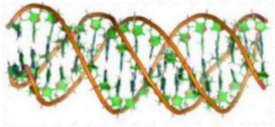


Rational for Appropriate Laboratory Testing

David Quig, PhD

To be covered…

- Objective laboratory testing for:
  - Retention of toxic elements
  - Deep oxidative stress
  - Glutathione
  - Aberrant methionine metabolism/”methylation,” and folates
  - Gastrointestinal paracellular permeability

Environmental Toxicants

C.D.C.

"The epidemic of epidemics of ***cardiovascular*** *disease* and ***immunological*** and ***neurological*** disease is likely associated with environmental toxicants."

ATSDR/CDC/USPHS monographs on specific toxic metals

Estimate of Net Retention

- Dates back to the 1950s (IV or IM, “lead mobilization test”)
- Nonlinear relationship between blood Pb and post Ca-EDTA urine Pb (NIEHS)*
- “Measurement of urine lead before $_2$ **AND** after **chelation** has been used as an indicator of significant lead **exposure**.”** [retention/body burden]
- Doctors treat (Ca-EDTA) adults with blood lead >45 µ/dL, and children who can't tolerate the drug used in *conventional chelation therapy (DMSA)***

*EHP(1995)103:1048-52 **www.mayoclinic.org/diseases-conditions/lead...treatment/treatment/txc-20275071 [4/18/17]
Ca-EDTA package insert

Estimate of Net Retention (cont'd)

- **<u>Bioacumulation/Body Burden</u>** acknowledged (C.D.C.)*, **but** "chelation is *useless* and *dangerous*" (ACMT)**

- For a given *individual*, ***toxic effects*** are elicited when the level of **retention** exceeds physiological tolerance.
  (typically vague and multiple diverse symptoms)

www.cdc.gov/nceh/lead [Accessed 6/29/16] *ATSDR/CDC Toxicological Profile for Lead (2007 update)
**J med Toxicol(2013)9:318-25

Interpretation of Provocation Test Results

- Provocation testing is valid when done correctly, and can serve as a ***component*** of *diagnostic judgement.*

- Consider results ***in context*** with amounts of all elements excreted, physical exam, symptoms, *complete occupational & environmental exposure history,* and other lab findings.

- **One cannot diagnose *"metal toxicity"* against unprovoked urine reference values (ACMT).***

J Med Toxicol(2013)9:318-25

Clinical Department of Laboratory Medicine and Pathology

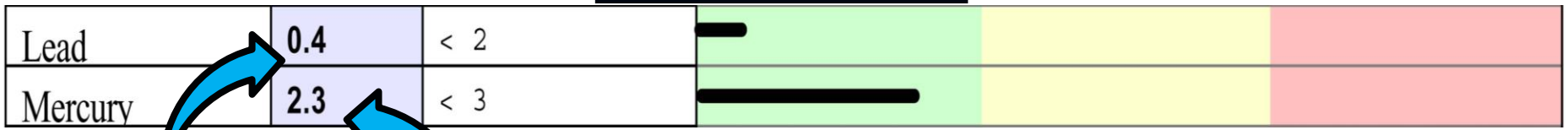
- "An increase in lead in the **post-chelation** specimen of **up to 6 times** the concentration in the **pre-chelation** specimen is *normal*."

(what if pre = 0.2 and post = 2.0 ug/gm creatinine?)

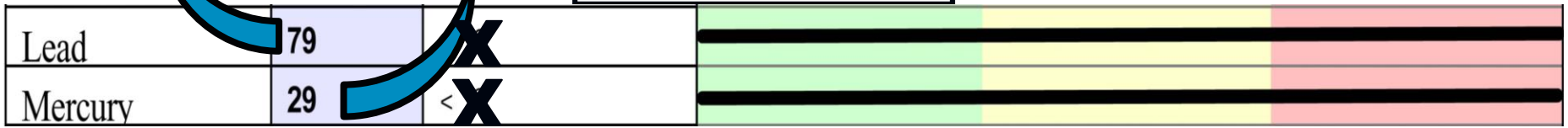
www.mayomedicallaboratories.com/test-catalog /Clinical+and+Interpretive/60246 [Accessed 7/14/18]

Urine Toxic Metals: Estimate of Retention

Unprovoked (Ongoing exposure)



Post DMSA



Compare Pre and Post RESULTS

Provocation Testing is Useful for Monitoring Metal Depuration Therapy

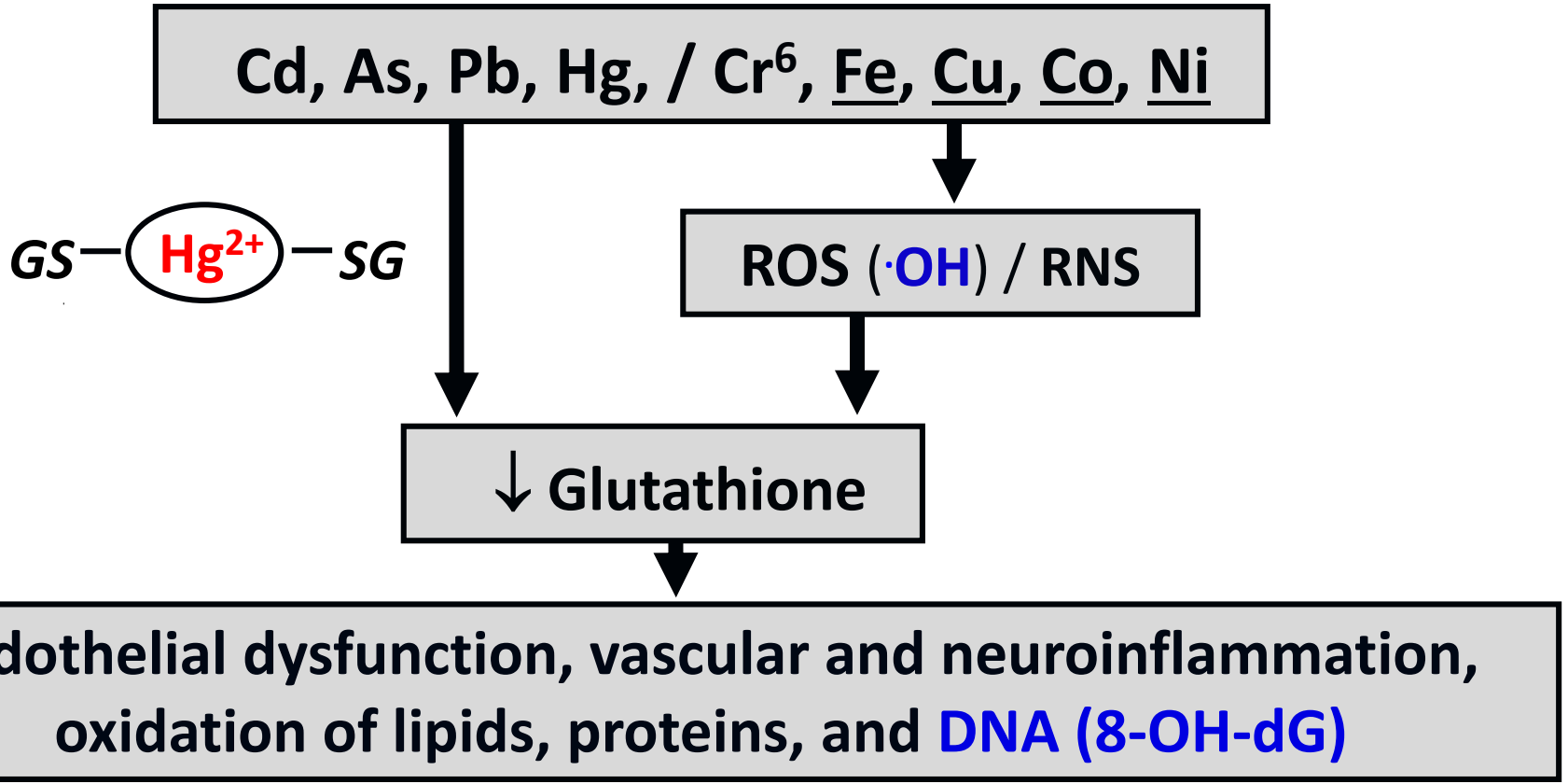
- Urine lead analysis is useful for monitoring chelation.*

- Follow up provocations should be performed **IDENTICALLY** to **monitor** elimination of toxic elements.

  (agent, dose, route of administration, collection time)

* www.aruplab.com [Accessed 2/3/17] www.labcorp.com [Accessed 2/3/17]

Metals, ROS, GSH and Oxidative Damage



Toxics(2015)3:20-62 Arch Toxicol(2010)84:825-89 Toxicol(2011)283:65-87 Curr Pharmacol(2010)8:259-75

Formation of Radical Oxygen and Nitrogen Species

- Hydroxy radical (·OH)

Fenton Reaction $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {\cdot}OH$

Haber-Weis Reaction $O^-_2{\cdot} + H_2O_2 \rightarrow O_2 + OH^- + {\cdot}OH$

- Peroxynitrite ($ONOO^-$)

$O^-_2{\cdot} + {\cdot}NO \rightarrow ONOO^-$

Oxidative Med Cell Longev (2011) DOI: 10.1155/2011/809696

“Deep” Oxidative Stress

- “The hyroxy radical is considered as the most destructive oxygen free radical causing damage to biomolecules”
- ·OH induces *nucleobase oxidation (8-OH deoxyguanosine; 8-OHdG)*.
- Induces C → T transversions which are among the most frequent *somatic* mutations found in human cancers
- 8-OHdG has a major role in *spontaneous mutagenesis* (rodent models)
- Also associated with CVD, mitochondrial dysfunction, T2D and global brain ischemia (post-MI)

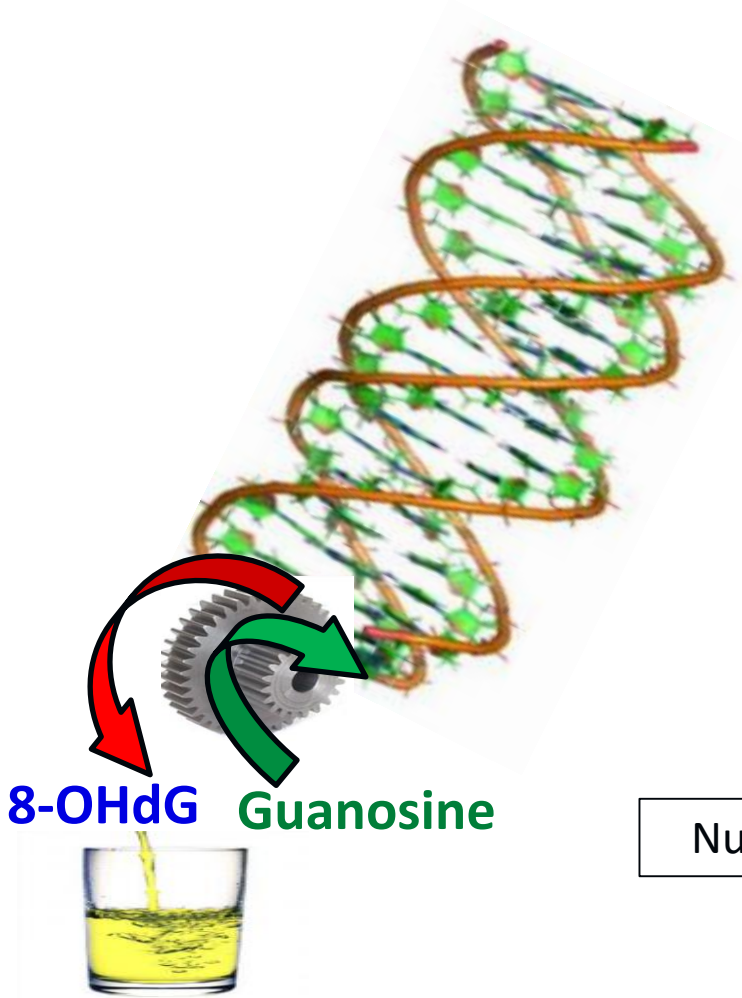
Oxidative Med Cell Longev (2011) doi: 10.1155/2011/809696 Cancer Epidemiol Biomarkers Prev(2008)17:3-14
Med Sci Monitor(2012)18CR409-14 Clin Biochem(2015) doi:10.1016/j.clinbiochem.2015.02.015

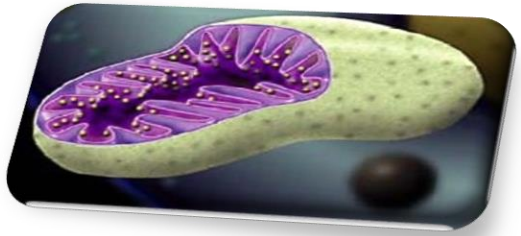
The Hydroxy Radical (·OH)

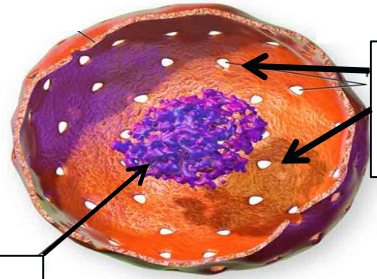
- $T^{1/2}$ (10^{-9} seconds)- *instantaneous nonselective attack*
  - **the hydroxy radical cannot be eliminated by any enzymatic reaction**
- 2-deoxyguanosine is *very* vulnerable to oxidation by **·OH**

  2-deoxyguanosine + **·OH** → **8-OH-2'-deoxyguanosine (8-OHdG)**
- Endonucleases remove the oxidized guanosine and replace it.
- The cleaved **8-OHdG** is excreted in the urine.
- **Levels in 1st AM voids and 24 hr. collections are highly correlated (r= 0.93, p<0.01)**

Oxidative Med Cell Longev (2011) DOI: 10.1155/2011/809696 Neurotox Res(2012)22:231-48 Clin Chim Acta(2012)413:1822-26 J Environ Sci Hlth(2009)27:120-39 Carcinogen(2002)23:1441-46


8-OHdG
Guanosine




Nuclear pores
Nucleolus

Measurement of Urine 8-OHdG is Indicative of Intracellular Levels

- Human study- 8-OHdG measured in lymphocytes and urine
  - HPLC-electrochemical detection, gel electrophoresis, and an immunoassay (ELISA)
- Levels of 8-OHdG in isolated ***lymphocytes*** and ***urine*** were ***highly correlated*** (all 3 methods).
- Highly *sensitive* and *specific* ELISAs are used extensively in clinical trials and by diagnostic laboratories.

Circ J(2012)76:117-26 Clin Chim Acta(2012)413:1822-26 Carcinogen(2002)23:1441-46

8-OH-dG : Predictor of Cardiac Events

- Sensitive biomarker of oxidative DNA damage, produced in cardiac muscle *(mitochondrial DNA)*
- Elevated urinary 8-OHdG was highly correlated with clinical status and cardiac dysfunction.

(*prospective study*, n=186 patients, 1.8 yr follow up)

High levels of 8-OHdG in atherosclerotic plaque (human)

> 12.4	**16.2**

Eur J Hrt Fail(2011)13:29-36 Circ J(2012)76:117-26 Clin Chim Acta(2004)339:1-9

Cancer and Elevated Urine 8-OHdG

DNA/RNA Oxidative Damage Assay; Urine

	RESULT / UNIT	REFERENCE INTERVAL	LOW MODERATE HIGH
8-hydroxy-2'-deoxyguanosine* (8-OHdG)	72.4 ng/mg creat	< 8.2	
			PERCENTILE 2.5th 16th 50th 84th 97.5th
Creatinine	226 mg/dL	35- 240	

- Colorectal, hepatocarcinogenesis, oral squamous cell carcinoma
- Breast and ovarian (adjusted for confounders like oral contraceptives)
- Prostate, bladder, and lung

J Exp Sci Environ Epidemiol(2014)24:17-26 Biomed Environ Sci(2008)21:286-289
J Gastroenterol(2008)23:1431-38

Quenching Reactive Oxygen Species (radical /non-radical)

- **Superoxide Dismutases** (cytosol; ***Cu, Zn***, mitochondria, ***Mn***)

  SOD + **2 $O_2^{\cdot}$** + 2 H^+ → **O_2** + **H_2O_2**

- **Catalase** (Heme Fe^{III}) **$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$**

- **GSH peroxidases** (***selenium***)

  $2\ H_2O_2 + 2\ {}_rGSH \rightarrow$ GS-SG + 2 H_2O

- **GSH reductase**

  GS-SG + NAD(P)H → **2 GSH**

  (***Mg*** and ˜10% of total body glucose “disposal”/day)

Curr Vascular Pharmacol(2010)8:259-75 Curr Pharm Des(2009)15:2988-3002 Biochim Biophys Acta(2009)1780:869-72
Arch Biochem Biophys(2009)485:56-62

Clinical Intervention to Decrease 8-OHdG

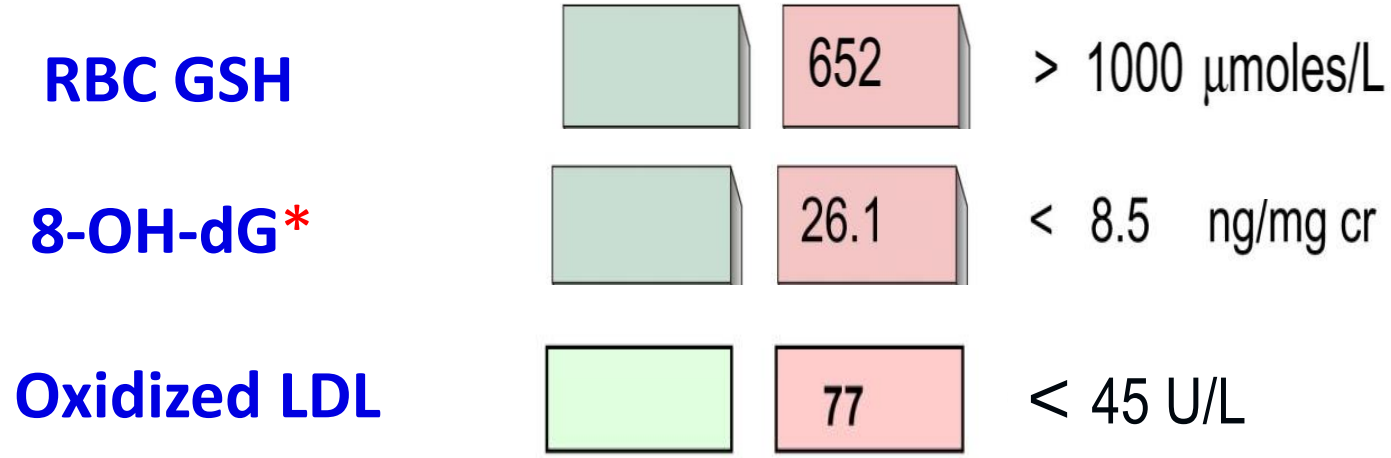
- 1st rule of toxicology
- Inconsistent results with vitamin C, carotenes, α-tocopherol or lycopene
- *Trend* for ↓ 8-OHdG after consuming 12 servings of fruits and vegetables vs. 5.8 for 14 days (*humans*)

Glutathione maintains the *redox state of proteins* necessary for the protection and repair of DNA

Cancer Epidemiol Biomarkers(2007)16:1428-36 Mutat Res(2005)574:58-66 Carcinogen(1999)12:2261-66
J Nutr Biochem(2005)16:577-86

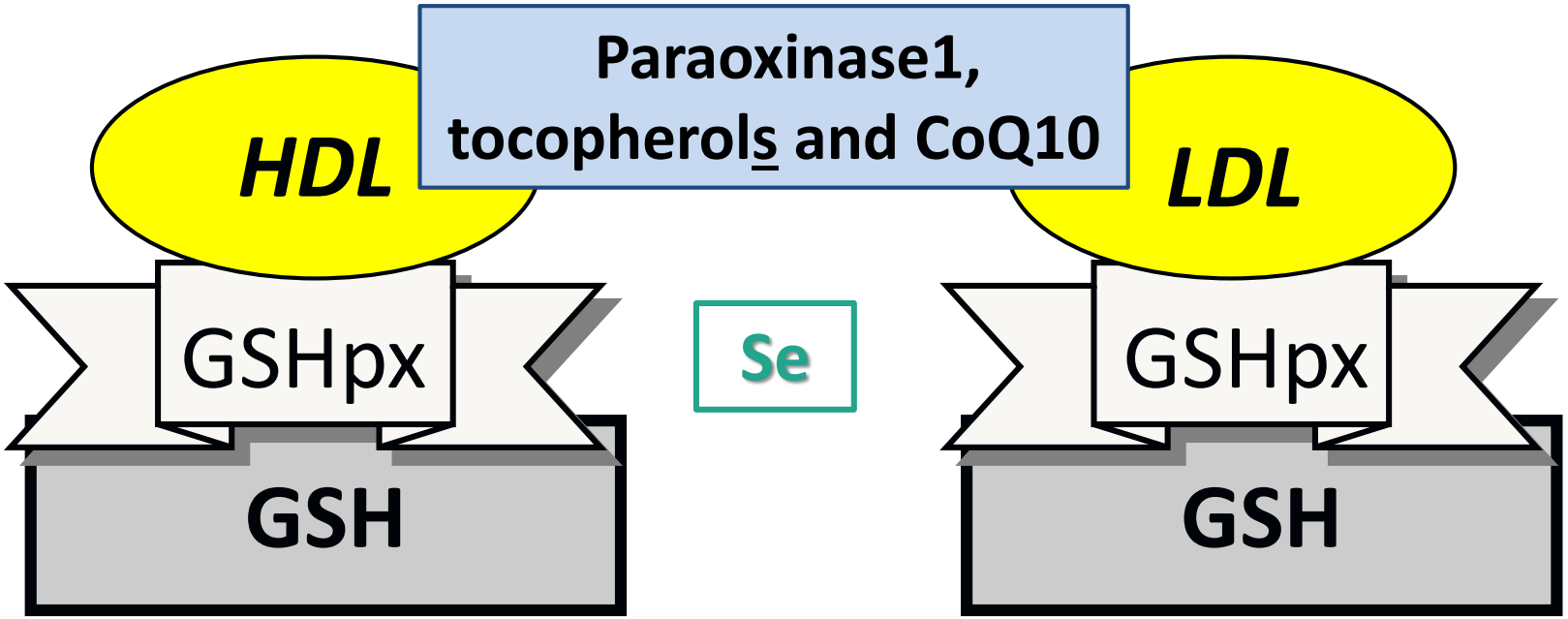
GSH Status and Oxidative Stress

59 yom, heavy smoker, hypertensive diabetic with elevated blood lead



*** First AM urine collection**

Protection of Plasma Lipoproteins



J Lipids(2012) doi:10.1155/2012/684010 Free Rad Biol Med(2009)46:607-15 Atherosclerosis(2007)195:e61-e68
Atheroscler(2005)181:9-15 Arch Biochem Biophys(2004)430:97-10 JBC(2002)277:4301-4308

Pilot Study- Liposomal GSH and RBC GSH

57 yof, 14 yrs. Lyme's disease, moderate Pb and Cd *retention*

	RBC GSH (umoles/L)*
Baseline	728, 739
2 weeks	925 (26%)
2 months	1,218 (66%)
4 months	1,680 **(128%)**

1 tsp. each AM w/o food. Blood drawn 24 hrs. after last dose.

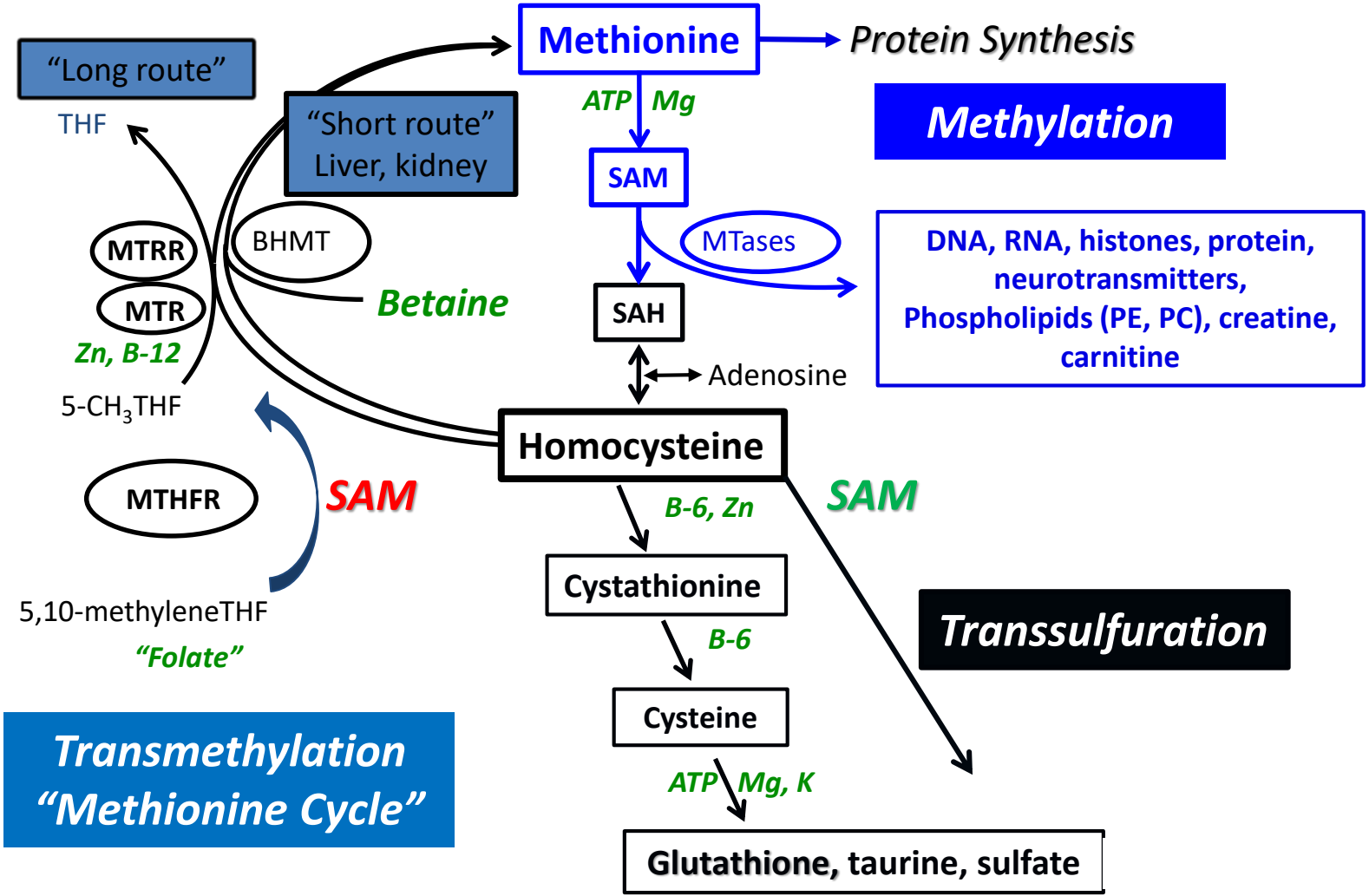
*(Reference range- >1,000 µg/L)

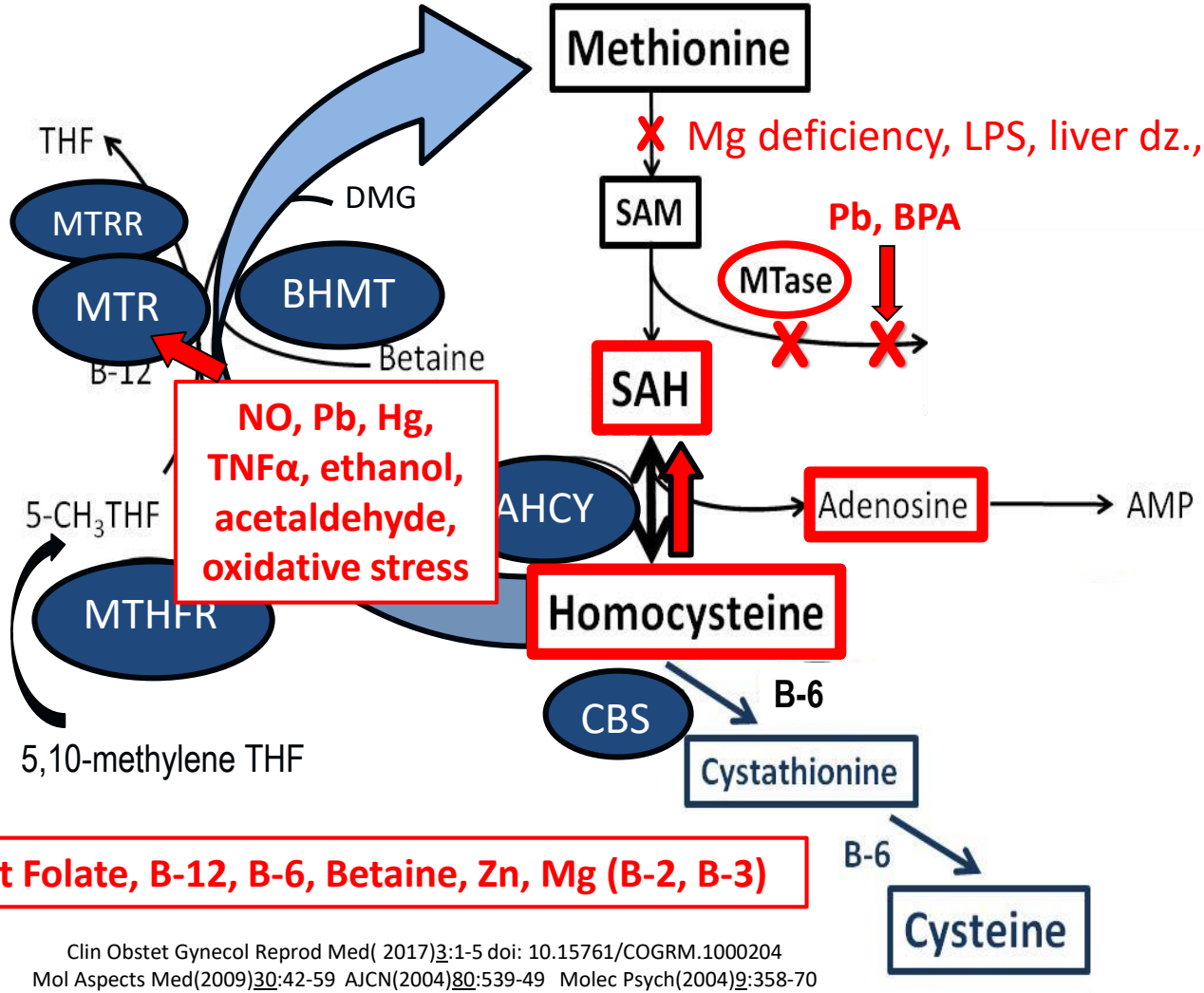
Quig, unpublished observations (2013)

Aberrant Methylation/Methionine/Folate Metabolism Increased Risk for:

Cardiovascular Disease
Certain Cancers (e.g. colorectal, prostate)
Immune Dysfunction
Asthma / Allergies
Birth Defects
Recurrent Pregnancy Losses
Central Nervous System Demyelination
Anxiety / Depression
Neuropsychiatric Disorders
(*schizophrenia, bipolar, autism*)
Developmental Delays
Poor endogenous detoxification

AJCN(2012)96:80-9 Nutr Rev(2014)7255-64 J Neurol Sci(2012)312:26-30 Neurol(2013)81:1298-1307 AJCN(2007)86:610-17


Methionine
Protein Synthesis
"Long route"
THF
"Short route"
Liver, kidney
ATP
Mg
Methylation
SAM
MTases
DNA, RNA, histones, protein, neurotransmitters, Phospholipids (PE, PC), creatine, carnitine
MTRR
MTR
BHMT
Betaine
Zn, B-12
SAH
Adenosine
5-CH3THF
Homocysteine
MTHFR
SAM
SAM
B-6, Zn
Cystathionine
Transsulfuration
B-6
Cysteine
5,10-methyleneTHF
"Folate"
ATP
Mg, K
Glutathione, taurine, sulfate
Transmethylation
"Methionine Cycle"



Insufficient Folate, B-12, B-6, Betaine, Zn, Mg (B-2, B-3)

Clin Obstet Gynecol Reprod Med(2017)3:1-5 doi: 10.15761/COGRM.1000204
Mol Aspects Med(2009)30:42-59 AJCN(2004)80:539-49 Molec Psych(2004)9:358-70

Assessment of Methionine Metabolism/Methylation

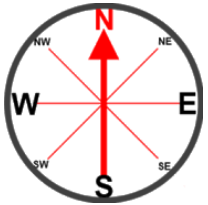
An integrated metabolic profile reflecting the influence of ***genetic and epigenetic*** factors on methionine metabolism / methylation potential *Jill S. James, PhD*

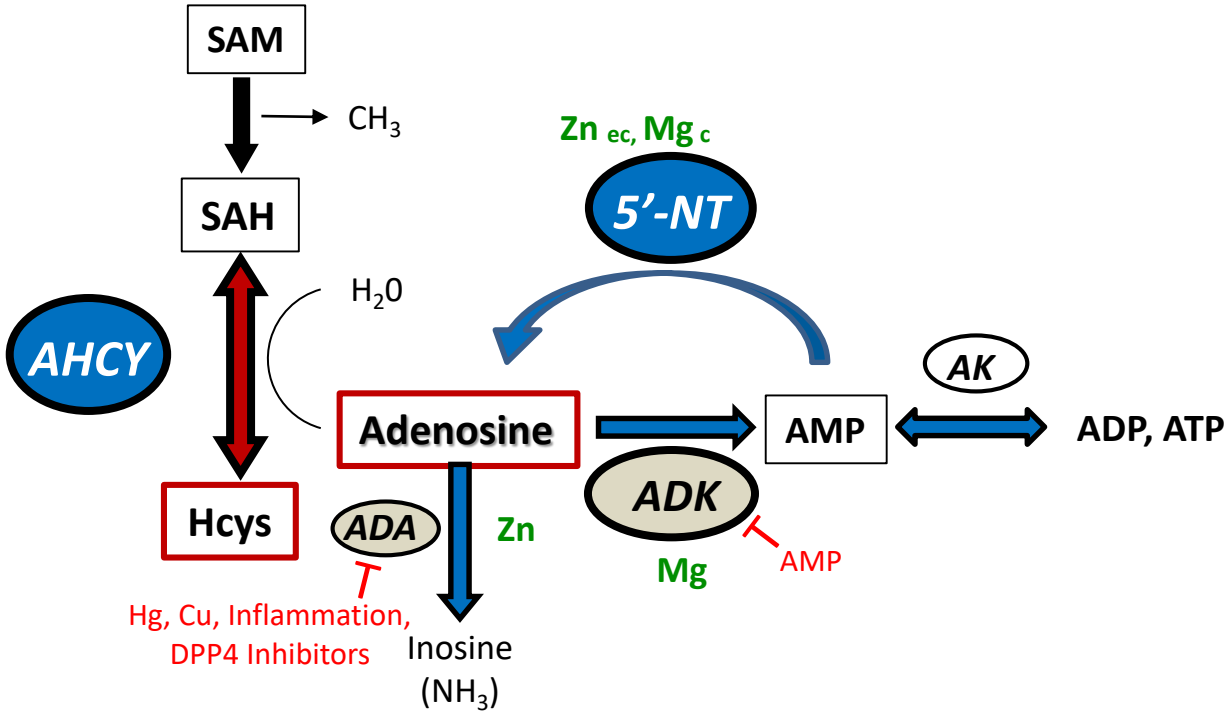
Methylation Profile; plasma

PRIMARY & INTERMEDIATE METABOLITES				
	RESULT/UNIT		REFERENCE INTERVAL	PERCENTILE 2.5th 16th 50th 84th 97.5th
Methionine	**2.6**	µmol/dL	1.6 - 3.6	
Cysteine	**22**	µmol/dL	20 - 38	
S-adenosylmethionine (SAM)	**91**	nmol/L	86 - 145	
S-adenosylhomocysteine (SAH)	**34.5**	nmol/L	10 - 22	
Adenosine *	**103**	nmol/L	20 - 80	
				68th 95th
Homocysteine	**5.9**	µmol/L	< 11	
Cystathionine	**0.01**	µmol/dL	< 0.05	

METHYLATION INDEX			
	RESULT	REFERENCE INTERVAL	PERCENTILE 68th 95th
SAM : SAH	**2.6**	> 4	

Forced To Go North: High Adenosine





ADK; adenosine kinase, ADA; adenosine deaminase, AK; adenylate kinase, 5'NT; 5'-nucleotidase

Immunol Rev(2017)276:121-44 Nature Comm(2017)8943 doi:10.1111/imr.12528 Pharm Rev(2013)65:906-43

Why Might Plasma Adenosine be Elevated?

- May be associated with-
  - Bacterial infection (LPS with GI permeability)
  - Asthma, COPD, sleep apnea (hypoxia)
  - Sleep deprivation
  - Insulin resistance / T2DM / Metabolic syndrome
  - Inflammation (proinflammatory cytokines TNF-α, IL-1β) e.g. Rheumatoid arthritis
  - Oxidative stress
  - Caffeine intake sufficient to promote oxidative stress- receptor antagonist *(>5 mg/kg)*
  - Chronic excessive *fructose* or *alcohol* intake
  - Mg or Zn deficiency
  - Tissue trauma, toxicants
- Medications- (DPP4 inhibitors), **diazepam**, theophylline, pentostatin, dipyridamole, cyclosporine

Purinergic Signaling(2014)10:51-70 Biomed Res(2014)25:489-93 Pharmcol Rev(2013)65:906-43 Ind J Clin Biochem(2013)28:52-4
Neuropharmacol(2013)68:116-21 Shock(2010)34:10-16 J Immunol(2010)65:1993-98 Arthritis Res(2011)13:R197

Synthetic Folic Acid Plus Impaired Transsulfuration

62 yom with Parkinson's disease patient with very poor diet.
Given **800 ug folic acid/day** for elevated hcy and **1,500 mg/day N-AC** for low RBC GSH

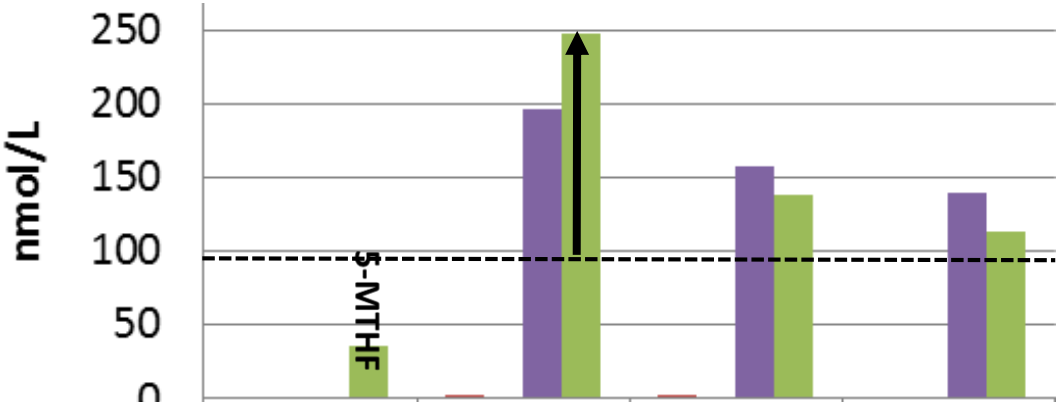
Methylation Profile; plasma

PRIMARY & INTERMEDIATE METABOLITES	RESULT/UNIT	REFERENCE INTERVAL	PERCENTILE (2.5th, 16th, 50th, 84th, 97.5th)
Methionine	2.3 µmol/dL	1.6 - 3.6	
Cysteine	39 µmol/dL	20 - 38	
S-adenosylmethionine (SAM)	124 nmol/L	86 - 145	
S-adenosylhomocysteine (SAH)	37.4 nmol/L	10 - 22	
Adenosine	47 nmol/L	20 - 80	
			68th, 95th
Homocysteine	16.2 µmol/L	< 11	
Cystathionine	0.09 µmol/dL	< 0.05	

METHYLATION INDEX	RESULT	REFERENCE INTERVAL	PERCENTILE (68th, 95th)
SAM : SAH	3.3	> 4	

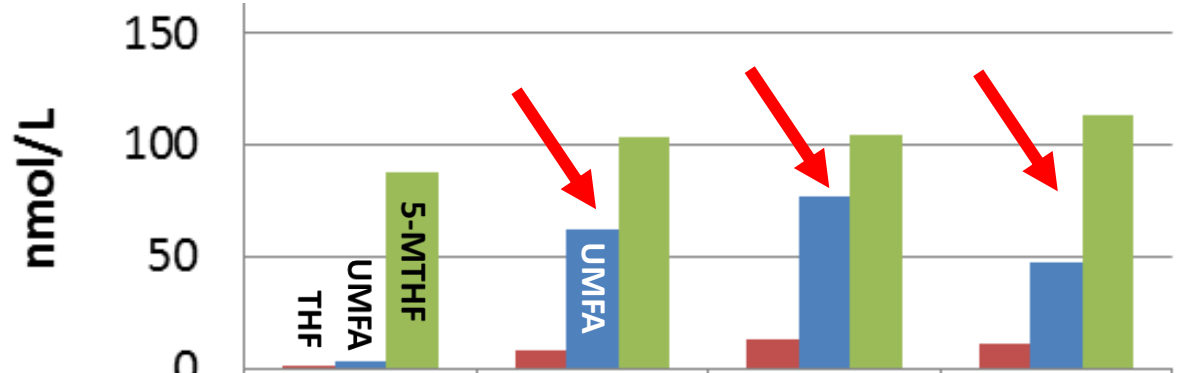
Intervention- **stop synthetic folic acid**, ↑uncooked green leafy greens, MeB-12, folinic acid or 5-MTHF, MeB-12, B-6, and Betaine (hcys)

*Response to 800 µg **Folinic Acid** (MTHFR C677C)*



	1100	1200	1300	1400	
Tetrahydrofolate	0.69	2.80	2.01	1.76	
Unmodified Folic Acid	1.16	1.36	1.12	1.27	< 2 (95th)
Folinic Acid	0.14	196.4	158.0	138.9	< 1
5-MTHF	35.3	247.5	138.1	112.9	8 - 72

*Response to 800 µg **Synthetic Folic Acid** (MTHFR C677C)*



	830	930	1030	1130	
Tetrahydrofolate	0.78	8.53	12.76	10.67	
Unmodified Folic Acid	3.60	61.96	76.91	47.86	< 2 (95th) < 44 (97.5th)
5-MTHF	88.0	103.6	104.3	113.5	8 - 72

Why Not folic Acid -"UMFA Syndrome"

- **All forms of "folate" must be *reduced* before conversion to active folate derivatives (1 or 2 steps to DHF and THF)**
- *Normal* Dihydrofolate reductase (DHFR) activity is low ("sluggish")
- Excess intake of synthetic folic acid (> about 200 µg/day) exceeds the enzymatic capacity of DHFR, *inhibits* DHFR, and blocks folate receptors and transporters;

"P*seudo MTHFR deficiency*"

- UMFA was detected in *fasted plasma* after supplementation with 400 µg/day (humans), and in about 40% of Americans > 1 year of age. (NHANES 2007/2008, n = 2,707)
- High UMFA was detected in pregnant women and cord blood (Canada)
- High levels of UMFA decrease the anti-viral/tumor activity of NK cells(rats)*
- High levels of UMFA *may* promote the growth of existing cancers, and has been associated with increased cancer risks (especially colorectal and prostate)

Clin Obsts Gynacol Reprod Med(2017) doi:10.15761/COGRM BMC Pub Hlth(2007)7:41 AJCN(2015)101:646-658 Cancer Epidemiol Biomarkers Prev(2008)17: 2220-25 *J Nutr Biochem(2016)30:102-107 J Nutr(2014) doi:10.3945jn.114.201210 AJCN(2015) doi:10.3945/ajcn.115.110783

Status Folate Derivatives and UMFA
(over night fast)

Folate Metabolism Profile; plasma

	RESULT / UNIT	REFERENCE INTERVAL	PERCENTILE 2.5th 16th 50th 84th 97.5th / 95th 97.5th
5-Methyltetrahydrofolate (5-MTHF)	**36** nmol/L	20– 66	
Folic Acid, unmodified **UMFA**	4.4 nmol/L	< 2.0	
Folinic Acid	**0.40** nmol/L	< 1.00	
Tetrahydrofolate (THF)	**0.41** nmol/L	< 1.00	

MTHFR wild type, good diet, but two supplements containing folic acid (400 µg/day)

Permeability of the Epithelium

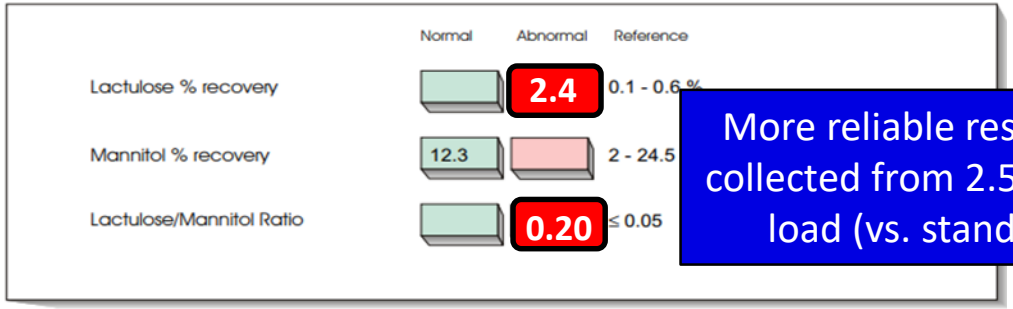
- *Dynamic, interconnected protein complexes* that regulate paracellular influx of **pro-inflammatory / antigenic macromolecules** for all epithelial cells *(GI, heart, brain).*
- Zonulin is the only known **physiological reversible modulator** of intercellular tight junctions.
- *Transient reversible* opening- clinically insignificant; "primes immune system"
- ***Sustained*** high serum zonulin levels occur with **autoimmune diseases**; Celiac (and NCGS), Crohn's, RA, T1DM, Lupus, **cancers**; gliomas, breast, ovarian, pancreatic, **neurological**; demyelinating polyneuropathy, MS, schizophrenia, *and NAFLD/NASH, asthma, and insulin resistance*

Int J Mol Sci(2017)18:582 Prac Lab Med(2017)9:39-44
World J Gastroenterol(2014)20:17107-17114 Transl Sci(2014)6:263ra158 Ann NYAS(2012)1258:25-33
Cell Microbiol(2010)12:31-41 & 654-64 Gastroenterol(2002)123:1607-15

Assessment Of Intestinal Permeability

	RESULT / UNIT	REFERENCE INTERVAL	LOW MOD HIGH
Zonulin*	70.6 ng/mL	< 45.0	

- ↑ **serum zonulin** antigen levels indicate increased GI epithelial permeability.
  - Highly correlated with **high urine lactulose to mannitol ratios** (L:M) and ↓TEER
- Similar percentage of high values for **serum zonulin (14.5%)** and **L:M (15.5%)** for suspect patients (2016-2018, Maggiore & Quig unpublished observations)



More reliable results when urine was collected from 2.5 - 4.0 hrs. post 15 gm load (vs. standard 6 hrs., n=40)*

World J Gastroenterol(2014) 20:17107-14 J Gastroenterol Motil(2015)21:33-50 Prac Lab Med(2017)9:39-44 Ann NYAS(2012)1258:25-33
Clin Gastroenterol Hepatol(2012)10:1096-1100 *PLOS ONE(2014) DOI:10.1371/journal.pone.0141399 Proc Lab Med(2017)9:39-44

Clinical Intervention to Restore the EC Barrier

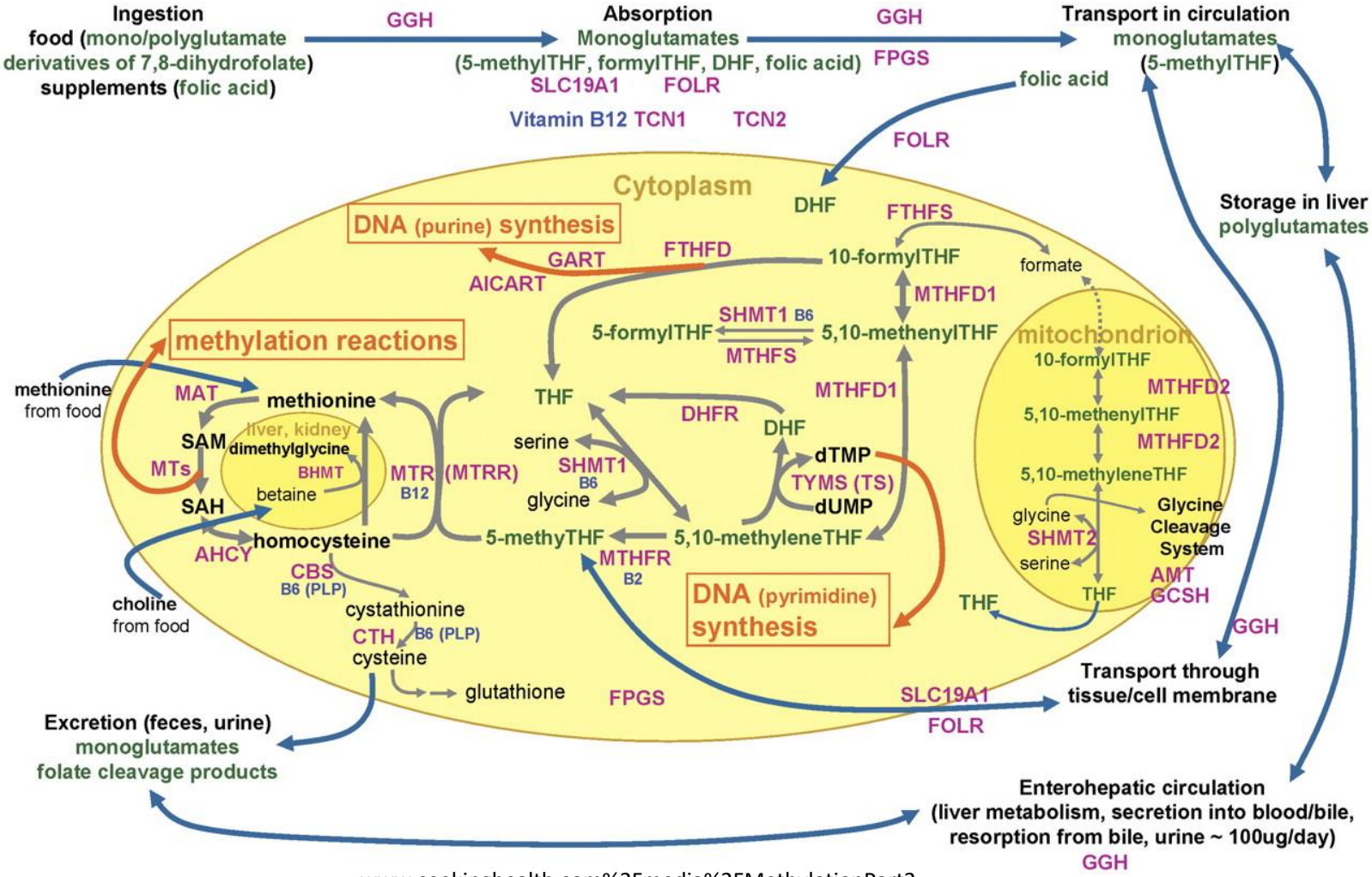
- **Eliminate triggers**
  - Gliadin *(related prolamines)*
  - Inflammation
  - Direct adherence of ***any*** bacteria to endothelial cells
  - Bacterial enterotoxins / proteases
  - Dietary protein fragments
  - Insulin resistance / Metabolic syndrome
  - Crohn's disease
  - Smoking (tobacco)

Proc Lab Med(2017)9:39-44 Int J Mol Sci(2017)18582 Ann NYAS(2012)1258:25-33
Clin Gastroenterol Hepatol(2012)10:1096-1100 Autoimun Rev(2015)14:479-89

Support Expression of Tight Junction Proteins

- Specific probiotics*
- Prebiotics and butyrate (from chickpeas, oatmeal, berries)
- Inulin** (DBPC human study- 11 gm/day, 5 days)
- Glutamine
- Curcumin
- Vitamin D_3 and retinol
- Quercetin, genistein
- γ- linoleic acid
- **Chitosan and ethanol *decrease* expression of tight junction proteins**

*BMC Gastroenterol(2014)14:189 Front Immunol(2015) doi: 10.3389/frimmu.2015.00612n Am J Clin Nutr (2013)97:117–26
**J Nutr(2012)940-46 Sci Transla Med(2014)6:263ra158



www.seekinghealth.com%2Fmedia%2FMethylationPart2.