Additional Documents

Identifying and resolving metabolic dysfunction and environmental toxicity

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William Shaw, PhD, Ellen Kassen, MT(ASCP), Enrique Chaves, MD

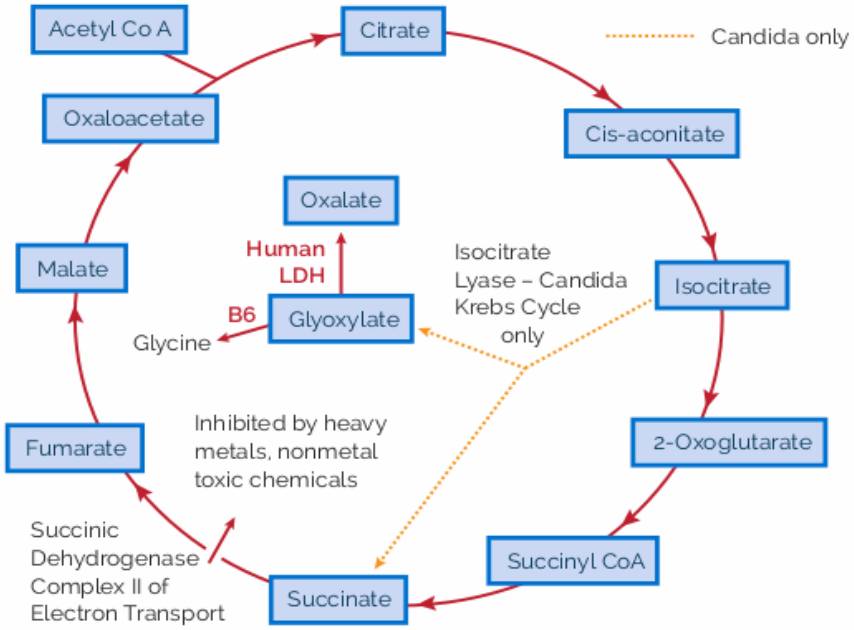
Requisition #:		*Physician:*	
Patient Name:		*Date of Collection:*	
Patient Age:	13	*Time of Collection:*	
Patient Sex:	M	*Print Date:*	

Organic Acids Test - Nutritional and Metabolic Profile

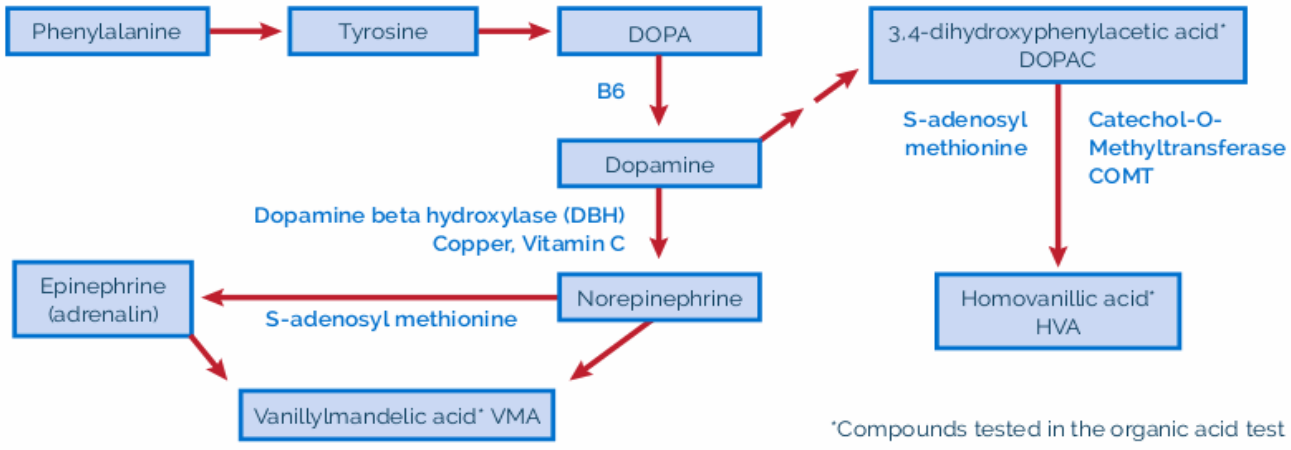
Metabolic Markers in Urine	Reference Range (mmol/mol creatinine)		Patient Value	Reference Population - Males Age 13 and Over
Intestinal Microbial Overgrowth				
Yeast and Fungal Markers				
1 Citramalic	0.11	- 2.0	H 4.2	4.2
2 5-Hydroxymethyl-2-furoic *(Aspergillus)*	≤ 18		11	11
3 3-Oxoglutaric	≤ 0.11		0	0.00
4 Furan-2,5-dicarboxylic *(Aspergillus)*	≤ 13		7.4	7.4
5 Furancarbonylglycine *(Aspergillus)*	≤ 2.3		0.05	0.05
6 Tartaric *(Aspergillus)*	≤ 5.3		H 814	814
7 Arabinose	≤ 20		H 103	103
8 Carboxycitric	≤ 20		2.1	2.1
9 Tricarballylic *(Fusarium)*	≤ 0.58		0.17	0.17
Bacterial Markers				
10 Hippuric	≤ 241		H 297	297
11 2-Hydroxyphenylacetic	0.03	- 0.47	0.40	0.40
12 4-Hydroxybenzoic	≤ 0.73		0.60	0.60
13 4-Hydroxyhippuric	≤ 14		8.7	8.7
14 DHPPA (Beneficial Bacteria)	≤ 0.23		0.17	0.17
Clostridia Bacterial Markers				
15 4-Hydroxyphenylacetic *(C. difficile, C. stricklandii, C. lituseburense & others)*	≤ 18		10	10
16 HPHPA *(C. sporogenes, C. caloritolerans, C. botulinum & others)*	≤ 102		H 130	130
17 4-Cresol *(C. difficile)*	≤ 39		H 53	53
18 3-Indoleacetic *(C. stricklandii, C. lituseburense, C. subterminale & others)*	≤ 6.8		0.68	0.68

Testing performed by The Great Plains Laboratory, LLC, Lenexa, Kansas. The Great Plains Laboratory has developed and determined the performance characteristics of this test. This test has not been evaluated by the U.S. FDA; the FDA does not currently regulate such testing.

Human Krebs Cycle showing Candida Krebs Cycle variant that causes excess Oxalate via Glyoxylate



Major pathways in the synthesis and breakdown of **catecholamine neurotransmitters** in the absence of microbial inhibitors



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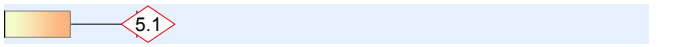
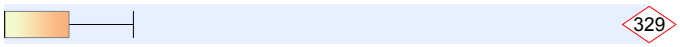
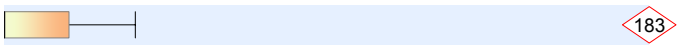
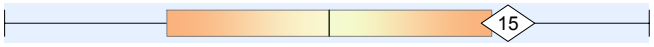
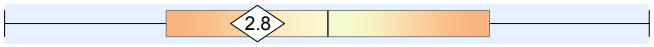
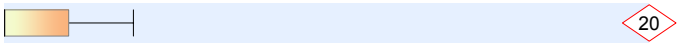
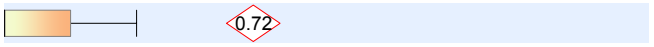
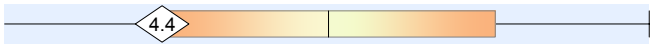
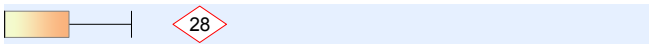
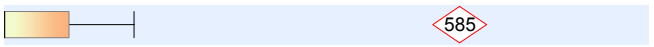
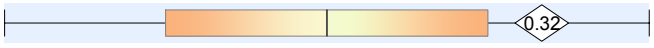
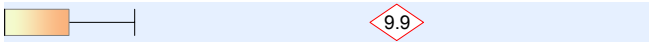
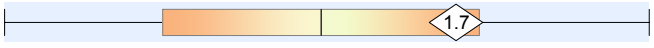
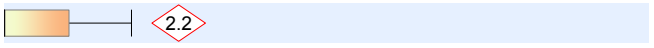
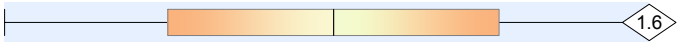
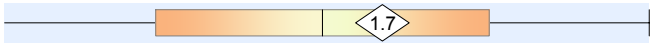
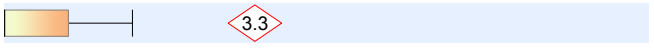
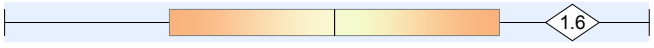
Requisition #: *Physician:*

Patient Name: *Date of Collection:*

Metabolic Markers in Urine	Reference Range (mmol/mol creatinine)	Patient Value	Reference Population - Males Age 13 and Over
Oxalate Metabolites			
19 Glyceric	0.21 - 4.9	H 5.1	
20 Glycolic	18 - 81	H 329	
21 Oxalic	8.9 - 67	H 183	
Glycolytic Cycle Metabolites			
22 Lactic	0.74 - 19	15	
23 Pyruvic	0.28 - 6.7	2.8	
Mitochondrial Markers - Krebs Cycle Metabolites			
24 Succinic	≤ 5.3	H 20	
25 Fumaric	≤ 0.49	H 0.72	
26 Malic	≤ 1.1	H 2.0	
27 2-Oxoglutaric	≤ 18	4.4	
28 Aconitic	4.1 - 23	H 28	
29 Citric	2.2 - 260	H 585	
Mitochondrial Markers - Amino Acid Metabolites			
30 3-Methylglutaric	0.02 - 0.38	0.32	
31 3-Hydroxyglutaric	≤ 4.6	H 9.9	
32 3-Methylglutaconic	0.38 - 2.0	1.2	
Neurotransmitter Metabolites			
Phenylalanine and Tyrosine Metabolites			
33 Homovanillic (HVA) *(dopamine)*	0.39 - 2.2	H 3.6	
34 Vanillylmandelic (VMA) *(norepinephrine, epinephrine)*	0.53 - 2.2	1.7	
35 HVA / VMA Ratio	0.32 - 1.4	H 2.1	
36 Dihydroxyphenylacetic (DOPAC) *(dopamine)*	0.27 - 1.9	H 2.2	
37 HVA/ DOPAC Ratio	0.17 - 1.6	1.6	
Tryptophan Metabolites			
38 5-Hydroxyindoleacetic (5-HIAA) *(serotonin)*	≤ 2.9	1.7	
39 Quinolinic	0.52 - 2.4	H 3.3	
40 Kynurenic	0.12 - 1.8	1.6	

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Requisition #: *Physician:*

Patient Name: *Date of Collection:*

Metabolic Markers in Urine	Reference Range (mmol/mol creatinine)	Patient Value	Reference Population - Males Age 13 and Over
Pyrimidine Metabolites - Folate Metabolism			
41 Uracil	≤ 6.9	H 8.9	8.9
42 Thymine	≤ 0.36	0.22	0.22
Ketone and Fatty Acid Oxidation			
43 3-Hydroxybutyric	≤ 1.9	1.6	1.6
44 Acetoacetic	≤ 10	0.90	0.90
45 Ethylmalonic	0.13 - 2.7	1.4	1.4
46 Methylsuccinic	≤ 2.3	H 3.4	3.4
47 Adipic	≤ 2.9	2.7	2.7
48 Suberic	≤ 1.9	H 5.9	5.9
49 Sebacic	≤ 0.14	0.03	0.03
Nutritional Markers			
Vitamin B12			
50 Methylmalonic ✱	≤ 2.3	2.2	2.2
Vitamin B6			
51 Pyridoxic (B6)	≤ 26	H 58	58
Vitamin B5			
52 Pantothenic (B5)	≤ 5.4	H 164	164
Vitamin B2 (Riboflavin)			
53 Glutaric ✱	≤ 0.43	0.21	0.21
Vitamin C			
54 Ascorbic	10 - 200	10	10
Vitamin Q10 (CoQ10)			
55 3-Hydroxy-3-methylglutaric ✱	≤ 26	H 28	28
Glutathione Precursor and Chelating Agent			
56 N-Acetylcysteine (NAC)	≤ 0.13	0.02	0.02
Biotin (Vitamin H)			
57 Methylcitric ✱	0.15 - 1.7	H 2.3	2.3

✱ ***A high value for this marker may indicate a deficiency of this vitamin.***

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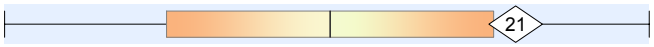
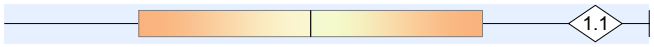
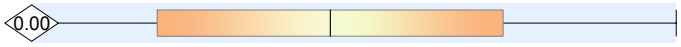
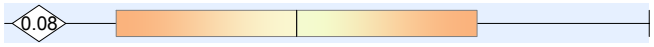
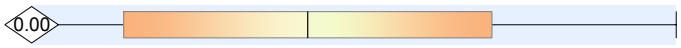
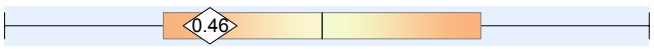
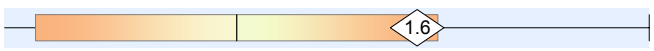
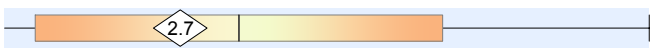
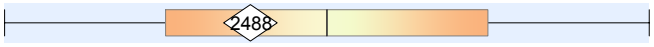
Requisition #: *Physician:*

Patient Name: *Date of Collection:*

Metabolic Markers in Urine		Reference Range (mmol/mol creatinine)		Patient Value		Reference Population - Males Age 13 and Over
Indicators of Detoxification						
Glutathione						
58 Pyroglutamic ✱	5.7	-	25	21		
Methylation, Toxic exposure						
59 2-Hydroxybutyric ✱✱		≤	1.2	1.1		
Ammonia Excess						
60 Orotic		≤	0.46	H	0.77	
Aspartame, salicylates, or GI bacteria						
61 2-Hydroxyhippuric		≤	0.86	H	1.6	

✱ *A high value for this marker may indicate a Glutathione deficiency.*

✱✱ *High values may indicate methylation defects and/or toxic exposures.*

Amino Acid Metabolites						
62 2-Hydroxyisovaleric		≤	0.41		0	
63 2-Oxoisovaleric		≤	1.5		0.08	
64 3-Methyl-2-oxovaleric		≤	0.56		0.53	
65 2-Hydroxyisocaproic		≤	0.39		0.01	
66 2-Oxoisocaproic		≤	0.34		0.05	
67 2-Oxo-4-methiolbutyric		≤	0.14		0	
68 Mandelic		≤	0.09		0	
69 Phenyllactic		≤	0.10		0.03	
70 Phenylpyruvic	0.02	-	1.4		0.46	
71 Homogentisic		≤	0.23		0.02	
72 4-Hydroxyphenyllactic		≤	0.62		0.24	
73 N-Acetylaspartic		≤	2.5		1.6	
74 Malonic		≤	9.9		2.7	
75 4-Hydroxybutyric		≤	4.3		3.6	
Mineral Metabolism						
76 Phosphoric	1,000	-	4,900		2,488	

Indicator of Fluid Intake

77	*Creatinine	174	mg/dL	

*The creatinine test is performed to adjust metabolic marker results for differences in fluid intake. Urinary creatinine has limited diagnostic value due to variability as a result of recent fluid intake. Samples are rejected if creatinine is below 20 mg/dL unless the client requests results knowing of our rejection criteria.

Explanation of Report Format

The reference ranges for organic acids were established using samples collected from typical individuals of all ages with no known physiological or psychological disorders. The ranges were determined by calculating the mean and standard deviation (SD) and are defined as ± 2SD of the mean. Reference ranges are age and gender specific, consisting of Male Adult (≥13 years), Female Adult (≥13 years), Male Child (<13 years), and Female Child (<13 years).

There are two types of graphical representations of patient values found in the new report format of both the standard Organic Acids Test and the Microbial Organic Acids Test.

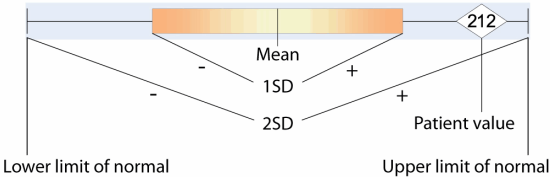
The first graph will occur when the value of the patient is within the reference (normal) range, defined as the mean plus or minus two standard deviations.

The second graph will occur when the value of the patient exceeds the upper limit of normal. In such cases, the graphical reference range is "shrunk" so that the degree of abnormality can be appreciated at a glance. In this case, the lower limits of normal are not shown, only the upper limit of normal is shown.

In both cases, the value of the patient is given to the left of the graph and is repeated on the graph inside a diamond. If the value is within the normal range, the diamond will be outlined in black. If the value is high or low, the diamond will be outlined in red.

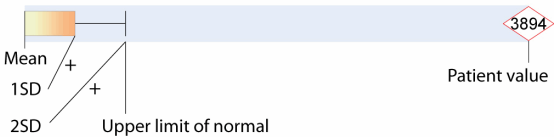
Example of Value Within Reference Range

Metabolic Markers in Urine	Reference Range (mmol/mol creatinine)		Patient Result	Reference Range - Males Age 13 and Under
Intestinal Microbial Overgrowth				
HPHPA (Clostridia marker)	<	219 .9	212	



Example of Elevated Value

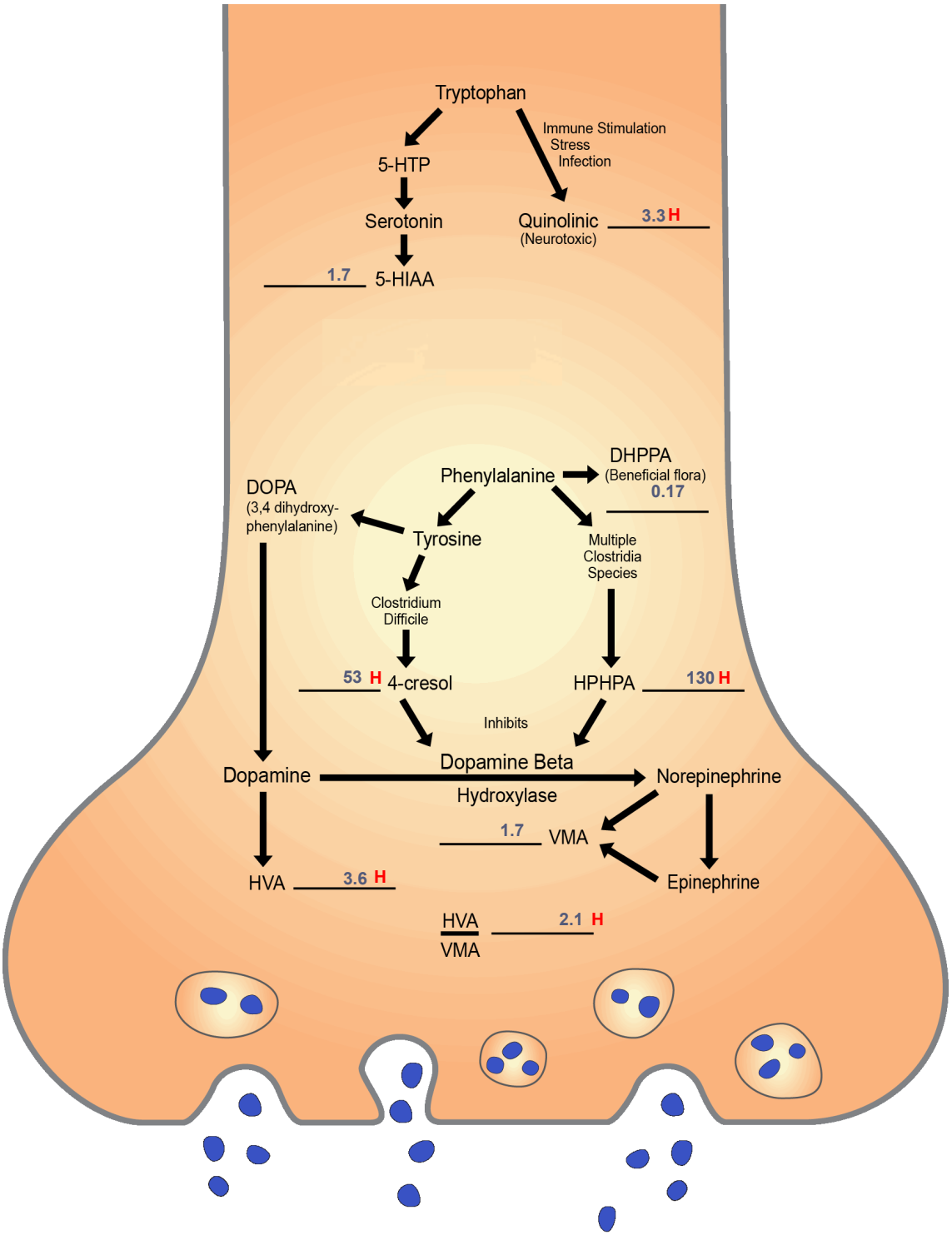
Metabolic Markers in Urine	Reference Range (mmol/mol creatinine)		Patient Result	Reference Range - Males Age 13 and Under
Intestinal Microbial Overgrowth				
HPHPA (Clostridia marker)	<	219 .9	H 3894	



Requisition #: *Physician:*

Patient Name: *Date of Collection:*

Neurotransmitter Metabolism Markers



The diagram contains the patient's test results for neurotransmitter metabolites and shows their relationship with key biochemical pathways within the axon terminal of nerve cells. The effect of microbial byproducts on the blockage of the conversion of dopamine to norepinephrine is also indicated.

Requisition #: ***Physician:***

Patient Name: ***Date of Collection:***

Interpretation

High yeast/fungal metabolites (1-8) Elevations of one or more metabolites indicate a yeast/fungal overgrowth of the gastrointestinal (GI) tract. Prescription or natural (botanical) anti-fungals, along with supplementation of high potency multi-strain probiotics, may reduce yeast/fungal levels.

High hippuric acid (10) may derive from food, GI bacterial activity, or exposure to the solvent toluene. Hippuric acid is a conjugate of glycine and benzoic acid formed in the liver. Most hippuric acid in urine is derived from microbial breakdown of chlorogenic acid to benzoic acid. Chlorogenic acid is a common substance in beverages and in many fruits and vegetables, including apples, pears, tea, coffee, sunflower seeds, carrots, blueberries, cherries, potatoes, tomatoes, eggplant, sweet potatoes, and peaches. Benzoic acid is present in high amounts in cranberry juice and is a food preservative. The workplace is the most common source of toluene exposure, but toluene may be absorbed from outgassing of new carpets and other building materials, or absorbed during recreational abuse of solvents such as glue-sniffing. Because most hippuric acid in urine is from GI sources, this marker is a poor indicator of toluene exposure and is being replaced by other markers in occupational safety testing. Bacterial overgrowth can be treated with natural anti-bacterial agents and/or probiotics (30-50 billion cfu's) that include *Lactobacillus rhamnosus*.

High HPHPA (3-(3-hydroxyphenyl)-3-hydroxypropionic acid) (16) is an abnormal phenylalanine metabolite produced when byproducts of *Clostridium* bacteria combine with human metabolites. High concentrations of this compound cause abnormal behavior by inhibiting metabolism of dopamine to epinephrine, resulting in high levels of the dopamine metabolite homovanillic acid (HVA) in the urine and insufficient epinephrine/norepinephrine in the body. It is associated with behavioral, gastrointestinal, and neuropsychiatric symptoms including tic disorders, depression, autism, schizophrenia, aggression, seizures, anorexia, obsessive compulsive disorder, and hyperactivity. Neuropsychiatric effects are more common when values exceed 500 mmol/mol creatinine.

The *Clostridia* species that cause the greatest quantities of urinary HPHPA are *C. sporogenes, C. caloritolerans,* and *C. botulinum*. Additionally, *C. mangenoti, C. ghoni, C. bifermentans, C. caproicum, and C. sordellii* are also capable of causing elevated urinary levels of HPHPA.

HPHPA precursors are not produced by *C.perfringens* -types A-F*, C.tetani, C.subterminale C.capitovale, C.septicum, C.difficile, C.histolyticum,* or *C.tertium.*

C. botulinum would appear to be an unlikely source unless clinical symptoms of botulism are present. The botulinum toxin can cause a severe flaccid paralytic <http://en.wikipedia.org/wiki/Flaccid_paralysis> disease in humans and animals and is the most potent toxin known to humankind, with a lethal dose of less than 1 µg in humans. Symptoms of botulism include weakness, impaired vision, fatigue, and impaired speech. This may then be followed by weakness of the arms, chest muscles and legs. Surprisingly, symptoms may sometimes be mild and the severity of symptoms appears to be modulated by the amount of beneficial flora in the intestinal tract. In food borne botulism, symptoms generally begin 18 to 36 hours after eating contaminated food, but they can occur as early as 6 hours or as late as 10 days. *C. caloritolerans* is so named because it can survive at the boiling point for 8 hours. Its extreme resistance to heat may allow common food borne transmission. *C. sporogenes* is the name given to strains of *Clostridium botulinum* that do not produce botulinum <http://en.wikipedia.org/wiki/Botulinum> neurotoxins. *C. sporogenes* differs from C. botulinum by a single gene. C. sporogenes is ubiquitous in nature and is commonly found in the flora of humans. *C. sordellii* can be pathogenic and has been implicated in fatal toxic shock syndrome among women of child bearing age.

Treatment with Metronidazole or Vancomycin is close to 100% effective at killing parent organisms but not their spores. At least three months of probiotic therapy is recommended after antimicrobial treatment due to spore formation by *Clostridia* species. *Clostridia* overgrowth can sometimes be controlled by supplementation with *Corebiotic*, *Lactobacillus rhamnosus GG* (Culturelle) or *Saccharomyces boulardii.* Phenalalanine or tyrosine supplements should be avoided because of the possibility of conversion to HPHPA or other toxic byproducts.

High 4-cresol (p-cresol) (17) in the urine is most commonly due to *C. difficile* and *C. scatologenes* in the gastrointestinal tract. Most other *Clostridia* species and all other bacteria species do not produce this metabolite. Since *C. scatologenes* is not commonly isolated from stool samples, *C. difficile* would be the most likely source of this compound.

The major clinical significance of 4-cresol is that it is a potent inhibitor of brain dopamine-beta-hydroxylase, the enzyme that converts dopamine to norepinephrine. High concentrations of this compound cause abnormal behavior by inhibiting metabolism of dopamine to norepinephrine, resulting in high levels of the dopamine metabolite homovanillic acid (HVA) in the urine and insufficient norepinephrine in the central nervous system. High urine values of 4-cresol are associated with the most severe clinical symptoms in autism, multiple sclerosis, neurotoxicity, hallucinations, and other neurological and psychiatric disorders. 4-Cresol is also a metabolite of toluene, wood tar creosote, and menthofuran (derived from the mint flavoring agent pennyroyal).

Treatment with Metronidazole or Vancomycin is almost 100% effective in killing parent organisms, including *C. difficile,* but not their spores. At least three months of probiotic therapy is recommended after antimicrobial treatment due to spore formation by *Clostridia* species. *Clostridia* overgrowth can sometimes be controlled by supplementation with *Lactobacillus rhamnosus GG* (Culturelle) or *Saccharomyces boulardi.*

High glyceric (19): may be due to microbial sources such as yeast (Aspergillus, Penicillium, Candida) or due to dietary sources containing glycerol/glycerine.

High glycolic (20): in the absence of oxalic is most likely a result of GI yeast overgrowth (Aspergillus, Penicillium, Candida) or due to dietary sources containing glycerol/glycerine. Glycolic acid had also been found to be a metabolite in Acetobacter, Acidithiobacillus, Alcanligenes, Corynebacterium, Cryptococcus, Escherichia, Gluconobacter, Kluyveromyces, Leptospirillum, Pichia, Rhodococcus, Rhodotorula and Saccharomyces (PMID: 11758919; PMID: 26360870; PMID: 14390024).

High oxalic (21) with or without elevated glyceric (19) or glycolic acids (20) may be associated with the genetic hyperoxalurias, autism, women with vulvar pain, fibromyalgia, and may also be due to high vitamin C intake. However, kidney stone formation from oxalic acid was not correlated with vitamin C intake in a very large study. Besides being present in varying concentrations in most vegetables and fruits, oxalates, the mineral conjugate base forms of oxalic acid, are also byproducts of molds such as *Aspergillus* and *Penicillium* and probably *Candida*. If yeast or fungal markers are elevated, antifungal therapy may reduce excess oxalates. High oxalates may cause anemia that is difficult to treat, skin ulcers, muscles pains, and heart abnormalities. Elevated oxalic acid is also the result of anti-freeze (ethylene glycol) poisoning. Oxalic acid is a toxic metabolite of trichloroacetic acid and other environmental pollutants. In addition, decomposing vitamin C may form oxalates during transport or storage.

Elevated oxalate values with a concomitant increase in glycolic acid may indicate genetic hyperoxaluria (type I), whereas increased glyceric acid may indicate a genetic hyperoxaluria (type II). Elevated oxalic acid with normal levels of glyceric or glycolic metabolites rules out a genetic cause for high oxalate. However, elevated oxalates may be due to a new genetic disorder, hyperoxaluria type III.

Regardless of its source, high oxalic acid may contribute to kidney stones and may also reduce ionized calcium. Oxalic acid absorption from the GI tract may be reduced by calcium citrate supplementation before meals. Vitamin B6, arginine, vitamin E, chondroitin sulfate, taurine, selenium, omega-3 fatty acids and/or N-acetyl glucosamine supplements may also reduce oxalates and/or their toxicity. Excessive fats in the diet may cause elevated oxalate if fatty acids are poorly absorbed because of bile salt deficiency. Unabsorbed free fatty acids bind calcium to form insoluble soaps, reducing calcium's ability to bind oxalate and increase its absorption. If taurine is low in a plasma amino acid profile, supplementation with taurine (1000 mg/day) may help stimulate bile salt production (taurocholic acid), leading to better fatty acid absorption and diminished oxalate absorption.

High levels of oxalates are common in autism. Malabsorption of fat and intestinal *Candida* overgrowth are probably the major causes for elevated oxalates in this disorder. Even individuals with elevated glyceric or glycolic acids may not have a genetic disease. To rule out genetic diseases in those people with abnormally high markers characteristic of the genetic diseases, do the following steps: (1) Follow the nutritional steps indicated in this interpretation for one month; (2) If *Candida* is present, treat *Candida* for at least one month; (3) Repeat the organic acid test after abstaining from vitamin C supplements for 48 hours; (4) If the biochemical markers characteristic of genetic oxalate disorders are still elevated in the repeat test, consider DNA tests for the most common mutations of oxalate metabolism. DNA testing for type I hyperoxaluria is available from the Mayo Clinic, Rochester, MN as test #89915 "*AGXT* Gene, Full Gene Analysis" and, for the p.Gly170Arg mutation only, as # 83643 "Alanine:Glyoxylate Aminotransferase [*AGXT*] Mutation Analysis [G170R], Blood"). Another option to confirm the genetic disease is a plasma oxalate test, also available from the Mayo Clinic (Phone 507.266.5700). Plasma oxalate values greater than 50 micromol/L are consistent with genetic oxalate diseases and may serve as an alternate confirmation test.

Bone tends to be the major repository of excess oxalate in patients with primary hyperoxaluria. Bone oxalate levels are negligible in healthy subjects. Oxalate deposition in the skeleton tends to increase bone resorption and decrease osteoblast activity.

Oxalates may also be deposited in the kidneys, joints, eyes, muscles, blood vessels, brain, and heart and may contribute to muscle pain in fibromyalgia. Oxalate crystal formation in the eyes may be a source of severe eye pain in individuals with autism who may exhibit eye-poking behaviors. High oxalates in the GI tract also may significantly reduce absorption of essential minerals such as calcium, magnesium, zinc, and others. In addition, oxalate deposits in the breast have been associated with breast cancer.

A low oxalate diet may also be particularly useful in the reduction of body oxalates even if dysbiosis of GI flora is the major source of oxalates. Foods especially high in oxalates include spinach, beets, chocolate, soy, peanuts, wheat bran, tea, cashews, pecans, almonds, berries, and many others. A complete list of high oxalate foods is available online at <http://www.greatplainslaboratory.com/home/eng/oxalates.asp>.

People with abnormally high markers characteristic of the genetic diseases should do the following:
1. Avoid spinach, soy, nuts, and berries for one month.
2. If *Candida* is present, treat *Candida* for at least one month.
3. Repeat the organic acid test having abstained from vitamin C supplements for 48 hours.
4. If the biochemical markers characteristic of genetic oxalate disorders are still elevated in the repeat test, consider DNA tests for the most common mutations of oxalate metabolism.

High succinic acid (24) The most common cause of elevated succinic acid is exposure to toxic chemicals which impairs mitochondria function. The most useful tests for confirming toxic chemical exposure are **The Great Plains Laboratory GPL-TOX test** on urine for 172 chemicals and the hair metals test. Succinic acid is metabolized by the mitochondrial enzyme succinic dehydrogenase, which is significant in that it is both a Krebs cycle enzyme and a component- complex 2-of the mitochondrial electron transport chain, making this metabolite a marker of mitochondrial complex 2 as well as Krebs cycle dysfunction. A sampling of toxic chemicals that have been associated with mitochondrial dysfunction include glyphosate, 2, 4-dichlorophenoxyacetic acid (2, 4-D), organophosphate pesticides, mercury, and lead. Approximately 95% of elevated succinic acid results are associated with toxic chemical exposure. Succinic acid in the organic acid test and tiglylglycine in the **GPLTOX test** are two of the most useful markers for mitochondrial dysfunction. Tiglylglycine is a marker for mitochondrial respiratory chain complex I dysfunction while elevated succinic acid indicates respiratory complex 2 dysfunction. Occasionally both succinic acid and tiglylglycine may be elevated in mitochondrial dysfunction. Other Krebs cycle markers may also be elevated when severe chemical toxicity is present. In general, the severity of the chemical toxicity is correlated with higher values of succinic acid.

Less common causes of elevated succinic acid are mitochondrial mutations which may be due to mutations in the nuclear or the mitochondrial DNA for mitochondrial proteins such as Kearns-Sayres disorder. Succinic acid is a metabolite of gamma aminobutyric acid (GABA) so supplementation with GABA may also increase succinic acid.

High fumaric acid (25) may be due to impaired Krebs cycle function, defect of the enzyme fumarase or a defect in mitochondrial function. Recommendations for supporting mitochondrial function include supplementation with coenzyme Q10, L-carnitine or acetyl-L-carnitine, riboflavin, nicotinamide, and vitamin E.* All of these supplements are known to improve mitochondrial dysfunction.

High malic acid (26) indicates a greater requirement for the nutrients niacin and coenzyme Q10.* Malic acid simultaneously elevated with citric, fumaric and alpha-ketoglutaric acids may indicate a possible Cytochrome C Oxidase deficiency. Mitochondrial energy pathway dysfunction would be expected.

High aconitic and citric acids (28, 29) may indicate a need for liposomal glutathione. Glutathione is required for the enzyme aconitase to metabolize citric and aconitic acids. This metabolite may also be associated with mitochondrial energy pathway dysfunction if elevated with other mitochondrial markers such as lactic, pyruvic, malic, fumaric, 3-methylglutaric, 3-hydroxyglutaric, and/or 3-methylglutagonic acids.

High 3-hydroxyglutaric (31) is a metabolite associated with the genetic disease glutaric aciduria type I, which is due to a deficiency of glutaryl CoA dehydrogenase, an enzyme involved in the breakdown of lysine, hydroxylysine, and tryptophan. Other organic acids elevated include glutaric and glutaconic. This disease has been associated with clinical symptoms ranging from near normal to encephalopathy, cerebral palsy, and other neurological abnormalities. Some individuals with glutaric acidemia have developed bleeding in the brain or eyes that may be mistaken for the effects of child abuse . This abnormality should be confirmed by additional testing of enzyme deficiencies and/ or DNA at a major pediatric medical genetics center (Morton et al. Glutaric aciduria type I: a common cause of encephalopathy and spastic paralysis in the Amish of Lancaster County, Pennsylvania. American J. Med. Genetics 41: 89-95, 1991). Elevated values may also be found in hepatic carnitine palmitoyltransferase I deficiency, short-chain acyl dehydrogenase deficiency (SCAD), and ketosis. Mitochondrial dysfunction induced by glutaric acid metabolites causes astrocytes to adopt a proliferative phenotype, which may underlie neuronal loss, white matter abnormalities and macrocephalia. Values in glutaric aciduria type I range from 60-3000 mmol/mol creatinine. Values higher than normal but less than 60 mmol/mol creatinine may be due to mild glutaric acidemia type I or to the other causes indicated above. Treatment of this disorder includes special diets low in lysine and supplementation with carnitine or acetyl-L-carnitine.

High HVA (33) High HVA is usually associated with Clostridia colonization or excess fusaric acid from fungus of the gastrointestinal tract and/or deficiencies of dopamine-beta-hydroxylase (DBH) activity due to single nucleotide polymorphisms (SNPs) or genetic deletions that code for enzymes with low activity. The Great Plains Laboratory now offers a test for the activity of the DBH enzyme on blood serum. The genetic deficiencies of DBH can be treated with the drug Droxidopa (L-*threo*-dihydroxyphenylserine). Droxidopa has the ability to cross the blood brain barrier and be converted to norepinephrine by an alternate biochemical pathway that bypasses the DBH genetic block. Individuals with genetic deficiencies of DBH may have orthostatic hypertension and hypoglycemia and may be more susceptible to attention deficit disorder, Alzheimer's disease, and Parkinson's disease, depression, and bipolar depression. The severity of ADHD symptoms is related to decreased DBH enzyme activity. Cocaine abusers with low-activity DBH SNPs have increased sensitivity to cocaine-induced paranoia and euphoria. The drugs disulfiram and Etamicastat inhibit DBH and the inhibition of alcohol, drug, and gambling addictions by disulfiram may be mediated by DBH inhibition.

If HVA is elevated and VMA is normal and the patient has elevated Clostridia markers, avoid supplementation with L-DOPA, phenylalanine or tyrosine until *Clostridia* is treated. Homovanillic acid (HVA), a dopamine metabolite, is often elevated due to stress-induced catecholamine output from the adrenal gland which depletes vitamin C. Supplementation with vitamin C (ascorbate) may be helpful in such cases.* Elevated HVA can result from the intake of L-DOPA, dopamine, phenylalanine, or tyrosine. Elevated HVA may also result from ingestion of aspartame (Nutrasweet®), salicylates (aspirin), and dietary salicylates. For more information about salicylates in foods go to <http://www.feingold.org/salicylate.php>. Elevated HVA may also result from toxic metal exposure (including lead, aluminum, manganese,arsenic, and mercury), presumably due to DBH inhibition. Heavy metal testing (blood or hair) might be useful to determine if such exposure is significant.

If values are more than double the upper limit of normal, toxoplasmosis and tumors such as neuroblastoma, or other catecholamine-secreting tumors should be ruled out. Catecholamine-secreting tumors can be ruled out by 24- hour VMA and/or HVA testing in urine. Even in this subgroup, the incidence of tumors is extremely rare.

High HVA/VMA ratio (35) the HVA/VMA ratio reflects the balance between dopamine and norepinephrine/epinephrine production by catecholamine producing neurons in the central nervous system, sympathetic nervous system, and adrenal gland. The most common reason for an elevation of the HVA/VMA ratio is a decreased conversion of dopamine to norepinephrine. The enzyme responsible for this conversion, dopamine beta-hydroxylase (DBH), is copper and vitamin C dependent so an elevated ratio could be due to deficiencies of these cofactors. **The most common reason** for this elevated ratio is inhibition of this enzyme by Clostridia byproducts including HPHPA, 4-cresol, or 4-hydroxyphenylacetic acid. Other causes of an increased ratio include inhibition of DBH by the mold metabolite fusaric acid, pharmaceuticals such as disulfiram, or food additives like aspartame. Another cause for an elevated ratio is a genetic variation (single nucleotide polymorphism or SNP) of the DBH enzyme. Alternatively, the activity of the DBH enzyme can be measured on blood serum. Individuals with low DBH activity can be treated with the drug Droxidopa™, which provides adequate norepinephrine by an alternate biochemical pathway. This DBH test on blood serum is now available at The Great Plains Laboratory. High ratios are common in a large number of neuropsychiatric diseases regardless of the reason for DBH deficiency.

High 3,4-dihydroxyphenylacetic acid (DOPAC) (36) 3,4-dihydroxyphenylacetic acid (DOPAC) is an intermediate in the metabolism of dopamine. Values may be elevated due to increased intake of amino acid precursors of DOPAC such as phenylalanine, tyrosine, or DOPA. Values may be elevated due to factors that inhibit dopamine beta hydroxylase (DBH) like Clostridia metabolites, the mold metabolite fusaric acid, pharmaceuticals such as disulfiram, or food additives like aspartame, or to deficiencies of the DBH enzyme due to copper deficiency, vitamin C deficiency, or malic acid deficiency. Single nucleotide polymorphisms (SNPs) of DBH or catechol-O-methyltransferase (COMT) that result in reduced enzyme activities also result in increased amounts of DOPAC. SNPs of COMT are available on **The Great Plains Laboratory DNA methylation pathway test** which can be performed on a cheek swab. Deficiencies of S-adenosylmethionine (S-ame) also are associated with high amounts of DOPAC. DOPAC may also be increased when bananas are ingested the day before urine collection.

High quinolinic acid (39) may be a sign of inflammation and/or neural excitotoxicity. Quinolinic acid is derived from the amino acid tryptophan and is neurotoxic at high levels. As an excitotoxic stimulant of certain brain cells that have NMDA-type receptors, high quinolinic acid may cause nerve cell death with continuous stimulation. Brain toxicity due to quinolinic acid has been implicated in Alzheimer's disease, autism, Huntington's disease, stroke, dementia of old age, depression, HIV-associated dementia, and schizophrenia. High levels of quinolinic acid may inhibit heart contractions, cause lipid peroxidation in the brain, and increase apoptosis (programmed cell death) of astrocytes in human brain. The level of quinolinic acid is also highly correlated with the degree of arthritis impairment.

Quinolinic acid is also a metal chelator, and inhibits enzymes that allow the body to produce glucose when needed. Excessive immune stimulation and chronic inflammation, resulting in overproduction of cytokines like interferon, stimulates overproduction of quinolinic acid. However, quinolinic acid is an important intermediate in making the essential nutritional cofactor nicotinamide adenine dinucleotide (NAD), which is also derived from niacin (B3). Phthalates inhibit the conversion of quinolinic acid to NAD.

Treatment of excessive levels of quinolinic acid can be achieved by multiple approaches: reducing tryptophan supplements, preventing repeated infections and subsequent immune overstimulation by: supplementation with colostrum, transfer factor and probiotics; reducing the use of immune modulators like interferon that increase quinolinic acid production; or reducing the numbers of vaccines given at one time or increasing the interval between vaccinations. The dietary supplements B6 (pyridoxine) and magnesium may reduce brain damage caused by quinolinic acid. A high quinolinic acid/ 5-hydroxyindoleacetic acid ratio would be indicative of immune overstimulation and/or phthalate toxicity.

High uracil (41) can be associated with disorders of folate metabolism, folate deficiency, and genetic disorders of pyrimidine metabolism. Genetic disorders of pyrimidine metabolism are more common when uracil exceeds 50 mmol/mol creatinine and thymine is also elevated. An autistic child with a uracil value >300 mmol/ mol creatinine and diffuse demyelination of the brain was treated with high levels of folate which normalized the uracil but did not improve the clinical symptoms.

High ethylmalonic, methylsuccinic, adipic, suberic, or sebacic acids (45,46,47,48,49) may be due to fatty acid oxidation disorders, carnitine deficiency, fasting, or to increased intake of the medium-chain triglycerides found in coconut oil, MCT oil, and some infant formulas. The fatty acid oxidation defects are associated with hypoglycemia, apnea episodes, lethargy, and coma. [An acyl carnitine profile (Duke University Biochemical Genetics Laboratory, http://medgenetics.pediatrics.duke.edu) can rule out fatty acid oxidation defects.] Regardless of cause, supplementation with L-carnitine or acetyl-L-carnitine may be beneficial.

High pyridoxic acid (51) indicates high recent intake of vitamin B6. Pyridoxic acid is a major metabolite of vitamin B6. Because some individuals may require very high doses of vitamin B6, high values do not necessarily indicate the need to reduce vitamin B6 intake.

High pantothenic acid (B5) (52) most commonly indicates recent intake of pantothenic acid as a supplement. Pantothenic acid is an essential B vitamin that is converted to coenzyme A (unrelated to vitamin A). Coenzyme A is needed for the synthesis of fatty acids, cholesterol, and acetyl choline and is also needed for the Krebs cycle and fatty acid catabolism. Because some individuals may require high doses of pantothenic acid, high values do not necessarily indicate the need to reduce pantothenic acid intake. However, if a patient who **does not take B-vitamin** supplements has high values of pantothenic acid, especially if the values are 20 or more times the upper limit of normal, the individual may have a genetic deficiency in the conversion of pantothenic acid to pantothenic acid-phosphate, which is the first step in the production of coenzyme A. It may be useful to retest after one week off all B-vitamin supplementation; individuals with PKAN would be expected to still have very elevated pantothenic acid levels even with no supplementation. This disease is called pantothenate kinase-associated neurodegeneration (PKAN), an inborn error of metabolism characterized by iron accumulation in the basal ganglia and by the presence of dystonia, dysarthria, Parkinson symptoms, and retinal degeneration. In mild variants of this disease, psychiatric illnesses such as schizoaffective disorder, hallucinations, obsessive compulsive disorder, speech defects, and depression are common. Mutations in pantothenate kinase 2 (PANK2), the rate-limiting enzyme in mitochondrial coenzyme A biosynthesis, represent the most common genetic cause of this disorder. Other biochemical abnormalities commonly found on the organic acid test in this disorder include elevated lactate, pyruvate, and Krebs cycle intermediates. Confirmation of mutant DNA requires special genetic testing. The University of Chicago does testing for PANK2 deletion for a price of $1000 in 2017.
The link is: <http://dnatesting.uchicago.edu/tests/pank2-deletionduplication-analysis>

Treatment for the illness is currently focused on giving high doses of pantothenic acid to stimulate any residual enzyme. Doses as high as 10 g per day have been ingested with few side effects. Other suggested therapies are increased supplementation with cholesterol, fat soluble vitamins, and bile salts. Since Lactobacillus species produce pantothenic acid phosphate, supplementation with high doses of probiotics might also be beneficial.

Ascorbic acid (vitamin C) levels below the mean (54) may indicate a less than optimum level of the antioxidant vitamin C. Individuals who consume large amounts of vitamin C can still have low values if the sample is taken 12 or more hours after intake. Supplementation with buffered vitamin C taken 2 or 3 times a day is suggested.

High 3-hydroxy-3-methylglutaric acid (55) is seen in the genetic disease 3-hydroxy 3-methylglutaric aciduria. Typical values observed in the genetic disease are 200-11,000 mmol/mol creatinine. The cause of less significant increases in this urinary metabolite is unknown. 3-Hydroxy-3-methylglutaric aciduria may cause vomiting, lethargy, hypotonia, and apnea, sometimes evolving to coma. Laboratory tests reveal metabolic acidosis with severe hypoketotic hypoglycemia on fasting or during acute illness, hyperammonemia, and abnormal liver function tests. Preliminary diagnosis is based on a pattern of organic acids in urine which includes 3-hydroxy-3-methylglutaric, 3-hydroxyisovaleric, 3-methylglutaconic, 3-methylglutaric, and 3-methylcrotonic acids. Because yeast also produces this compound and yeast metabolites are frequently elevated along with this compound, slight increases may be yeast-related. Reduced activity of 3-hydroxy 3-methylglutaryl Co A reductase, a critical enzyme at the beginning of the cholesterol synthesis pathway, may also elevate this compound. Check cholesterol values when this compound is elevated up to 300 mmol/mol creatinine. Slight elevations may result from coenzyme Q10 deficiency. Supplementation with coenzyme Q10 may be beneficial.

High methylcitric acid (57) is commonly due to biotin deficiency. Biotin is an essential B vitamin. Biotin deficiency may be due to malabsorption, excessive intake of raw egg white, dietary deficiency, or dysbiosis. Methylcitric values greater than 100 mmol/mol creatinine may be due to inborn errors of metabolism involving biotin-dependent enzymes and may require biotin supplementation at very high doses. A high quality multivitamin with biotin or biotin as a single supplement is recommended.

Slightly elevated orotic acid (60) levels less than 1.5 mmol/mol creatinine are commonly associated with dysbiosis. In this case, the use of probiotics may be beneficial.

High 2-hydroxyhippuric acid (61) may result from ingestion of aspartame (Nutrasweet®), salicylates (aspirin), dietary salicylates, or from GI bacteria converting tyrosine or phenylalanine to salicylic acid. For more information about salicylates in foods go to <http://www.feingold.org/salicylate.php>. 2-Hydroxyhippuric acid is a conjugate of hydroxybenzoic acid (salicylic acid) and glycine. Very high 2-hydroxyhippuric also inhibits dopamine beta-hydroxylase resulting in elevated HVA, decreased VMA, and elevated HVA/VMA ratio.

High quality nutritional supplements can be purchased through your practitioner or at New Beginnings Nutritionals, www.NBNUS.com <http://www.NBNUS.com> , or call 877-575-2467.

The nutritional recommendations in this test are not approved by the US FDA. Supplement recommendations are not intended to treat, cure, or prevent any disease and do not take the place of medical advice or treatment from a healthcare professional.

Candida/Yeast Intervention Suggestions

By Kurt N. Woeller, D.O.

Mild and/or Sensitive Individual

Bot = Botanical *(typically use lower dose)*

Nt = Nystatin *(meant to be low dose, e.g., 125,000 units to 250,000 units)*. Nystatin comes in oral suspension at 100,000 units/ml. However, different strength suspensions can be specially prepared by compounding pharmacies, e.g. 250,000 units/ml. Minimal dosing three times daily is suggested.

ALL remedies can be taken with or without food, although away from food is preferred.

NOTE: 5ml = one teaspoon

// = repeat dose

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Breakfast	Bot or Nt	Bot or Nt	Bot or Nt	Bot or Nt	Bot or Nt	Bot or Nt	Bot or Nt
Lunch	//	//	//	//	//	//	//
Dinner	//	//	//	//	//	//	//
Bedtime	Probiotic	Probiotic	Probiotic	Probiotic	Probiotic	Probiotic	Probiotic

Moderate

Bot = Botanical *(typically use moderate/higher dose than for sensitive individual listed above).*

Nt = Nystatin *(meant to be a moderate dose, e.g. 375,000 units to 500,000 units)* - Nystatin comes in oral suspension at 100,000 units/ml. However, different strength suspensions can be specially prepared by compounding pharmacies, e.g. 250,000 units/ml. Minimal dosing three times daily is suggested.

ALL remedies can be taken with or without food, although away from food is preferred.

NOTE: 5ml = one teaspoon

// = repeat dose

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Breakfast	Bot and/or Nt	Bot and/or Nt	Bot and/or Nt	Bot and/or Nt	Bot and/ or Nt	Bot and/or Nt	Bot and/or Nt
Lunch	//	//	//	//	//	//	//
Dinner	//	//	//	//	//	//	//
Bedtime	Probiotic	Probiotic	Probiotic	Probiotic	Probiotic	Probiotic	Probiotic

Severe

Dfc = Diflucan *(fluconazole)*

Bot = Botanical *(typically use higher dose compared to mild/sensitive or moderate individual).*

Nt = Nystatin *(meant to be a high dose, e.g. 750,000 units to 1.5 to 2 million units)* - Nystatin comes in oral suspension at 100,000 units/ml. However, different strength suspensions can be specially prepared by compounding pharmacies, e.g. 250,000 units/ml.

ALL remedies can be taken with or without food, although away from food is preferred.

NOTE: 5 ml = one teaspoon

// = repeat dose

2 Weeks of Diflucan *(or similar systemic antifungal)*

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Breakfast	Dfc	Dfc	Dfc	Dfc	Dfc	Dfc	Dfc
Lunch	Nt or Bot	Nt or Bot	Nt or Bot	Nt or Bot	Nt or Bot	Nt or Bot	Nt or Bot
Dinner	//	//	//	//	//	//	//
Bedtime	Probiotic	Probiotic	Probiotic	Probiotic	Probiotic	Probiotic	Probiotic

2 Weeks OFF Diflucan (or similar systemic antifungal)

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Breakfast	Nt & Bot	Nt & Bot	Nt & Bot	Nt & Bot	Nt & Bot	Nt & Bot	Nt & Bot
Lunch	//	//	//	//	//	//	//
Dinner	//	//	//	//	//	//	//
Bedtime	Probiotic	Probiotic	Probiotic	Probiotic	Probiotic	Probiotic	Probiotic

Keys to Chronic Candida Intervention.

- Dietary control through eliminating reactive and toxic foods
- Improving digestive system health and microbiome diversity
- Eradicating opportunistic infections such as parasites, bacteria, including clostridia
- Identifying and elimination of gut colonization of mold
- Eliminating or reducing environmental toxin exposures such as chemicals and heavy metals.
- Consistent and ongoing antifungal intervention through medication and/or botanical remedies.
- Confirm eradication of candida/yeast by checking Organic Acids Test or Microbial Organic Acids Test markers every 90 days.

Recurrent Clostridia Antibiotic or Natural Remedy Intervention Protocols (examples)

By Kurt N. Woeller, D.O.

These intervention options are typically used for individuals with recurrent *clostridia* problems seen on the Organic Acids Test and without severe illness and/or digestive disease secondary to *Clostridia difficile* infection. People suffering with severe health problems such as fever, weight loss, abdominal cramping, loose and/or bloody stools should be evaluated and treated medically.

Antibiotic Option:

The goal with this intervention approach for recurrent *clostridia* is to hit the *clostridia* bacterial colonies with a 10-day course of an antibiotic, then stop for a few days before hitting the bacterial colonies again with a series of cyclical treatment days. This cycle then repeats itself over a 3 week period of time. The total program is approximately 3-1/2 weeks. The typical dose for Flagyl or Vancocin is 30-40mg/kg split dose three times daily. Vancocin can be given four times per day, but compliance is an issue and three times daily has worked well, particularly when the dosing schedule is spread out over time:

- One dose of Vancocin or Flagyl three times daily for ten days straight, then
- Every 3rd day thereafter administer another treatment day (at three doses for that one day) for an additional 3 weeks.

Additional Nystatin Treatment Option: The addition of Nystatin may prove beneficial to combat yeast overgrowth secondary to antibiotic use. A typical dose is 500,000 units three times daily. Smaller dosage amounts can be used in children, e.g. 125,000 units to 250,000 units. It is recommended to repeat the Organic Acids Test (OAT) or Microbial Organic Acids Test (mOAT) from Great Plains Laboratory during the last week of Vancocin or Flagyl if possible.

Natural Remedy Option:

This program is intended to mimic the dosing sequence of Vancocin or Flagyl but using natural remedies instead. There are documented cases where the *clostridia* counts have normalized using the combination botanical supplement called Biocidin (from BioBotanical Research). Other supplement options may provide treatment support as well.

All products are available from New Beginnings Nutritionals (NBN) – https://nbnus.com:

- **Biocidin** (capsule or liquid) – 1 to 3 capsules three times daily or 5 to 15 drops of the liquid three times daily for 14 days. Then every 3rd day thereafter give one day dosing (three times per day as either the capsule or oral liquid) for an additional 4 weeks.
- **CoreBiotic** – 1 to 4+ capsules nightly away from Biocidin
- **Additional Options:**

    - **Grapefruit Seed Extract** (Nutribiotic) or **Garlic Extract** (Allimax) – 3 to 5+ drops of either in juice or water three times daily throughout the entire course of treatment (optional*)*, or some other botanical remedy, e.g., Berberine Complex.
    - **Culturelle** – 2 to 4 capsules daily. This is a probiotic that has been used too for clostridia bacteria.

NOTE: It is recommended to repeat the Microbial Organic Acids Test (mOAT) or Organic Acids Test (OAT) from Great Plains Laboratory during the last week of botanical remedy program.

Research article

Increased urinary excretion of a 3-(3-hydroxyphenyl)-3-hydroxypropionic acid (HPHPA), an abnormal phenylalanine metabolite of *Clostridia* spp. in the gastrointestinal tract, in urine samples from patients with autism and schizophrenia

William Shaw

The Great Plains Laboratory, Inc., Lenexa, Kansas, USA

A compound identified as 3-(3-hydroxyphenyl)-3-hydroxypropionic acid (HPHPA) was found in higher concentrations in urine samples of children with autism compared to age and sex appropriate controls and in an adult with recurrent diarrhea due to *Clostridium difficile* infections. The highest value measured in urine samples was 7500 mmol/mol creatinine, a value 300 times the median normal adult value, in a patient with acute schizophrenia during an acute psychotic episode. The psychosis remitted after treatment with oral vancomycin with a concomitant marked decrease in HPHPA. The source of this compound appears to be multiple species of anaerobic bacteria of the *Clostridium* genus. The significance of this compound is that it is a probable metabolite of *m*-tyrosine (3-hydroxyphenylalanine), a tyrosine analog which depletes brain catecholamines and causes symptoms of autism (stereotypical behavior, hyperactivity, and hyperreactivity) in experimental animals.

Keywords: *m*-tyrosine, 3-hydroxyphenylalanine, metronidazole, enkephalins, Lactobacillus acidophilus

Introduction

For the past 10 years, I have evaluated by gas-chromatography mass-spectrometry biochemical abnormalities that appear to be of microbial origin in urine samples of children with autism and other developmental disorders as well as adults with a wide variety of disorders, after the discovery that certain putative microbial metabolites appeared in higher than normal values in urine samples of two brothers with autism.[1] These findings were of especial interest to me because of a report that autistic children have a greater incidence of ear infections than age-matched peers; that lower functioning autistic children had an earlier onset of ear infections than their higher functioning autistic peers; and that the ears of children with autism were anatomically positioned differently than those of normal children, perhaps leading to greater ear infection susceptibility.[2] Intestinal overgrowth of yeast and anaerobic bacteria are well-documented sequelae of the common oral antibiotics used to treat ear infections.[3–6] Therefore, it is possible that abnormally elevated biochemical products of

Correspondence to: William Shaw PhD, The Great Plains Laboratory, Inc., 11813 W. 77th Street, Lenexa, KS 66214, USA
Tel: +1 913 341 8949; Fax: +1 913 341 6207;
E-mail: williamsha@aol.com

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abnormal micro-organisms in the gastrointestinal tract may play a role in the etiology of autism just as abnormal elevations of phenylalanine and its metabolites cause the disorder phenylketonuria (PKU). During testing, an unusual compound was detected in high concentrations in samples from children with autism, child psychosis, attention deficit hyperactivity, and in adults with severe depression, seizures, or schizophrenia. Since this compound in urine has not been adequately characterized, I began an intense investigation to identify it and determine its source.

Subjects and methods

Low-resolution electron impact gas-chromatography/mass spectrometry was performed as previously described.[1] In order to identify unknown compounds, the same procedure was performed except that perdeuterated *N,O-bis*(trimethylsilyl) acetamide (BSTFA) was used in place of non-deuterated BSTFA. High resolution gas-chromatography/mass spectrometry was performed on a VG 70-250S double focusing mass spectrometer interfaced with a Hewlett Packard model 5890 gas chromatograph. The column used was a 15-m, DB-1 capillary column with a 0.25 mm internal diameter and a 1.0 micron film from J & W Scientific (Folsom, CA, USA). The injection mode was splitless, and the injection volume was 0.5 μl. Initial mass calibration was performed using perfluorinated kerosene (PFK) over the mass range of 35–650 Da. PFK was also used over a limited mass range to calibrate for the high-resolution mass measurements. Once the compound of interest was located by low-resolution analysis, the resolution of the instrument was increased to 10,000 (10% valley criterion). The theoretical mass for 3-(3-hydroxyphenyl)-3-hydroxypropionic-TMS3 is 398.1765 Da. Two PFK peaks of known accurate mass (392.9760 and 404.9760 Da), which bracket the expected mass of the unknown, were located. The mass spectrometer was set to scan only this limited mass range. The sample was then injected and, at the proper retention time, the data acquisition system was started. 3,4-Dihydroxyphenylpropionic acid and 3-(4-hydroxyphenyl)-2-hydroxypropionic acid were obtained from Sigma Chemical Company (St Louis, MO, USA). Other common names for 3,4-dihydroxyphenyl-propionic acid are hydrocaffeic acid and 3,4-dehydroxy-hydrocinnamic. The minimum purity claimed by the manufacturers was 98%. Both Sigma and Fluka provided NMR analyses on the lots of materials to confirm the isomeric composition.

For quantitative analysis of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid, the response factor for 3,4-dihydroxyphenylpropionic acid was used as a surrogate calibration standard for the 3-(3-hydroxyphenyl)-3-hydroxypropionic acid using the reconstructed ion chromatograph signal of the ion at *m/z* 398 for quantitation. Undecanoic acid was used as the internal standard as described previously.[1] All results were normalized to urine creatinine as a way of minimizing variability due to differences in fluid intake.

Urine samples were obtained from both in-patients and out-patients at a pediatric hospital as well as from physicians submitting samples for my reference laboratory service. Urine samples (14 from males and 14 from females) were obtained from babies under 2 months old at a well-baby clinic at a local pediatric hospital. Urine samples from normal control children, 30 of each sex between the ages of 2–13 years, were obtained from children of local hospital employees. Normal adult values (n = 19) were obtained from adult volunteers (11 females and 8 males). Urine samples were obtained from children with autism between the ages of 2–13 years (211 males and 51 females). The ratio of male-to-female samples reflects the approximate male-to-female ratio of autism incidence in the population. The autistic children were either out-patients at the hospital or were referred for testing at the hospital. We requested first morning urine samples but did not verify compliance with this request. Baby urine samples were collected into tape-on urine collection bags during the night. Pediatric neurologists, developmental pediatricians, or child psychiatrists, using DSM-IV criteria, had made the diagnosis of autism. Dr Walter Gattaz at the Central Mental Health Institute at Mannheim, Germany collected samples from 12 drug-free patients with schizophrenia (4 males and 8 females). An additional sample from a drug-free patient with first onset of schizophrenia with auditory hallucinations was submitted by the attending physician. Urine samples were randomly collected in plastic screw-cap containers and stored at –20ºC until tested. The procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 1983.

Results

A typical urine chromatogram from a child with autism is shown in Figure 1A. A large peak from the TMS derivatives of urine extracts of patients with autism, all 12 drug-free patients with schizophrenia, and one patient with child psychosis and eluting shortly after the citric acid trimethylsilyl (TMS)

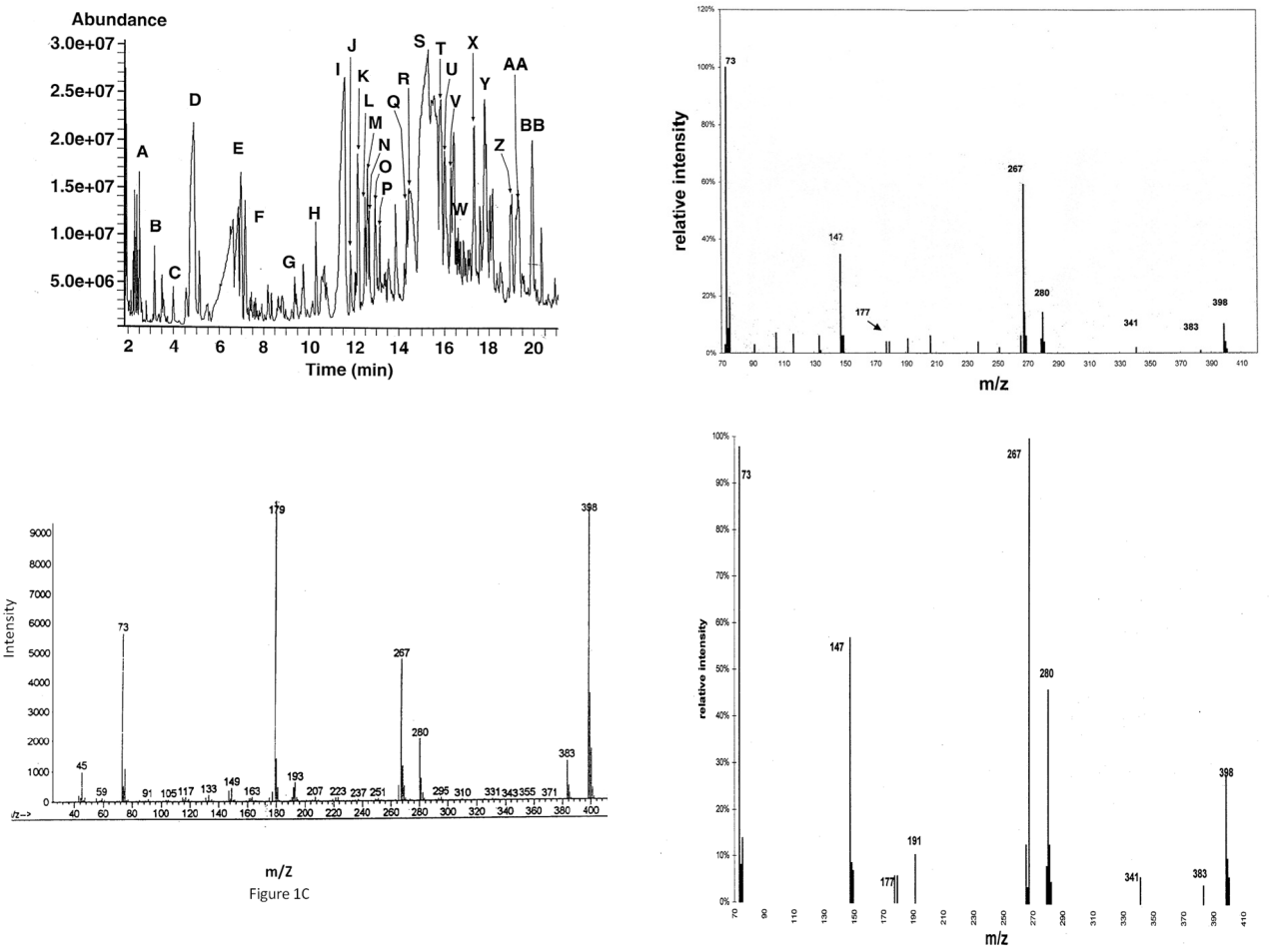

Figure 1 (A) Total ion current GC/MS chromatogram of the derivatized urine extract of child with autism. Peaks are identified as follows: A, glycolic; B, oxalic; C, 3-hydroxyisobutyric; D, urea; E, phosphoric; F, succinic; G, deoxytetronic; H, citramalic; I, undecanoic (internal standard); J, unidentified; K, 3-hydroxyphenylacetic; L, 2-oxoglutaric; M, 4-hydroxyphenylacetic; N, furandicarboxylic; O, furancarbonylglycine; P, tartaric; Q, arabinose; R, aconitic; S, hippuric; T, citric; U, 3-(3-hydroxyphenyl)-3-hydroxypropionic acid; V, vanillylmandelic; W, 3-indoleacetic; X, ascorbic; Y, citric analog; Z, uric; AA, unidentified; BB, hydroxyhippuric. Modified from Shaw W et al Clin Chem 41:1094-1104,1995 with permission. (B) Electron-impact mass spectrum of unknown compound in the urine sample extract of a child with autism. (C) Electron-impact mass spectrum of 3,4-dihydroxyphenylpropionic acid TMS derivative. (D) Electron-impact mass spectrum of authentic 3-(3-hydroxyphenyl)-3-hydroxypropionic acid TMS derivative.

derivative has an electron-impact mass spectrum with prominent ions at *m/z* 73,147,267, 280, 341,383, and 398. The mass spectrum in Figure 1B was from a child with autism. This mass spectrum is suggestive of a dihydroxy-substituted phenylpropionic acid tri-TMS derivative. The mass spectrum of 3,4-dihydroxyphenylpropionic acid tri (TMS) derivative (Fig. 1C) is very similar to the compound in urine but has a very intense ion at *m/z* 179, which is weak in the urine compound. Small quantities (< 1 mmol/mol creatinine) of 3,4 dihydroxyphenylpropionic acid derivative were sometimes found in a variety of urine samples. Furthermore, the major isomer in urine does not match the retention time of commercial 3,4-dihydroxyphenylpropionic acid tri (TMS) derivative. None of the published mass spectra of all of the other isomers of dihydroxyphenylpropionic acid tri (TMS) derivatives[7] or that of 3-(4-hydroxyphenyl)-2-hydroxypropionic acid tri(TMS) derivative match the spectrum of the compound in urine. However, the spectrum is an exact match for the mass spectrum of a 3-(3-hydroxyphenyl)-3-hydroxypropionic acid tri (TMS) derivative (Fig. 1D) obtained from the laboratory that synthesized the compound. This compound was designated β-*p*-hydroxyphenylhydracrylic acid in older nomenclature. Furthermore, the compound does not match the spectra of authentic 3-(4-hydroxyphenyl)-3-hydroxypropionic acid tri (TMS) or 3-(3-hydroxyphenyl)-2-hydroxypropionic acid tri(TMS) derivatives obtained in the same laboratory.[8]

To identify the compound of interest in urine further, an identical urine extract was prepared and

Table 1Interpretation of mass spectra of urine compound derivative prepared with deuterated and non-deuterated BSTFA

Loss from 398	Major ions with TMS	Major ions with d_2 TMS	Δ	TMS content	Interpretation
325	73	82	9	1 TMS	TMS
251	147	162	15	1 TMS 1 DMS	TMS-O-DMS
131	267	285	18	2 TMS	M-CH_2COOTMS
118	280	298	18	2 TMS	M-COOHTMS
57	341	365	24	2 TMS 1 DMS	M-CO-CH_2-CH_3
15	383	407	24	2 TMS 1 DMS	M-CH_3
0	398	425	27	3 TMS	M

The values in columns 1–4 are in daltons.
Δ is the difference in molecular weight (Da) of major ions of the compound derivatized with deuterated BSTFA (column 3) compared to non-deuterated BSTFA (column 2). Thus, Δ = value in column 3 – value in column 2.
TMS, trimethylsilyl; DMS, dimethylsilyl.
In the last column, M is the molecular ion.

then derivatized with perdeuterated BSTFA. The ions in the mass spectrum of the non-deuterated derivative were then compared to the ions in the mass spectrum of the perdeuterated derivative (Table 1). Since the deuterium of perdeuterated BSA is covalently bonded to the TMS groups, the transfer of the perdeuterated TMS groups to the accepting molecule increases the mass of the resulting deuterated derivative in proportion to the number of functional groups derivatized. Functional groups derivatized by the derivatizing reagent include carboxyl, hydroxyl, sulfhydryl and amino groups. Thus the transfer of one deuterated TMS group would add 9 deuterium atoms to the derivatized molecule, increasing the mass by 9 Da compared to the derivative containing ordinary hydrogen. Two deuterated TMS units would add 18 Da, and so forth. The ion at *m*/*z* 398 in the spectrum of the non-deuterated TMS derivative shifted to *m*/*z* 425 (a shift of 27 Da) in the spectrum of the perdeuterated derivative indicating that this ion contained three TMS groups (27/9 = 3) and was likely the molecular ion. The ion at *m*/*z* 383 in the spectrum of the non-deuterated TMS derivative shifted to *m*/*z* 407 (a shift of *m*/*z* 24) in the spectrum of the perdeuterated TMS derivative indicating the presence of two TMS groups (shift of 18 Da) and one dimethylsilyl group (shift of 6 Da), indicating a loss of a methyl group from a TMS group, a loss extremely common in TMS derivatives. The ion at *m*/*z* 341 in the spectrum of the non-deuterated BSTFA derivative shifted to *m*/*z* 365 in the spectrum of the perdeuterated derivative consistent with migration of –OTMS combined with a loss of –CH_3 from a TMS group and a loss of CH_2CO. All of the data support the identification of the urine compound as 3-(3-hydroxyphenyl)-3-hydroxy-propionic acid tri (TMS) derivative.

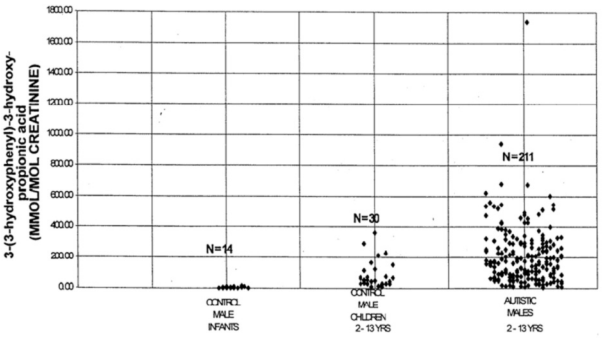

Figure 2 Distribution of 3-(3-hydroxyphenyl)-3-hydroxy-propionic values in urine samples of male infants, male control children, and male autistic children

With the high-resolution mass spectrometer, the mass of the putative molecular ion was measured three times, yielding an average of 398.1766 Da. The theoretical accurate mass for 3-(3-hydroxyphenyl)-3-hydroxy-propionic acid-TMS [3] is 398.1765 Da. The average of the measured values agree to within < 1 ppm of theoretical for

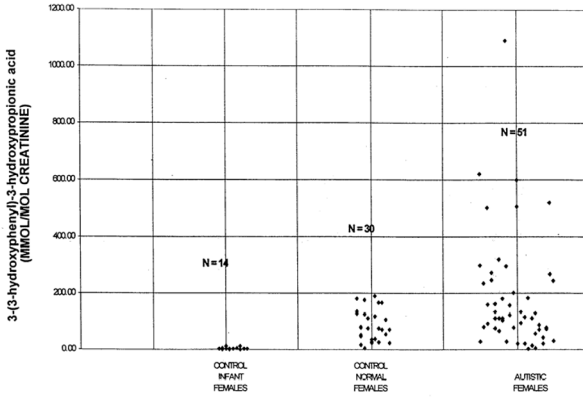

Figure 3 Distribution of 3-(3-hydroxyphenyl)-3-hydroxypropionic values in urine samples of female infants, female control children, and female autistic children

Table 2 Urinary excretion of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid in a drug-free patient with first onset of schizophrenia symptoms

Compound in urine of patient during acute psychosis (mmol/mol creatinine)	7500
Compound in urine of same patient 6 months after antimicrobial treatment (mmol/mol creatinine)	673
Compound in urine samples of normal adult controls (n = 19) (mmol/mol creatinine)	Mean 39.8, median 24.6; SD 50.2

an elemental composition of $C_{18}O_4Si_3H_{34}$, the elemental composition of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid or 3-(hydroxyphenyl)-3-hydroxypropionic acid TMS [3] derivative. The identity of the compound was later confirmed by comparison of both retention time and mass spectrum of the authentic compound.

Concentration of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid in urine samples of humans

The excretion of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid in urine was very low in a group of infants attending a well-baby clinic at 6 weeks of age (Figs 2 and 3). The mean value for all infants is 3.7 mmol/mol creatinine with a standard deviation of 3.6 mmol/mol creatinine and a range from 0.3–12.7 mmol/mol creatinine. In normal male control children, the mean value is 91.5 mmol/mol creatinine with a standard deviation of 90.4; the median value in this group is 51.1 mmol/mol creatinine. In autistic male children, the mean value is double that of the controls: 192.4 mmol/mol creatinine with a standard deviation of 90.4; the median value in the autistic male children group is 143.5 mmol/mol creatinine, nearly triple the value of the control group. In normal female control children, the mean value is 85.5 mmol/mol creatinine with a standard deviation of 55.9; the median value in this group is 74.5 mmol/mol creatinine. In autistic female children, the mean value is double that of the controls: 182.4 mmol/mol creatinine with a standard deviation of 200.6; the median value in this group is 111 mmol/mol creatinine, a value 49% greater than the control females. The differences between autism and control groups of the appropriate sex are statistically significant by the t-test at $P < 0.005$.

The highest value for this compound (Table 2) in over 7000 urine samples tested was a value of 7500 mmol/mol creatinine in a 21-year-old female with acute schizophrenia during an acute psychotic episode. Normal adult values (n = 19) for the compound are: mean, 39.8 mmol/mol creatinine; median, 24.6 mmol/mol creatinine; SD, 50.2 mmol/mol creatinine. Thus, the value in the urine of the schizophrenic patient was 300 times the median normal value. The patient was treated with oral vancomycin for one week, resulting in normalization of the psychotic behavior without the use of neuroleptic drugs. Re-testing the patient 6 months later indicated the compound in urine to be 673 mmol/mol creatinine, a value still significantly higher (nearly 27 times the median normal value) than normal values. Presumably, some recolonization of the *Clostridia* may have occurred after antimicrobial treatment ceased. The value in a child with child psychosis during hospitalization was probably even greater than that in this schizophrenic patient since the peak in the patient with child psychosis was so large it obscured about half the chromatogram. However, exact quantitation was not done on this patient.

Effect of metronidazole on urinary excretion of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid

Testing was performed on several patients at the attending physician's request who had suspected or confirmed clostridial infections and were treated with metronidazole, an antibacterial agent with specificity toward anaerobic bacteria and no antifungal properties.[9,10] I tested several of these patients before and after metronidazole therapy at standard age-appropriate dosages and found a substantial decrease in the concentration of this compound from baseline in these patients after drug therapy (Table 2). As shown in Table 3, there is a marked decrease in the urinary concentration of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid following the oral administration of the antibiotic metronidazole at 50 mg/kg/24-h divided into three doses. In all four patients, the concentrations of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid decreased 99% or more after 2–3 weeks on this drug. In the first patient in the above series, 3-(3-hydroxyphenyl)-3-hydroxypropionic acid rapidly increased following the cessation of metronidazole treatment. There was a severe Herxheimer or 'die-off' reaction (toxin release as the bacteria die) for several days with the use of this drug that includes fever, lethargy, profuse sweating, and heart palpitations.

Discussion

The marked decrease in 3-(3-hydroxyphenyl)-3-hydroxypropionic acid (Table 2) following treatment with metronidazole is consistent with the production of this

Table 3 Effect of metronidazole therapy on urinary excretion of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid

Diagnosis and sex	Age (years)	Duration of time metronidazole therapy (days)	Urinary 3-(3 hydroxyphenyl)-3-hydroxypropionic acid (mmol/mol creatinine)
Autism, male	4	0	435
		6	184
		16	1
		21 (stop metronidazole)	5
		24	2
		43	236
		93	274
Previous *C. difficile* infection and uncontrolled diarrhea, adult female	54	0	396
		13	1
Autism, male	3	0	549
		19	1
		30	3
Autism, male	4	0	1362
		11	28
		15	3

compound by one or more species of anaerobic bacteria. Phenylpropionic acid and/or monohydroxyphenylpropionic acid, which are very closely related biochemically to this compound, are produced by multiple species of *Clostridia* including *C. sporogenes*, *C. botulinum*, *C. caloritolerans*, *C. mangenoti*, *C. ghoni*, *C. bifermentans*, *C. difficile*, and *C. sordellii* while *C. tetani*, *C. sticklandii*, *C. lituseburense*, *C. subterminale*, *C. putifaciens*, *C. propionicum*, *C. malenomenatum*, *C. limosum*, *C. lentoputrescens*, *C. tetanomorphum*, *C. coclearium*, *C. histolyticum*, *C. aminovalericum*, and *C. sporospheroides* do not produce these compounds.[11]. Bhala *et al*.[12] found that *Clostridia* were the only organisms that produced phenylpropionic acid after they evaluated 67 different isolates of microbes from nine different genera of bacteria and *Candida albicans*. Furthermore, they found that metronidazole, clindamycin, and combined therapy of ticarcillin, clavulanate, and oxacillin abolished gut flora producing phenylpropionic acid. Cefalzolin, cefuroxime, ampicillin, chloramphenicol, and gentamicin did not abolish phenylpropionic production. Since a large group of intestinal bacteria including *E. coli*, *Streptococci*, *Salmonella*, *Shigella*, *Proteus*, *Pseudomonas*, and *Klebsiella* are killed by one or more of these agents,[13] the persistence of phenylpropionic acid in the presence of these agents appears to eliminate them as potential sources of this compound. (This latter group of drugs is generally ineffective against *Clostridia* species in mixed cultures like those in the gastrointestinal tract; the other species may inactivate antibiotics such as penicillin even though *Clostridia* in pure cultures may be susceptible to these antibiotics.) The increase in 3-(3-hydroxyphenyl)-3-hydroxypropionic acid in the urine of the child with autism after cessation of metronidazole treatment (Table 2) is consistent with the frequent recurrence of gastrointestinal *Clostridia* due to germination of resistant spores following antibiotic treatment. Alternatively, drug-resistant organisms may have been present that might have required longer therapy or the dose of drug may not have been adequate for total clearance of the organism.

I did not find 3-(3-hydroxyphenyl)-3-hydroxypropionic acid in multiple culture media samples in which multiple species of *Clostridia* were cultured. The lack of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid in pure cultures of *Clostridia* is almost surely due to the production of precursors of the compound such as phenylpropionic and monohydroxyphenylpropionic acids that are then converted to 3-(3-hydroxyphenyl)-3-hydroxypropionic acid (Fig. 4) by human metabolism.

The origin of this compound (Fig. 4) is almost surely dietary phenylalanine in the intestinal tract that is converted to 3-hydroxyphenylpropionic acid by two possible routes. The hydroxylation of the phenylalanine ring at the 3-position may occur before or after removal of the amino group (deamination). If deamination occurs first, phenylpropionic acid would be formed. If deamination occurs after hydroxylation, *m*-tyrosine (3-hydroxyphenylalanine) would be formed (Fig. 4). *m*-Tyrosine induces a characteristic behavioral syndrome in rats consisting of forepaw padding, head weaving, backward walking, splayed hind limbs, wet dog shakes, hyperactivity and hyper-reactivity and depletes the brain of catecholamines.[14] Thus, this compound might play a direct role in causing abnormal behaviors in autism, schizophrenia, and other disorders. It is also possible that this compound might form an analog of dopamine, if *m*-

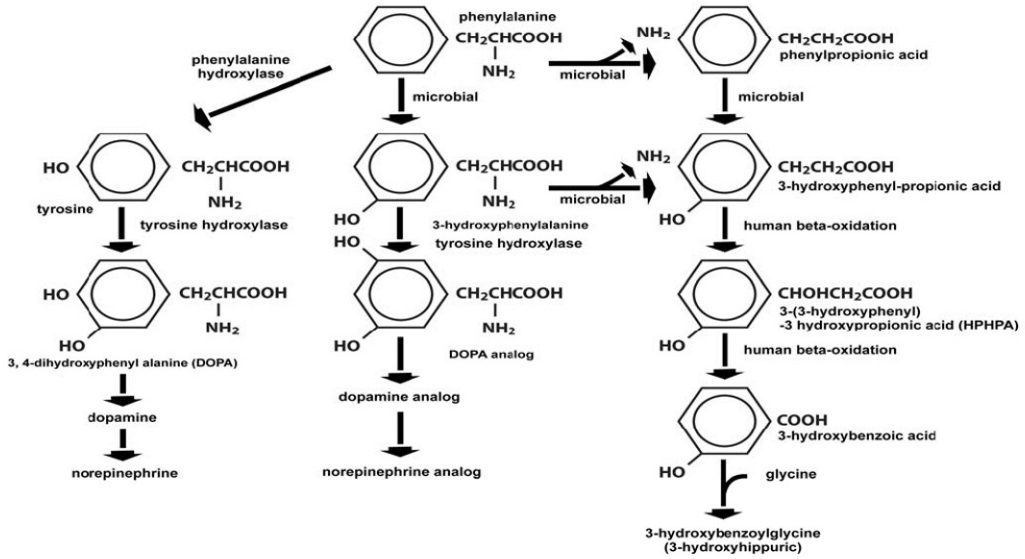

Figure 4 Suggested pathway for the metabolism of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid

tyrosine is metabolized by the same enzymes that convert tyrosine to dopamine.

Male and female autistic children both excreted more 3-(3-hydroxyphenyl)-3-hydroxypropionic acid than the appropriate control group. The difference is especially prominent in autistic males in which the median value is nearly triple that of the control children. The other metabolic routes for this compound might be expected to include sulfation and/or glucuronidation of the phenolic groups (Fig. 4). A similar compound, 3-phenylpropionic acid, is converted to benzoic acid by the enzymes of fatty acid oxidation.[15] These enzymes convert phenylpropionic acid to benzoic acid, which is then conjugated with glycine in the liver to form hippuric acid (Fig. 4). If these same enzymes metabolize 3-(3-hydroxyphenyl)-3-hydroxypropionic acid, increased hydroxybenzoic acid and its glycine conjugate hydroxyhippuric acid would be expected to be formed when excessive 3-(3-hydroxyphenyl)-3-hydroxypropionic acid is produced. Hydroxyhippuric acid TMS derivative, usually a very small peak in urine extracts of normal individuals, is indeed present as a large peak in the chromatogram of the urine sample extract of the child with autism in Figure 1A. The finding of elevated median values of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid in urine samples of autistic children might indicate they harbor bacteria that produce more of this compound than in normal children. Previous studies have indicated lower activity of phenolsulfotransferase in autistic children,[16] which might result in decreased detoxification of this compound by the sulfation pathway characteristic of phenolic compounds.

This compound might also have importance as a marker for the overgrowth of *Clostridia* in the gastrointestinal tract. The very low values of this compound in both male and female infants (Figs 2 and 3) are consistent with the age at which *Clostridia* normally colonize the gastrointestinal tract.[16] Breast-fed infants are colonized with almost no *Clostridia* but extensive anaerobic bacterial colonization begins with the introduction of solid foods.[17] Bennett *et al*.[18] found that phenylpropionic acid was not detected in 84% of stool samples from infants younger than 4 months but was present in 67% of stool samples from infants 4 months or older. Intestinal overgrowth of *C. botulinum* and *C. difficile* both cause serious illness,[19] and *C. botulinum* in infant botulism produces a neurotoxin that is apparently absorbed from the gastrointestinal tract.[20] Increased frequency of ear infections in children with autism has been documented and the severity of the autism has been related to both the frequency of such ear infections and the age of onset of ear infections.[2] Since many species of *Clostridia* are not susceptible to some of the common antibiotics used to treat ear infections, antibiotic therapy might be selecting harmful species of *Clostridia*. It is also possible that these children may be susceptible to harmful *Clostridia* as a consequence of their environmental exposure in early life and/or their specific genetic make-up, enabling these organisms to proliferate.

Phenylpropionic acid has only been detected in the culture media of bacterial species of the *Clostridium* genus,[18] indicating that a closely related compound, 3-

(3-hydroxyphenyl)-3-hydroxypropionic acid is probably a product of *Clostridia* species. I am currently examining stool samples of autistic children to determine if there are certain species of *Clostridia* that are prevalent in these children. Bolte[20] has hypothesized that gastrointestinal tetanus due to *C. tetani* may be a major etiological agent in autism and compares symptoms of autism to subacute gastrointestinal tetanus infection; animals with tetanus exhibit many of the same characteristics as individuals with autism: stereotyped behavior, hypotonia, difficulties in chewing and swallowing, reduced learning ability, and seizures. Sandler *et al*.[21] reported that vancomycin treatment of a group of autistic children resulted in a significant decrease in autistic symptoms. However, the benefits of therapy were lost after vancomycin treatment ended. This regression is consistent with possible *Clostridia* overgrowth of the intestinal tract in which *Clostridia* commonly recur after discontinuation of antibiotic use because of formation of resistant spores.[22] Bolte[20] demonstrated that a similar number of *Clostridia* species was harbored by autistic spectrum disorder (ASD) patients and healthy controls. However, nine *Clostridium* species were exclusively isolated from stool samples of autistic children (*i.e*. not found in the predominant fecal microflora of healthy controls). In addition, three species were only found in healthy samples. In a subsequent study, Song *et al*.[23] identified significantly higher levels of *C. bolteae* and *Clostridium* clusters I and XI in autistic children than in healthy controls. The fecal flora of patients with ASDs was studied by Helena *et al*.[24] and compared with those of two control groups (healthy siblings and unrelated healthy children). Fecal bacterial populations were assessed through the use of a culture-independent technique, fluorescence *in situ* hybridization, using oligonucleotide probes targeting predominant components of the gut flora. The fecal flora of ASD patients contained a higher prevalence of the *C. histolyticum* group (*Clostridium* clusters I and II) of bacteria than that of healthy children.

Phenylpropionic acid, the probable precursor of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid, is an *in vitro* inhibitor of both carboxypeptidase and enkephalinase activities;[25] administration of this compound to mice raises brain enkephalin concentrations[26] and causes analgesia when injected intraperitoneally into mice. I have detected phenylpropionic acid in many of the same urine samples in which 3-(3-hydroxyphenyl)-3-hydroxypropionic acid is elevated, and I suspect that elevation of enkephalins due to enkephalinase inhibition by phenylpropionic acid in humans might also contribute to abnormal behaviors. An evaluation of the possible role of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid in inhibiting these same enzymes may be worthwhile because of the biochemical similarity of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid and phenylpropionic acid.

High doses of the GG strain of *Lactobacillus acidophilus* have been used to control *C. difficile*.[27] *L. acidophilus* therapy has no reported toxicity, and treatment with *L. acidophilus* GG of individuals with an elevated concentration of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid in their urine markedly reduces the concentration of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid in subsequent urine samples (unpublished data). Bolte[20] reported a marked decrease in symptoms of autism in children treated with antibiotics effective against *Clostridia*, indicating treatment of abnormal microbial overgrowth may be a promising new therapy for the treatment of autism in individuals with this abnormality. The observation that elevated amounts of this compound in urine samples were associated with mental illnesses in general was made 50 years ago but has been completely ignored since then.[28] Significant decreases in symptoms of schizophrenia, tic disorders, depression, chronic fatigue syndrome, and attention deficit hyperactivity have been reported by the attending physicians (personal communications, see Addendum) following antimicrobial treatment of individuals with elevated urinary concentrations of this compound, indicating that this compound may be of importance to many other mental diseases in addition to autism but also indicating that these probable *Clostridia* species are not specific for the etiology of autism or other diseases.

3,4-Dihydroxyphenylpropionic acid (DHPPA), a compound I found at very low amounts in the urine, is a by-product of chlorogenic acid, a common substance found in beverages and in many fruits and vegetables including apples, pears, tea, coffee, sunflower seeds, carrots, blueberries, cherries, potatoes, tomatoes, eggplant, sweet potatoes, and peaches.[29–40] Because of the chemical similarities, similar retention times in many chromatographic systems, and the similar mass spectra of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid (HPHPA) and DHPPA, it is important to differentiate the sources of these compounds. The breakdown of chlorogenic acid is mediated mainly by harmless or beneficial bacteria such as *Lactobacilli*, *Bifidobacteria*, and *E. coli*.[41]. In addition, one clostridial species, *C. orbiscindens*, can convert the flavanoids luteolin and

eriodictyol, that occur only in a relatively small food group that includes parsley, thyme, celery, and sweet red pepper to 3,4-dihydroxyphenylpropionic acid.[42] In addition, DHPPA is also produced from wine phenols and catechin, a constituent in chocolate. The quantity of *C. orbiscindens* in the gastrointestinal tract is negligible (approximately 0.1% of the total bacteria) compared to the predominant flora of *Lactobacilli*, *Bifidobacteria*, and *E. coli*.[43] Thus, elevated amounts of this compound are due primarily to beneficial microbial breakdown of chlorogenic acid which is present in abundance in common major foods and is overwhelmingly an indicator of beneficial bacteria presence and/or a diet high in foods containing phenolic flavanoid compounds.

Elevated values of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid (data not shown) also occur in children with attention deficit hyperactivity and child psychosis, adults with depression, and in some children and adults with seizure disorders, chronic fatigue syndrome, obsessive-compulsive disorders, and tic disorders (unpublished data). The compound is not drug derived since it is found in the urine of many drug-free persons, including the drug-free schizophrenic patients obtained from Dr Gattaz.

Acknowledgements

The author would like to thank Dr M. Duran and his associates at the Biochemical Genetics Laboratory of Queen Wilhelmina Children's Hospital in Utrecht, The Netherlands for providing the mass spectra of the compounds synthesized in their laboratory, and Dr Walter Gattaz at the Central Mental Health Institute at Mannheim, Germany for the drug-free urine samples from patients with schizophrenia.

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Addendum

Physicians who have used the HPHPA marker in their clinical practices and their addresses:

Jeff Bradstreet MD and Dan Rossingnol MD, ICDRC, 3800 W. Eau Gallie Blvd, Suite 105 , FL 32934, USA

Jeremy Baptist MD PhD, Allergy Link, 6806 W.83rd St, Overland Park, KS 66204, USA

Arnold Brenner MD, 5400 Old Court Rd, #105, Randallstown, MD 21133, USA

David Berger MD, 3341 W. Bearss Ave, Tampa, FL 33618, USA

Kurt Woeller DO, 41660 Ivy St #A, Murrieta, CA 92562, USA

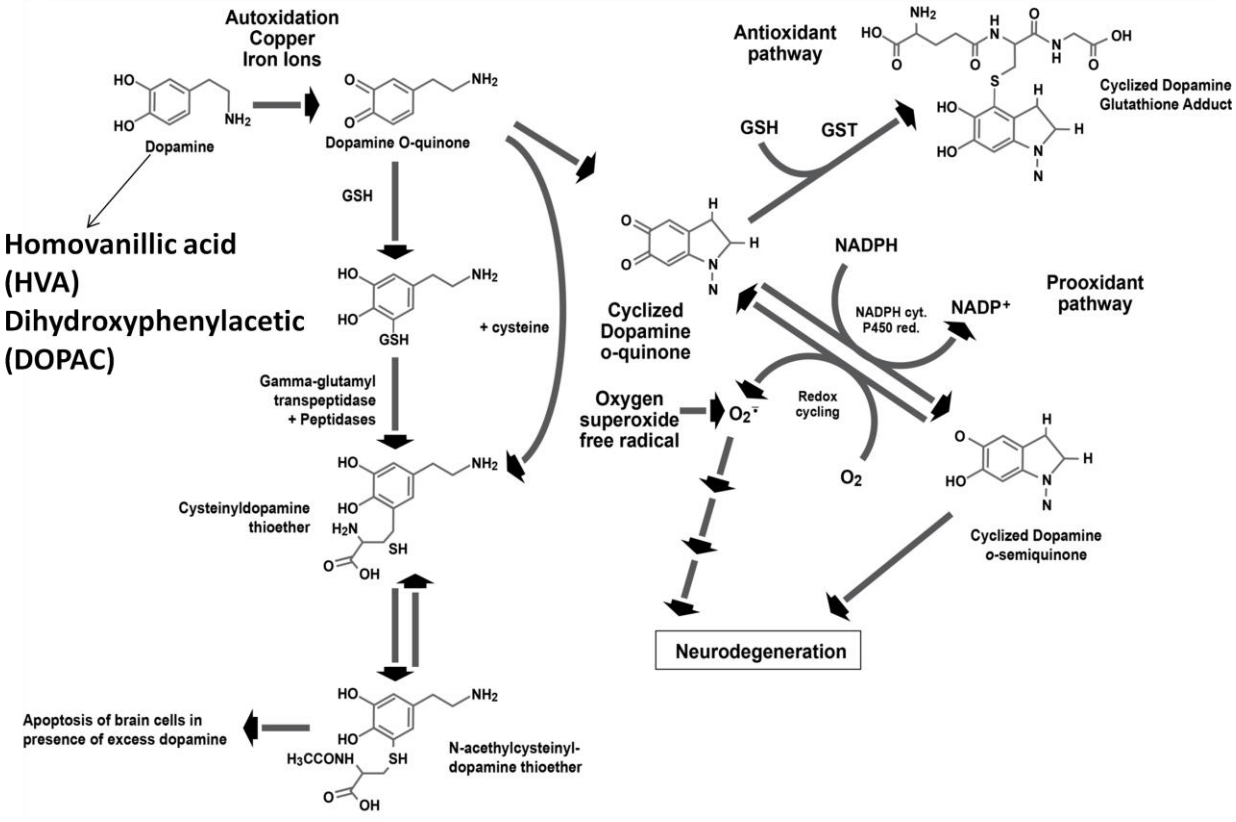
Inhibition of dopamine conversion to norepinephrine by *Clostridia* metabolites appears to be a (the) major cause of autism, schizophrenia, and other neuropsychiatric disorders. All these factors can now be monitored in The Great Plains Laboratory organic acid test.

William Shaw Ph.D.

The Great Plains Laboratory, Inc

Concentrations of the dopamine metabolite homovanillic acid, or HVA, have been reported to be much higher in the urine of children with autism compared to controls. In the same study, severity of autism symptoms was directly related to the concentration of HVA. There was a relation between the urinary HVA concentration and increased agitation, stereotypical behaviors, and reduced spontaneous behavior. Furthermore, vitamin B_6, which has been shown to decrease autistic symptoms, decreases urinary HVA concentrations. Excess dopamine has been implicated in the etiology of psychotic behavior and schizophrenia for over 40 years. Drugs that inhibit dopamine binding to dopaminergic receptors have been some of the most widely used pharmaceuticals used as antipsychotic drugs and have been widely used in the treatment of autism. Recent evidence reviewed below indicates that dopamine in high concentrations may be toxic to the brain.

Figure 1. Toxicity of excess dopamine



Dopamine is a very reactive molecule compared with other neurotransmitters, and dopamine degradation naturally produces oxidative species (Figure 1). More than 90 percent of dopamine in dopaminergic neurons is stored in abundant terminal vesicles and is protected from degradation. However, a small fraction of dopamine is cytosolic, and it is the major source of dopamine metabolism and presumed toxicity. Cytosolic dopamine (Figure 1) undergoes degradation to form 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) via the monoamine oxidase pathway. Alternatively, dopamine undergoes oxidation in the presence of excess iron or copper (common in autism and schizophrenia) to form dopamine cyclized o-quinone, which is then converted to dopamine cyclized o-semiquinone, depleting NADPH in the process. Dopamine cyclized o-semiquinone then reacts with molecular oxygen to form oxygen superoxide free radical, an extremely toxic oxidizing agent. In the process, dopamine cyclized o-quinone is reformed, resulting in a vicious cycle extremely toxic to tissues producing dopamine, including the brain, peripheral nerves, and the adrenal gland.

It is estimated that each molecule of dopamine cyclized o-quinone produces thousands of molecules of oxygen superoxide free radical in addition to depleting NADPH. The *o*-quinone also reacts with cysteine residues on glutathione or proteins to form cysteinyl-dopamine conjugates (Figure 1). One of these dopamine conjugates is converted to N-acetylcysteinyl dopamine thioether, which causes apoptosis (programmed cell death) of dopaminergic cells. These biochemical abnormalities cause severe neurodegeneration in pathways that utilize dopamine as a neurotransmitter. Neurodegeneration is due to depletion of brain glutathione and NADPH as well as the overproduction of oxygen superoxide free radicals and neurotoxic N-acetylcysteinyl dopamine thioether. In addition, the depletion of NADPH also results in a diminished ability to convert oxidized glutathione back to its reduced form.

What is the likely cause of elevated dopamine in autism? A significant number of studies have documented increased incidence of stool cultures positive for certain species of *Clostridia* bacteria in the intestine in children with autism using culture and PCR techniques. All these studies have indicated a disproportionate increase in various *Clostridia* species in stool samples compared to normal controls. In addition, metabolic testing has identified the metabolites 3-(3-hydroxyphenl)-3-hydroxypropionic acid (HPHPA) and 4-cresol from *Clostridia* bacteria at significantly higher concentrations in the urine samples of children with autism and in schizophrenia.

Treatment with antibiotics against *Clostridia* species, such as metronidazole and vancomycin, eliminates these urinary metabolites with reported concomitant improvement in autistic symptoms. In addition, I had noticed a correlation between elevated HPHPA and elevated urine homovanillic acid (HVA). The probable mechanism for this correlation is that certain *Clostridia* metabolites have the ability to inactivate dopamine beta-hydroxylase, which is needed for the conversion of dopamine to norepinephrine (Figure 2).

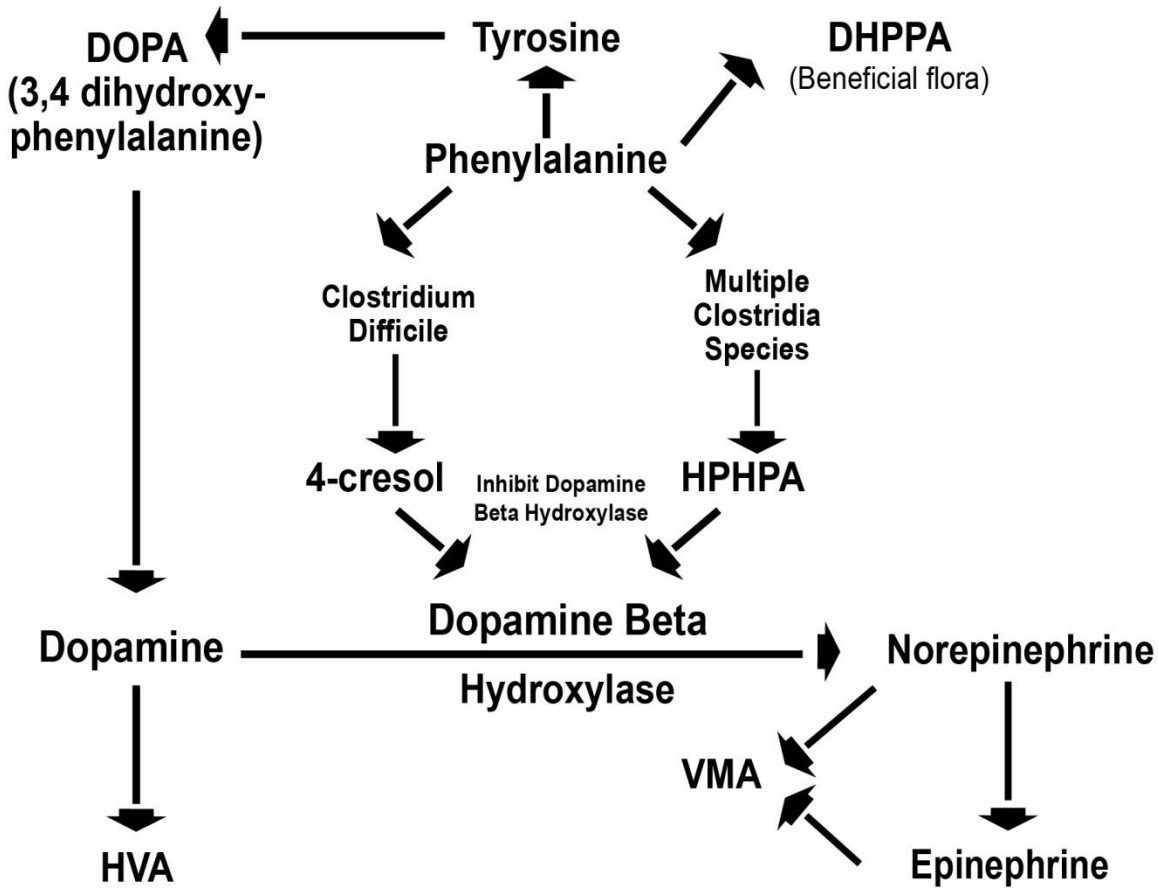


Figure 2. Effect of *Clostridia* metabolites on human catecholamine metabolism. DHPPA, 4-cresol, HPHPA, HVA, and VMA are all measured in The Great Plains Laboratory organic acid test.

Such metabolites are not found at only trace levels. The concentration of the *Clostridia* metabolite HPHPA in children with autism may sometimes exceed the urinary concentration of the norepinephrine metabolite vanillylmandelic acid (VMA) by a thousand fold on a molar basis and may be the major organic acid in urine in those with severe gastrointestinal *Clostridia* overgrowth, and *even exceed the concentration of all the other organic acids combined*. Dopamine beta hydroxylase that converts dopamine to norepinephrine in serum of severely retarded children with autism was much lower than in those who were higher functioning. Decreased urine output of the major norepinephrine metabolite meta-hydroxyphenolglycol (MHPG) was decreased in urine samples of children with autism, consistent with inhibition of dopamine beta hydroxylase.

Many physicians treating children with autism have noted that the severity of autistic symptoms is related to the concentration of the *Clostridia* marker 3-(3-hydroxyphenyl)-3-hydroxypropionic acid (HPHPA) in urine. These are probably the children with autism with severe and even psychotic behavior treated with Risperdal® and other anti-psychotic drugs, which block the activation of dopamine receptors by excess dopamine. I have identified a number of species of *Clostridia* species that produce HPHPA including *C. sporogenes*, *C.botulinum*, *C. caloritolerans*, *C. mangenoti*, *C. ghoni*, *C.bifermentans*, *C. difficile*, and *C.*

sordellii. All species of *Clostridia* are spore formers and thus may persist for long periods of time in the gastrointestinal tracts even after antibiotic treatment with oral vancomycin and metronidazole.

How do the changes in brain neurotransmitters caused by *Clostridia* metabolites alter behavior? The increase in phenolic *Clostridia* metabolites common in autism significantly decreases brain dopamine beta hydroxylase activity. This leads to overproduction of brain dopamine and reduced concentrations of brain norepinephrine, and can cause obsessive, compulsive, stereotypical behaviors associated with brain dopamine excess and reduced exploratory behavior and learning in novel environments that are associated with brain norepinephrine deficiency. Such increases in dopamine in autism have been verified by finding marked increases in the major dopamine metabolite homovanillic acid (HVA) in urine. The increased concentrations of HVA in urine samples of children with autism are directly related to the degree of abnormal behavior. The concentrations of HVA in the urine of some children with autism are markedly abnormal.

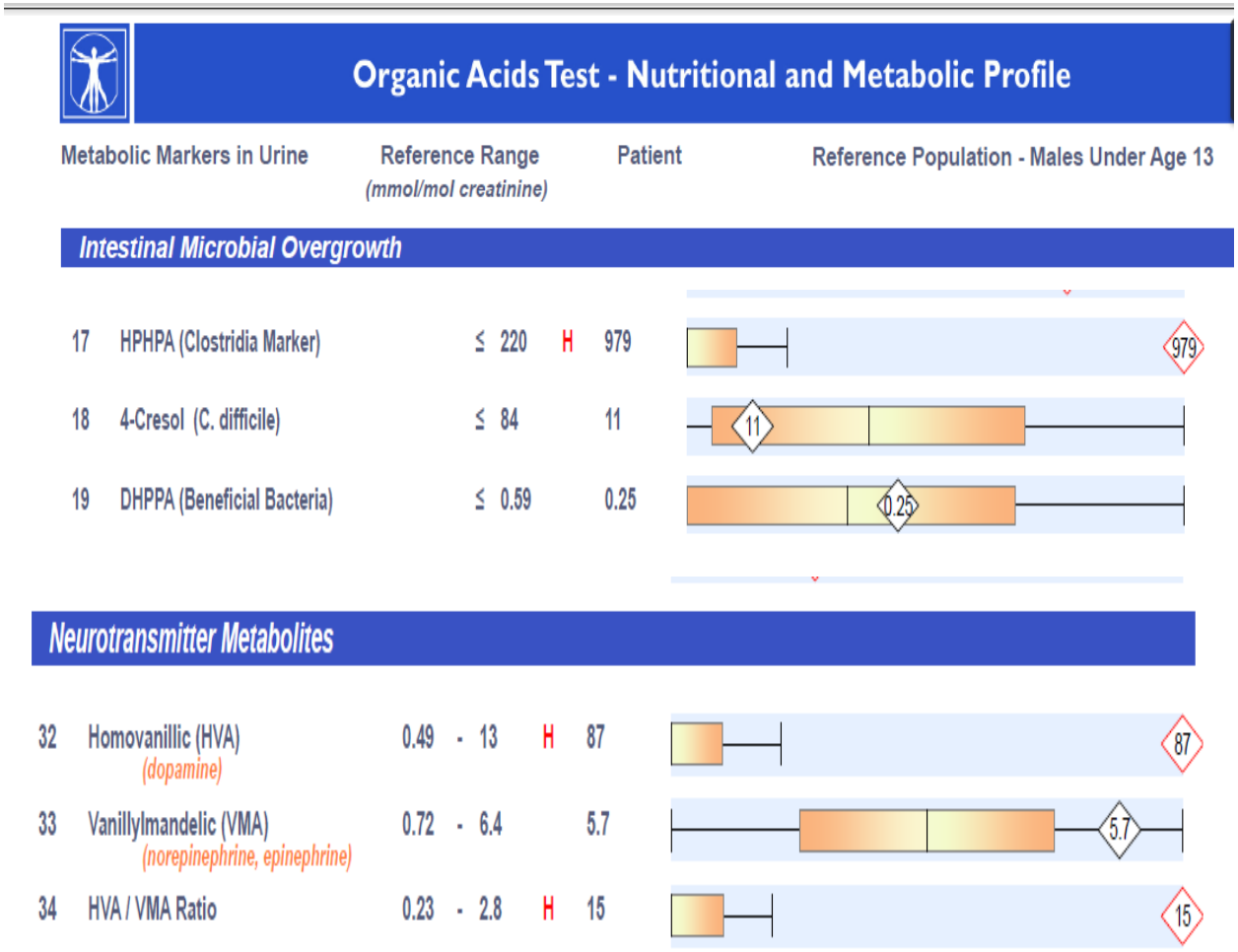
In addition to alteration of brain neurotransmitters, the inhibition of the production of norepinephrine and epinephrine by *Clostridia* metabolites may have a prominent effect on the production of neurotransmitters by the sympathetic nervous system and the adrenal gland. The major neurotransmitter of the sympathetic nervous system that regulates the eyes, sweat glands, blood vessels, heart, lungs, stomach, and intestine is norepinephrine. An inadequate supply of norepinephrine or a substitution of dopamine for norepinephrine might result in profound systemic effects on physiology. The adrenal gland which produces both norepinephrine and epinephrine might also begin to release dopamine instead, causing profound alteration in all physiological functions. In addition to abnormal physiology caused by dopamine substitution for norepinephrine and dopamine, dopamine excess causes free radical damage to the tissues producing it, perhaps leading to permanent damage of the brain, adrenal glands, and sympathetic nervous system if the *Clostridia* metabolites persist for prolonged periods of time, if glutathione is severely depleted, and if there is apoptotic damage caused by the dopamine metabolite N-acetylcysteinyl dopamine thioether.

Depletion of glutathione can be monitored in The Great Plains Laboratory organic acid test by tracking the metabolite pyroglutamic acid, which is increased in both blood and urine when glutathione is depleted. In addition, The Great Plains Laboratory also tests the other molecules involved in this toxic pathway, the dopamine metabolite homovanillic acid (HVA), the epinephrine and norepinephrine metabolite VMA and the *Clostridia* metabolites HPHPA and 4-cresol.

In summary, gastrointestinal *Clostridia* bacteria have the ability to markedly alter behavior in autism and other neuropsychiatric diseases by production of phenolic compounds that dramatically alter the balance of both dopamine and norepinephrine. Excess dopamine not only causes abnormal behavior but also depletes the brain of glutathione and NADPH and causes a vicious cycle producing large quantities of oxygen superoxide that causes severe brain damage. Such alterations appear to be a (the) major factor in the causation of autism and schizophrenia. The organic acid test (see sample organic acid test report below) now has the ability to unravel a major mystery in the causation of autism, schizophrenia, and other neuropsychiatric diseases, namely the reason for dopamine excess in these disorders.

In the past, some physicians would order the organic acid test once a year or less. With the new knowledge of the mechanism of *Clostridia* toxicity via inhibition of dopamine beta-hydroxylase, it seems that the control of such toxic organisms needs to monitored much more frequently to prevent serious brain, adrenal gland, and sympathetic nervous system damage caused by excess dopamine and oxygen

superoxide. Below is a test report of a child with autism tested with The Great Plains Laboratory Organic acid test.



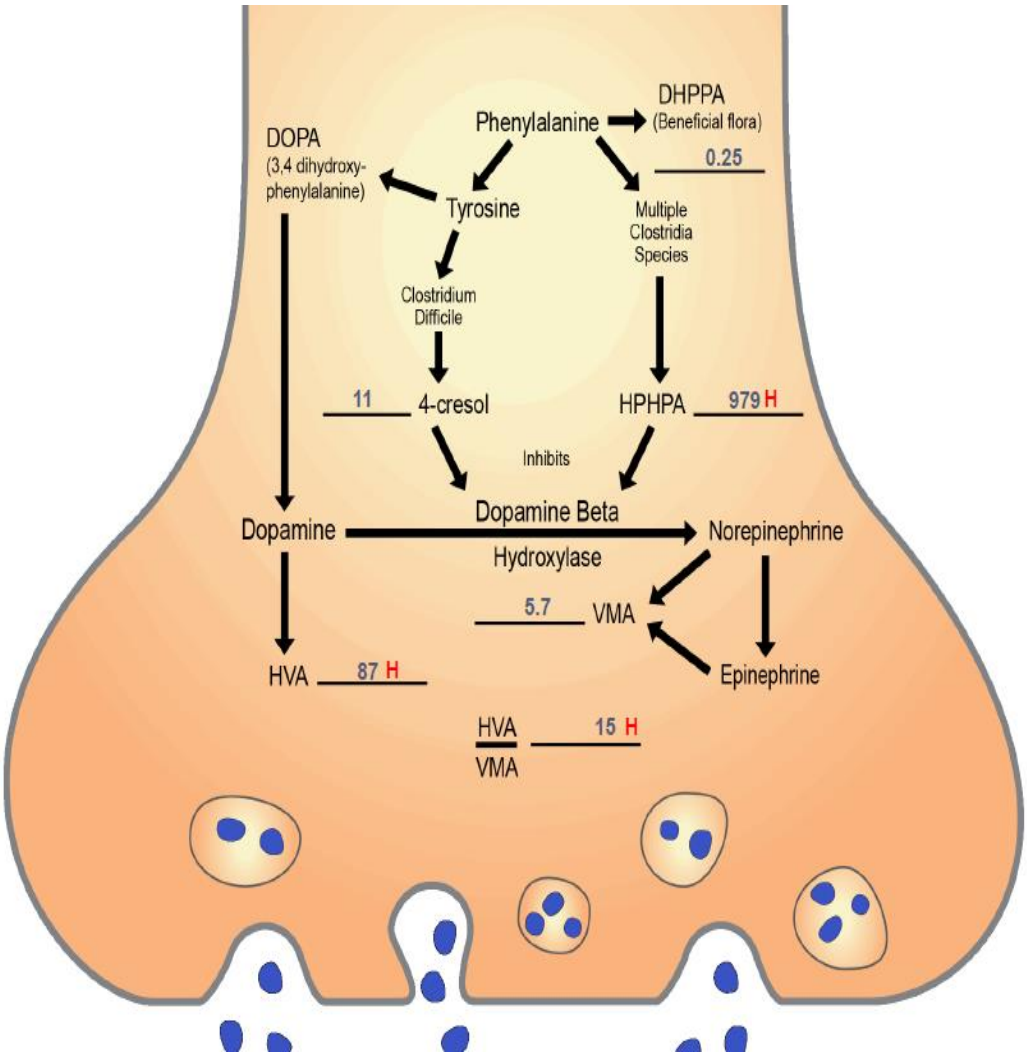
Organic Acids Test - Nutritional and Metabolic Profile

	Metabolic Markers in Urine	Reference Range (mmol/mol creatinine)		Patient	Reference Population - Males Under Age 13
Intestinal Microbial Overgrowth					
17	HPHPA (Clostridia Marker)	≤ 220	H	979	979
18	4-Cresol (C. difficile)	≤ 84		11	11
19	DHPPA (Beneficial Bacteria)	≤ 0.59		0.25	0.25
Neurotransmitter Metabolites					
32	Homovanillic (HVA) (dopamine)	0.49 - 13	H	87	87
33	Vanillylmandelic (VMA) (norepinephrine, epinephrine)	0.72 - 6.4		5.7	5.7
34	HVA / VMA Ratio	0.23 - 2.8	H	15	15

Discussion of patient results

In the graph above, the vertical bar is the upper limit of normal and the patient's value is plotted inside a diamond (red for abnormal, black for normal). The above results were from a boy with severe autism. The HPHPA *Clostridia* marker was very high (979 mmol/mol creatinine), about 4.5 times the upper limit of normal. However, the metabolite due to *Clostridium difficile* was in the normal range, indicating that *Clostridium difficile* was unlikely to be the *Clostridium* bacteria producing the high HPHPA. In other words, a different *Clostridia* species was implicated. The major dopamine metabolite homovanillic acid (HVA) was extremely high (87 mmol/mol creatinine), almost 7 times the upper limit of normal. The major metabolite of epinephrine and norepinephrine, VMA was in the normal range. The HVA/VMA ratio was 15, more than five times higher than the upper limit of normal, indicating a severe imbalance in the production of epinephrine/norepinephrine and that of dopamine. The very high dopamine metabolite, HVA, indicates that the brain, adrenal glands, and sympathetic nervous system may be subject to severe oxidative stress due to superoxide free radicals and that brain damage due to severe oxidative stress might result if the *Clostridia* bacteria are left untreated. Below the same patient's results are displayed in a form

that is related to the metabolic pathways. This graphical result now appears on all organic acid results from The Great Plains Laboratory, Inc.



Reference:

Shaw W. Increased urinary excretion of a 3-(3-hydroxyphenyl)-3-hydroxypropionic acid (HPHPA), an abnormal phenylalanine metabolite of *Clostridia* spp. in the gastrointestinal tract, in urine samples from patients with autism and schizophrenia. Nutr Neurosci. 2010 Jun;13(3):135-43.

Low Oxalate Program (example)

By Kurt N. Woeller, D.O.

Low Oxalate Diet:

Commonly consumed foods high in oxalates are Spinach, Berries (including juice), Nuts (including nut butters) and Soy. There are many other high oxalate foods as well that individual patients/clients may be consuming. There are various online resources available for more information regarding high oxalate foods and the incorporation of a low oxalate diet:

- *Low Oxalate.info – https://lowoxalate.info.*
- *Great Plains Laboratory Oxalate Control brochure – https://greatplainslaboratory.com. Their brochure regarding low oxalate information is found in the 'Organic Acids Test (OAT)' section.*
- *The Vulvar Pain (VP) Foundation – https://thevpfoundation.org.*
- *Nourishing Hope (Julie Matthews, CNC.) – https://nourishinghope.com.*

Reducing the consumption of high oxalate foods is essential for a low oxalate program. Have patient/client access one or more of the above listed websites for a more thorough analysis of their consumption of high oxalate foods. Also, incorporating certain supplements can help with the elimination of oxalates.

These supplements are available from **New Beginnings Nutritionals** – https://nbnus.com. Other supplement companies of your choosing may provide similar options.

- **Cal/Mag Citrate** *(capsules or chewable tablets)* – 1 to 2 capsules or chewable tablets with meals
- **Visbiome** – 1 to 2 capsules daily
- **Vitamin B6 Tablet** *(50mg)* – 1 to 2 tablets daily
- **Additional Options:**
    - ***Magnesium*** *– oral magnesium, e.g. Magnesium Chelate (1 to 2+ capsules daily) and/or magnesium sulfate, aka. Epsom Salt cream (daily application). The Epsom Salt cream is used primarily if having body pain with high oxalates or oxalate dumping.*
    - ***Biotin*** *(5mg) – 1 to 4+ capsules daily. Used primarily if having body pain with oxalates or oxalate dumping.*

ORIGINAL PAPER *Clinical Practice of Alternative Medicine* 1(1): 15-26, 2000

Assessment of Antifungal Drug Therapy in Autism by Measurement of Suspected Microbial Metabolites in Urine with Gas Chromatography-Mass Spectrometry

William Shaw, PhD, Ellen Kassen, MT(ASCP), Enrique Chaves, MD

ABSTRACT: Context-Certain compounds found by gas chromatography-mass spectrometry in urine samples of children with autism might be produced by yeast in the gastrointestinal tract. Therefore, treatment with antifungal drugs might reduce clinical symptoms of autism.

Objective-To determine if symptoms of autism and chemical compounds in urine samples of children with autism decreased after antifungal treatment.

Design-A number of urinary organic acids first characterized as elevated in 2 brothers with autism were tested in urine samples of 23 children with autism (21 boys and 2 girls) before and after treatment with the antifungal drug nystatin.

Patients-Twenty-one boys and 2 girls with a mean age of 7.1 years with diagnosis of autism. Twenty boys and 17 girls with a mean age of 7.7 years, who were normal children of hospital employees, served as controls for urine testing.

Interventions-Children with abnormal baseline organic acid results received oral nystatin suspension at a dose of 100,000 units 4 times a day for 10 days. If abnormalities were still present after 10 days, an additional 60 days of antifungal treatment were offered. An additional urine organic acid test was then performed.

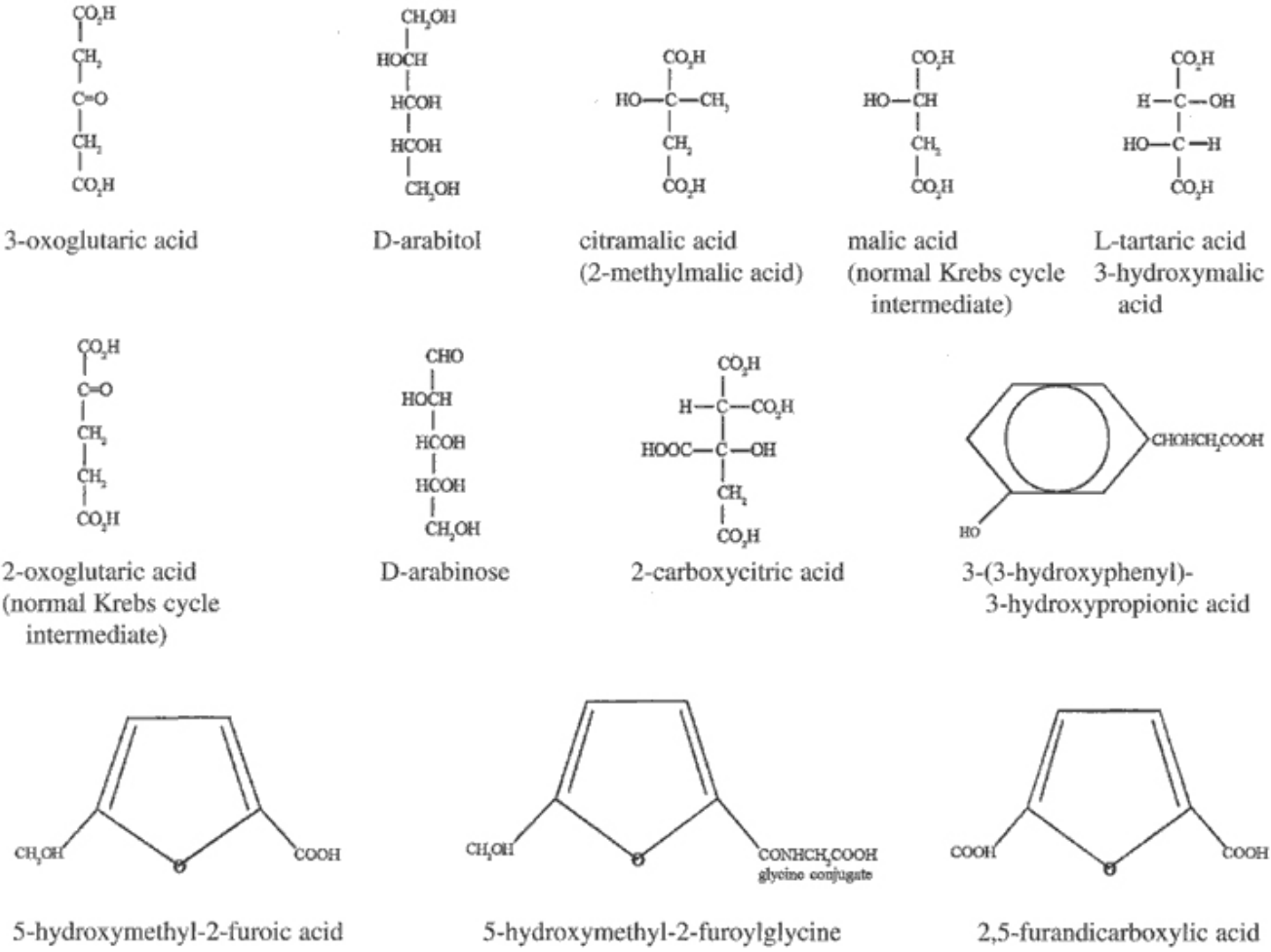
Results-The mean childhood autism rating scale, an indicator of the severity of autism, improved significantly after antifungal treatment based on the paired *t* test (P=.037). Seven of the urine markers were significantly higher in autistic males than in the control males. The concentration of several of the urine markers of autistic children decreased after antifungal treatment.

Conclusions-Antifungal drug therapy may be a promising therapeutic method for the treatment of autism. Organic acid testing appears to be a useful diagnostic test to indicate an overgrowth of yeast and bacteria in the gastrointestinal tract of children with autism and to predict the response of antifungal drug therapy, and may indicate relapse following the cessation of antifungal drug therapy. (*Clinical Practice of Alternative Medicine* 1(1): 15-26, 2000)

Our interest in a possible metabolic cause for autism was stimulated by the referral of 2 brothers with autism, on whom numerous urinary organic acid profiles were performed over a 2-year period.[1] These tests revealed a consistent excretion of a number of compounds of possible microbial origin identified as the carbohydrate arabinose, analogs of normal Krebs cycle intermediates including 3-oxoglutaric acid, tartaric (3-hydroxymalic) acid, citramalic (methylmalic) acid, and a new tentatively identified analog of citric acid (carboxycitric acid) not previously reported in the medical literature.[1] High concentrations of a compound identified as a phenylcarboxylic acid were also found; the mass spectrum of this compound did not correspond to the mass spectrum of any known compound in gas chromatography/mass spectrometry (GC/MS) libraries.[1] Hydroxymethylfurancarboxylic acid, furandicarboxylic acid, furancarbonylglycine, and 3-(3-hydroxyphenyl)-3-hydroxypropionic acid were also found in urine samples of the 2 autistic brothers. Elevations of the latter 4 compounds were not previously reported by Shaw et al[1]; each of these compounds was positively identified by comparison to mass spectra in GC/MS libraries, published spectra, or by mass spectra provided by other GC/MS laboratories. The structures of the above compounds and closely related compounds are shown in Figure 1.

These findings of chemicals of possible microbial origin are of particular interest because of a report that autistic children have a greater incidence of ear infections than peers of the same age; that lower functioning autistic children had an earlier onset of ear infections than their higher functioning autistic peers; and that the ears of children with autism were anatomically positioned differently than those of normal children, perhaps leading to greater ear infection susceptibility.[2] The use of oral antibiotics is the most prevalent therapy for ear infections in the US and many other nations. Furthermore, yeast and pathogenic bacterial overgrowth of the gastrointestinal (GI) tract commonly follows the use of oral antibiotics.[3-15] Therefore, compounds produced by antibiotic-resistant bacteria, yeast, or fungi in the GI tract and then absorbed into the bloodstream might be involved in the etiology of autism just as abnormal elevations of phenylalanine and its metabolites cause the disease phenylketonuria (PKU). The production of microbial compounds due to microbial overgrowth

FIGURE 1
Structures of putative microbial metabolites and human metabolites evaluated in this study



of the GI tract would be acquired rather than genetic, although genetic susceptibility could be important in both reduced immunity or reduced ability to detoxify microbial products.

A review of the medical records from a group of autistic patients at the outpatient clinic of the pediatric hospital where this study was performed revealed a high frequency of multiple ear infections or sore throats treated with multiple courses of broad-spectrum antibiotics in infancy. Thrush in infancy was also common. A 2-year old, developmentally normal child had a rapid onset of autistic symptoms that coincided with *Candidiasis* following multiple courses of antibiotic therapy for otitis media. This child excreted large quantities of tartaric acid, which decreased with nystatin therapy concomitant with improved clinical symptoms such as improved eye contact, decreased hyperactivity, and increased vocalization.

Based on these initial results, we decided to undertake a formal evaluation of the possible relationship between these elevated metabolites, possible yeast overgrowth, and autism. The objectives of the study were (1) to determine if the urinary excretion of abnormal Krebs cycle metabolites, abnormal carbohydrates, and other compounds of possible microbial origin are biochemical characteristics in autistic children, and (2) to determine if antifungal treatment results in decrease or elimination of abnormal metabolites in autistic children and/or improvement in autistic symptoms.

Methods and Materials

The proposed study comprised 23 autistic children, including the 2 siblings previously reported,[1] from the outpatient department of a pediatric hospital. The study was reviewed and approved by the Institutional Review Board. Each child was examined by a pediatric neurologist, and then classified as autistic according to the latest criteria proposed by the American Psychiatric Association's *Diagnostic Statistical Manual of Mental Disorders*.[16] After informed consent, a random urine sample was collected without special preparation for organic acid analysis by GC/MS for the presence of abnormal metabolites. A solvent extract of the urine sample was derivatized by trimethylsilylation and then analyzed with a GC/MS, as described by Shaw et al.[1] No analytical standards were available for the furan compounds, and quantification of these compounds was performed by assigning the response of an average size ion chromatogram peak as 100 units and then calibrating all other peaks of the same compounds against these arbitrary calibrators; each of these compounds was positively identified by comparison to mass spectra in a GC/MS library. For quantitative analysis of 3-(3-hydroxyphenyl)-3-hydroxypropionic acid, 3,4 -dihydroxyphenylpropionic acid was used as a surrogate calibration standard since 3-(3-hydroxyphenyl)-3-hydroxypropionic acid is not commercially available. The reconstructed ion chromatograph signal of the ion at mass to charge ratio 398 was used for quantitation. Similar use of surrogate standards is used extensively in the GC/MS analysis of environmental compounds in methods approved by the United States Environmental Protection Agency.

If the presence of abnormal metabolites was detected in the baseline urine sample (the sample collected before antifungal therapy), the child was offered treatment of suspected yeast infection with nystatin 100,000 units 4 times daily orally for 10 days. Then, another random urine sample was obtained and analyzed for organic acids. If the presence of abnormal metabolites was detected in the second urine sample, the child was offered a second course of treatment with nystatin for 2 months, and another urine sample was tested.

The patients' baseline urine values served as controls. Urine samples from normal children of hospital employees served as additional controls. All of the control children were in good health and had no significant diseases or developmental disorders. An assessment of the severity of autistic behaviors was done by both a staff psychologist working with parents and by teachers using the Childhood Autism Rating Scale (CARS) scale.[17] This assessment was done both at the beginning and at the end of nystatin therapy. The use of 2 evaluators was included to increase the reliability of this test. Furthermore, parents and teachers observed the children for extensive periods of time.

All 23 children were evaluated and their baseline urine was evaluated. One of the families moved shortly after the study began and was lost to follow-up. Thus, only 22 of 23 children completed the first course of nystatin therapy. Seven children did not complete the second course of nystatin for a variety of reasons. One of the children was dropped from the study after the first nystatin trial because the parent feared that the nystatin might exacerbate a preexisting colitis condition for which the child was hospitalized. One of the children refused to take the drug. In another case, the personnel at a child's school were uncooperative in administering the medication.

Statistical analysis of the data was done as follows: The concentrations of each organic acid or other compounds in the groups of male or female children with autism were compared to the control groups using *t* tests that assumed unequal variances. For comparison of patients following treatment with nystatin, or after completion of therapy, paired *t* tests were performed on each metabolite concentration so that the value of each metabolite in the treatment groups was paired with the same patient's baseline value. This statistical technique is sensitive in detecting small differences due to treatment.

Results

Characteristics of Patient and Control Populations

The patients who were initially recruited included 21 males and 2 females. None of these children had organic acid abnormalities characteristic of any of the well defined inborn errors of metabolism based on baseline organic acid testing. Patients were admitted into the study in the order in which the parents or guardians applied. The sex distribution of the controls included 17 females and 20 males. The mean age of the autistic children at the time of admission into the study was 7.1 years (SD=3.2), with a median age of 7.4 years and a range of 2.4 to 12.7 years. The mean age of the normal children at the time of urine collection was 7.7 years (SD=3.0), with a median age of 7.7 years and a range of 3.1 to 12.9 years. The mean age of the male autistic children at the time of admission into the study was 7.1 years (SD=3.3), with a median age of 7.4 years and a range of 2.4 to 12.7 years. The mean age of the male normal children at the time of urine collection was 8.1 years (SD=2.7), with a median age of 8.2 years and a range of 3.3 to 12.8 years. The mean age of the female autistic children at the time of admission into the study was 6.7 years (SD=2.1), with a median age of 6.7 years and a range of 5.2 to 8.2 years. The mean age of the female normal children at the time of urine

collection was 7.8 years (SD=3.3), with a median age of 7.3 years and a range of 3.3 to 12.9 years.

Differences in Concentrations of Metabolites in Normal and Autistic Children

Because of the high percentage of autistic males entered into the study (91.7%), comparisons of the urine metabolite concentrations in autistic children were made only to controls of the same sex. Comparison of the urine metabolite concentrations of autistic males and control males (Table 1) revealed that the mean values for all of the urine metabolite concentrations except carboxycitric acid were higher in the autistic group than in the control group. Eight of these 9 mean values of the autistic males were significantly higher than those of the control males ($P<.05$). The median values of the autistic males for 8 of the 10 metabolites were also higher in the autistic male group. The mean difference between the autistic and normal males was greater than 60% for all of the metabolites except 3-(3-hydroxyphenyl)-3-hydroxypropionic acid and carboxycitric acid. The mean arabinose concentration of the autistic males was 5 times greater than that of the control males, and the median value for the arabinose concentration was 6 times that of the control males. The median value for urinary tartaric acid concentration in the autistic males was over 6 times that of the control males, but the *t* test significance was borderline ($P=.097$). The limited data for the autistic females (n = 2) restricted any generalizations about this group compared to normal females.

Effect of Antifungal Therapy on Concentrations of Metabolites

First Course of Nystatin Therapy. Of the 23 autistic children admitted into the antifungal drug study, 22 completed nystatin therapy and had their urine retested following this therapy. Eight of the 10 mean values for the urine metabolite concentrations decreased from baseline values following the first 10 days of nystatin therapy (Table 2). The mean concentrations of 5-hydroxymethyl 2-furoic acid, furan-2,5-dicarboxylic acid, and arabinose decreased significantly ($P<.05$). The mean values for the urine metabolite concentrations of phenylcarboxylic acid and 3-(3-hydroxyphenyl)-3-hydroxypropionic acid increased from baseline values following nystatin therapy; the mean concentration of phenylcarboxylic acid increased by 61.9% ($P=.031$).

Second Course of Nystatin Therapy. Fifteen of the 23 subjects admitted into the antifungal drug study completed the second course of nystatin therapy (an additional 60 days), and their urine was retested following this therapy. Nine of the 10 mean values for the urine metabolite concentrations decreased from baseline values following the second course of nystatin therapy (Table 3). Baseline values in Table 3 are different from those in Table 2 because only baseline values for those completing the second course of nystatin were included in Table 3. The mean concentrations of

TABLE 1
Compounds in urine samples of male children with autism and normal male children

	Normal Males n=20			Autistic Males n=21				Autism Mean as Multiple of Normal
Compounds	Mean	Median	SD	Mean	SD	Median	*t* test	Mean
Citramalic*	1.10	0.80	0.80	3.84	5.27	1.70	0.011	3.491
5-hydroxymethyl-2-furoic†	49.50	50.50	32.00	135.26	177.57	58.00	0.013	2.733
3-oxoglutaric*	0.00	0.00	0.00	0.66	1.24	0.00	0.015	NA
Furan-2,5-dicarboxylic†	28.00	27.10	18.00	51.28	73.44	23.00	0.038	1.831
Tartaric*	9.60	24.30	2.50	24.36	69.62	3.70	0.097	2.538
Furancarbonylglycine†	27.70	33.20	15.00	59.85	77.85	39.00	0.012	2.161
Arabinose*	85.80	85.40	50.00	377.66	464.46	271.00	0.004	4.402
3-(3-hydroxyphenyl)-3-hydroxypropionic*	106.60	81.60	75.00	141.48	150.90	99.00	0.038	1.327
Carboxycitric†	48.30	45.20	37.00	29.36	63.10	9.80	0.441	0.608
Phenylcarboxylic†	12.20	16.70	3.50	28.39	36.72	8.70	0.012	2.327

*Unit of measure is mmol/mol creatinine.
†Unit of measure is U/mol creatinine.

TABLE 2
Effect of 10 days of nystatin therapy on urine concentrations of metabolites in children with autism

Compounds	Baseline			After First Nystatin Therapy				
	Mean	SD	Median	Mean	SD	Median	*t* test	Mean % Decrease
Citramalic*	3.61	5.08	1.60	2.82	3.99	1.45	0.218	21.88
5-hydroxymethyl-2-furoic†	130.15	170.28	58.00	56.33	129.90	17.75	0.037	56.72
3-oxoglutaric*	0.64	1.19	0.00	0.35	1.14	0.00	0.051	45.31
Furan-2,5-dicarboxylic†	48.82	70.50	23.00	17.99	19.86	10.50	0.017	63.15
Tartaric*	23.20	66.54	3.70	16.50	54.77	1.80	0.069	28.88
Furancarbonylglycine†	59.25	74.34	41.00	44.65	84.03	11.50	0.233	24.64
Arabinose*	356.86	448.18	257.00	183.00	136.91	146.50	0.034	48.72
3-(3-hydroxyphenyl)-3-hydroxypropionic*	137.53	145.88	99.00	161.02	139.89	138.50	0.324	-17.08
Carboxycitric†	30.72	60.45	11.87	18.32	26.46	7.15	0.229	40.36
Phenylcarboxylic†	28.36	35.17	9.30	45.90	57.61	13.50	0.031	-61.85

*Unit of measure is mmol/mol creatinine.
†Unit of measure is U/mol creatinine.

5-hydroxymethyl-2-furoic acid and furan-2,5-dicarboxylic acid decreased significantly ($P<.05$), but the significance for the arabinose decrease was $P=.085$. The mean value for the urine concentration of the phenylcarboxylic acid compound increased 74.4 % ($P=.117$) from baseline values following the second course of nystatin therapy.

TABLE 3
Effect of 70 days of Nystatin therapy on urine metabolite concentrations of children with autism

Compounds	Baseline			After First Nystatin Therapy				
	Mean	SD	Median	Mean	SD	Median	*t* test	Mean % Decrease
Citramalic*	4.50	6.15	1.60	2.68	2.29	1.70	0.119	40.44
5-hydroxymethyl-2-furoic†	113.20	114.68	58.00	52.36	75.83	23.50	0.019	53.75
3-oxoglutaric*	0.90	1.38	0.00	0.85	2.33	0.00	0.477	5.56
Furan-2,5-dicarboxylic†	52.60	66.19	26.00	23.48	29.93	14.00	0.049	55.36
Tartaric*	34.20	81.13	5.80	4.99	11.45	1.20	0.064	85.41
Furancarbonylglycine†	79.20	85.05	58.00	50.22	73.02	21.00	0.148	36.59
Arabinose*	394.50	528.11	271.00	184.88	185.80	130.00	0.085	53.14
3-(3-hydroxyphenyl)-3-hydroxypropionic*	182.30	161.88	133.00	164.94	130.64	140.00	0.443	9.52
Carboxycitric†	40.40	72.31	23.00	16.29	27.24	2.60	0.137	59.68
Phenylcarboxylic†	26.30	33.02	17.00	45.86	84.17	8.30	0.117	-74.37

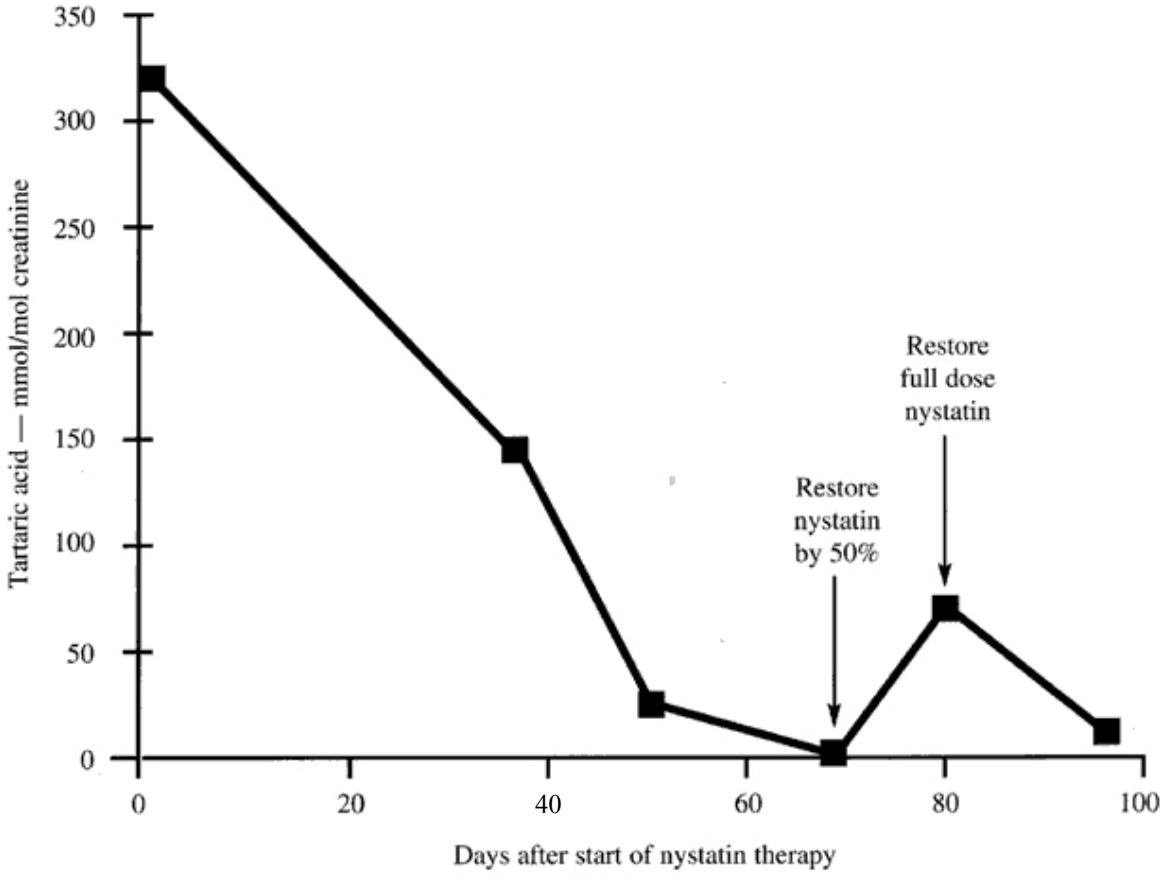
*Unit of measure is mmol/mol creatinine.
†Unit of measure is U/mol creatinine.

The response to nystatin is well-illustrated (Figure 2) in a child that had the highest baseline excretion of tartaric acid (300 mmol/mol creatinine), an abnormal value that was about 20 times the median normal value. Following the treatment with the nystatin, the tartaric acid continued to decrease. After 68 days, the parent began administering only 50% of the dose to avoid running out of nystatin completely. During that time, the tartaric acid began to increase. When the nystatin prescription was refilled and full doses of nystatin were administered, the tartaric acid again decreased.

CARS Evaluations. The CARS evaluations were completed for 18 of the children, 16 males and 2 females. The key to the CARS score scale is as follows: 15 to 30.0, normal; 30.1 to 37.0, mild to moderate autism; 37.1 to 60, severe autism. The baseline mean and median scores were 39.07 and 38.00, respectively, indicating that the group as a whole was in the severe autism range. The range in baseline scores is 30.5 to 53.5.

Eleven of the children were scored in the severe autism range, whereas 7 were scored in the mild to moderate range. Nine of the 18 children were reevaluated after the completion of nystatin therapy; the mean and median scores were 34.57 and 32.50, respectively. The mean decrease in the CARS score was statistically significant (P=.037) using the paired t test. Improvements in the clinical symptoms of autism that were cited by the parents and teachers of the autistic children included decreased hyperactivity, increased eye contact and vocalization, better sleep patterns and concentration, increased imaginative play, reduced stereotypical behaviors such as spinning objects, and better academic performance. Several parents reported a loss of gains made by their child during antifungal drug therapy when the antifungal drug therapy was stopped and elected to reinstate the antifungal drug therapy. Some of the children have stayed on this therapy for more than 2 years and the parents report continued benefits to their child.

FIGURE 2
Concentration of urinary tartaric acid following initiation of antifungal therapy in a child with autism



Discussion

Based on the reduction of the concentration of these compounds in urine after antifungal therapy, it seems likely that the furan compounds and arabinose are yeast or fungal metabolites and that tartaric, citramalic, and carboxycitric acids are metabolites of yeast or fungi.

Two of the compounds, 3-(3-hydroxyphenyl)-3-hydroxypropionic acid and the partially identified phenylcarboxylic acid that was commonly elevated in the urine of children with autism, were initially suspected of being yeast metabolites. However, these 2 compounds increased in the urine samples of children with autism after antifungal drug therapy. Later research established that these compounds were phenylalanine metabolites of several clostridia species (W. Shaw, unpublished data, 1999), which may also proliferate in the GI tract after most of the common broad-spectrum oral antibiotics.[11-15] Berg[13] reported that oral antibiotic treatment disrupts the normal ecology of the GI tract, allowing certain antibiotic-resistant indigenous bacteria to overpopulate while there is as much as a thousand fold decrease in the total number of anaerobic bacteria; following this overpopulation, these bacteria translocate from the GI tract to the mesenteric lymph nodes and possibly to other organs. Organisms of the *Clostridium* family, such as *C botulinum, C. tetani,* and *C perfringens,* are resistant to most of the broad-spectrum antibiotics commonly used to treat otitis media.

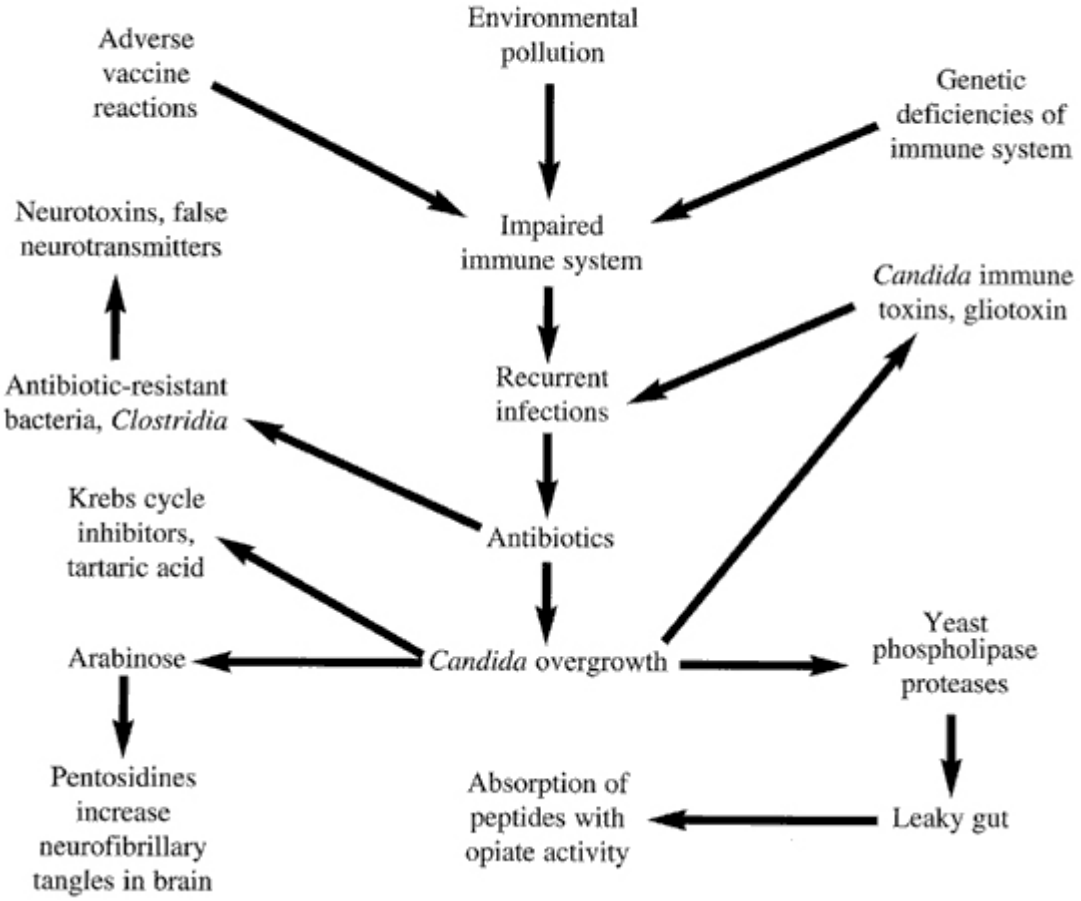
Yeast cultures on blood were not included on the protocol because none of these children had symptoms that are associated with systemic yeast infection, such as fever. Although stool testing for yeast was not performed on this study group, results of stool yeast enumeration and putative yeast metabolites in urine have been compared in a substantial number of children with autism since this study; a general correspondence has been found in the 2 methods, indicating that the increased metabolites are likely to be derived from a GI yeast over growth. Both elevated arabinose and tartaric acid in the urine are associated with increased numbers of multiple species of *Candida* in stool samples.

The metabolites evaluated in this study are not specific for autism. Elevations of both the putative yeast and clostridia metabolites have been found to be common in the urine samples of many patients with many neuropsychiatric diseases, including pervasive developmental delay, child and adult psychosis, depression, Rett syndrome, seizure disorders, attention deficit hyperactivity, and Alzheimer's disease. The findings in this study may open the investigation of the immune system and metabolites from the GI flora in these neuropsychiatric diseases as well. The putative yeast and clostridia metabolites were not elevated in urine samples of children with Down's syndrome, but were elevated in urine samples of children with Down's syndrome who also have autism.

The furan compounds are known fungal metabolites, [18-20] but the origin of these compounds in urine is controversial.[21-24] Speculation includes the possibility that they are artifacts derived from certain sugars, compounds formed from bakery products, and food precursors. Furan fatty acids have been detected in almost all living creatures including fish, crayfish, amphibians, reptiles, and mammals (including man), as well as in plants.[23] The reduction of these compounds after nystatin therapy in this study seems consistent with a significant fungal origin for at least a portion of these compounds in urine. A large number of studies have indicated a wide number of abnormalities of the immune system in autism,[25-29] including IgG deficiency, IgA deficiency, IgG subclass deficiency, myeloperoxidase deficiency (a genetic defect in an enzyme of the leukocytes that produces hypochlorite ions to kill yeast), and a deficiency in complement C4b. The 2 brothers with autism in which these metabolites were first reported[1] both had abnormally low concentrations of serum IgG. Autism has also been diagnosed in other children with defined inborn errors of metabolism, such as biotinidase deficiency and isovaleric acidemia, in which yeast infections are common (J. Lombard, oral and written communication, 1997). Furthermore, the success of Gupta et al[25] in treating the symptoms of autistic children with gamma globulin therapy indicates an immune abnormality in autism.

Based on these findings and the findings of abnormal organic acids in this study, we propose a model for autism (Figure 3). According to this model, immune deficiencies, which may be genetic or acquired, lead to an increased frequency of infections that are almost always treated with broad-spectrum oral antibiotics. As a result of these antibiotics, a proliferation of yeasts and bacteria such as *Clostridium* occur in the GI tract; these organisms may eventually colonize the regional lymph nodes surrounding the GI tract. Furthermore, many isolates of *Candida albicans* produce gliotoxins[30,31] and other immunotoxins[32,33] that impair the immune system and increase the likelihood of additional infections, which lead to additional antibiotic usage and greater proliferation of yeasts and antibiotic resistant bacteria, setting up a vicious cycle. These organisms produce high amounts of abnormal carbohydrates, such as arabinose; Krebs cycle analogs, such as tartaric and citramalic acids; and compounds derived from phenylalanine (3-(3-hydroxyphenyl-3-hydroxypropionic acid) that are biochemically similar to the phenylalanine-and tyrosine-derived neurotransmitters. These compounds are absorbed into the bloodstream and may alter behavior; they may also produce neurotoxins in both the GI tract and in the regional lymph nodes that are absorbed into the

FIGURE 3
Biochemical model for autism based on current research findings



circulation, also affecting behavior. If this model is correct, efforts to locate a single autism gene would fail since any genetic factor that severely impairs the immune system may eventually lead to the proliferation of antibiotic-resistant yeasts and bacteria, which then alter behavior through the excretion of their products. A portion of children with autism do not have a history of frequent infections. These children might have been exposed to yeast infection prenatally or developed abnormal microbial flora because of immune deficiencies rather than from repeated antibiotic therapy. This model is not inconsistent with other research findings in autism such as abnormal neuroanatomical findings, abnormal electroencephalogram results, and abnormal brain scans.

Similar abnormalities were found in PKU, even though the primary abnormality is a genetic defect in a single enzymatic reaction. There is no inherent reason that dramatic biochemical changes in multiple biochemical systems caused by microorganisms would not be expected to alter brain structure and function. In PKU, correction of the metabolic defect by restriction of phenylalanine during infancy allows for normal development; retardation occurs if dietary intervention occurs too late. If abnormally elevated metabolites cause autism, it is reasonable that elevations of these compounds would have maximum negative impact during periods of critical brain growth and development. As in PKU, metabolic intervention in autism might only be possible in the early stages of the disorder before the brain has matured. The differences in severity of disease and individual differences in symptoms might be due to different combinations of metabolites, how elevated they are, the duration of the elevation, the age at which the metabolites become abnormally elevated, and the susceptibility of the individual developing nervous system to the different microbial metabolites. Indeed, these differences may even determine which disease is manifested. Furthermore, the concentration of these microbial products is not trace amounts on the metabolic scale.

One nonstudy child with autism evaluated in our laboratory had a urine tartaric acid concentration (6000 mmol/mol creatinine) that was nearly 400 times the upper limit of normal after the use of multiple oral

antibiotics. The value normalized after several weeks of antifungal therapy. Many of the concentrations of the microbial compounds reported here may exceed even the concentrations of the predominant mammalian organic acids in urine. Furthermore, many other metabolic products of microbial origin such as amino acids and lipids were not measured with the present organic acid test and should also be evaluated. It is likely that these abnormalities, some of which have been known for decades, have been ignored for so long by almost all of the researchers in the field of metabolic diseases because of the intense focus on finding new inborn errors of metabolism. By definition, abnormal microbial products are not due to a genetic defect in a human biochemical pathway. However, most researchers in the field of metabolic disorders also make the unwarranted assumption that microbial metabolites are metabolically and physiologically inert. Instead, the human and the microorganisms in the GI tract are an integrated biochemical system, the functions of which are interdependent.

The exact biochemical role of arabinose is unknown, but a closely related yeast alcohol arabitol has been used as a biochemical indicator of invasive *Candidiasis*.[34-36] Elevated arabitol was not found in any of the urine samples tested in this study and arabinose was not found in the culture media of multiple isolates of *Candida albicans* isolated from stool samples of autistic children (W. Shaw, unpublished data, 1995). Arabitol produced by yeast in the intestinal tract may be absorbed into the portal circulation and then converted to arabinose by the liver. A nonstudy autistic child with the highest level of urine arabinose (over 40 times the upper limit of normal) had chronic hypoglycemia (blood glucose 20-50 mg/dL) for nearly 2 years. This child's urine arabinose remained extremely elevated in repeated testing over the same period. The blood sugar of the child returned to normal for the first time in 2 years after 1 week of nystatin therapy concomitant with a marked decrease in urine arabinose, indicating a possible inhibition of gluconeogenesis by the arabinose. Similar hypoglycemia occurs in inborn errors of fructose metabolism, in which fructose inhibits gluconeogenesis,[37] and it is possible that children with autism might be deficient in one or more enzymes involved in the metabolism of pentoses. Elevated protein-bound arabinose has been found in the serum glycoproteins of schizophrenics,[38] and in children with conduct disorders[39] and alteration of protein function by arabinose might be another mechanism by which arabinose might affect biochemical processes.

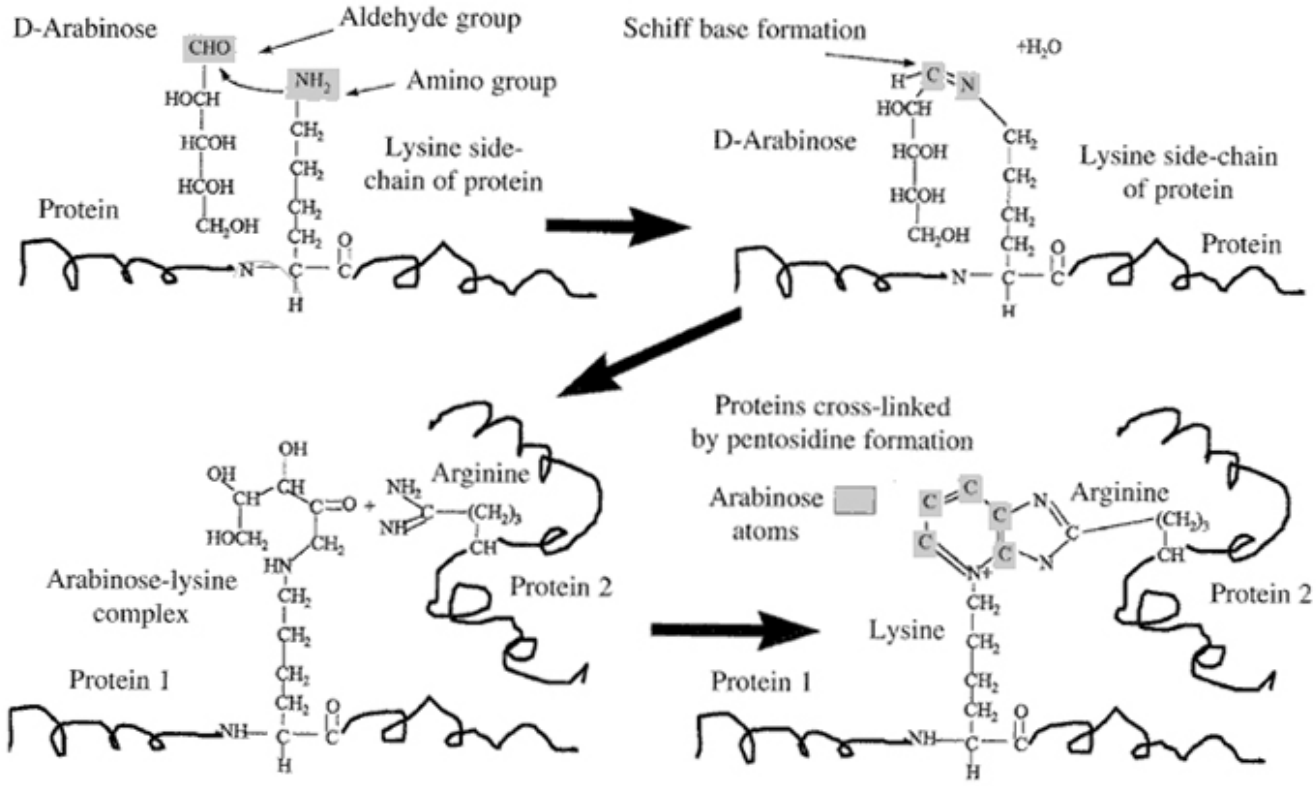
Arabinose, a sugar aldehyde or aldose, reacts with the epsilon amino group of lysine in a wide variety of proteins and may then form cross-links with arginine residues in an adjoining protein,[40] thereby cross-linking the proteins and altering both biological structures and functions of a wide variety of proteins (Figure 4). This adduct of arabinose, lysine, and arginine is called a pentosidine (Figure 4). The epsilon amino group of lysine is a critical functional group of many enzymes to which pyridoxal (vitamin B6), biotin, and lipoic acid are covalently bonded during coenzymatic reactions[41]; the blockage of these active lysine sites by pentosidine formation may cause functional vitamin deficiencies even when nutritional intake is adequate. In addition, this epsilon amino group of lysine may also be important in the active catalytic site of many enzymes. Protein modification caused by pentosidine formation is associated with crosslink formation, decreased protein solubility, and increased protease resistance. The characteristic pathological structures, called neurofibrillary tangles associated with Alzheimer's disease contain modifications typical of pentosidine formation. Specifically, antibodies against pentosidine react strongly to neurofibrillary tangles and senile plaques in brain tissue from patients with Alzheimer's disease.[42] In contrast, little or no reaction is observed in apparently healthy neurons of the same brain. Thus, it appears that the neurofibrillary tangles of Alzheimer's disease may be caused by the pentosidines. The modification of protein structure and function caused by arabinose could account for the biochemical and insolubility properties of the lesions of Alzheimer's disease through the formation of protein cross-links. Similar damage to the brains of autistic children might also be due to the pentosidines; neurofibrillary tangles have been reported in the brain tissue of an individual with autism.[43] Since vitamin B_6 reacts with the same critical epsilon amino group of lysine, the beneficial effects of vitamin B_6 in autism reported in multiple studies[44] may be mediated by prevention of further pentosidine formation. Analysis of brain tissue of people with autism for increased brain pentosidines could be invaluable in the confirmation of this hypothesis.

Horowitz et al[45] found that women with vulvovaginitis due to *Candida* had elevated arabinose in the urine; restriction of dietary sugar brought about a dramatic reduction in the incidence and severity of the vulvovaginitis. Thus, one of the mechanisms of action of antifungal drug therapy for autism might be to reduce the concentration of an abnormal carbohydrate produced by the yeast that can not be tolerated by the child with defective pentose metabolism. Arabinose tolerance tests should be used to determine rapidly if such biochemical defects are present in children with autism.

Some of the other metabolites may also be due to a new inborn error of mammalian metabolism and/or a reduced ability to detoxify the microbial compounds. An analog of the Krebs cycle intermediate 2-oxoglutaric acid, 3-oxoglutaric acid, was first reported

by Shaw et al[1] as elevated in 2 brothers with autism,

FIGURE 4
Conversion of the pentose sugar arabinose to a pentosidine



Pentosidine is a compound linking 2 different proteins through arginine and lysine side chains. Pentosidine formation in the brain appears to be the cause of neurofibrillary tangles in the brains of patients with Alzheimer's disease.

but was not previously known. This compound is not unique to autism, and in this study, it was found to be elevated in urine samples of several nonautistic children with seizure disorders, A child with seizures but not autism had the highest concentration of 3-oxoglutaric acid in a urine sample (40 mmol/mol creatinine), and had severe brain-demyelinating disease as judged by magnetic resonance imaging. This compound was elevated (concentration > 0.5 mmol/mol creatinine) in 5 of 23 (21.7%) of the children with autism in this study, and it was elevated in 25.6 % of children with autism (n=255) evaluated a laboratory for inborn errors of metabolism (W. Shaw, unpublished data, 1996), The highest value for a child with autism measured in this laboratory is a value of 30 mmol/mol creatinine. A closely related compound, 3-hydroxyglutaric acid, a metabolite of the amino acids tryptophan, lysine, and hydroxylysine, is elevated in the genetic disease glutaric acidemia type I,[46] a neurological genetic disease due to a deficiency of glutaryl-CoA dehydrogenase, Abnormal elevations of the plasma l amino acids tryptophan and lysine have been reported in children with autism,[47-50] The conversion of 3-hydroxyglutaryl CoA to 3-oxoglutaric would presumably be catalyzed by a 3-hydroxyacyl CoA dehydrogenase, one of the enzymes present on both a peroxisomal enzyme complex, as well as in the mitochondrion, which functions in the beta-oxidation of fatty acids.[51] In other peroxisomal diseases, there is frequently a late onset of symptoms similar to many cases of autism, in which there is frequently developmental losses between 18 to 24 months of age. In the peroxisomal disorders, there is an accumulation of long-chain fatty acids.

Autism is not the only developmental disorder associated with increased incidence and severity of otitis media, A number of studies have also indicated that increased incidence of attention deficit hyperactivity is associated with increased incidence of otitis media.[52-56]

Conclusions

Antifungal drug therapy may be a promising therapeutic method for the treatment of autism; however, a double-blind placebo study will be required to prove the effectiveness of this therapy unequivocally.

Nystatin is not absorbed into the bloodstream at the doses employed in this study. If yeast or fungi are the source of abnormal metabolites, the significant reduction of some of these metabolites after nystatin therapy appears to indicate fungal overgrowth of the

GI tract as the primary site of yeast or fungal colonization.

Organic acid testing appears to be a useful diagnostic test to indicate an overgrowth of yeast and bacteria in the GI tract of children with autism, to predict the response to antifungal drug therapy, and to indicate relapse following the cessation of antifungal drug therapy.

We are presently involved in evaluating the species of clostridia in stool samples of autistic children and in determining which species produce the 3-(3-hydroxyphenyl)-3-hydroxypropionic acid.

Much additional research is needed to determine which species and/or subtypes of yeast or fungi and bacteria produce the compounds that are elevated in autism, to determine which compounds may be most important in the etiology of autism and how they are metabolized, to determine whether potential adverse effects caused by these compounds can be reversed by other therapies, to determine how antifungal drug and immunological therapy can be modified or combined to produce therapeutic results that have a greater therapeutic duration, and to determine if dietary measures can be used to potentiate the effects of antifungal drug therapy.

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