and/or oxidative inhibition of the methionine synthase enzyme. Because these three metabolic pathways are mutually interdependent, genetic or environmental perturbation of folate or methionine metabolism will indirectly impact glutathione synthesis, and conversely, alterations in glutathione synthesis will alter flux through pathways of folate and methionine metabolism. This interdependency translates into broader impact on essential cellular functions.

As illustrated in Figure 1, Pathway 1 is the folate cycle, Pathway 2 is the methionine cycle (transmethylation), and Pathway 3 is the transsulfuration pathway leading to glutathione synthesis. The functional importance of these pathways is underscored by their essentiality for error-free DNA synthesis, proliferation, and immune function (Pathway 1); for cellular methylation reactions including DNA, RNA, protein, and phospholipid methylation, epigenetic control of gene expression as well as membrane signaling (Pathway 2); and for the maintenance of cellular redox homeostasis and detoxification capacity (Pathway 3). The ratio of glutathione to oxidized glutathione (GSH:GSSG) reflects the redox potential of the intracellular environment, which is critical for maintenance of normal redox signaling and antioxidant and detoxification capacity. Glutathione is present in millimolar concentrations inside the cell and is the major determinant of intracellular redox homeostasis and cell detoxification/antioxidant capacity. Cell functions affected by glutathione deficit include cell proliferation (eg, immune function, DNA synthesis and repair), essential methylation (eg, DNA, RNA, protein, phospholipid, neurotransmittors, creatine) and redox homeostasis (eg, cell signaling, detoxification, stress response, cell cycle progression, apoptosis). Clearly, these are essential pathways for cell viability and normal cell function.

Now let's get back to my pathway to autism. A colleague asked me if I was aware that cystathionine beta synthase (CBS), an enzyme in the transsulfuration pathway, was on chromosome 21 and could influence the pathogenesis of Down syndrome (trisomy 21). My head was immediately filled with all sorts of possibilities of how CBS overexpression (3 copies) could impact the metabolic profile of children with Down syndrome. I started by testing lymphoblastoid cell lines derived from individuals with Down syndrome, and sure enough, their transmethylation and transsulfuration metabolites were abnormal. We then wanted to test whether plasma levels of these metabolites from fresh blood samples from the children with Down syndrome also were abnormal compared to those of unaffected control children. One of the major problems with doing pediatric case-control studies is finding control children who are willing to stick out their arms for research. I came up with a solution: the unaffected siblings with two copies of chromosome 21 would be perfect controls for Down syndrome. To my amazement, the moms and siblings were more than willing to help, and we easily got enough control samples for our study.[1]

One of the case-control pairs was very odd. I could usually tell which profile was from the Down syndrome sibling and which was from the control sibling. For this particular pair, I could not tell which was which and actually had to call the Mom to inquire. It turned out that these two boys were twins. One had Down syndrome, and the control sibling had autism! I knew nothing about autism. And this was an n of 1, so the question was whether it was a real finding or just a technical fluke. Allocation of time and resources is always a major issue in research in an era of tight funding. I could have easily written it off as a technical error not worthy of pursuit (it happens)—except for the insistence of one incredible mom, Laurette Janak, who encouraged me to pursue this odd result by insisting that she could get me 20 more samples from

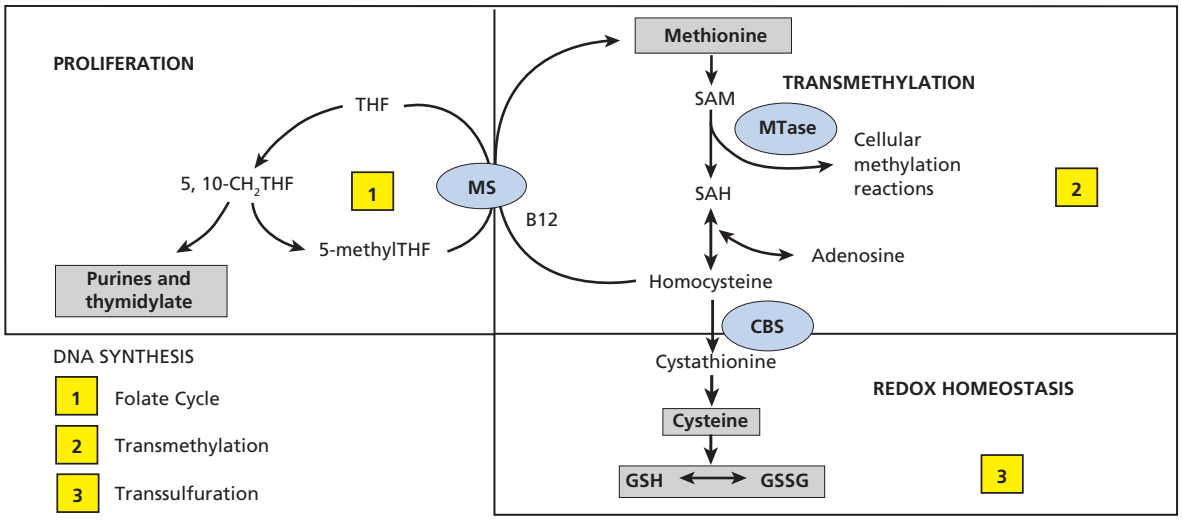

Figure 1 A diagram of tetrahydrofolate (THF)-dependent methionine transmethylation and glutathione synthesis. The methionine cycle (transmethylation) involves the regeneration of methionine from homocysteine via the B_{12}-dependent transfer of a methyl group from 5-methyl-tetrahydrofolate (5-CH3THF) via the methionine synthase (MS) reaction. Methionine is then activated to S-adenosylmethionine (SAM), the methyl donor for multiple cellular methyltransferase (MTase) reactions and the methylation of essential molecules such as DNA, RNA, proteins, phospholipids, creatine, and neurotransmittors. The transfer of the methyl group from SAM results in the demethylated product S-adenosylhomocysteine (SAH). The reversible hydrolysis of SAH to homocysteine and adenosine by the SAH hydrolase (SAHH) reaction completes the methionine cycle. Homocysteine can then be either remethylated to methionine or irreversibly removed from the methionine cycle by cystathionine beta synthase (CBS). This is a one-way reaction that permanently removes homocysteine from the methionine cycle and initiates the transsulfuration pathway for the synthesis of cysteine and glutathione. Glutathione is shown in its active reduced form (GSH) and its inactive oxidized disulfide form (GSSG). Glutathione is present in millimolar concentrations inside the cell and is the major determinant of intracellular redox homeostasis. Cell functions affected by perturbations in these interwoven pathways include proliferation (eg, immune function, DNA synthesis and repair), essential methylation (eg, DNA, RNA, protein, phospholipid, neurotranmittors, creatine), and redox homeostasis (eg, cell signaling, detoxification, stress response, cell cycle progression, and apoptosis).

children with autism to test. She did, we did the testing, and the outcome was our first article on the abnormal metabolic profile in children with autism.[2] She was the first of many parents who have been the inspirational source and passion for our continuing research in autism. Their incredible dedication to their children and their relentless search for answers and treatment for their children has touched my heart deeply and has provided the passion to do what I can to try to find answers for these special parents. I remember when I presented our first data at an Autism Research Institute (ARI) parent-physician conference in 2004, and to my shock and amazement, the parents gave me a standing ovation after my talk—something that does not typically occur at scientific conferences. To me, this was more an expression of their gratitude to someone trying to find answers for them than it was a reaction to anything I said. Their gratitude and encouragement have been the guiding force behind our work to this day.

The decision to pursue this single unexpected observation in a control child for an unrelated study was what I call a "branch-point" decision that easily could have been a dead end and waste of resources but instead became a major turning point and refocused our future research efforts. In my years of research experience, I have learned that the unexpected result can be very disconcerting and disheartening (nothing like an ugly fact to ruin a beautiful hypothesis), but often it is the unexpected results that lead to more interesting and innovative outcomes than could have been anticipated if you have the "willingness to risk failure." This idea was discussed in a commencement address at Princeton by Harold T. Shapiro that was published as an editorial in *Science* and has been a source of inspiration to me over the years:

> *Let me focus for a moment on the willingness to take informed risks. The willingness to risk failure is an essential component of most successful initiatives. The unwillingness to face the risks of failure—or an excessive zeal to avoid all risks—is, in the end, an acceptance of mediocrity and an abdication of leadership. To use a sports metaphor, if you do not come to bat at all, or, when at bat, wait hopefully for a walk, you cannot hit a homerun. At best, you can get to first base. The risk of failure is intrinsic to significant accomplishment. Successful change depends on experimentation with uncertain results. A willingness to occupy new ground always involves the risk of losing your footing along the way. We must also beware of raising the flag of failure too quickly. The world too often calls it failure if we do not immediately reach our goals; true failure lies, rather, in giving up on our goals. When 10000 experiments with a storage battery failed to produce results, Thomas Edison said, "I have not failed. I've just found 10000 ways that won't work." Few battles result in immediate and full victory. History, however, has shown us that these seeming failures are often just threads of a larger tapestry; until this tapestry is on the wall, the nature of the final pattern is difficult to discern. Let me be clear,*

I do not recommend failure. Nor am I attracted to the idea that failure builds character. But a willingness to accept the risk of failure is one of the costs of leadership and, therefore, the price of all success.[3]

This editorial was published in 1990, when government funds were more readily available. Today, the willingness to risk failure can be detrimental for investigator-initiated National Institutes of Health (NIH)Research Project Grant (R01) proposals. The peer review process and programmatic priorities too often favor investment in incremental advancements in knowledge over out-of-the-box, high-risk, high-reward research proposals. So the willingness to risk failure takes on new meaning and takes even greater courage. One approach to this challenge is to include the idea with greatest risk as the third aim of an otherwise safe proposal that is an incremental advancement of knowledge. If the reviewers support the first two aims, they are more likely to let you venture out of the box with the third aim.

I was fortunate to get a 5-year NIH R01 grant funded, which resulted in 12 peer reviewed articles.[2,4-14] Unfortunately, the grant was not renewed last year. Coming to our rescue with "bridging" funds was the philanthropy of the Jane Botsford Johnson Foundation (New York). Jane is a very intelligent and unassuming woman with uncanny perception and dedication to autism research and finding answers. She has kept many autism researchers alive with her foundation's funding, and she is another of my parent heroes.

Without the dedication and expertise of our laboratory staff, we would have no research to publish. I have been fortunate to have Stepan Melnyk, MD, PhD, as my laboratory director for the last 17 years. It has been like a professional marriage, with all the ups and downs and compromises needed for any long-term relationship. We make a great team, and any successes are equally shared. Stefanie Jernigan, MD, has been my project manager for 10 years. Over the years, we have developed a mother-daughter relationship and friendship that extends beyond the work. In fact, everyone in the lab, including research associates Lesya Pavliv and Teresa Evans; my predoctoral student, Shannon Rose; and my study coordinator Hannah Field, are like family. I think this is the secret to team dedication and productivity: we all care about each other and share the passion for what we do and hope to accomplish.

So what have we learned about autism during the past 10 years that has shaped our thinking about pathophysiology and pathogenesis? We think that the metabolic phenotype of an individual reflects the combined influence of both endogenous and exogenous factors on genotype and provides a window through which the cumulative impact of genes and environment may be viewed. While both genetic and environmental factors appear to be necessary, in the majority of cases, neither factor is independently sufficient for the autistic phenotype. A metabolic endophenotype provides an intermediate biomarker that is influenced by both genes and environment and can offer insights into relevant candidate genes and pathways. Moreover, chronic or systemic metabolic imbalance can leave a metabolic footprint that can be followed analytically to gain mechanistic insights into the pathophysiology and pathogenesis of autism and thereby open new windows for therapeutic intervention. While most mainstream clinicians define autism by the abnormal behavior and attempt to treat the behavioral symptoms with psychotropic medication, scientists view the behavior as a symptom of an underlying process that has gone wrong at some point in the patient's development. Our quest is to identify the biological mechanism driving the behavior in order to target the root of the behaviors. This approach assumes a final common pathway in most children with autism that has many converging roots that vary with the individual child. The goal is to define the subtypes and individual routes that lead to autism for each child. Just as there are many forms of cancer that require different treatment approaches despite the common final pathway of uncontrolled proliferation, it is highly likely that future autism research will take a similar path.

As illustrated in Figure 2, both gene and environment are necessary but not sufficient to cause autism in most cases. The metabolic redox ratio (GSH:GSSG) and the methylation ratio (SAM:SAH) are relevant examples of metabolic endophenotypes that could provide insights into potential mechanisms (oxidative stress and methylation, respectively) that contribute to neurological and behavioral abnormalities associated with autism. More importantly, because oxidative inhibition of metabolic pathways is inherently reversible, candidate pathways can provide targets for intervention and treatment strategies.

Although cell-specific differences in metabolism account for tissue-specific functions, there are also interwoven metabolic pathways present in all cells that are fundamental for cell survival. Metabolically based vital cell functions include pathways for the stress response, nutrient uptake and metabolism, redox homeostasis, energy production and regulation of gene expression, proliferation, and cell death. Perturbation of these common pathways by genetic, nutritional, or environmental factors would not target a single system in isolation but would be expected to have a broader multisystem impact in the body.

The metabolic footprint we have observed in many autistic children will negatively affect proliferative potential (immune function, DNA synthesis and repair); essential methylation reactions (DNA, RNA, protein, lipids, neurotransmitters, creatine); and glutathione-dependent redox homeostasis (detoxification, signal transduction, gene expression, cell cycle control, and cell death). The link between these basic functions and folate-dependent methylation and glutathione metabolic pathways is summarized in Figure 1. Because the autism diagnosis is based solely on behavioral symptoms, it is logical although perhaps too simplistic to assume that brain abnormalities are the sole cause of autism. Clearly, brain involvement is a major consideration; however, many

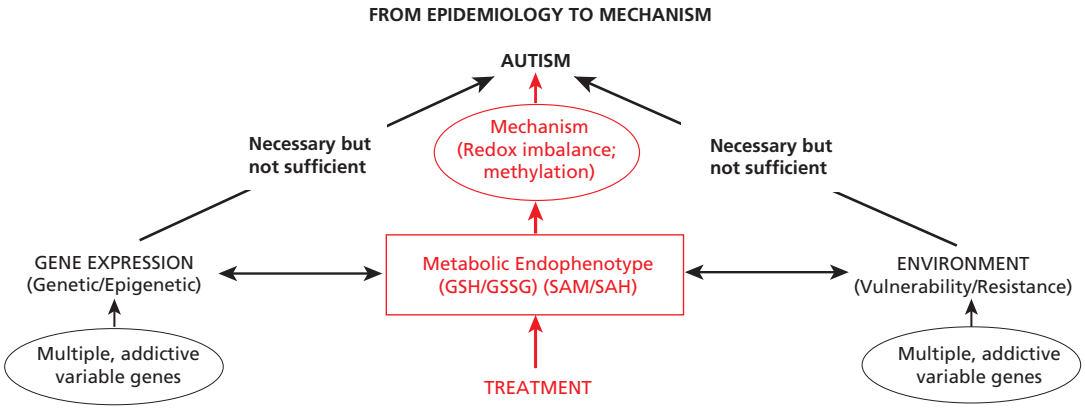

Figure 2 Role of metabolic endophenotype as a reflection of both genetic and environmental influences contributing to autism that is treatable and can provide clues to the pathophysiology of autism and targeted treatment options.

Abbreviations: GSH, glutathione; GSSG, glutathione disulfide; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine.

autistic children also exhibit evidence of gastrointestinal (GI) and immunological pathology that is consistent with systemic involvement.

Based on escalating direct and indirect evidence, it is plausible to propose that some forms of autism could be a manifestation of a genetically based inability to control or resolve oxidative stress. Metabolic evidence from an increasing number of studies supports the possibility that a significant proportion of autistic children may be under chronic oxidative stress. Because the response to oxidative stress is programmed into every cell, it is likely that the plasma biomarkers observed in autistic children reflect a systemic redox imbalance rather than tissue-specific response. A systems approach to autism biology suggests the provocative possibility that some autistic behaviors could, in part or in parallel, reflect a neurological manifestation of a genetically based systemic metabolic derangement. Such a paradigm shift from a primary neurodevelopmental disorder to a broader systemic disorder with neurological sequelae would widen the net in the quest to understand the biological basis of autism. A more systemic approach would encompass not only the neurological manifestations but also the evidence for GI and immunological pathology associated with autism. In addition to impaired central nervous system function, abnormalities in folate-dependent methionine and glutathione metabolism also occur with GI and immunological dysfunction. Moreover, pro-oxidant environmental exposures are known to affect all three systems; thus, it fits the definition of a "final common pathway" at least for some of the children.

Much of my thinking over the years has been profoundly influenced by attending the think tank meetings and conferences organized by ARI. In particular, I would like to acknowledge the inspiration and support of Bernie Rimland, PhD; Sidney Baker, MD; Jon Pangborn, PhD; Richard Deth, PhD; and Jane Johnson. Dr Rimland was the founding father of ARI and the originator of the biological theory of autism and an inspiration for all who were fortunate enough to know him. Drs Baker, Pangborn, and Deth launched the one-carbon biochemistry of autism, and Ms Johnson, as executive director of ARI and president of the Jane Botsford Johnson Foundation, is a driving force for autism research.

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