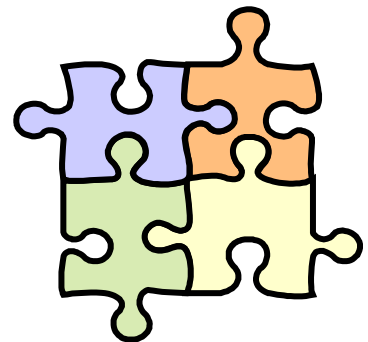
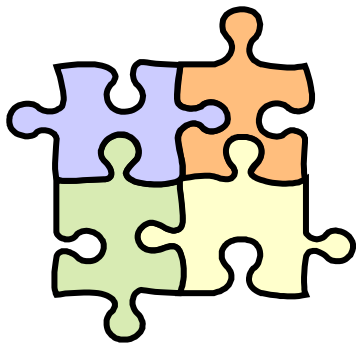


# Pathologic Implications of Low Glutathione Levels And Oxidative Stress in Children with Autism: Metabolic Biomarkers and Genetic Polymorphisms

**S. Jill James, Ph.D.**

Department of Pediatrics  
Arkansas Children's Hospital Research Institute  
University of Arkansas for Medical Sciences  
Little Rock, AR



# Overview

**Pathways of Folate/Methionine/Glutathione metabolism;  
Impact of Oxidative Stress**

**Mechanisms and Functions of Glutathione**

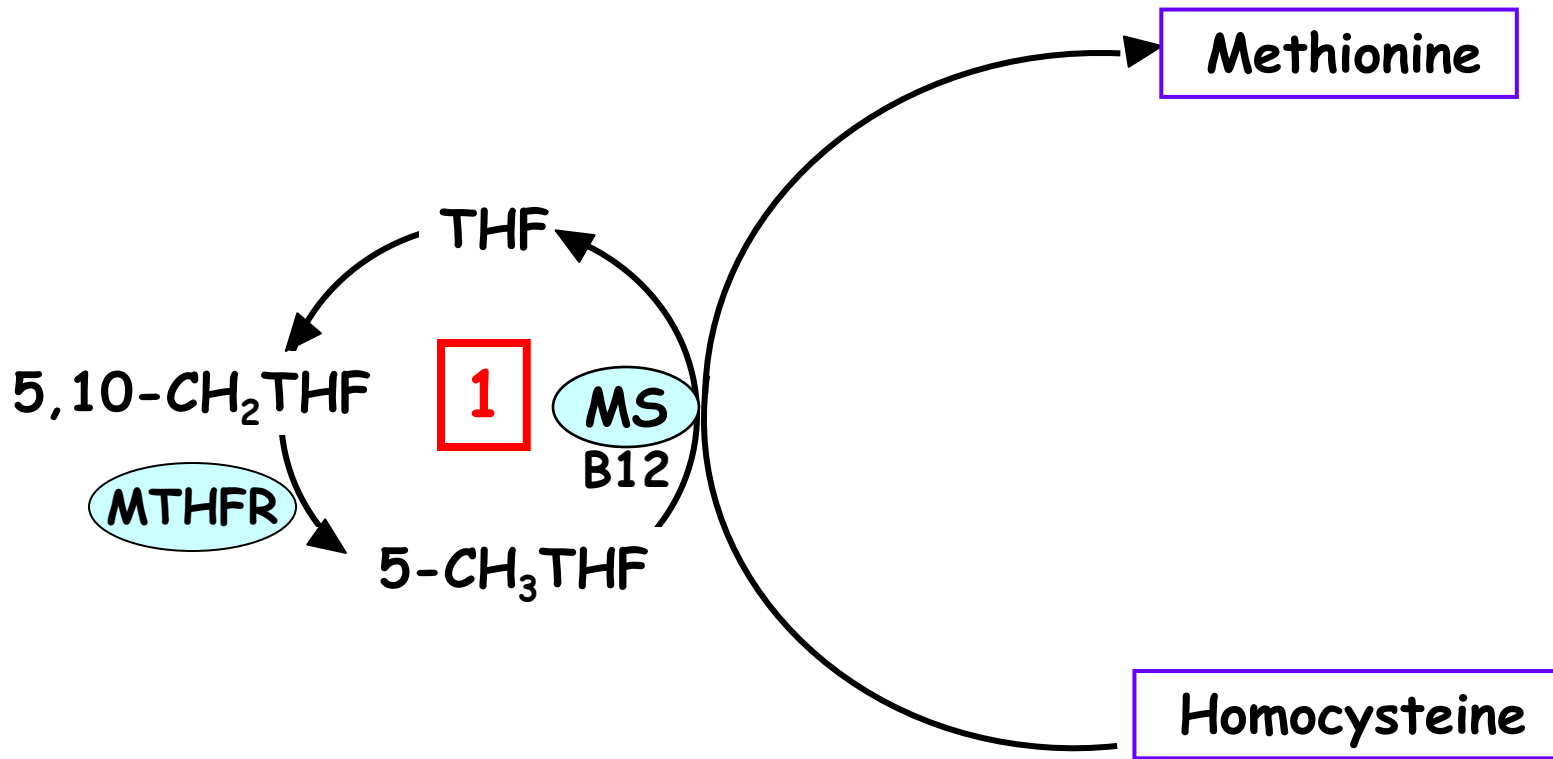
**Depletion of glutathione with Thimerosal in vitro**

**Abnormal Methylation and Oxidative Stress in Autistic  
Children: Treatment with Methyl B-12, Folinic Acid, and TMG**

**Selected Genetic Polymorphisms Associated with the Abnormal  
Metabolic Profile in Autism**

**Implications of Oxidative Stress in the Pathogenesis of Autism**

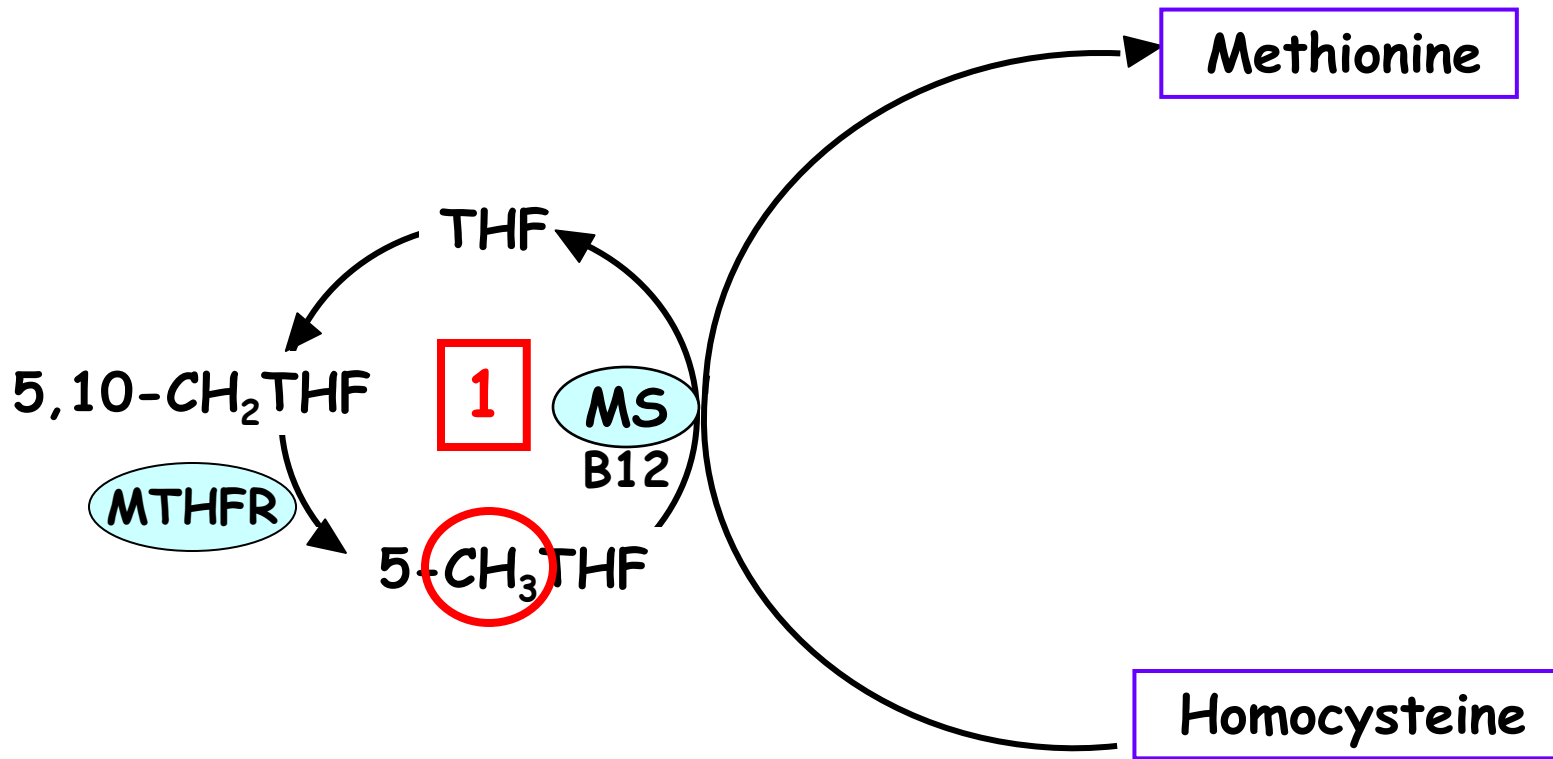
# Methionine Transsulfuration to Cysteine and Glutathione



THF: tetrahydrofolate

Enzymes

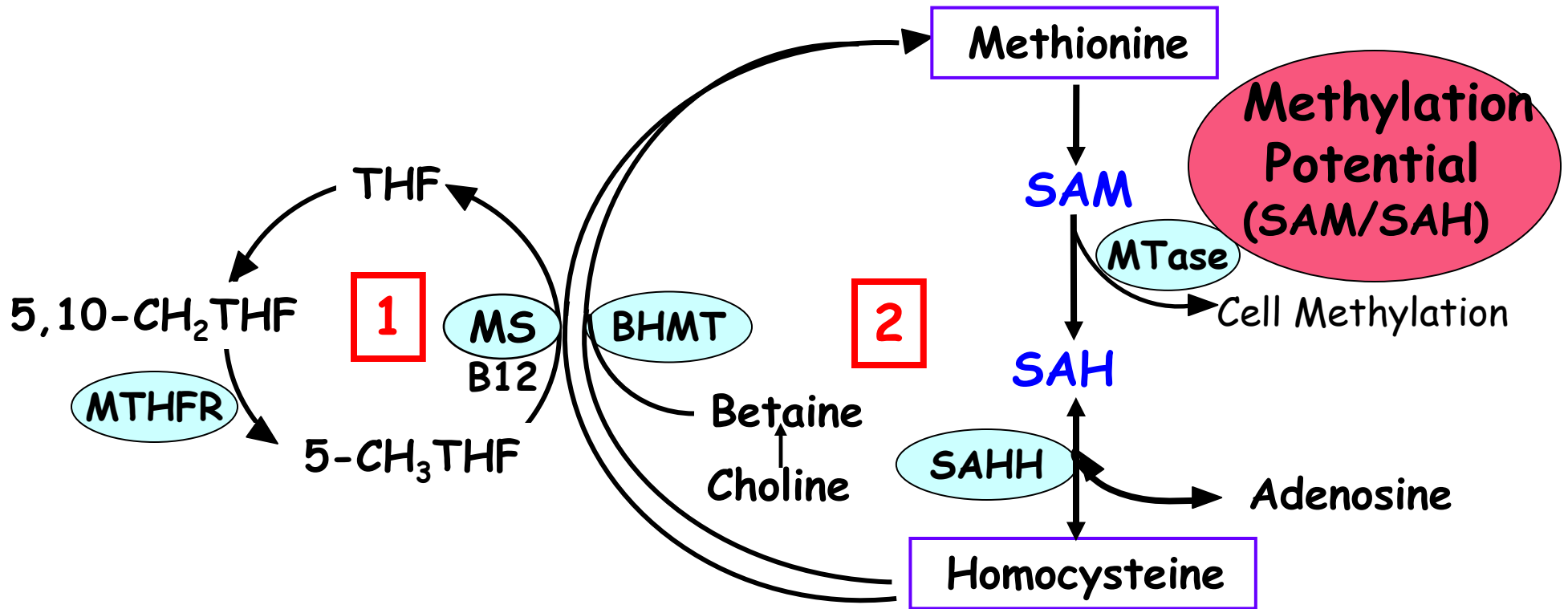
# Methionine Transsulfuration to Cysteine and Glutathione



THF: tetrahydrofolate

Enzymes

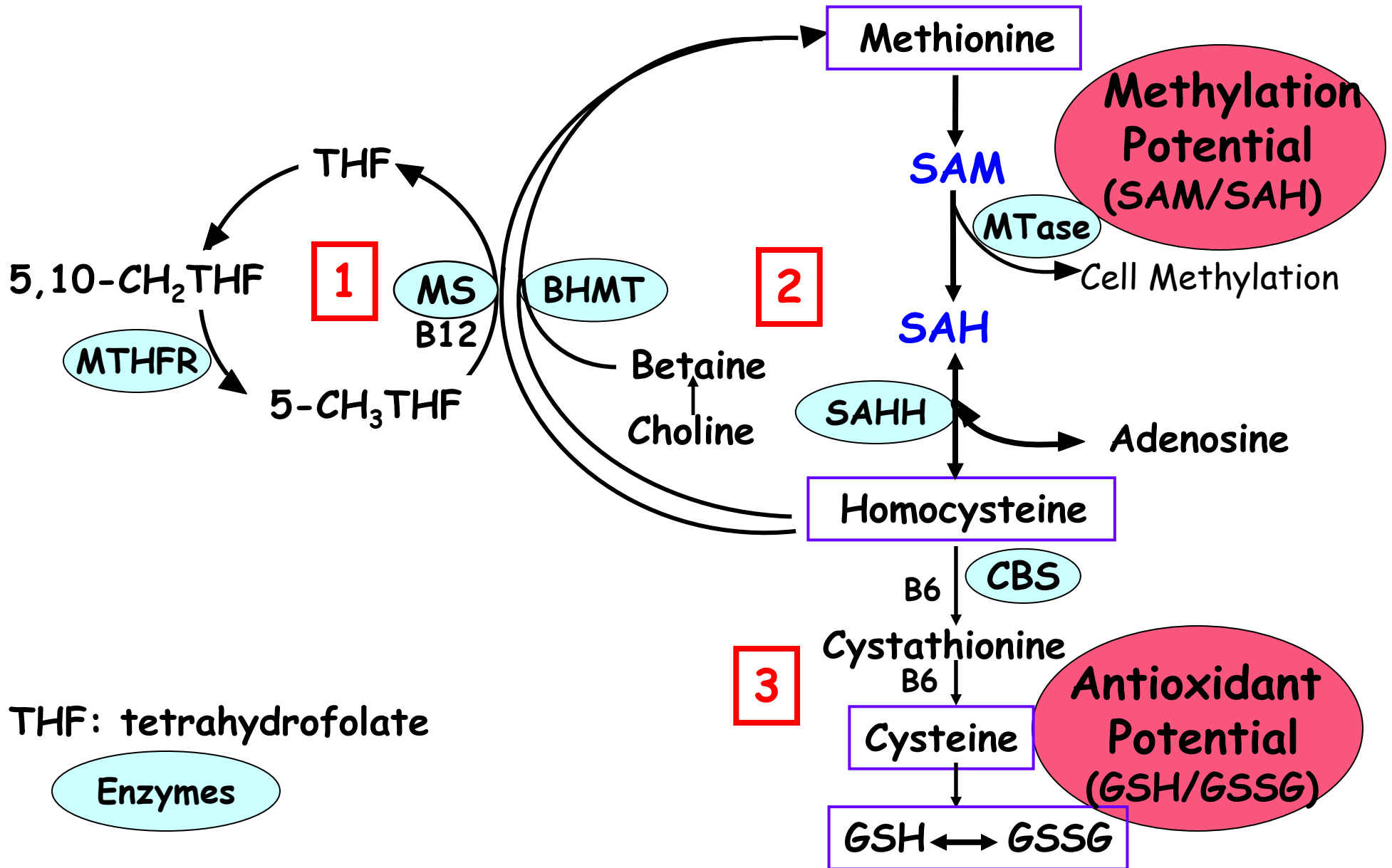
# Methionine Transsulfuration to Cysteine and Glutathione



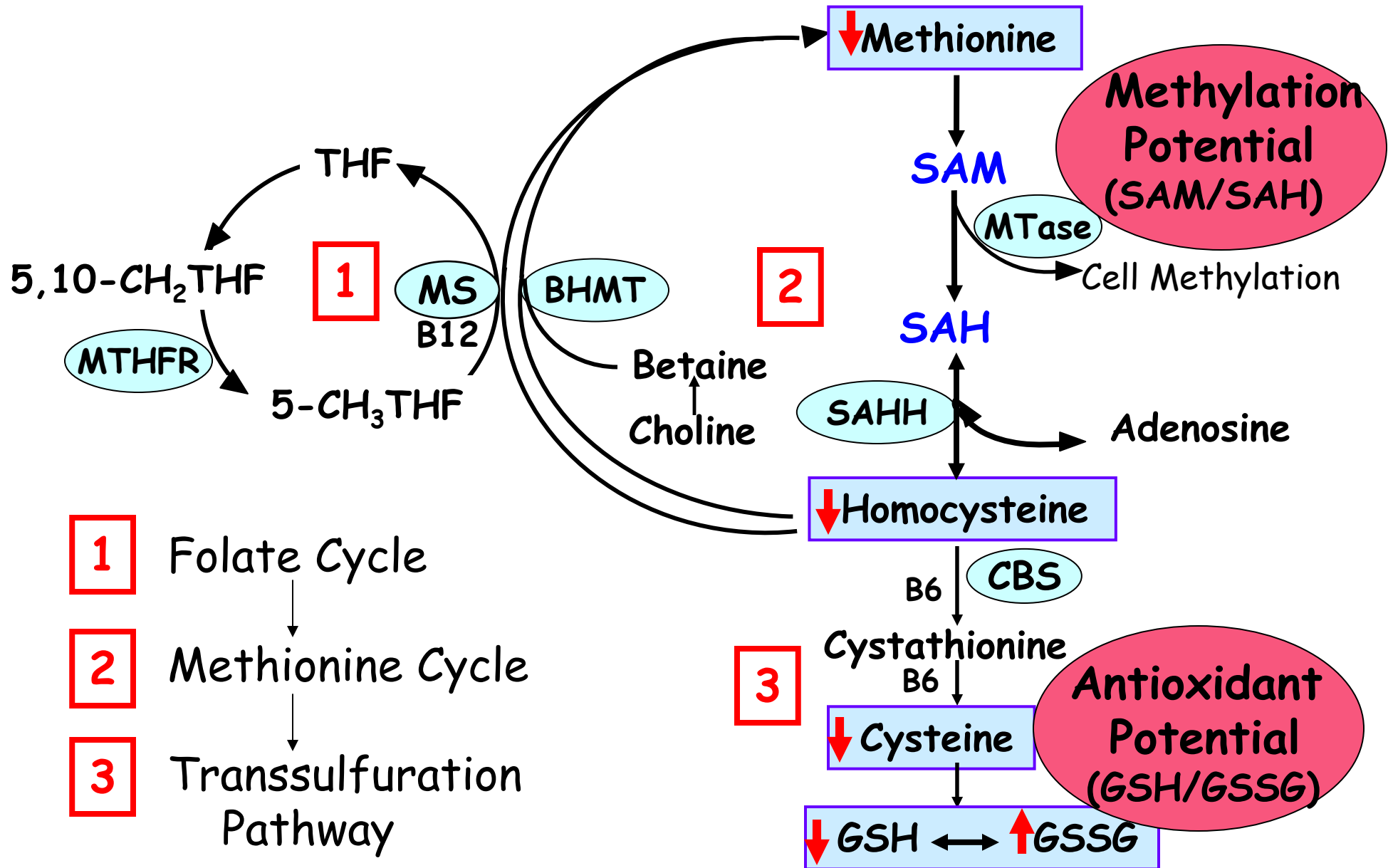
THF: tetrahydrofolate

Enzymes

# Methionine Transsulfuration to Cysteine and Glutathione



# Methionine Transsulfuration to Cysteine and Glutathione



## RELEVANT BACKGROUND INFORMATION

**Methionine is an essential AA:** Methionine Cycle conserves methionine

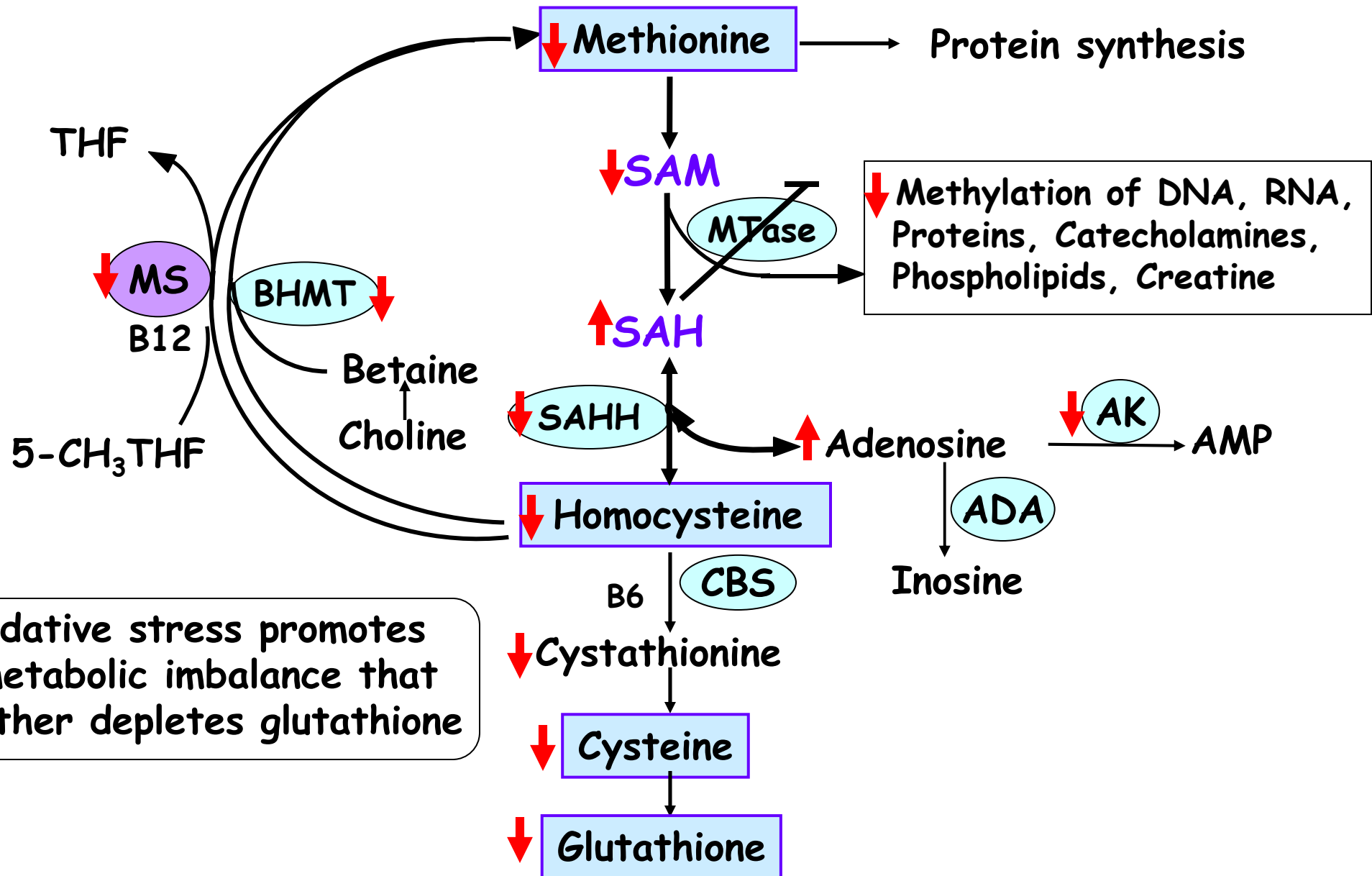
**Cysteine is a “conditionally” essential AA:** >50% of cysteine is derived from methionine via the transsulfuration pathway; normal cysteine levels depend on normal methionine levels.

**Cysteine may be an essential amino acid in children with autism!**

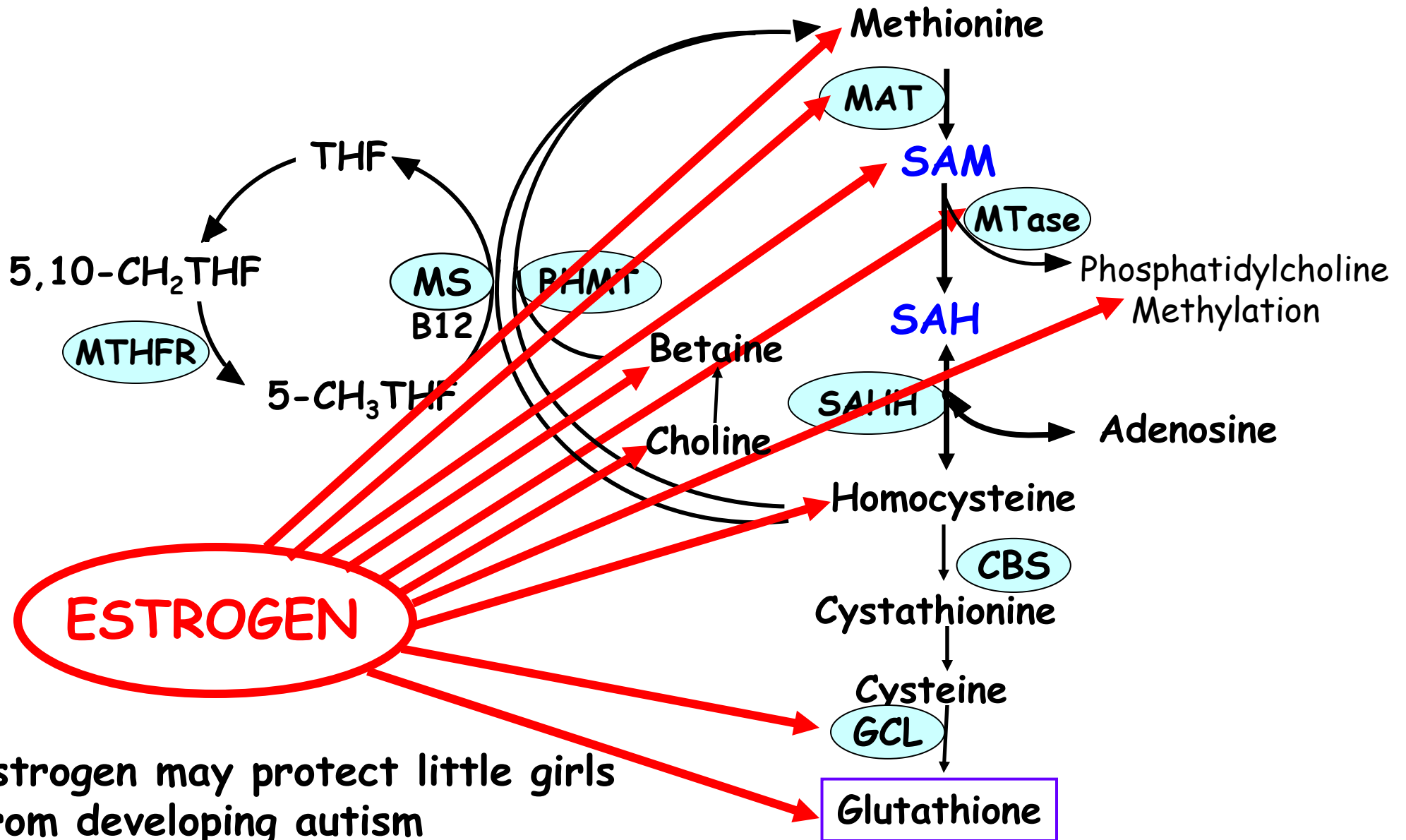
**Cysteine is the rate-limiting amino acid for glutathione (Glu-Gly-Cys) synthesis:** the thiol (-SH) group of cysteine is the active antioxidant component of glutathione.

# How Does Oxidative Stress Influence Methionine Metabolism?

# Impact of Oxidative Stress on Methionine Transsulfuration

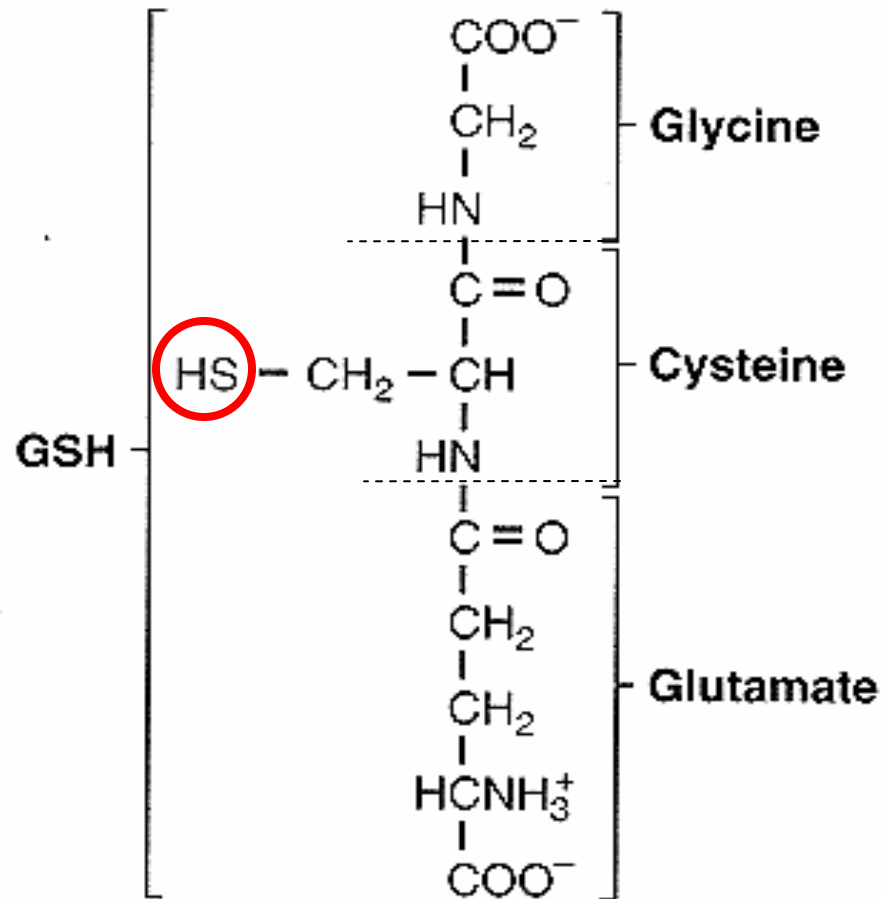


# ESTROGEN: THE FEMALE ADVANTAGE IN THIOL CHEMISTRY



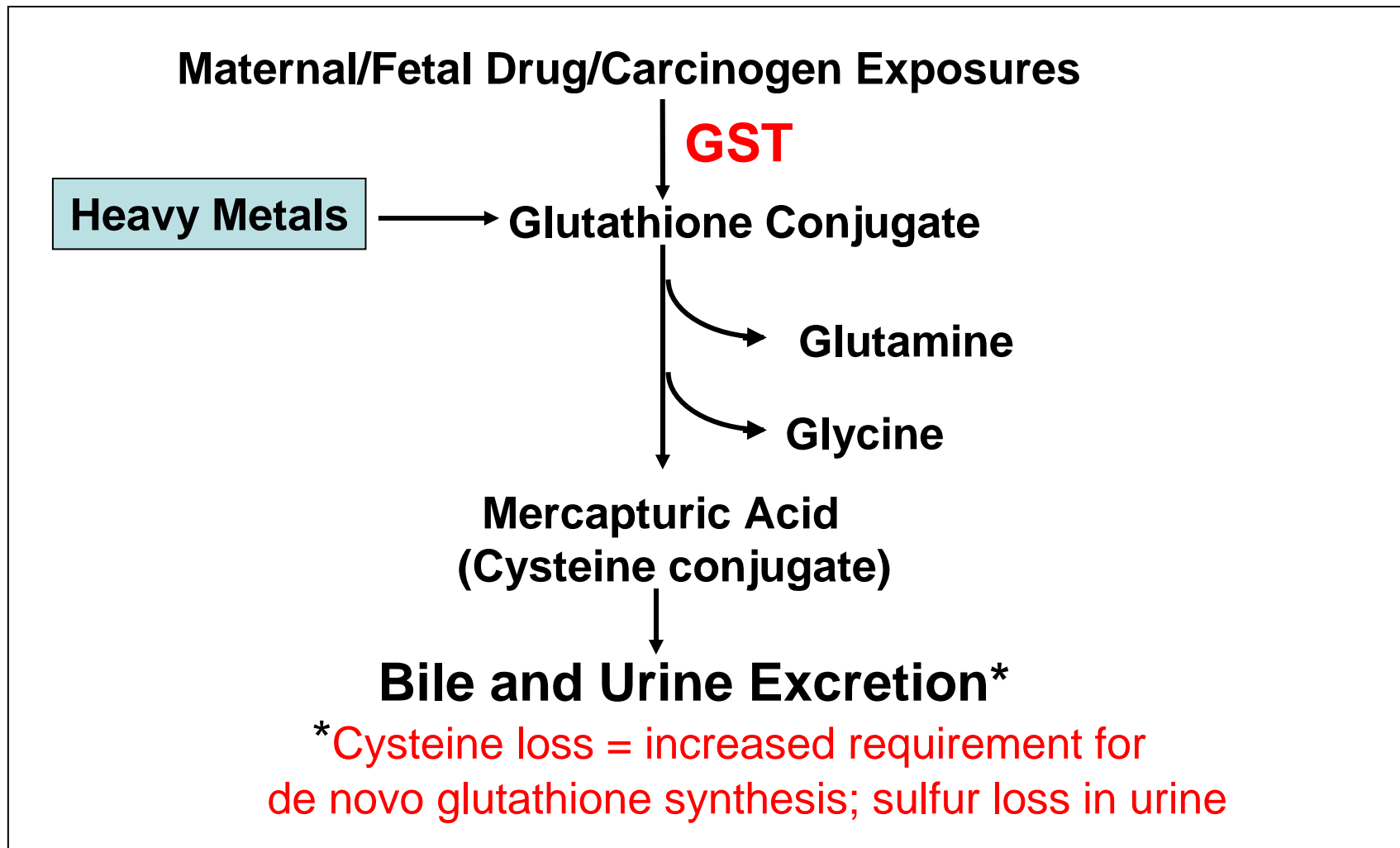
Estrogen may protect little girls from developing autism

# ANTIOXIDANT FUNCTION OF GLUTATHIONE



Major intracellular antioxidant: H<sub>2</sub>O<sub>2</sub>, superoxide, hydroxyl radical, peroxynitrite, membrane lipid peroxidation

# DETOXIFICATION FUNCTIONS OF GLUTATHIONE



Detoxification: Hg, As, Pb, Cd bind to thiol (SH) group; Metal-cysteine conjugates excreted

## Functional Consequences of Low GSH/GSSG and Increased Oxidative Stress

Reduced ability to detoxify environmental toxicants and heavy metals:  
Neurotoxicity; Immunotoxicity

Oxidation of active site cysteine (-SH groups) in enzymes: altered structure/function:

Abnormal methionine metabolism; Altered membrane signaling

Decreased liver GSH synthesis: reduced export of cysteine to brain:  
Reduced astroglial/neuronal GSH synthesis: increased sensitivity to heavy metals; Neurotoxicity

Integrity of the gut epithelium compromised:

Increased mucosal membrane permeability; malabsorption

Altered T-cell subpopulations:

Decreased Th1:Increased Th2; autoimmunity; gut inflammation

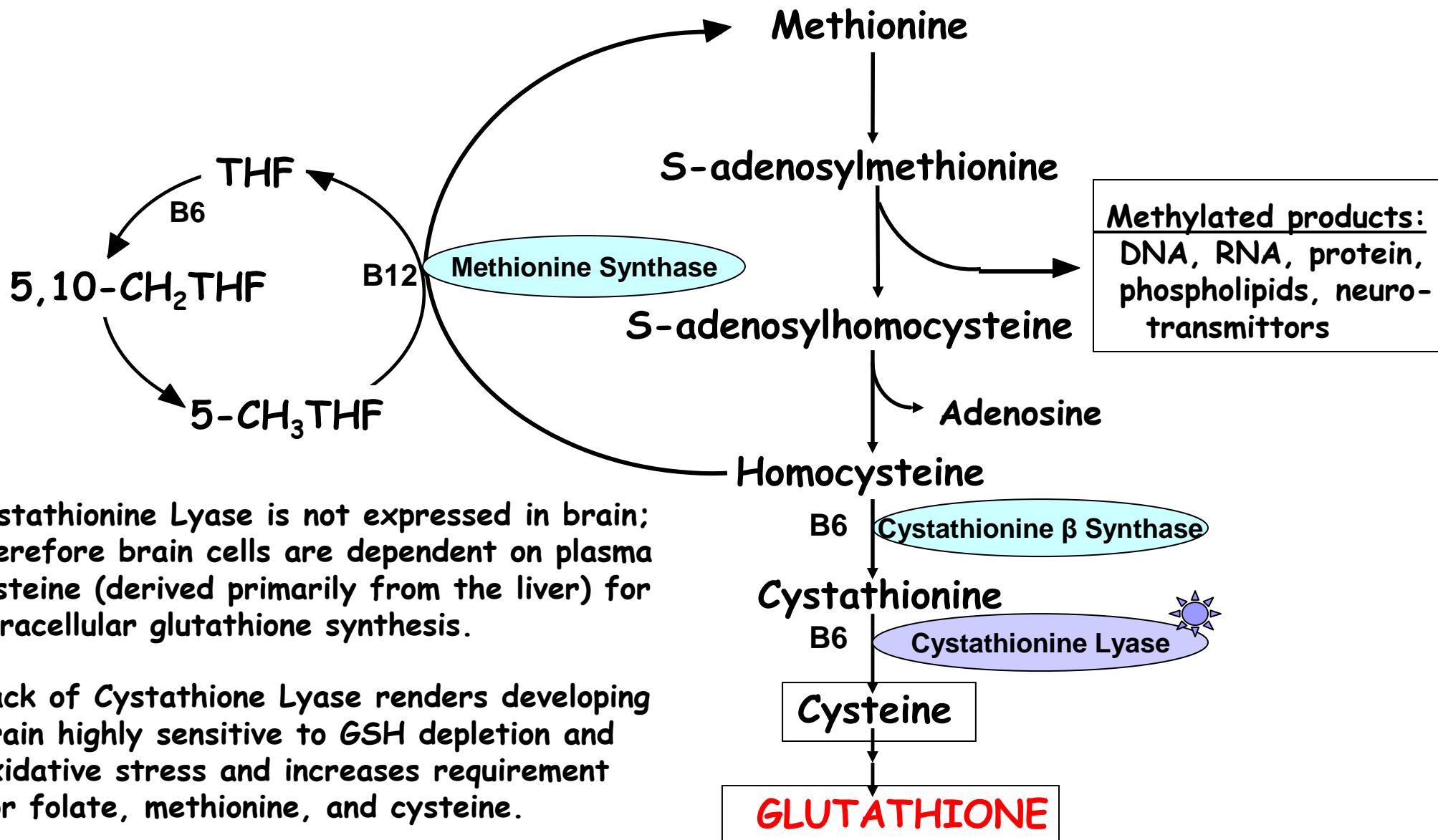
**Neurotoxicity of Thimerosal in Human Brain Cells  
is Associated with Glutathione Depletion:**

**Protective Effect of N-Acetyl Cysteine or Glutathione**

S. Jill James, William Slikker, Elizabeth New,  
Stefanie Jernigan, Stepan Melnyk

**Neurotoxicology, volume 26: pp1-8, 2005**

# GLUTATHIONE SYNTHESIS IN THE BRAIN IS DEPENDENT ON HEPATIC CYSTEINE SYNTHESIS AND EXPORT



☀️ Cystathionine Lyase is not expressed in brain; therefore brain cells are dependent on plasma cysteine (derived primarily from the liver) for intracellular glutathione synthesis.

Lack of Cystathionine Lyase renders developing brain highly sensitive to GSH depletion and oxidative stress and increases requirement for folate, methionine, and cysteine.

## WORKING HYPOTHESIS

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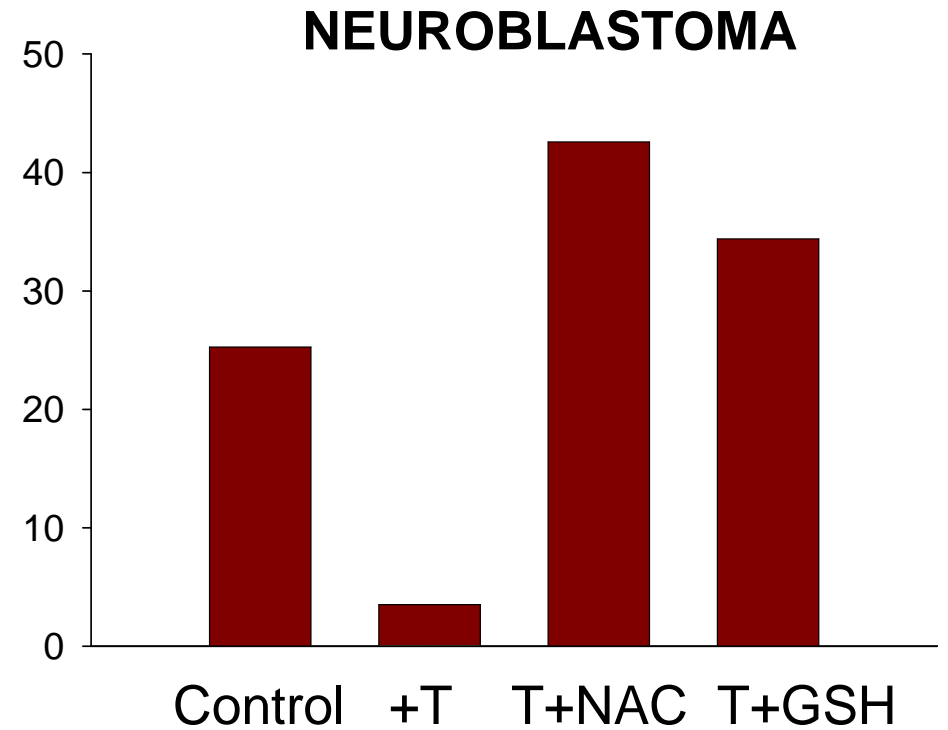
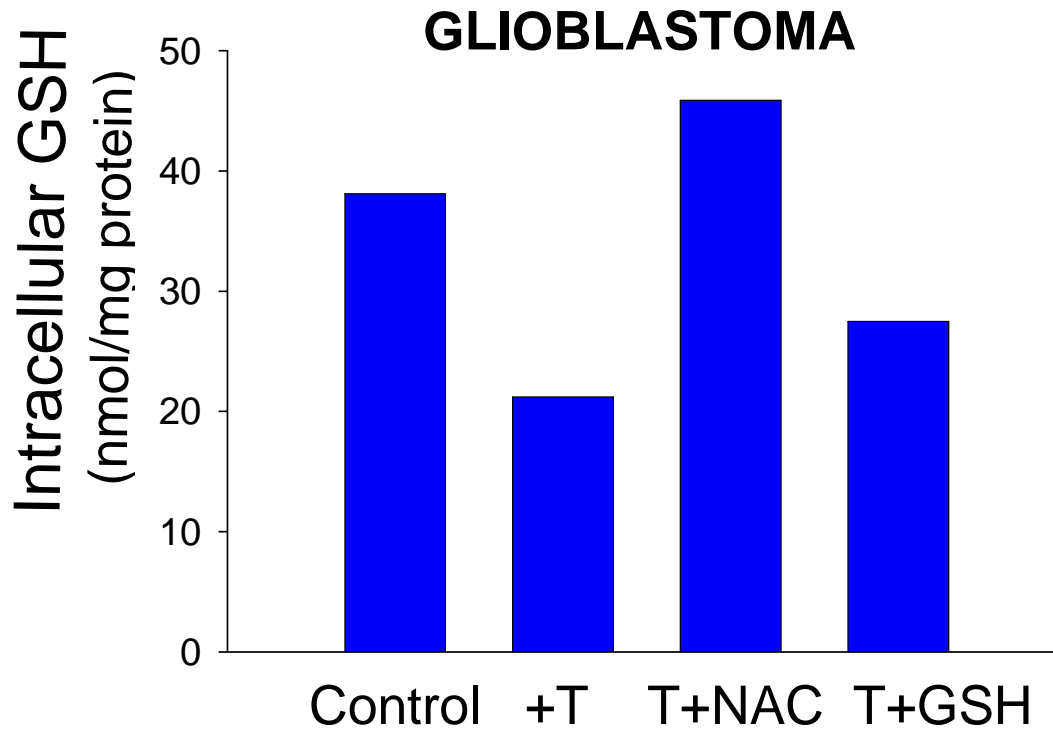
The neurotoxicity of Thimerosal is associated with depletion of glutathione, the major intracellular antioxidant

Ethyl mercury in Thimerosal binds to cysteine thiol (-SH) groups on intracellular proteins and inactivates function.

The cysteine-SH group of glutathione, binds mercury and protects essential proteins from functional inactivation.

**Glutathione is the major mechanism of mercury detoxification**

Intracellular glutathione levels in cells exposed to 15  $\mu\text{M}$  Thimerosal (T) in presence of 100  $\mu\text{M}$  N-acetylcysteine (NAC) or glutathione ethyl ester (GSH)



# Relative sensitivity to oxidative stress between Autistic and Control Lymphoblastoid cells

A Project Funded by SafeMinds

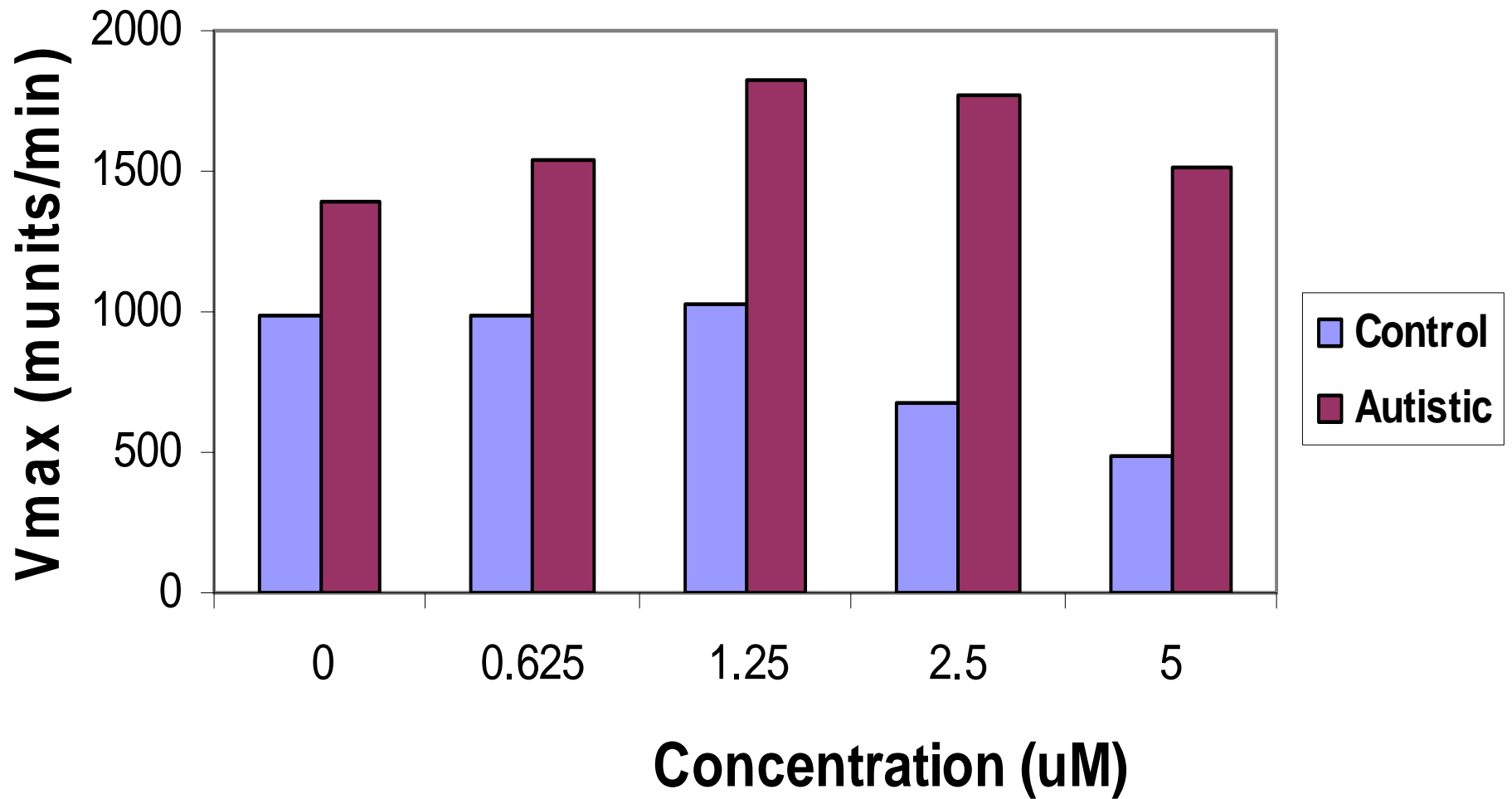
## Experimental Procedures

Lymphoblastoid cell lines from autistic children with at least one affected sibling were obtained from AGRE.

Unaffected healthy control lymphoblastoid cell lines were obtained from ATCC

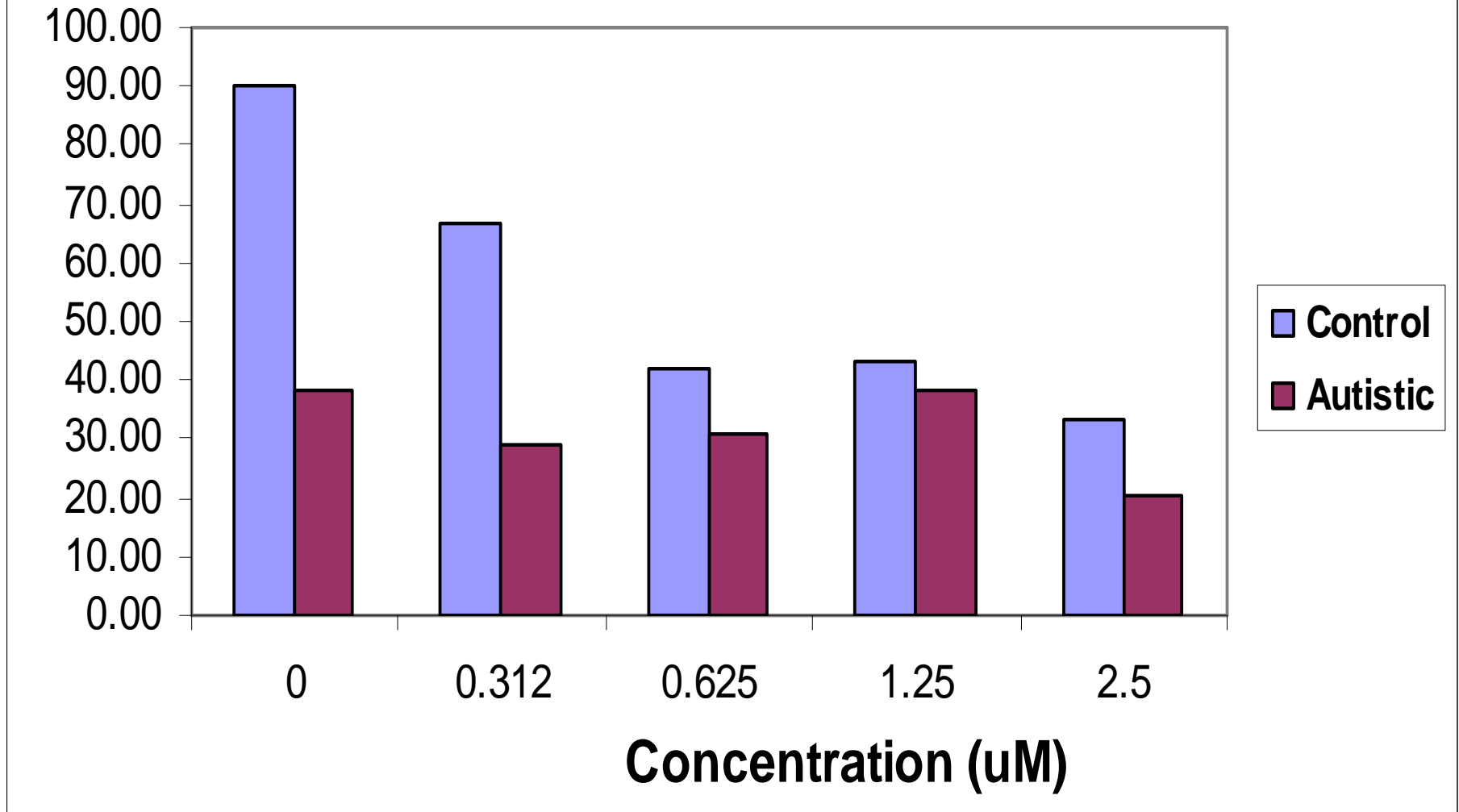
Pairs of autistic and control cells lines were cultured under identical conditions. Rate of free radical generation, GSH/GSSG, , and mitochondrial caspase-3 were measured at baseline and after exposure to oxidative stress.

# ROS generation



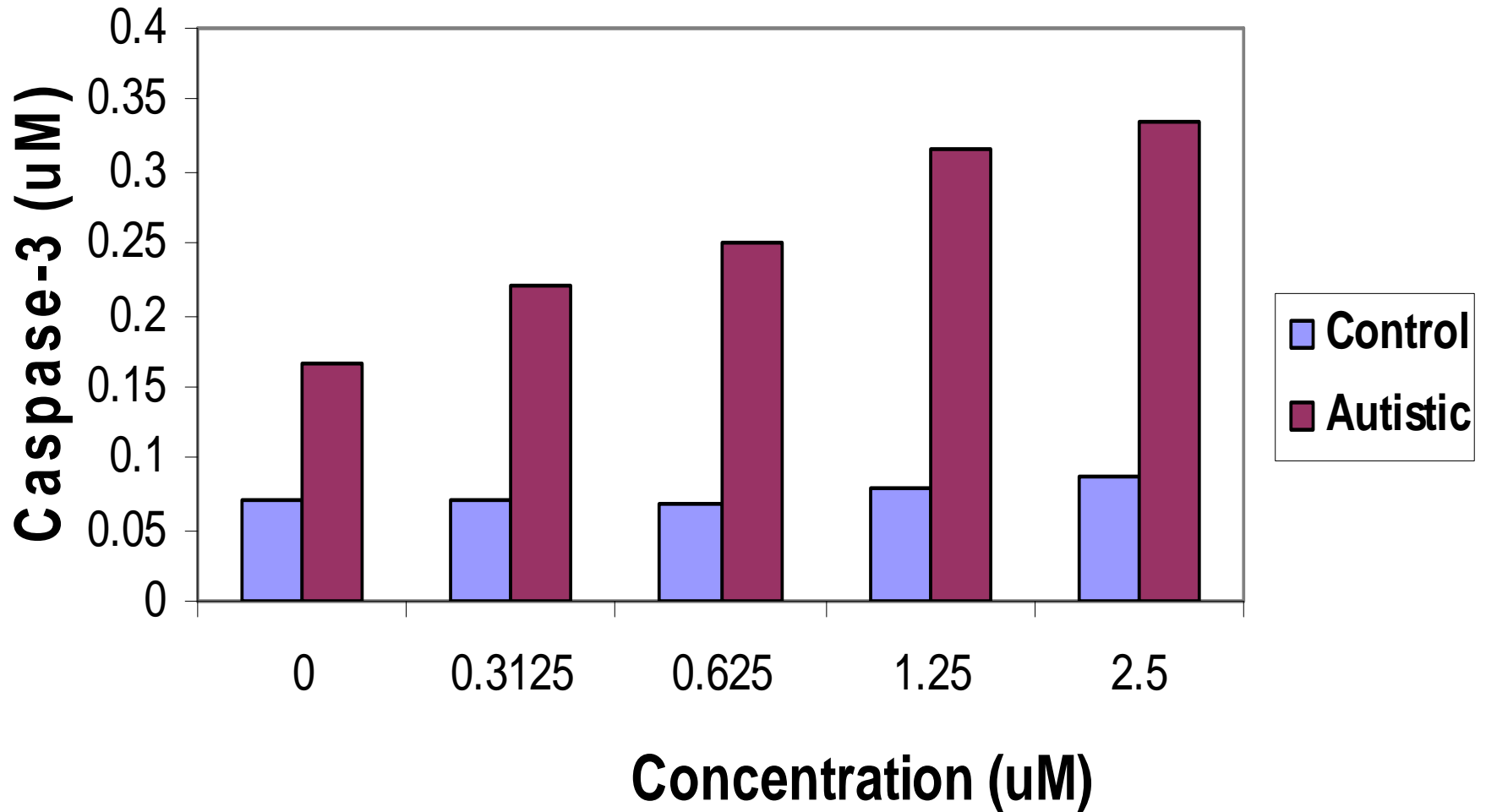
Cells from autistic children generate more free radicals than control cells

# fGSH:fGSSG



Cells from autistic children have lower *GSH/GSSG* ratio than control cells

## uM Caspase-3/mg protein



Cells from autistic children have higher cell death rate than control cells

## CONCLUSION

Since both cell lines were cultured at the same time under identical conditions with identical media, the differences at baseline and after exposure to oxidant stress must reflect inherent genetic differences.

These results provide experimental evidence that autistic children may be genetically more sensitive to pro-oxidant environmental exposures.

# Increased Oxidative Stress and Impaired Methylation Capacity in Children with Autism: Metabolic Biomarkers and Genetic Predisposition

## Results of Intervention Trial with Folinic Acid, TMG, and Methyl-B12

S. Jill James, Ph.D., Laurette Janak, M.O.M.,  
Stepan Melnyk, Ph.D., Stefanie Jernigan, Paul Cutler, M.D.

**American Journal of Clinical Nutrition volume 80: pp 611-17,2004**

## Baseline Methionine Cycle Metabolites: Subset means within the overall means

	Autistic n=80	Control n=75	p value
Methionine ( $\mu\text{M}/\text{L}$ ) <b>Subset: 51% <math>&lt;20\mu\text{M}/\text{L}</math></b>	21.2 <b>16.8</b>	28.0	$<0.0001$
SAM (nM/L) <b>Subset: 28% <math>&lt;75\mu\text{M}/\text{L}</math></b>	81.5 <b>65.7</b>	94.2	$<0.0001$
SAH (nM/L) <b>Subset: 20% <math>&gt;28 \mu\text{M}/\text{L}</math></b>	23.4 <b>36.6</b>	19.5	$<0.0001$
SAM/SAH Ratio <b>Subset: 61% <math>&lt;4</math></b>	3.9 <b>3.0</b>	5.1	$<0.0001$

## INTERPRETATION

1. The significant decrease in methionine, SAM and homocysteine levels in autistic children is consistent with reduced turnover of the methionine cycle.
2. The decrease in SAM and increase in SAH (decreased SAM/SAH ratio) provides metabolic evidence that methylation capacity may be reduced in autistic children.

## Baseline Transsulfuration Metabolites: Subsets within the overall means

	Autistic n=80	Control n=75	p value
Cysteine ( $\mu\text{M}/\text{L}$ )	161	205	<0.0001
<b>Subset: 71% &lt;170<math>\mu\text{M}/\text{L}</math></b>	<b>153</b>		
Total GSH ( $\mu\text{M}/\text{L}$ )	5.1	7.5	<0.0001
<b>Subset: 66% &lt;5<math>\mu\text{M}/\text{L}</math></b>	<b>4.4</b>		
Free GSH ( $\mu\text{M}/\text{L}$ )	1.4	2.1	<0.0001
<b>Subset: 86% &gt;2<math>\mu\text{M}/\text{L}</math></b>	<b>1.2</b>		
GSSG ( $\mu\text{M}/\text{L}$ )	0.4	0.24	<0.0001
<b>Subset: 61% &lt;0.3<math>\mu\text{M}/\text{L}</math></b>	<b>0.5</b>		

## INTERPRETATION

1. The significant decreases in homocysteine, cystathionine, cysteine and glutathione suggests that the transsulfuration pathway is insufficient for adequate glutathione synthesis.
2. The increase in GSSG (oxidized inactive glutathione) and decrease in GSH (active antioxidant glutathione) is strong evidence that oxidative stress is increased in autistic children.

# MINIMUM ESSENTIAL THIOL PROFILE AVAILABLE COMMERCIALY

**(FASTING)**

**Methionine:** Low methionine is correlated with low SAM

**Total Homocysteine:** Elevated homocysteine is correlated with elevated adenosine and SAH

**Cysteine:** Low cysteine is correlated with low GSH and taurine

**GSH:** Low GSH indicates low detox and antioxidant capacity

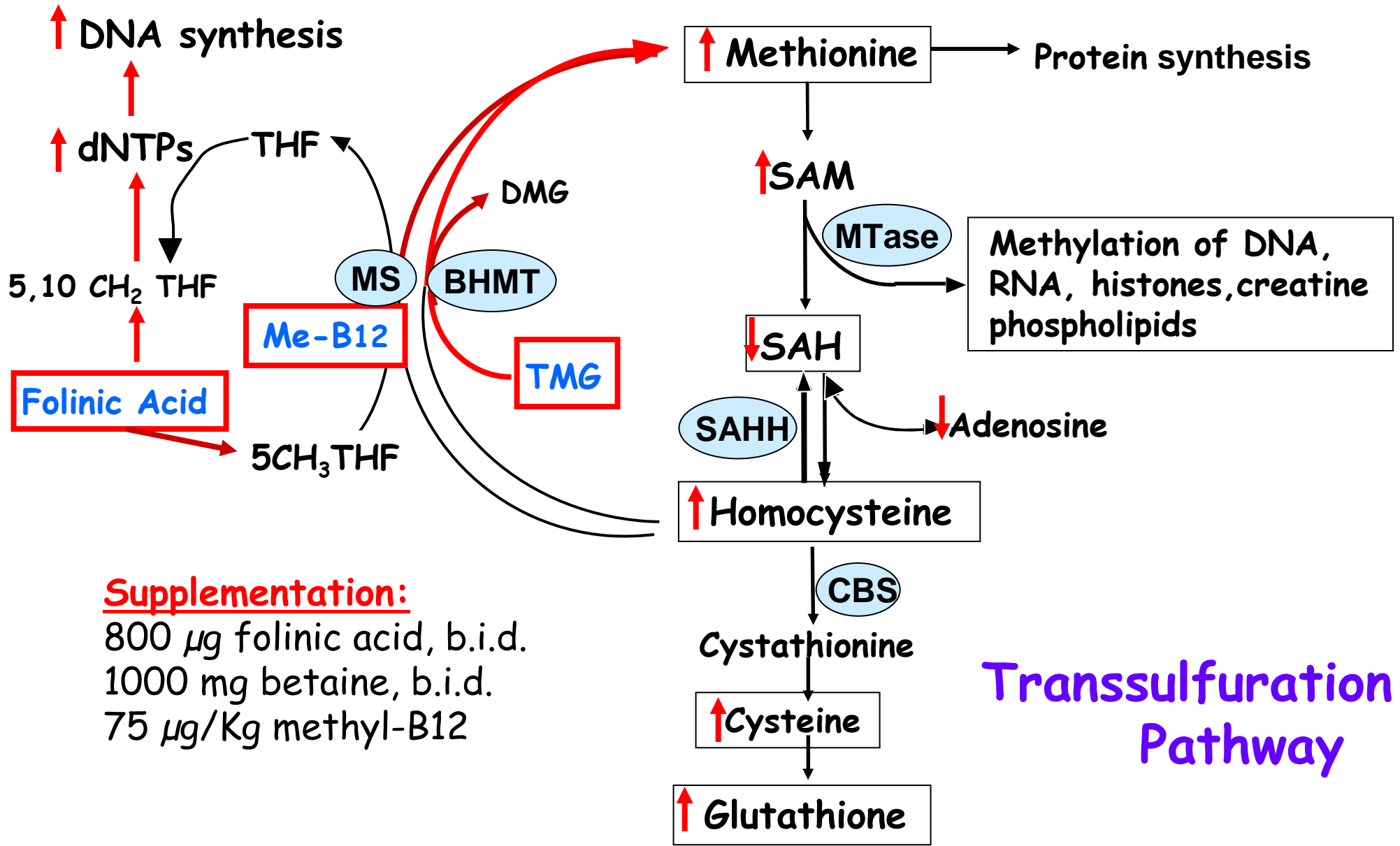
Note: AA profiles measure "free" homocysteine which is usually negligible: Ask for "total" homocysteine (protein bound plus free)

**Pharmacologic doses of nutrient cofactors can break the vicious cycles, restore normal flux, and release metabolic blocks by mass action**

## Folinic Acid, Betaine, and Methyl B-12 Supplementation in Children with Autism

Eight of the children participated in an intervention trial and were given 800  $\mu\text{g}$  folinic acid and 1000 mg betaine b.i.d. for 3 months and the plasma metabolites were re-measured.

The children were then given injectible methyl-B12 (75  $\mu\text{g}/\text{Kg}$  2x/week) and the plasma profile was repeated after 4 weeks of combined folinic acid, betaine, and methyl B12



## Proportion of Autistic Children within Normal Range Before and After Supplementation

Metabolite	Normal Range <sup>a</sup>	Baseline	Folinic+Betaine	Folinic+Betaine +methylB12
Methionine (μmol/L)	> 24	1/8	5/8	<b>7/8</b>
SAM (nmol/L)	> 80	2/8	<b>8/8</b>	8/8
SAH (nmol/L)	< 23	2/8	<b>7/8</b>	7/8
SAM/SAH	> 4	1/8	<b>7/8</b>	7/8
Adenosine (μmol/L)	< 0.3	4/8	<b>8/8</b>	8/8
Homocysteine (μmol/L)	< 5.5	3/8	<b>8/8</b>	8/8
Cysteine (μmol/L)	<180	0/8	2/8	<b>7/8</b>
GSH (μmol/L)	> 5.4	0/8	2/8	<b>7/8</b>
GSSG (μmol/L)	< 0.33	0/8	2/8	<b>8/8</b>
GSH/GSSG	> 16	0/8	3/8	<b>8/8</b>

<sup>a</sup> Range estimated to include 90% of control children

## INTERPRETATION

Folinic Acid and Betaine brought all the methionine cycle metabolites into normal range.

The combined regimen of Folinic Acid, Betaine, and Methyl B12 brought all the transsulfuration metabolites into the normal range.

Best predictors of impaired methylation are low methionine and SAM/SAH ratio OR elevated adenosine.

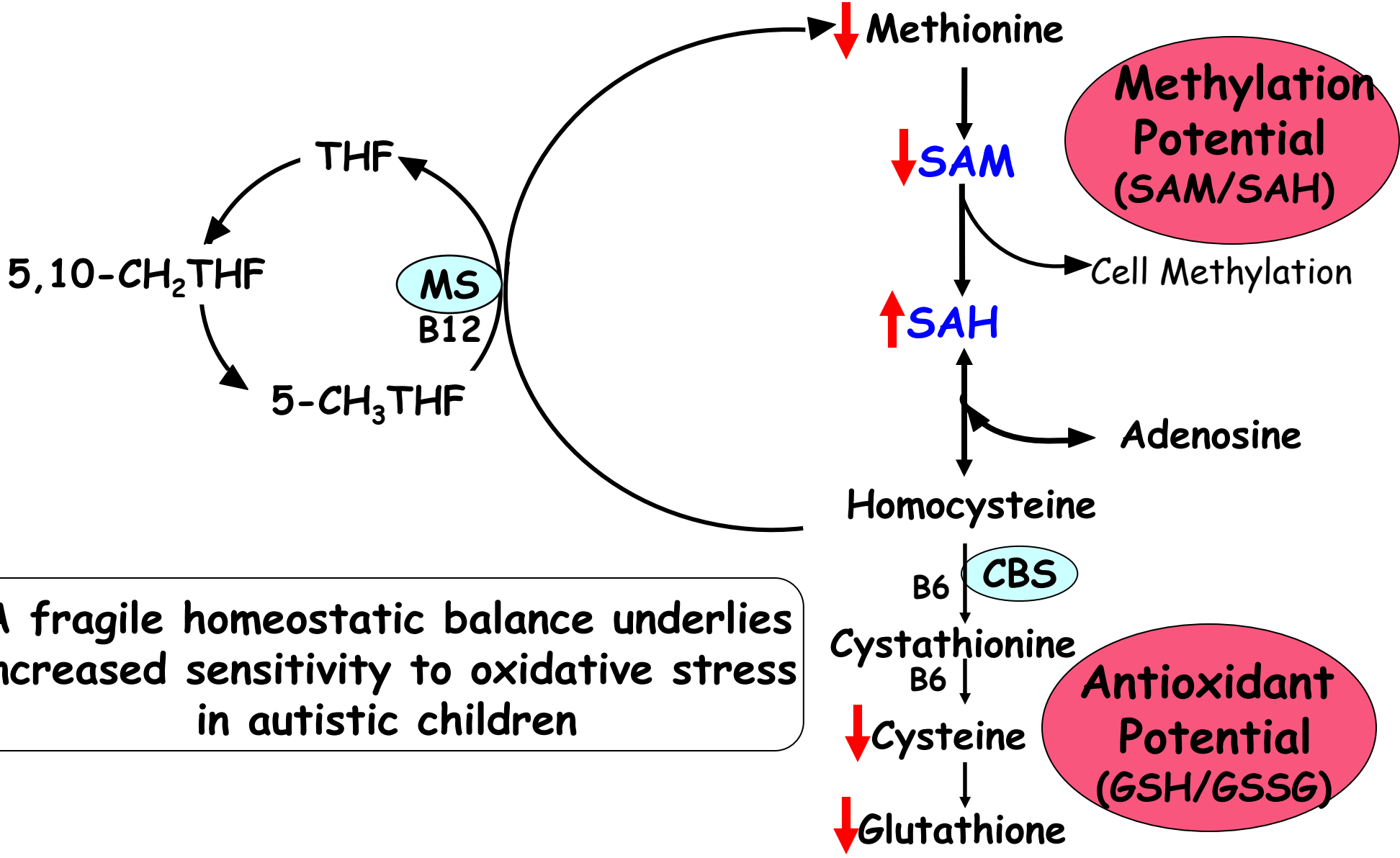
Best predictors of impaired antioxidant defense are low cysteine, and low glutathione (low GSH/GSSG ratio).

# Expanded Baseline Data and Lessons Learned

We now know after analyzing baseline metabolites from over 100 autistic children:

1. Each child is unique - one size (dose) does not fit all
2. Low cysteine, low free glutathione, low GSH/GSSG Ratio (reduced antioxidant capacity) are present in over 80% of autistic children.
3. Increased SAH and elevated adenosine (reduced methylation capacity) only occurs in a subset of about 21% of autistic children.
4. About 20% of children with autism do not tolerate TMG (become hyperactive) and about 5% do not tolerate folic acid or methylB12.

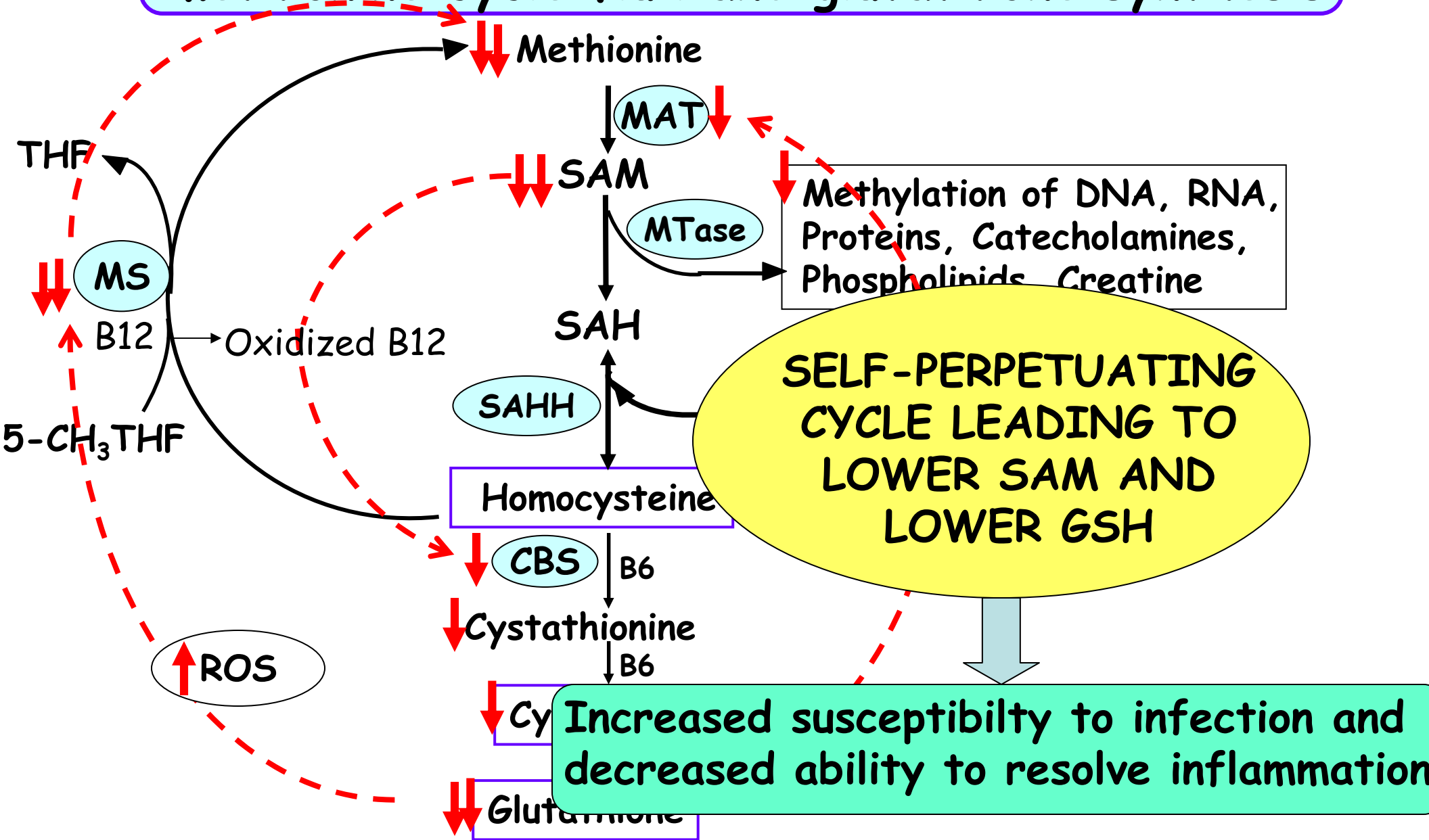
# Methionine Transsulfuration to Cysteine and Glutathione



A fragile homeostatic balance underlies increased sensitivity to oxidative stress in autistic children

Chronic oxidative stress decreases both methionine cycle flux and glutathione synthesis creating a self-perpetuating vicious cycle in autistic children

**Chronic oxidative stress decreases both methionine cycle flux and glutathione synthesis**



# GLUTATHIONE AND VIRAL INFECTION

Viral infection depletes GSH and increases oxidative stress

Influenza, herpes, measles virus infection induce GSH export

GSH blocks cytokine induction required for viral replication, reduces viral reverse transcriptase activity and blocks expression of viral proteins

Maintains cell-mediated immune response (Th1/Th2 ratio)

**GSH is a potent anti-viral agent!**

# AUTISM, GSH, AND VIRAL INFECTION

Low SAM and low GSH self-amplify and increase susceptibility to viral infection, increase virulence, and impair timely resolution of the viral infection

Many autistic children have low SAM and low GSH

High levels of SAM and GSH are anti-viral

Treatment strategies that increase SAM and GSH are protective against viral infection

# Is there a genetic basis for increased vulnerability to oxidative stress?

e.g.,

Mercury and other heavy metal toxicity; cell death

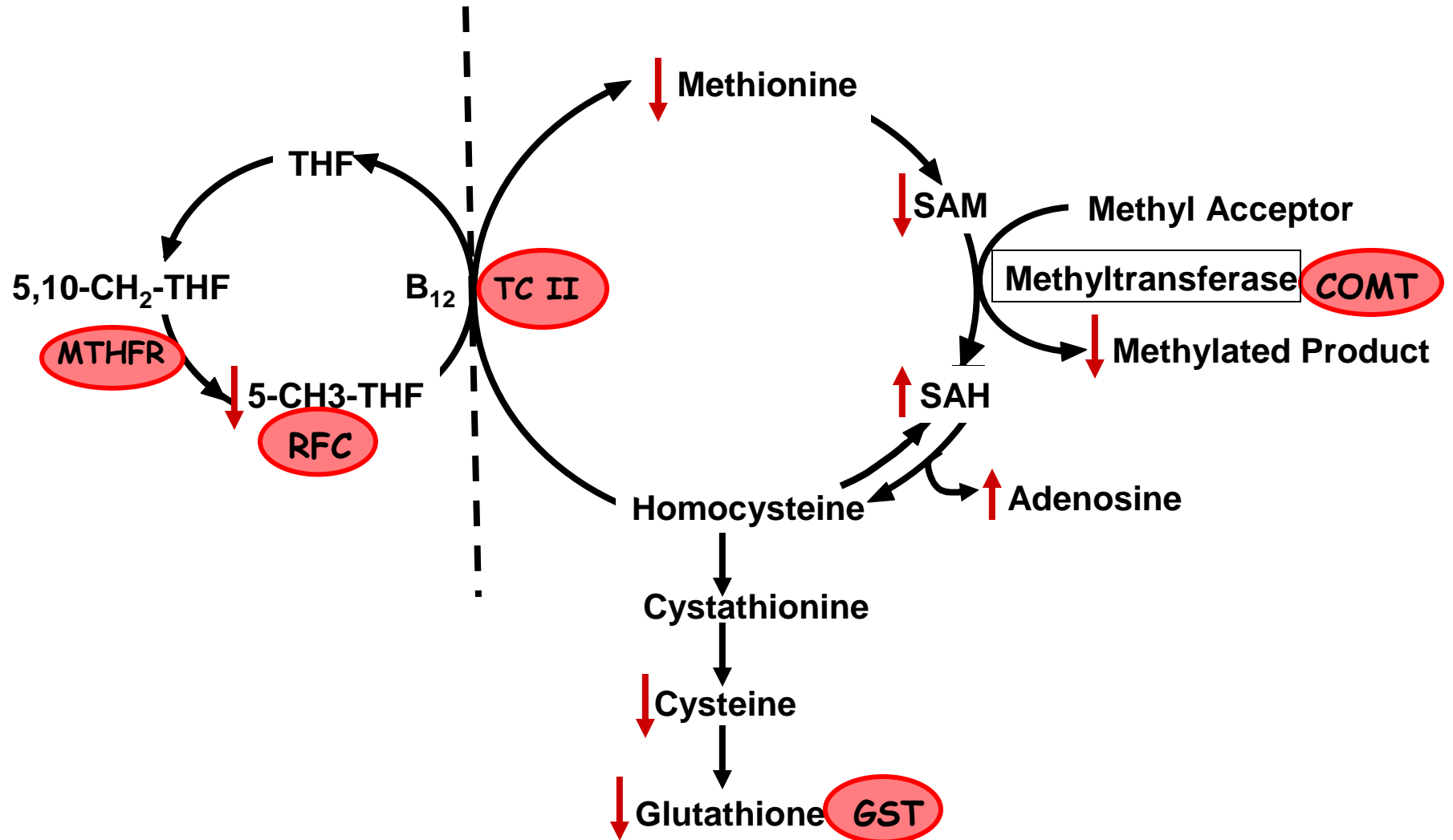
Autoimmunity: increased T helper2 cells

Gut Inflammation: increased inflammatory cytokines

Redox imbalance in the brain: inflammation; cell death

Redox enzyme inhibition: excess dopamine, glutamate

# Metabolic Response to Genetic Polymorphisms in the Methionine Cycle



# Functions of Polymorphic Genes Analyzed

MTHFR: Methylene tetrahydrofolate reductase  
Transfers methyl groups for methionine synthesis

MTRR: Methionine synthase reductase  
May reduce methionine synthase activity

RFC: Reduced Folate Carrier; Transports folate into the cell

GSTs: Glutathione-S-Transferase  
Important for glutathione detoxification capacity

TCII: Transcobalamin II; Transports methyl B12 into the cell

COMT: Catechol-O-methyltransferase; Methylates dopamine and prevents dopamine-induced oxidative stress in the brain

APO E4: May help detoxify mercury

# Polymorphisms in the Methionine Cycle Pathway

## Methylenetetrahydrofolate Reductase (677C→T;1298A→C)

	<u>Frequency</u>	<u>Odds Ratio</u>	<u>p value</u>
1. <u>MTHFR 677 TT</u>			
Control Individuals (203):	10.7%		
<b>Autistic Children (360):</b>	13.1%	1.47	NS**
2. <u>MTHFR 677 CT</u>			
Control Individuals (205):	43.9%		
<b>Autistic Children (360):</b>	49.2%	1.35	NS
3. <u>MTHFR 677CT/1298AC</u>			
Control Individuals (205):	19.1%		
<b>Autistic Children (360):</b>	<b>23.7%</b>	<b>1.7</b>	<b>NS; 0.07</b>

\*\*NS: Not statistically significant

# Polymorphisms in the Methionine Cycle Pathway

## Transcobalamin II (TCII 776 66C→G)

	<u>Frequency</u>	<u>Odds Ratio</u>	<u>p value</u>
1. <u>TCII 776 GG</u>			
Control Individuals (203):	16.0%		
<b>Autistic Children (360):</b>	<b>25.8%</b>	<b>1.8</b>	<b>0.05**</b>
2. <u>TCII 776 CC+CG</u>			
Control Individuals (205):	84.0%		
<b>Autistic Children (360):</b>	<b>74.2%</b>	<b>0.55</b>	<b>0.01**</b>

\*\*Statistically significant

**TCII G allele is significantly more frequent in autistic children**

**Theoretically, this could reduce B12 transport inside the cell and could decrease methionine synthase activity**

## Polymorphisms in the Methionine Cycle Pathway

Reduced Folate Carrier RFC1 80A→G

<u>RFC1 80 GG</u>	<u>Frequency</u>	<u>Odds Ratio</u>	<u>95% C.I.</u>
Control Individuals (203):	30%		
<b>Autistic Children (360):</b>	<b>39%</b>	<b>2.0</b>	<b>1.15, 3.33*</b>

A decrease in the transport of folate into the cells would produce a functional folate deficiency

# Polymorphisms Glutathione-S-Transferase (GST) Affecting Antioxidant Capacity

## GST M1 and GST T1

	<u>Frequency</u>	<u>Odds Ratio</u>	<u>p value</u>
1. <u>GST M1 Null</u>			
Control Individuals (205):	57.2%		
Autistic Children (360):	49.2%	1.4	0.07, NS
2. <u>GST T1 Null</u>			
Control Individuals (183):	25.8%		
Autistic Children (233):	22.7%	0.86	NS

# Polymorphisms Affecting Methylation and Increased Oxidative Stress

Catechol-O-Methyltransferase (COMT 1947A→G)

## COMT 472GG:

(high activity variant)

	<u>Frequency</u>	<u>Odds Ratio</u>	<u>95% C.I.</u>
Control Individuals (205):	16.3%		
<b>Autistic Children (360):</b>	<b>26%</b>	<b>2.34</b>	<b>1.06, 2.85*</b>

## Gene-Gene Interactions

### Affecting Methylation and Increased Oxidative Stress

Combined TCII GG plus COMT GG in the same individual

<b>TCII GG/COMT GG</b>	<u>Frequency</u>	<u>Odds Ratio</u>	<u>95% C.I.</u>
Control Individuals (203):	2.5%		
<b>Autistic Children (360):</b>	<b>9.7%</b>	<b>7.0</b>	<b>2.32, 21.2*</b>

The TCII and COMT gene polymorphisms may interact to synergistically increase the risk of autism

## Gene-Gene Interactions

### Affecting Methylation and Increased Oxidative Stress

Combined TCII GG plus GSTM1 null in the same individual

<b>TCII GG/GSTM1 null</b>	<u>Frequency</u>	<u>Odds Ratio</u>	<u>95% C.I.</u>
Control Individuals (203):	7.6%		
<b>Autistic Children (360):</b>	<b>13.4%</b>	<b>2.2</b>	<b>1.05, 4.4*</b>

The TC II and GSTM1 null gene polymorphisms interact to increase the risk of autism

# Gene-Gene Interactions Affecting Methylation and Increased Oxidative Stress

Combined RFC1 GG plus COMT AG in same individual

<b>RFC1 GG/COMT GG</b>	<u>Frequency</u>	<u>Odds Ratio</u>	<u>95% C.I.</u>
Control Individuals (203):	6.7%		
<b>Autistic Children (360):</b>	<b>13.8%</b>	<b>2.5</b>	<b>1.05, 5.75*</b>

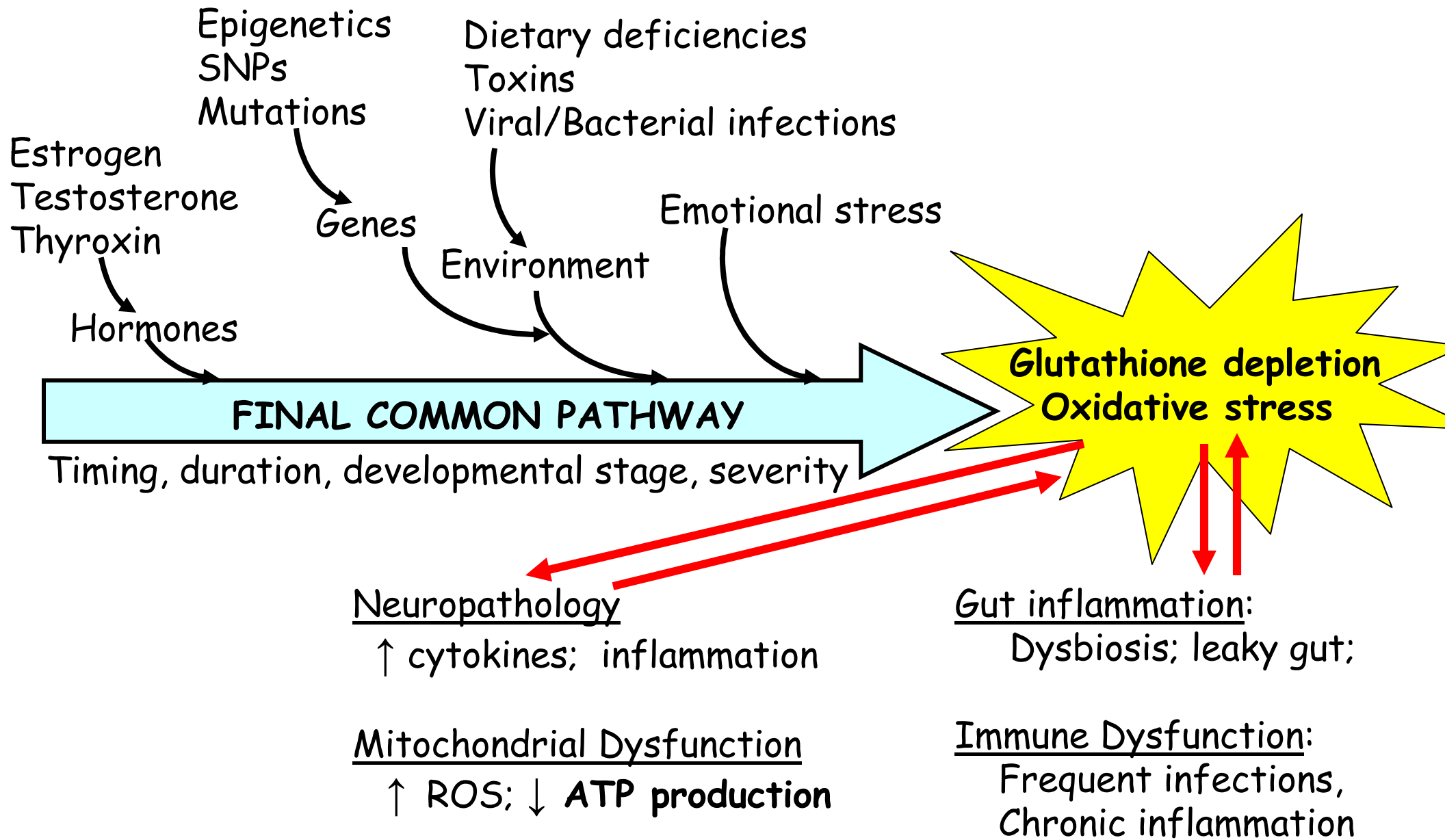
Polymorphisms in the RFC1 and COMT genes interact to increase risk of autism

## Important Caveat

No single polymorphism alone can predict increased risk of autism because, by definition, polymorphisms are highly prevalent in normal people as well. It is possible, however, that specific combinations of these polymorphisms interact to shift specific metabolic pathways that are important in the pathogenesis of autism.

To determine if a relationship exists between metabolic profile and genetic profile will require statistical analysis of ~1500 cases and ~1500 control children.

**PUTTING IT ALL TOGETHER.....**



# Metabolic Indicators

**Reduced Methionine**

(Oxidative inhibition of MS)

**Reduced SAM**

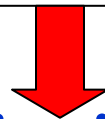
(Low methionine)

**Reduced Cysteine**

(Low methionine, Homocysteine)

**Reduced Glutathione**

(Low Cysteine)



**Reduced Cellular Antioxidant Capacity in Autism  
and Increased Vulnerability to Oxidative Stress**



# OVERALL CONCLUSION

## MAY 2006

The abnormal metabolic profile in children with autism strengthens the hypothesis that a genetic inability to control oxidative stress may be central to the development of neurologic, immunologic, and gastrointestinal dysfunction that occurs with autism.

# Our Kids



Dr. Moms

Researchers  
And Physicians

2.4.56

# Factors Contributing to Oxidative Stress in Autistic Children

