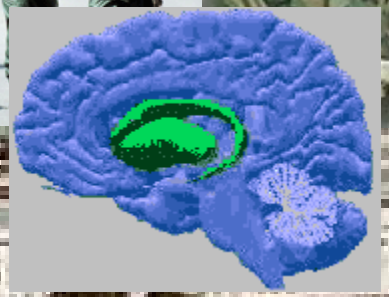
PON1 Q192R, Nerve Agent and Gulf War Illness:

The Power of Gene-Environment Interaction to Establish Causation



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Sources of Support

- U.S. Army Medical Research and Materiel Command grant number DAMD17-01-1-0741 (PI: R. Haley)
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Outline of Presentation

- Hypothesis-framing studies
- The Pre-Stated Hypothesis
- The New Study
- What about Recall Bias?
- What about Unmeasured Confounding?
- The Interpretation
- The Accompanying Commentary
- Conclusion

Hypothesis-Framing Studies

1991 Gulf War Environmental Exposures Identified by the Defense Science Board, 1994*

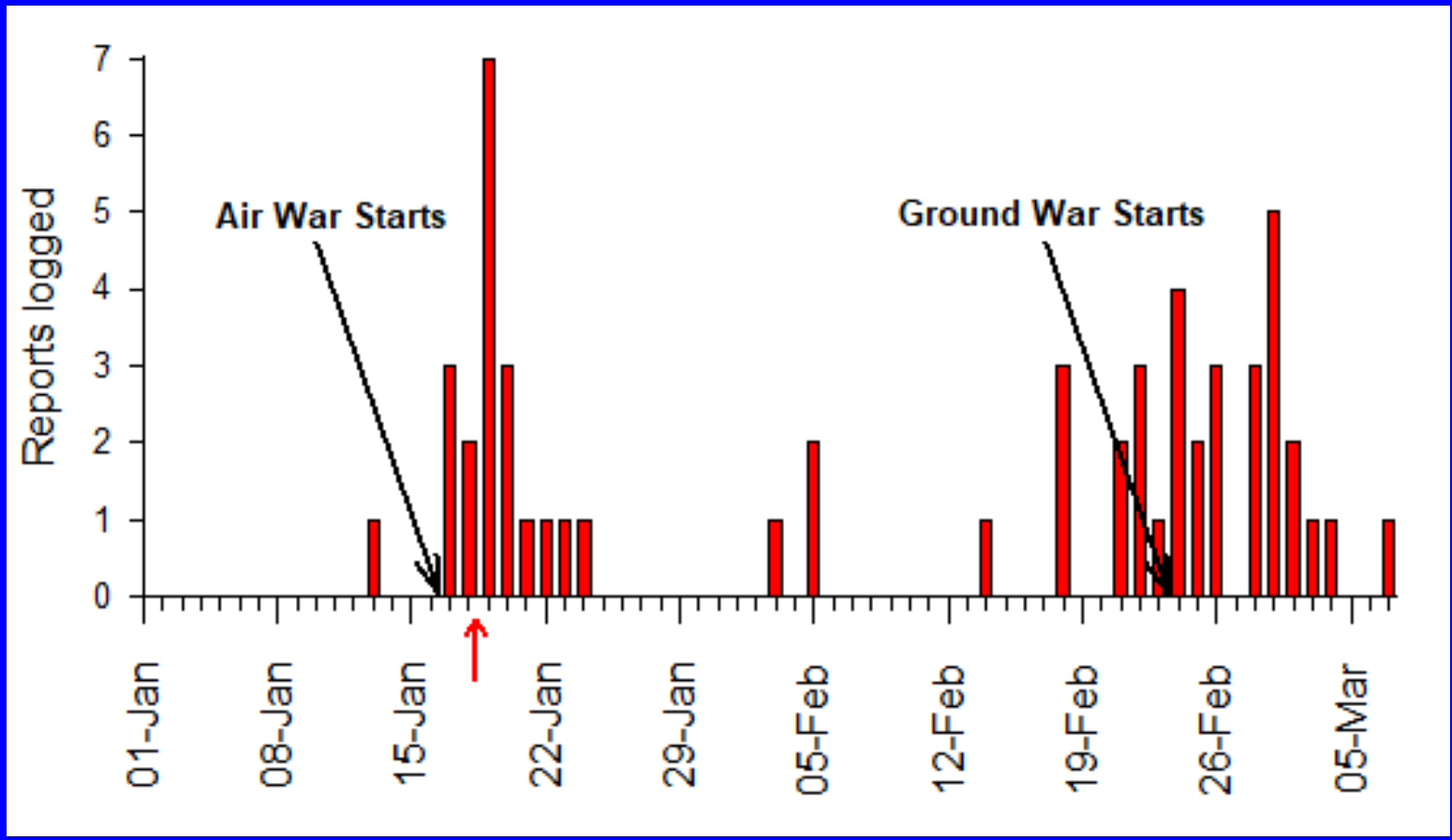
- **OP chemical warfare agents (sarin, cyclosarin)****
- OP pesticide spraying
- OP pesticides on uniforms
- DEET insect repellants
- Pyridostigmine bromide
- Ciprofloxacin
- Chloroquine
- Multiple immunization including anthrax vaccine
- Smoke from oil well fires
- Fumes from jet fuel sprayed on roads
- Fumes from burning jet fuel in tent stoves
- Petroleum in drinking water
- Depleted uranium
- CARC pain
- Combat stress/PTSD (the official explanation in 1994)

*Also by the NIH Consensus Conference 1994; etc.

**Pentagon officially denied that chemical weapons were in theater.

Number of CW Alarms Logged with the NBC Cells of the Central Command, Army Central Command and VII Army Corps During Conflict Period of Gulf War

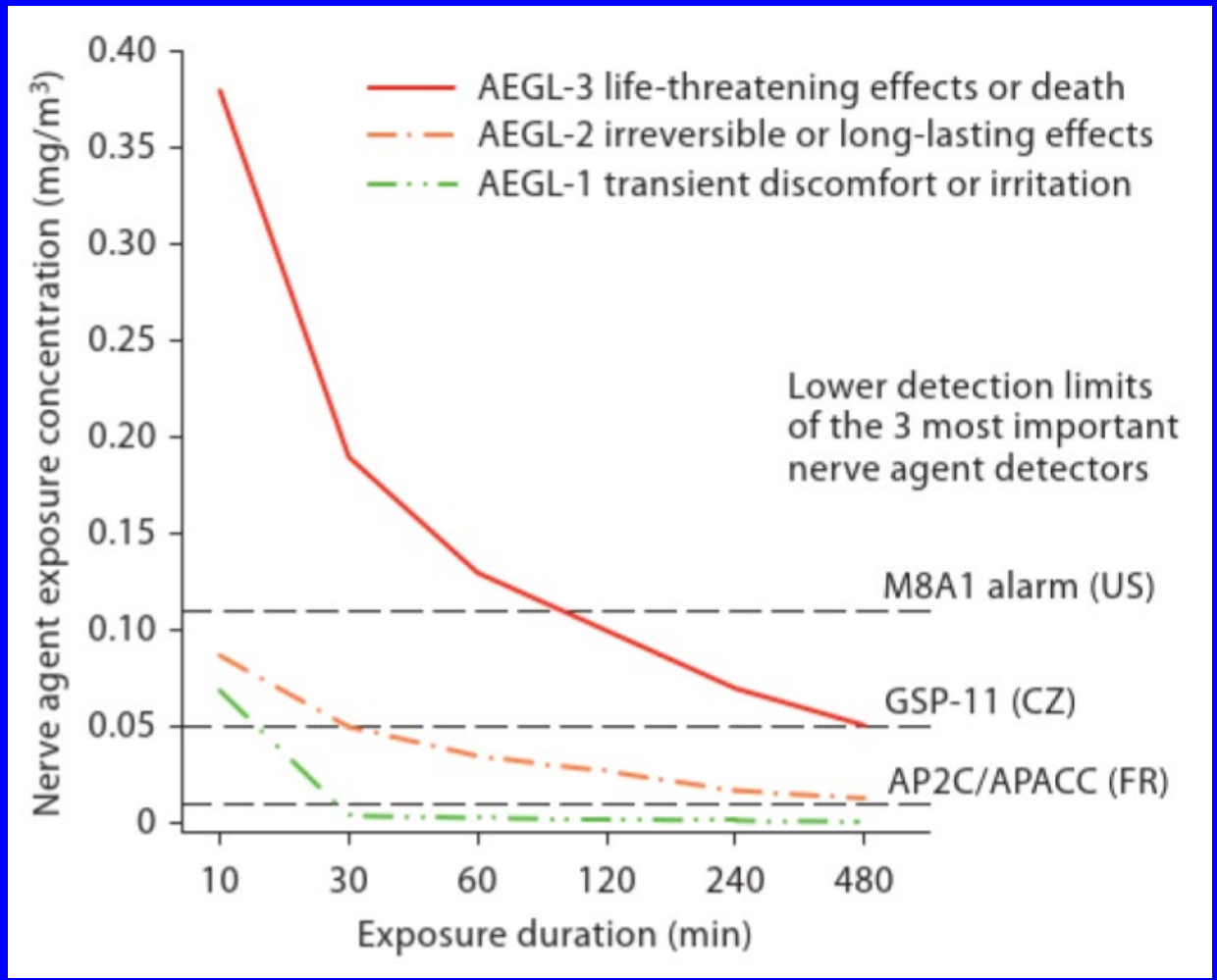
M8A1 organophosphate detector used at the unit level



Tuite, Haley. *Neuroepidemiology* 2013;40:160-177

Detection Threshold of the M8A1 OP Detector is Above EPA's Acute Exposure Guideline Level 2 for Sarin

When soldiers heard alarms, they were being exposed to sarin levels (AEGL-2) sufficient to cause "irreversible or serious long-lasting health effects."*



*National Research Council. *Acute Exposure Guidelines for Selected Airborne Chemicals, Vol 3*. National Academy Press 2012

Tuite, Haley. *Neuroepidemiology* 2013;40:160-177

May 25, 1994

U.S. Senate's "Riegle Report" Details Credible Chemical Weapon Exposures During Gulf War

103d Congress
2d Session

SENATE

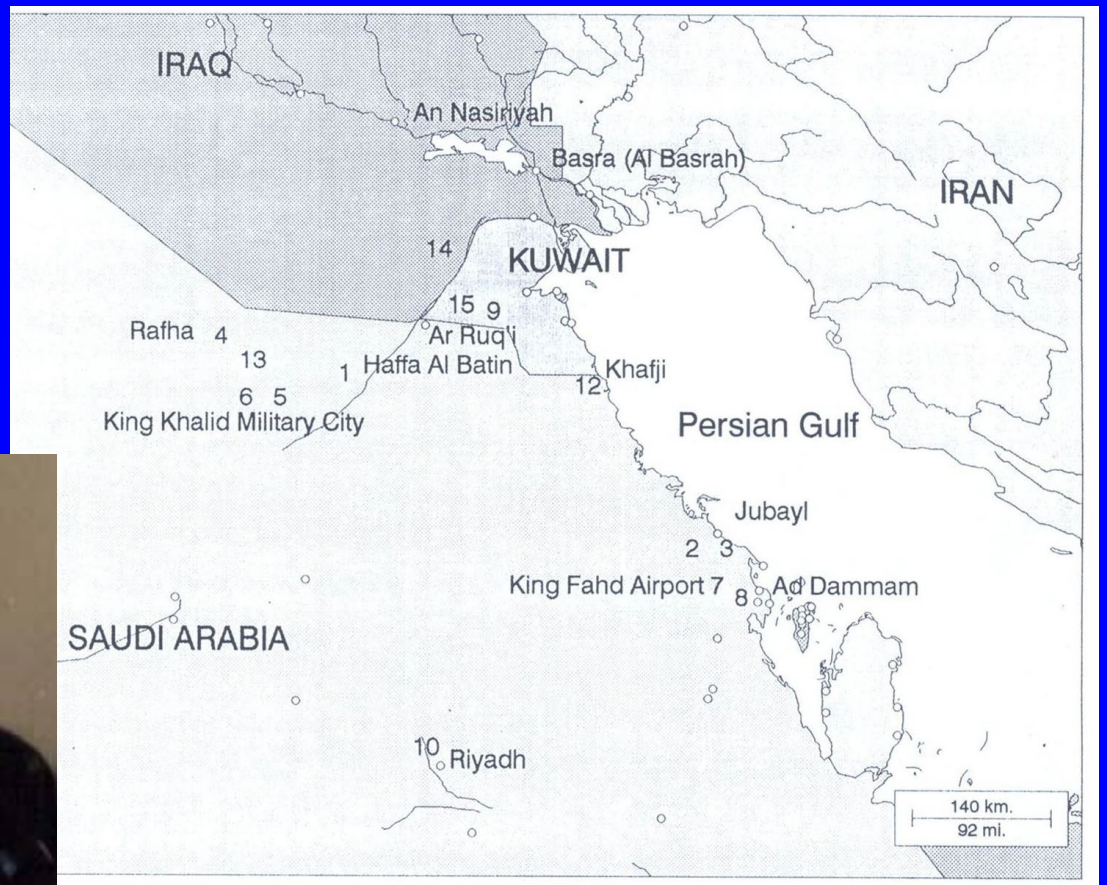
U.S. CHEMICAL AND BIOLOGICAL WARFARE-RELATED DUAL USE EXPORTS TO IRAQ AND THEIR POSSIBLE IMPACT ON THE HEALTH CONSEQUENCES OF THE PERSIAN GULF WAR

A REPORT
OF
CHAIRMAN DONALD W. RIEGLE, JR.
and
RANKING MEMBER ALFONSE M. D'AMATO
OF THE
COMMITTEE ON BANKING, HOUSING
AND URBAN AFFAIRS
WITH RESPECT TO
EXPORT ADMINISTRATION

UNITED STATES SENATE

May 25, 1994

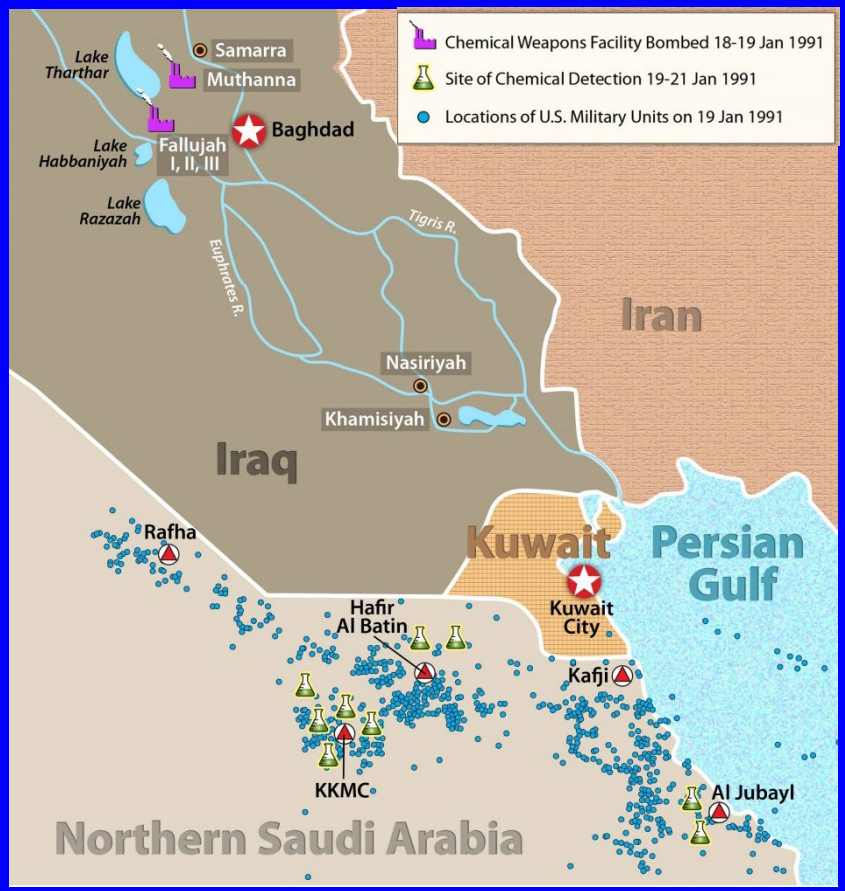
James J. Tuite, III



Pentagon responds with denial of any chemical weapons in theater.

Detections of Sarin Among U.S. Troop Positions

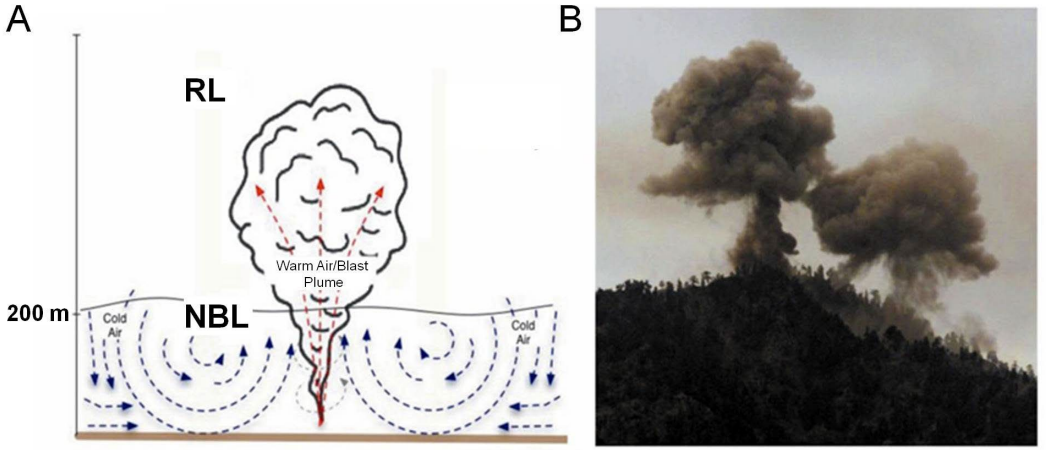
On third night of the Air War **18-19 Jan**, Coalition bombers destroyed chemical weapons storage sites at Muthanna and Fallujah, the next morning 10,000 chemical alarms started sounding and continued intermittently for over a week.

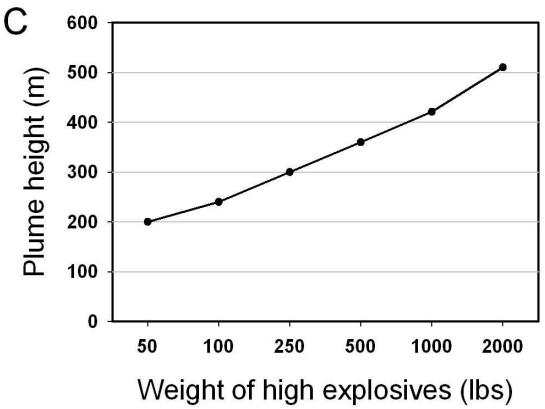


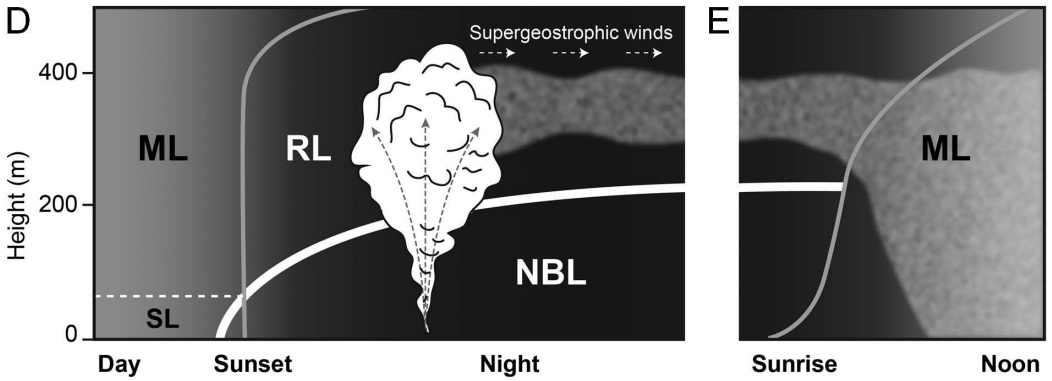
Tuite, Haley. *Neuroepidemiology* 2013; 40: 160-177

Explanation for How Sarin Transited Hundreds of Kilometers from Bombing Sites to U.S. Troop Positions

James J. Tuite, III
Intelligence Expert
Head Staffer for Senator Riegel's
1994 Investigation



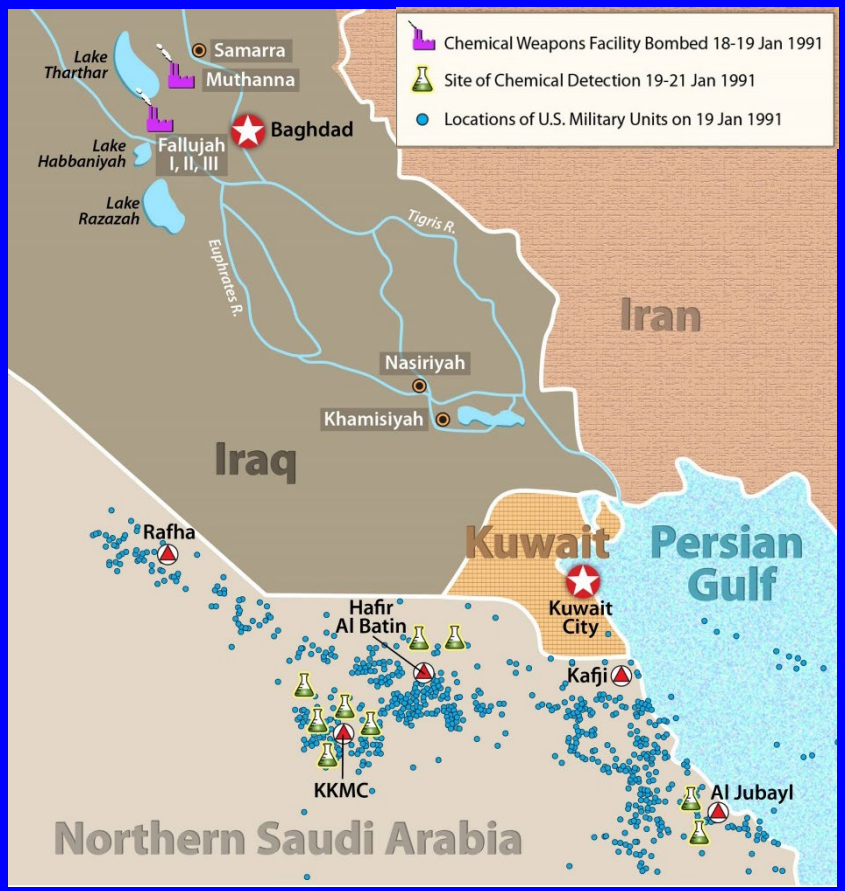


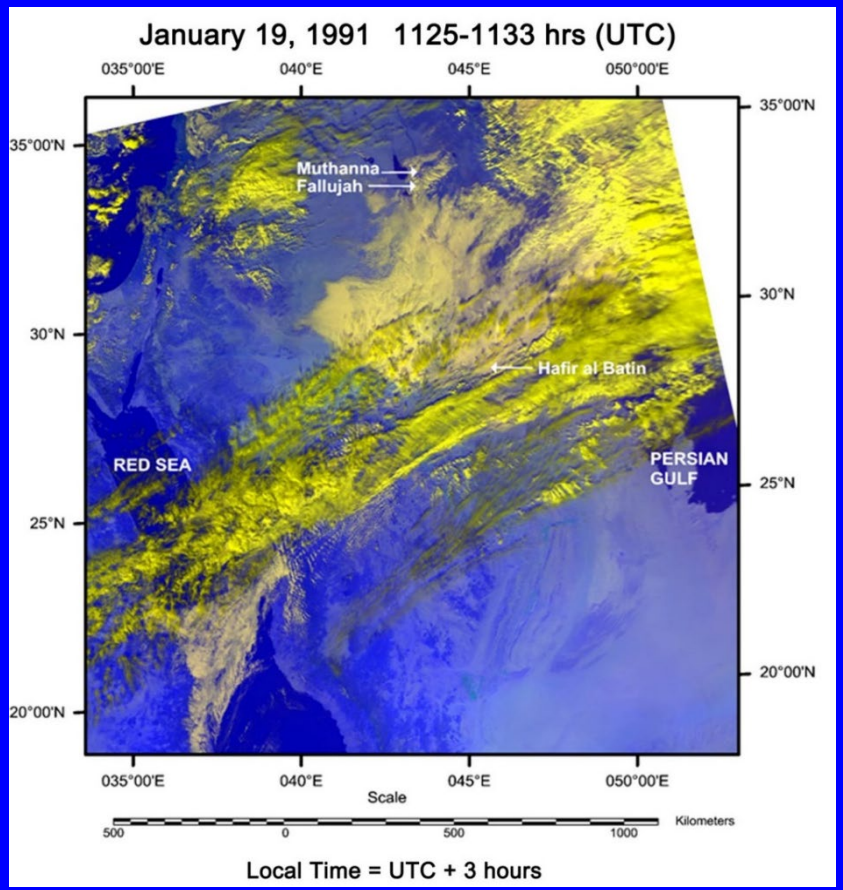


Tuite, Haley. *Neuroepidemiology* 2013; 40: 160-177

Alarms were Due to Low Level Nerve Gas Exposure

On third night of the Air War 18-19 Jan, Coalition bombers destroyed chemical weapons storage sites at Muthanna and Fallujah, the next morning 10,000 chemical alarms started sounding and continued intermittently for over a week.





Tuite, Haley. *Neuroepidemiology* 2013; 40: 160-177

First Multivariable Analysis of Risk Factors For GWI (N=249)

Syndrome	*Exposure*	*RR*	*P value*
1	Wore flea collar (chlorpyrifos)	8.2	.001
Impaired cognition	Military security	6.4	.007
2	Chemical nerve agent exposure	7.8	<.0001
Confusion-ataxia	Many advanced side effects of PB	32.4	<.0001
	N.E. Saudi on 4th day of Air War*	4.3	.004
3	Many advanced side effects of PB	5.1	<.0001
Central pain	Index of DEET insect repellant use	7.8	<.0001

*Paths crossed near Khafji on Jan. 19-20, 1991.

Haley RW, Kurt TL. *JAMA* 1997;277:215-222.

10 of 11 epidemiologic studies that included a nerve agent risk factor found an association with GWI.

Table S18. Methods and results of prior epidemiologic and clinical studies of the association of chemical weapons with GWI.

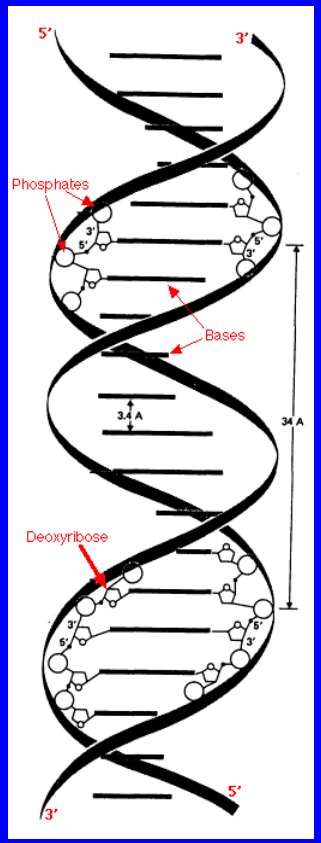
Reference	Ascertainment method	Study design	Reported question	Outcome association
Haley and Kurt 1997[6]	Written questionnaire	Supervised survey of a battalion sample	"experienced likely chemical weapons attack"	PRR 7.8 (2.3-25.9) for GWI (syndrome 2)
Nisenbaum et al. 2000[7]	Written questionnaire	Study of an Air National Guard unit and airmen at 3 U.S. Air Force bases	"belief that biological or chemical weapons were being used against them"	OR 6.05 (3.43-19.68 for severe GWI; 2.52 (1.83-3.48) for mild-moderate GWI
White et al. 2001[8]	Written questionnaire	Supervised survey and neuropsycho-logical testing and interviews of 3 cohorts: 2 Gulf-deployed and 1 deployed to Germany	"poison gas or germ warfare"	Neuropsychological measures of mood, memory, and attention/executive function, P<0.05
Kang et al. 2002[9]	Mailed questionnaire survey	Mailed survey of random sample of GWV population	Checklist of exposures: "Nerve gas"	RR 9.17 (7.69-10.93) for GWI (4 most typical symptoms)
Lindem et al. 2003[10]	Written questionnaire	Supervised survey and neuropsycho-logical testing and interviews in a subset from the White et al. study	Checklist: "Chemical or biological warfare agents"	Neuropsychological measures of attention, executive function, and memory, p<0.01
Proctor et al. 2006[11]	Khamisiyah computer exposure plume model	Supervised survey and neuropsycho-logical testing and interviews in a subset from the White et al. study	Not applicable (nerve agent exposure estimated by unit location in computer-modeled atmospheric dispersion from demolition of ammunition depot)	Neuropsychological measures of psychomotor function and visuospatial abilities, P<0.01
Heaton et al. 2007[12]	Khamisiyah computer exposure plume model	Volumetric analysis of brain MRI in GWI cases and controls from White et al. study	Not applicable	White matter and brain volume reduction associated with estimated sarin/cyclo-sarin exposure
Steele et al. 2012[13]	Telephone interview questionnaire	Cases and controls recruited from Kansas GW veterans	"Heard chemical alarms sounded"	OR 1.31 (0.83-2.07) for GWI
Haley and Tuite 2013[14]	CATI questionnaire telephone interview	National telephone interview survey (USMHS) of a random sample of 1991 U.S. military population	"Did the alarms on the chemical warfare detection devices in areas where you were living or working ever go off while you were present there?" if yes, "on how many days . . ."	aOR 4.13 (2.51-6.80) Trend test p<0.001 for overall GWI
Chao et al. 2010,2011, 2014,2015,2016,2018[15-20]	Written questionnaire	Volunteer GW veterans recruited by public ads in Northern California	"Did you hear chemical alarms sound?" If yes, "How many days did you hear chemical alarms?"	Various measures of abnormal brain structure and function and white matter integrity in those who recalled hearing alarms
Barth et al. 2017[21]	Khamisiyah computer exposure plume model	National random sample survey	Not applicable	aRR for brain cancer 2.71 (1.25-5.87)

15 studies identified mechanisms by which low-level, subclinical sarin (or DFP) exposure causes chronic cellular pathology with behavioral changes resembling GWI.

Table S19. Prior studies identifying biochemical mechanisms by which low-level subclinical sarin exposure similar to that experienced in the 1991 Persian Gulf War causes chronic cellular pathology with behavioral changes resembling GWI.

Reference(s)	Experimental model	Finding
Spiegelberg 1961[22]	Hypothesis-raising clinical description	Description of a previously unsuspected chronic encephalopathic symptoms similar to GWI in workers who had repetitive subclinical sarin exposures in German nerve agent factories during World War II.
Duffy et al. 1979[23]	Hypothesis-raising clinical description	Description of a previously unsuspected chronic encephalopathic symptoms similar to GWI in workers who had repetitive subclinical sarin exposures in U.S. nerve agent factories during the Cold War, associated with unusual EEG changes.
Burchfiel et al. 1976, 1982[24,25]	Laboratory experiments	Administration of subclinical doses of sarin to Rhesus monkeys (1 µg/kg i.m. weekly x 10) produced chronic electroencephalographic (EEG) changes similar to those reported in the Duffy et al. study.
Henderson et al. 2001, 2002[26,27]	Laboratory experiments	Inhalation administration of subclinical doses of sarin to rats (0, 0.2, or 0.4 mg/m^3 of sarin for 1 h/day for 1, 5, or 10 days; follow-up at 30 d) produced persistent alteration in the numbers of muscarinic cholinergic M1 and M3 receptors in cortical and hippocampal brain regions, compatible with cognitive dysfunction.
Kassa et al. 2001,2001[28,29]	Laboratory experiments	Inhalation administration of subclinical doses of sarin to rats (1.25 µg/L x 3 over 7 d; follow-up at 3 mo) resulted in increased CNS excitability and impaired gait and mobility, memory and cognitive behavior and altered immune function.
Scremin et al. 2003[30]	Laboratory experiments	Administration of subclinical doses of sarin to rats (62.5 µg/kg [0.5 LD_{50}] s.c. 3x per wk x 3 wks; follow-up at 16 wks) altered behavioral measures associated with down-regulation of muscarinic receptors in hippocampus, caudate putamen, and mesencephalon, not seen after PB alone or PB plus sarin.
Pena-Phillippides et al. 2007[31]	Laboratory experiments	Inhalation administration of subclinical doses of sarin to rats (0.4 mg/m^3/day x 5d; follow-up at 2-4 wks) suppressed serum corticosterone and ACTH levels.
Van Helden et al. 2003,2004[32,33]	Laboratory experiments	Inhalation administration of sarin vapor to marmosets at concentration-time doses below the dose producing miosis or detectable by military field devices (≤150 µg/m^3 for 5 h; follow-up at 1 yr) produced persisting EEG changes like those reported by Duffy and Burchfiel (above) that increased in severity over time.
Mach et al.2008[34]	Laboratory experiments	Administration of subclinical doses of sarin (64 µg/kg [0.4 LD_{50}] s.c. daily x 3; follow-up at 21 d) with shaker stress to rats produced delayed behavioral change and catecholamine depletion in adrenal glands, suggesting autonomic dysfunction.
Morris et al.2007[35]	Laboratory experiments	Administration of subclinical doses of sarin to mice (8 µg/kg [0.05 LD_{50}] s.c. on 2 consecutive days; follow-up at 10 wks) produced delayed chronic reduction in high frequency heart rate variability and increased tyrosine hydroxylase mRNA in locus coeruleus and dorsal vagal complex of brain, indicating abnormal central autonomic activity similar to that in GWI.[36,37]
Shewale et al. 2012[38]	Laboratory experiments	Administration of subclinical doses of sarin to mice (64 µg/kg [0.4 LD_{50}] s.c. on 2 consecutive days; follow-up at 8-12 wks) produced reduced cardiac responsive-ness to beta-adrenergic stimulation, reduced adrenal tyrosine hydroxylase mRNA, corticosterone, and stress response in HPA axis indicating autonomic impairment.
Oswal et al. 2013[39]	Laboratory experiments	Administration of subclinical doses of sarin to mice (64 µg/kg [0.4 LD_{50}] s.c. on 2 consecutive days; follow-up at 4-8 wks) produced alterations in dopamine turnover in the frontal cerebral cortex, amygdala and caudate nuclei of the brain capable of mediating long-term behavioral and neuropsychological changes.
O'Callahgan et al. 2015; Ashbrook et al. 2018; Belgrad et al. 2019; Michalovicz et al. 2020[40-43]	Laboratory experiments	Administration of corticosterone in drinking water daily x 5 or 7 d followed by sarin surrogate DFP (diisopropyl fluorophosphate, 1.5 mg/kg s.c.) initiated chronic neuroinflammation in the brains of mice with adverse effects on oligodendrocytes and epigenetic modification of genes related to the brain's immunologic and cognitive systems.
Alshelh et al. 2020[44]	Clinical study	Neuroinflammation was recently demonstrated in veterans with GWI by in vivo positron-emission-tomography (PET) imaging of the brain.
Deshpande et al. 2010, 2016, 2018, 2020[45-48]	Laboratory experiments	Administration of a subclinical dose of DFP to rats (0.5 mg/kg daily s.c. x 5d; follow-up at 3-6 mo) was followed by behavioral abnormalities analogous to chronic depression, anxiety and memory impairment as well as hippocampal neuronal damage leading to a chronic elevation of intracellular calcium concentration, all largely corrected by 2 previously FDA-approved drugs.

Genetic Predisposition to Sarin Toxicity: *Paraoxonase-1 (PON1)* Q192R and Isoenzyme Assay



Dr. Bert La Du
U. of Michigan
"Father of PON Biochemistry"

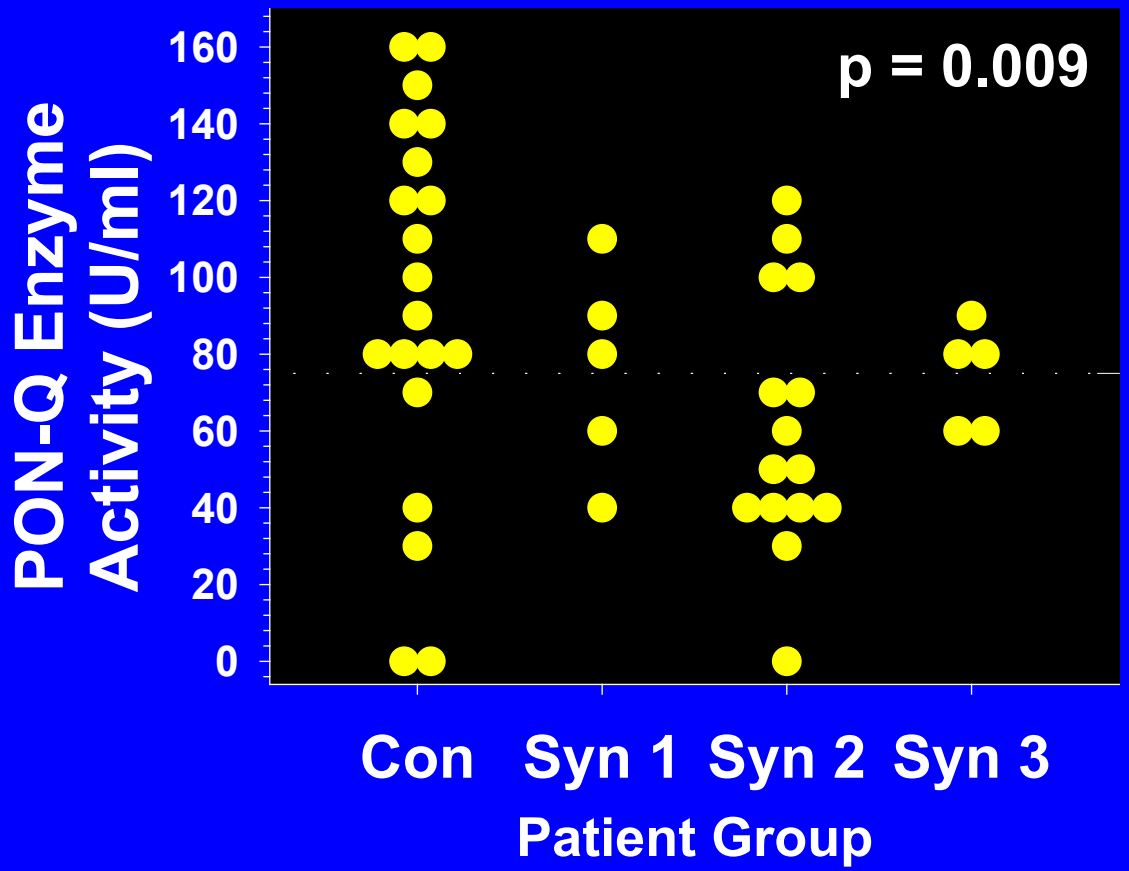
PON1 Q192R Substrate Specificity

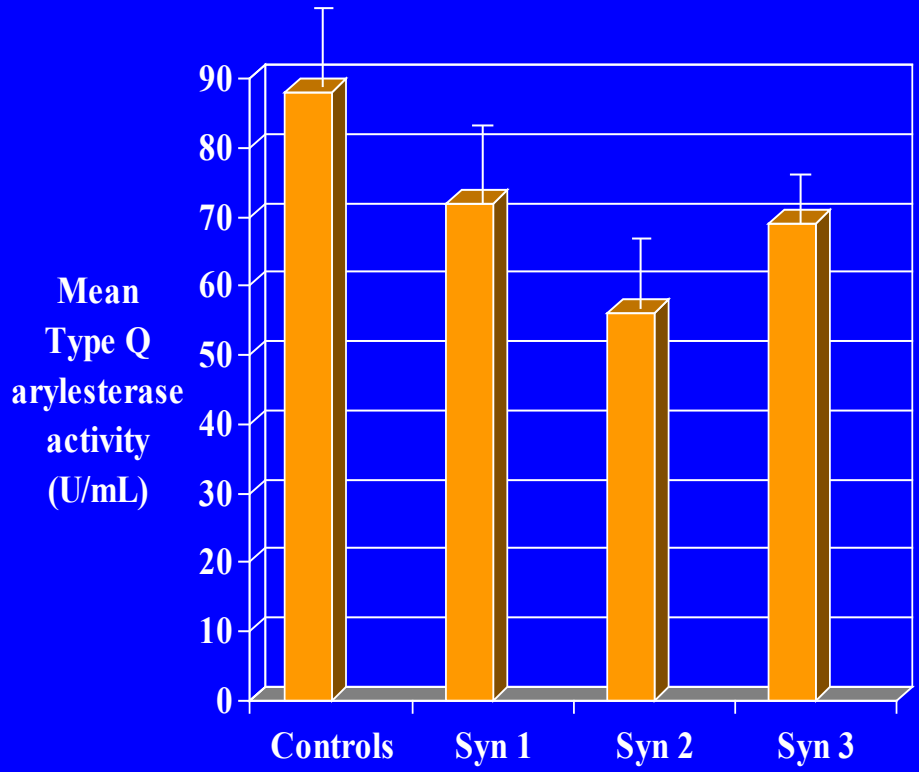
- The PON1 gene directs production of the PON1 family of serum isoenzymes that hydrolyze:
  - OP pesticides (parathion, diazinon, chlorpyrifos, etc.)
  - OP warfare nerve agents (sarin, tabun, soman, VX, Novichok)

- The Q192R polymorphism strongly affects the hydrolytic efficiency for the different substrates.
  - The Q isoenzyme efficiently hydrolyzes nerve agents.
  - The R isoenzyme efficiently hydrolyzes pesticides.

- Q192R provides a natural experiment to differentiate etiologies.
  - GWI associated with having 192R allele (low Q isoenzyme) supports nerve agent.
  - GWI associated with having 192Q allele (high Q isoenzyme) supports pesticides.

Lower PON1 Type Q Isoenzyme Levels in Blood of Ill Gulf War Veterans than Controls (N=43)





Haley RW, Billecke S, La Du BN. *Toxicol Appl Pharmacol* 1999; 157: 227-233

5 experimental studies established that the PON1 192Q isoenzyme protects from neurotoxic effects of low-level sarin

Table S20. Prior experimental evidence establishing that the PON1 Q192R type Q isoenzyme activity is the property of the *PON1* gene that best protects the brain from the neurotoxic effects of low-level sarin nerve agent.

Reference(s)	Experimental model	Finding
Davies et al. 1996[50]	In vitro assays	From assays of the rate of hydrolysis of sarin by the plasma from 93 human volunteers, plasma from *PON1* QQ homozygotes had a mean hydrolysis rate of sarin 9.3 times that of RR homozygotes.
La Du et al. 2001[51]	In vitro assays	Sera from 25 veterans with GWI and 20 well control veterans were assayed for rate of hydrolysis of sarin (sarinase activity) as well as serum hydrolytic activity of the PON1 Q and R isoenzymes. Sarinase activity was correlated with Q isoenzyme activity but not with R isoenzyme activity. The catalytic efficiency of the purified Q isoenzyme with sarin was over 4-fold greater than with the R isoenzymes. This study is particularly relevant because it shows that the Q isoenzyme can effectively hydrolyze sarin in blood at the low physiologic concentrations expected with low-level sub-symptomatic sarin exposure.
Kanamori-Kataoka and Seto 2009[52]	In vitro assays	The maximum rate of hydrolysis of sarin with purified PON1 Q and R isoenzymes from plasma of 63 civilian volunteers was 3.5 times greater with the Q isoenzyme than with the R isoenzyme, confirming the finding of Davies et al.
Valiyaveetti et al. 2010[53]	In vitro assays	Acetylcholinesterase (AChE) is exceptionally sensitive to inhibition by sarin nerve agent and considered its primary target. In a series of vitro assays, purified human PON1 type Q isoenzyme, at physiological concentrations present in blood, was shown to potently prevent inhibition of AChE by sub-micromolar concentrations of sarin.
Valiyaveetti et al. 2011,2011[54,55]	In vivo experiments	Intravenous treatment of guinea pigs with purified human PON1 type Q isoenzyme significantly increased survival, reduced physiologic signs of nerve agent exposure, and attenuated brain AChE inhibition after microinstillation inhalation exposure to 1.2 x LC_{50} of sarin.

Conclusion: The above experimental research has established that the *PON1* Q192R is a gene that biologically modifies the pathological effects of organophosphate exposure and is not merely serving as a proxy marker.[49]

The Pre-Stated Hypothesis

Pre-stated Hypothesis:

If GWI was caused by low-level sarin, it will be associated with a gene-environment (*G*x*E*) interaction between *G* having the *PON1* 192R allele (low 192Q isoenzyme) and *E* having heard nerve agent alarms in the war.

Note: The PON1 enzyme was named for its ability to hydrolyze paraoxon ("paraoxonase"), but this is a property of the 192R isoenzyme and thus does not hydrolyze sarin efficiently.

Pre-stated Hypothesis:

If GWI was caused by low-level sarin, it will be associated with a gene-environment (*GxE*) interaction between *G* having the *PON1* 192R allele (low 192Q isoenzyme) and *E* having heard nerve agent alarms in the war.

Hypothetical logistic regression model of GWI*

Variable	*LR coef*	*OR*	*P*
Heard nerve agent alarms (N=0/Y=1)	1.1094	3.03	<0.0001
PON1 genotype (QQ=0/RR=1)	0.0402	1.04	0.92
Interaction (GxE)	1.2267	3.41	0.001

**Adjusted by the confounding variables: age, sex, rank, active duty/reserve, service branch, and combat exposure scale.*

The New Study

Research

A Section 508–conformant HTML version of this article
is available at https://doi.org/10.1289/EHP9009.

Evaluation of a Gene–Environment Interaction of *PON1* and Low-Level Nerve Agent Exposure with Gulf War Illness: A Prevalence Case–Control Study Drawn from the U.S. Military Health Survey's National Population Sample

Robert W. Haley,[1] *Gerald Kramer,*[1] *Junhui Xiao,*[1] *Jill A. Dever,*[2] *and John F. Teiber*[1]

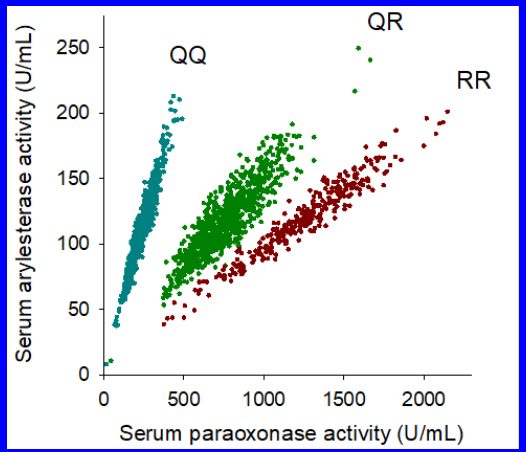
[1]Division of Epidemiology, Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, Texas, USA
[2]RTI International, Washington, District of Columbia, USA

U.S. Military Health Survey, 2007-2009

- **RTI International** selected a stratified random sample of GW-era veterans from 1991 U.S. Military personnel file (DMDC, Seaside, CA)
- Trained RTI interviewers performed computer-assisted telephone interviews of 8,020 veterans.
- Battery of symptom questions included all required to construct the 3 most used GWI case definitions: Original Research, CDC and Modified Kansas.
- The question measuring the environmental exposure of interest:

  "During the time period from August 2, 1990, to July 31, 1991, did the alarms on the chemical warfare detection devices in areas where you were living or working ever go off while you were present there?"

- Collected serum, plasma, DNA and RNA from a nested case-control subsample of all who met any of the case definitions and a random subsample of non-GWI, for a total N = 2,021.
  - Genotyped DNA for the *PON1* Q192R polymorphism
  - Assayed serum for Q and R isoenzyme activity levels



Iannacchione et al. *Neuroepidemiology* 2011; 37: 129-140

Rothman's Solution: 3 Tests for Additive (Biological) Interaction

- RERI
  - Relative Excess Risk due to Interaction
- AP(AB), just AP
  - Attributable Proportion due to interaction
- S
  - Synergy index

K. J. Rothman. Synergy and antagonism in cause-effect relationships. *Am J Epidemiol* 1974; 99(6): 385-388

The Solution: 3 Tests for Additive (Biological) Interaction

- RERI (Relative Excess Risk due to Interaction)

$$RERI = RR(AB) - RR(A\bar{B}) - RR(\bar{A}B) + 1$$

- AP (Attributable Proportion due to interaction)

$$AP(AB) = \frac{RERI}{RR(AB)}$$

- S (Synergy index)

$$S = \frac{RR(AB) - 1}{[RR(A\bar{B}) - 1] + [RR(\bar{A}B) - 1]}$$

	$\bar{A}$	A
$\bar{B}$	1.2	3.7
B	3.2	8.3

K. J. Rothman. Synergy and antagonism in cause-effect relationships. *Am J Epidemiol* 1974; 99(6): 385-388

The Solution: 3 Tests for Additive (Biological) Interaction

- RERI (Relative Excess Risk due to Interaction)

$$RERI = RR(AB) - RR(A\bar{B}) - RR(\bar{A}B) + 1$$

Distribution: $-\infty$ to ∞ (RERI > 0 → Synergy, RERI < 0 → Antagonism)

- AP (Attributable Proportion due to interaction)

$$AP(AB) = \frac{RERI}{RR(AB)}$$

Distribution: -1 to 1 (AP > 0 → Synergy, AP < 0 → Antagonism)

- S (Synergy index)

$$S = \frac{RR(AB) - 1}{[RR(A\underline{B}) - 1] + [RR(\underline{A}B) - 1]}$$

Distribution: 0 to ∞ (S > 1 → Synergy, S < 1 → Antagonism)

Final Results Presented According to Knoll & VanderWeele*

Table 2. Interaction on the additive and multiplicative scales of hearing nerve agent alarms and *PON1* Q192R genotype on GWI.

	PON1 Q192R genotype						PORs for *PON1* Q192R genotypes within strata of alarms	
	QQ		QR		RR			
Heard nerve agent alarms	N cases/ controls	POR (95%CI)	N cases/ controls	POR (95%CI)	N cases/ controls	POR (95%CI)	QR vs QQ	RR vs QQ
No	43/130	1.0	50/120	1.26 (0.78-2.03) p=0.34	18/37	1.47 (0.76-2.85) p=0.25	1.26 (0.78-2.03) p=0.34	1.47 (0.76-2.85) p=0.25
Yes	129/104	3.75 (2.44-5.77) p<0.001	177/96	5.57 (3.64-8.53) p<0.001	91/21	13.10 (7.29-23.55) p<0.001	1.49 (1.04-2.13) p=0.03	3.49 (2.04-6.00) p<0.001
POR (95% CI) for alarms within strata of genotypes		3.75 (2.44-5.77) p<0.001		4.43 (2.93-6.69) p<0.001		8.91 (4.27-18.60) p<0.001		
Additive scale: Synergy index (95% CI)								
Unadjusted		1.0		1.52 (0.93-2.48) p=0.09		3.76 (1.91-7.37) p<0.001		
Adjusted for confounders		1.0		1.87 (0.95-3.67) p=0.07		4.71 (1.82-12.19) p=0.001 [a]		
Multiplicative scale: POR (95% CI) from LR interaction term								
Unadjusted		1.0		1.18 (0.65-2.14) p=0.59		2.38 (1.01-5.57) p=0.047		
Adjusted for confounders		1.0		1.45 (0.70-2.97) p=0.32		3.41 (1.20-9.72) p=0.02		

Note: The synergy index is a measure of interaction on the additive scale; it has the same distribution as the POR, viz., 0 to plus infinity with 1.0 as the equivalency point indicating no association. The ratio of the PORs, obtained from the interaction term in a logistic regression analysis, is a measure of interaction on the multiplicative scale. The potential confounders controlled for in the adjusted models include: age (years), sex (M, F), service branch (Army [referent], Navy, Air Force, Marines), rank (officer, enlisted), active duty vs Guard/Reserve, special strata (yes, no), Combat Exposure Scale (0=missing, 1=light [referent], 2=light to moderate, 3=moderate to heavy and heavy). One subject's missing age was imputed to the mean age of the sample.. The analyses included 508 cases and 508 controls. Abbreviations: aRERI, relative excess risk due to interaction adjusted for measured confounding; CI, confidence interval; LR, logistic regression; PON1, paraoxonase-1; POR, prevalence odds ratio.

[a] aRERI = 7.69 (2.71-19.13)

*Knoll MJ, VanderWeele TJ. Recommendations for presenting analyses of effect modification and interaction. *Int J Epidemiol* 2012; 41: 514-520.

Final Results Presented According to Knoll & VanderWeele

Table 3. Interaction on the additive and multiplicative scales of hearing nerve agent alarms and PON1 type Q isoenzyme level on GWI.

	PON1 type Q isoenzyme activity level (quartiles)								POR (95% CI) for PON-Q quartiles within strata of alarms		
	4th quartile (lowest risk)		3rd quartile (mid-low risk)		2nd quartile (mid-high risk)		1st quartile (highest risk)				
Heard nerve agent alarms	N cases/ controls	POR (95% CI)	N cases/ controls	POR (95% CI)	N cases/ controls	POR (95% CI)	N cases/ controls	POR (95%CI)	3rd vs 4th quartile	2nd vs 4th quartile	1st vs 4th quartile
No	29/83	1.0	29/74	1.12 (0.61-2.05) p=0.71	25/77	0.93 (0.45-1.72) p=0.82	28/53	1.51 (0.81-2.82) p=0.19	1.12 (0.61-2.05) p=0.71	0.93 (0.50-1.72) p=0.82	1.52 (0.81-2.82) p=0.19
Yes	74/70	3.03 (1.77-5.16) p<0.001	89/62	4.10 (2.41-7.00) p<0.001	88/50	5.04 (2.92-8.71) p=0.001	146/39	10.71 (6.18-18.59) p<0.001	1.36 (0.86-2.15) p=0.19	1.67 (1.03-2.68) p=0.04	3.54 (2.19-5.73) p<0.001
PORs (95% CI) for alarms within strata of PON-Q activity		3.03 (1.77-5.16) p<0.001		3.66 (2.14-6.27) p<0.001		5.42 (3.73-9.58) p<0.001		7.09 (3.97-12.64) p<0.001			
Additive scale: Synergy index (95% CI)											
Unadjusted		1.0		1.45 (0.71-2.96) p=0.31		2.07 (0.95-4.47) p=0.07		3.83 (1.94-7.55) p<0.001			
Adjusted for confounders		1.0		1.38 (0.57-3.35) p=0.48		2.48 (0.96-6.39) p=0.06		3.89 (1.60-9.49) p=0.003 [a]			
Multiplicative scale: POR (95% CI) from LR interaction term											
Unadjusted		1.0		1.21 (0.57-2.58) p=0.62		1.79 (0.82-3.91) p=0.14		2.34 (1.07-5.15) p=0.034			
Adjusted for confounders		1.0		1.07 (0.43-2.68) p=0.88		2.30 (0.90-5.89) p=0.08		2.78 (1.08-17) p=0.03			

Note: The synergy index is a measure of interaction on the additive scale; it has the same distribution as the OR, viz., 0 to plus infinity with 1.0 as the equivalency point indicating no association. The ratio of the PORs, obtained from the interaction term in a logistic regression analysis, is a measure of interaction on the multiplicative scale. The potential confounders controlled for in the adjusted models include: age (years), sex (M, F), service branch (Army [referent], Navy, Air Force, Marines), rank (officer, enlisted), active duty vs Guard/Reserve, special strata (yes, no), Combat Exposure Scale (0=missing, 1=light [referent], 2=light to moderate, 3=moderate to heavy and heavy). One subject's missing age was imputed to the mean age of the sample. The analyses included 508 cases and 508 controls. Comparable tables for the PON1 R isoenzyme, diazoxonase, arylesterase, paraoxonase, and BChE enzyme are given in **Tables S8-S15**. Abbreviations: aRERI, relative excess risk due to interaction adjusted for measured confounding; CI, confidence interval; LR, logistic regression: PON1, paraoxonase-1; POR, prevalence odds ratio.

[a] aRERI = 5.91 (95% CI 2.49-13.45)

Knoll MJ, VanderWeele TJ. Recommendations for presenting analyses of effect modification and interaction. *Int J Epidemiol* 2012; 41: 514-520.

How strongly does low PON1 Q isoenzyme potentiate the neurotoxic effects of nerve agent at different exposure levels?

Table 3. Interaction on the additive and multiplicative scales of hearing nerve agent alarms and PON1 type Q isoenzyme level on GWI.

	PON1 type Q isoenzyme activity level (quartiles)								POR (95% CI) for PON-Q quartiles within strata of alarms		
	4th quartile (lowest risk)		3rd quartile (mid-low risk)		2nd quartile (mid-high risk)		1st quartile (highest risk)				
Heard nerve agent alarms	N cases/ controls	POR (95% CI)	N cases/ controls	POR (95% CI)	N cases/ controls	POR (95% CI)	N cases/ controls	POR (95%CI)	3rd vs 4th quartile	2nd vs 4th quartile	1st vs 4th quartile
No	29/83	1.0	29/74	1.12 (0.61-2.05) p=0.71	25/77	0.93 (0.45-1.72) p=0.82	28/53	1.51 (0.81-2.82) p=0.19	1.12 (0.61-2.05) p=0.71	0.93 (0.50-1.72) p=0.82	1.52 (0.81-2.82) p=0.19
Yes	74/70	3.03 (1.77-5.16) p<0.001	89/62	4.10 (2.41-7.00) p<0.001	88/50	5.04 (2.92-8.71) p=0.001	146/39	10.71 (6.18-18.59) p<0.001	1.36 (0.86-2.15) p=0.19	1.67 (1.03-2.68) p=0.04	3.54 (2.19-5.73) p<0.001
PORs (95% CI) for alarms within strata of PON-Q activity		3.03 (1.77-5.16) p<0.001		3.66 (2.14-6.27) p<0.001		5.42 (3.73-9.58) p<0.001		7.09 (3.97-12.64) p<0.001			
Additive scale: Synergy index (95% CI)											
Unadjusted		1.0		1.45 (0.71-2.96) p=0.31							
Adjusted for confounders		1.0		1.38 (0.57-3.35) p=0.48							
Multiplicative scale: POR (95% CI) from LR interaction term											
Unadjusted		1.0		1.21 (0.57-2.58) p=0.62							
Adjusted for confounders		1.0		1.07 (0.43-2.68) p=0.88							

Note: The synergy index is a measure of interaction on the additive scale; it has t
the PORs, obtained from the interaction term in a logistic regression analysis, is a
age (years), sex (M, F), service branch (Army [referent], Navy, Air Force, Marines
[referent], 2=light to moderate, 3=moderate to heavy and heavy). One subject's r
tables for the PON1 R isoenzyme, diazoxonase, arylesterase, paraoxonase, and
measured confounding; CI, confidence interval; LR, logistic regression: PON1, pa

[a] aRERI = 5.91 (95% CI 2.49-13.45)

Effect of low PON1 Q isoenzyme level on GWI by estimated nerve agent exposure

	Serum PON1 Q isoenzyme level						
	Below median		Above median				
Number of nerve agent alarms heard	Cases	Controls	Cases	Controls	Odds ratio	95% CI	P
0	53	130	58	157	1.10	0.71-1.71	0.74
1	49	20	31	42	3.31	1.65-6.66	<0.001
2-9	108	50	79	69	1.88	1.18-3.01	0.01
≥10	77	19	53	21	1.61	0.79-3.27	0.21

Total N = 1016

Conclusion: High PON1 Q isoenzyme activity is most protective at low nerve agent exposure levels but is overwhelmed at high exposure levels.

What about Recall Bias?

Recall bias may occur because:

sick people tend to recall environmental exposures more vividly and perhaps embellish (higher sensitivity but lower specificity);

whereas,

well people tend to recall less vividly and under-report (lower sensitivity but higher specificity).

Sensitivity Analysis: Effect of Recall Bias in Self-Reported Nerve Agent Alarms data on the GxE Interaction

Table 4. Sensitivity analysis of the effect of differential misclassification of the environmental variable (hearing nerve agent alarms) on the association of GWI with the GxE interaction between the PON1 RR vs. QQ genotype and having heard nerve agent alarms on the additive and multiplicative scales.

		Interaction on the additive scale[a]				
		Controls				
Cases		Se: 1.00	0.90	0.85	0.80	0.80
Se	Sp	Sp: 1.00	0.90	0.90	0.90	0.95
1.00	1.00	3.76 (1.91, 7.37)[b]	—	—	—	—
			—	—	—	—
0.90	0.90	—	4.45 (2.35, 8.41)	4.57 (2.40, 8.68)	4.74 (2.46, 9.14)	4.73 (2.40, 9.32)
0.90	0.80	—	4.70 (2.43, 9.10)	4.86 (2.48, 9.52)	5.09 (2.53, 10.23)	5.13 (2.46, 10.73)
0.90	0.70	—	5.10 (2.53, 10.29)	5.34 (2.58, 11.03)	5.69 (2.63, 12.32)	5.85 (2.51, 13.62)
0.95	0.80	—	4.55 (2.25, 9.19)	4.70 (2.27, 9.72)	4.92 (2.28, 10.65)	4.93 (2.14, 11.37)
0.95	0.70	—	4.90 (2.29, 10.48)	5.12 (2.29, 11.41)	5.45 (2.27, 13.11)	5.55 (2.07, 14.91)

Note: —, no data; GxE, gene-environment interaction; GWI, Gulf War illness; Se, sensitivity; Sp, specificity.
[a]Cells of the upper table contain the unadjusted synergy index (95% CI).
[b]From Table 2.
[c]Cells of the lower table contain the unadjusted prevalence odds ratio (95% CI) of the interaction term from logistic regression.

Conclusion: Correcting for recall bias in measurement of nerve agent exposure **increased** the strength of the interaction (Synergy index). Thus, recall bias had caused us to underestimate the GxE interaction rather than manufacturing a false one.

Conclusion on the Effects of Recall Bias on the GxE Interaction

With GxE independence and the absence of confounding, measurement error in the environmental variable always biases the GxE interaction toward the null, and . . .

Conversely, a statistically significant GxE interaction cannot be due to misclassification of the environmental variable.

Garcia-Closas et al. *American Journal of Epidemiology* 1998; 147: 426-433
VanderWeele et al. *Statistics in Medicine 2012; 31: 2552-2564*

Conclusion on the Effects of Recall Bias on the GxE Interaction

With GxE independence and the absence of confounding, measurement error in the environmental variable always biases the GxE interaction toward the null, and . . .

Conversely, a statistically significant GxE interaction cannot be due to misclassification of the environmental variable.

Controlling for the measured confounders in our multivariable models only strengthened the association of the GxE interaction with GWI, but . . .

Garcia-Closas et al. *American Journal of Epidemiology* 1998; 147: 426-433
VanderWeele et al. *Statistics in Medicine 2012; 31: 2552-2564*

What about Unmeasured Confounding?

Sensitivity Testing for Effect of Unmeasured Confounding

How strong would unmeasured confounding have to be to nullify the GxE interaction?

Conclusion: 90% of those who heard alarms would have to have the unmeasured confounder (UC), and the UC would have to be at least 7 times more common in the GWI veterans than the control veterans.

If such extreme conditions were present, the UC would be obvious to everyone.

Table S16. Sensitivity analysis for correcting for unmeasured confounding the adjusted RERI for the effect of the GxE interaction of hearing alarms and *PON1* RR vs QQ genotype[a] on GWI on the *additive scale*.

Stipulated			Calculated			Stipulated			Calculated		
P_0	P_1	PRR_{UD}	k	$aRERI_c$	95% CI	P_0	P_1	PRR_U	k	$aRERI_c$	95% CI
1	1.0	1	1.000	7.69[b]	3.64-18.64[b]	0.3	0.9	5	2.714	2.81	1.23-6.68
						0.3	0.9	7	3.250	2.34	0.95-5.58
0.5	0.7	1	1.000	7.69	3.64-18.64	0.3	0.9	9	3.667	2.07	0.80-4.93
0.5	0.7	3	1.250	6.15	2.91-14.86						
0.5	0.7	5	1.364	5.63	2.65-13.60	0.1	0.3	1	1.000	7.69	3.64-18.64
0.5	0.7	7	1.429	5.37	2.53-12.96	0.1	0.3	3	1.167	6.59	3.12-15.94
0.5	0.7	9	1.471	5.22	2.46-12.57	0.1	0.3	5	1.211	6.35	3.00-15.35
						0.1	0.3	7	1.231	6.24	2.95-15.09
0.5	0.9	1	1.000	7.69	3.64-18.64	0.1	0.3	9			
0.5	0.9	3	1.667	4.60	2.17-11.07				1.242	6.18	2.93-14.95
0.5	0.9	5	2.143	3.57	1.63-8.55	0.1	0.5	1	1.000	7.69	3.64-18.64
0.5	0.9	7	2.500	3.05	1.36--7.28	0.1	0.5	3	1.400	5.48	2.58-13.23
0.5	0.9	9	2.778	2.74	1.196.53	0.1	0.5	5	1.533	5.00	2.36-12.03
						0.1	0.5	7	1.600	4.79	2.26-11.53
0.3	0.5	1	1.000	7.69	3.64-18.64	0.1	0.5	9	1.640	4.67	2.21-11.25
0.3	0.5	3	1.200	6.40	3.03-15.49						
0.3	0.5	5	1.267	6.06	2.87-14.66	0.1	0.7	1	1.000	7.69	3.64-18.64
0.3	0.5	7	1.300	5.91	2.79-14.28	0.1	0.7	3	1.750	4.38	2.06-10.53
0.3	0.5	9	1.320	5.82	2.75-14.07	0.1	0.7	5	2.091	3.66	1.68-8.77
						0.1	0.7	7	2.286	3.34	1.52-7.97
0.3	0.7	1	1.000	7.69	3.64-18.64	0.1	0.7	9	2.412	3.17	1.42-7.55
0.3	0.7	3	1.500	5.11	2.42-12.32						
0.3	0.7	5	1.727	4.44	2.08-10.68	0.1	0.9	1	1.000	7.69	3.64-18.64
0.3	0.7	7	1.857	4.12	1.91-9.93	0.1	0.9	3	2.333	3.27	1.49-7.81
0.3	0.7	9	1.941	3.94	1.82-9.50	0.1	0.9	5	3.286	2.31	0.94-5.52
						0.1	0.9	7	4.000	1.89	0.71-4.51
0.3	0.9	1	1.000	7.69	3.64-18.64	0.1	0.9	9	4.556	1.66	0.57-3.95
0.3	0.9	3	2.000	3.83	1.769-.20						

Abbreviations: PRR_{UD}, stipulated prevalence rate ratio in the underlying population for the association of the unmeasured confounder (U) with GWI; P_0, stipulated probability of U in those in the underlying population who did not hear alarms; P_1, stipulated probability of U in those in the underlying population who heard alarms; PRR_{EU}, the association of U with hearing alarms, assumed equal to PRR_{UD}; *k*, adjustment factor calculated by the first equation below; aOR, the odds ratio from a logistic regression for the gene-environment interaction adjusted for the measured confounders; $aRERI_c$, relative excess risk due to interaction on the additive scale, adjusted for measured confounders and corrected for unmeasured confounding, calculated by the second equation below; 95% CI, asymmetrical 95% confidence limits of $aRERI_c$ calculated by bootstrapping with 5,000 repetitions; plausible values of P_0 and P_1 are >0 to <1 and of PRR_U, >1 to <10.

Assumption: $PRR_{UD} = PRR_{EU}$

[a] Equations for calculating $aRERI_c$ adapted from *Corollary 3B* in section 5 and the second example in section 6 of VanderWeele et al.[5]

$$\kappa = \frac{1+(1/PRR_{EU}-1)(P_0)}{1+(1/PRR_{EU}-1)(P_1)}$$

$$aRERI_c = \frac{1}{\kappa}aOR_{11} - aOR_{10} - \frac{1}{\kappa}aOR_{01} + 1$$

[b] This row, using 1.0 for the 3 stipulated parameters for validation, represents the values uncorrected for unmeasured confounding. This aRERI agrees exactly with the RERI adjusted for measured confounders in Table 2 calculated by Zou's SAS macro; whereas, its asymmetrical 95% CI from bootstrapping is slightly less conservative than that from Zou's method.

The Interpretation

The findings indicate that a true GxE interaction is present. How strongly then does this support a causal role of low-level sarin in GWI?

Causal Inference about GxE Interaction from RERI

Assuming Independence and Monotonicity of G and E Variables

Monotonicity assumption	RERI > 0	RERI > 1	RERI > 2
None	Statistical	Statistical	Mechanistic
One of G or E monotonic	Statistical	Mechanistic	Mechanistic
Both G and E monotonic	Mechanistic	Mechanistic	Mechanistic

Note: Assumes that adjustments have been made for confounding.
Interpretation:

1. **Statistical interaction** carries no implication of a causal interaction.
2. **Mechanistic interaction** (Rothman's "sufficient cause") means that there are individuals who would have the outcome if both exposures are present but not if only one is.
3. For Mechanistic interaction to imply **Biological (Functional) interaction** requires evidence from biochemical or animal experiments.

VanderWeele TJ, Knol MJ. A tutorial on interaction. *Epidemiologic Methods* 2014; 3; 33-72.

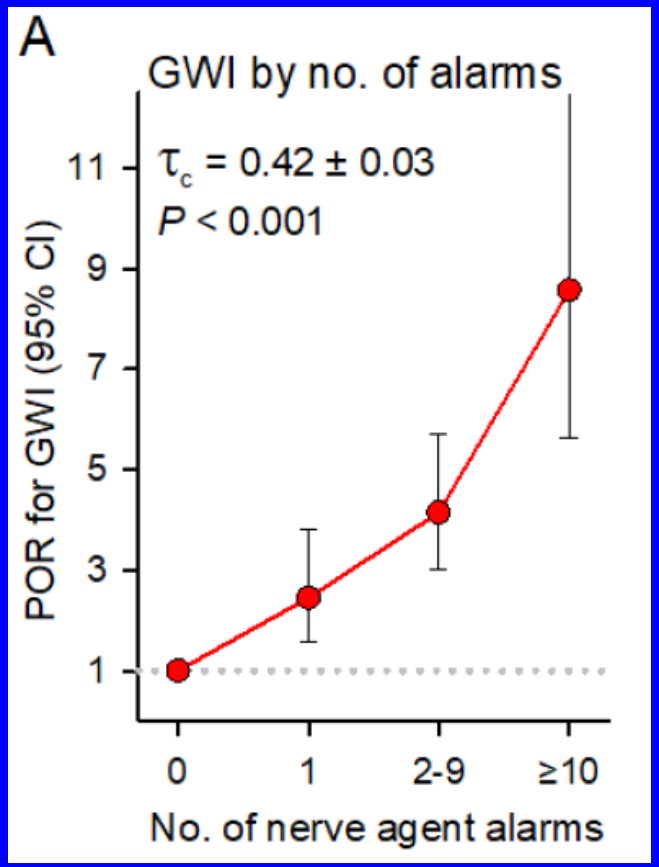
Gene-Environment Independence

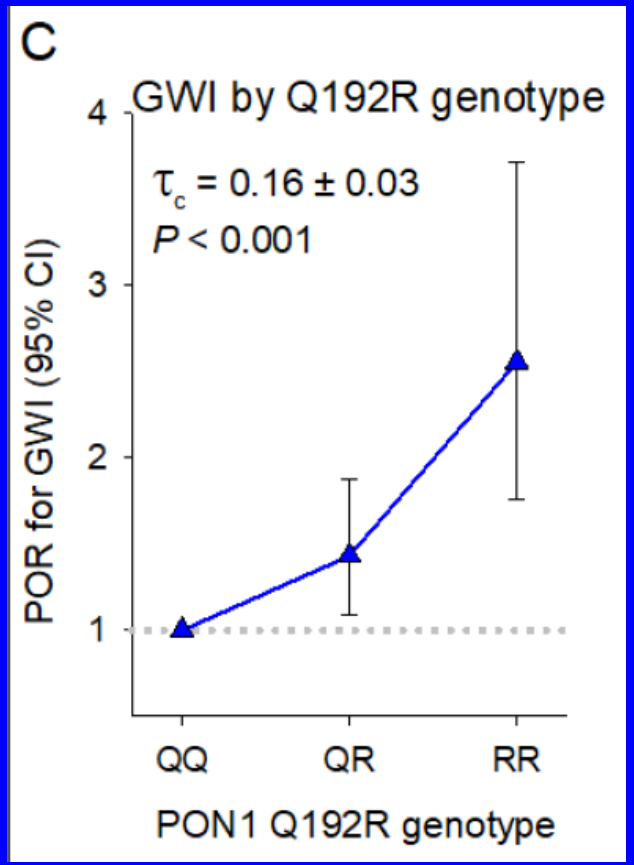
In the 508 controls, the association between G (having the R allele) and E (having heard nerve agent alarms), controlling for the confounding variables*:

OR = 1.18 (95% CI 0.81-1.73, p = 0.35)

*The confounding variables were age, sex, service branch, military rank, active duty/reserve status, special strata, and combat exposure.

Monotonically* Increasing Risk of GWI over Number of Nerve Agent Alarms and PON1 Q192R Genotypes





**Monotonic* means relentlessly increasing or decreasing, i.e., never increasing and then decreasing.

Causal Inference about GxE Interaction from RERI

Assuming Independence and Monotonicity of G and E Variables

Monotonicity assumption	RERI > 0	RERI > 1	RERI > 2
None	Statistical	Statistical	Mechanistic
One of G or E monotonic	Statistical	Mechanistic	Mechanistic
Both G and E monotonic	Mechanistic	Mechanistic	Mechanistic

Note: Assumes that adjustments have been made for confounding.

Interpretation:

1. **Statistical interaction** carries no implication of a causal interaction.
2. **Mechanistic interaction** (Rothman's "sufficient cause") means that there are individuals who would have the outcome if both exposures are present but not if only one is.
3. For Mechanistic interaction to imply **Biological (Functional) interaction** requires evidence from biochemical or animal experiments.

Conclusion: Meeting both assumptions, our finding of RERI=7.69 (95% CI 2.71-9.13) constitutes a *mechanistic interaction* and, with the many studies showing brain cell pathology from low-level sarin (or DFP) exposure, it strongly indicates a *biological interaction*.

VanderWeele TJ, Knoll MJ. A tutorial on interaction. *Epidemiologic Methods* 2014; 3; 33-72.

The Accompanying Commentary

Invited Perspective

A Section 508–conformant HTML version of this article is available at https://doi.org/10.1289/EHP11057.

Invited Perspective: Causal Implications of Gene by Environment Studies Applied to Gulf War Illness

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https://doi.org/10.1289/EHP11057

Refers to https://doi.org/10.1289/EHP9009

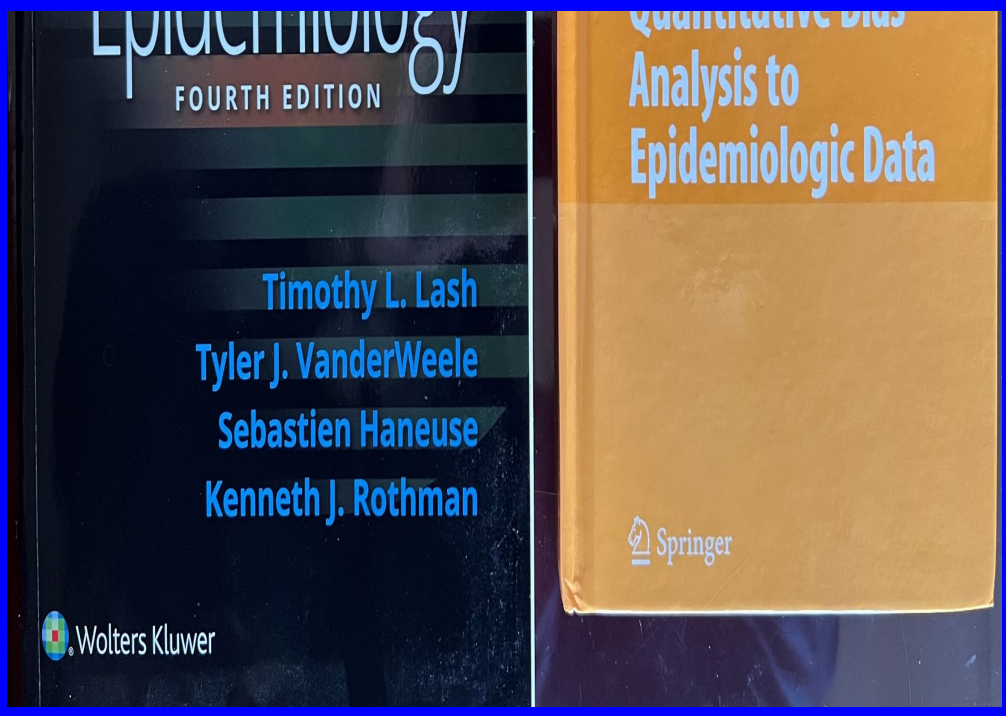
"In summary, the authors' exploration of a gene-environment interaction between presumed nerve agent exposure and the *PON1* gene offers some strong arguments that there is a true causal effect at work. . . . It also suggests, at least in part, why some soldiers who were presumably exposed to toxicants like nerve agents suffer from GWI and some do not."

The Conclusion

Conclusion

- Findings supporting our pre-stated hypothesis.
  - Weather satellite imagery confirms sarin exposure from Coalition bombing.
  - Strong dose-related association of GxE interaction with GWI on the additive scale (RERI > 2) establishes a **mechanistic interaction**.
  - Large random sample avoided selection bias.
  - Controlled for measured confounders in the analysis.
  - Sensitivity analysis ruled out unmeasured confounders.
  - Sensitivity analysis demonstrated that misclassification of self-reports of hearing nerve agent alarms (recall bias) biased against finding the association with GWI.
  - Prior biochemical and toxicological experimental findings have demonstrated neurotoxicity from sarin and the protective effects of the PON1 Q isoenzyme from sarin, thus qualifying the mechanistic interaction as a **biological interaction**.
- These findings constitute strong evidence for a causal role of low-level sarin nerve agent in Gulf War illness.

Methodologic Resources for This Study



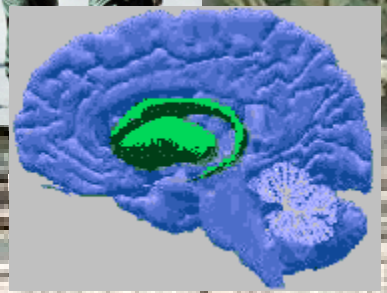
Programs for Calculating Tests for Interaction on the Additive Scale

- Hosmer and Lemeshow. *Epidemiol* 1992;29(5):452-456.
  - Methods for CI of RERI, AP(AB) and S from output of LR software.
  - Wald CI have been criticized.
- Assmann, Hosmer, Lemeshow, Mundt. *Epidemiol* 1996;7(3):286-290.
  - Further developed methods including delta method and bootstrap CI.
- Lundberg et al. *Epidemiol* 1996;6:655-656.
  - SAS program implementing original Hosmer & Lemeshow method.
  - Distributes program on request (Program has trouble with antagonism).
- Andersson et al. *Europ J Epidemiol* 2005;20:575-579.
  - Broadened H&L method to both LR and Proportional Hazards output.
  - Provided website to input parameters from SAS to calculate RERI, AP and S.
- Li. *Ann Epidemiol* 2007;17(3):227-236.
  - Further extension to Proportional Hazards analysis without automated link to additivity analysis
- Zou. *Am J Epidemiol* 2008;168(2):212-224. (My preference)
  - Best all-around method; most accurate, accommodates multivariable models, all 3 measures
  - Program in appendix of the paper; email the author for more versatile version.
- Richardson and Kaufman. *Am J Epidemiol* 2009;169(6):756-760.
  - Novel method using linear odds ratio model with Proc NLMIXED, gives only RERI not AP or S
  - Program in the online attachment on journal's website.

Programs for Calculating Tests for Interaction on the Additive Scale

- Mathur and VanderWeele. *Epidemiol* 2018;29(1):e6-e6. doi:10.1097/EDE.0000000000000752.
  - R function for calculating all measures of additive interaction and testing mechanistic interaction.
  - Confidence intervals and P values calculated by the Delta method, which may be symmetrical?

PON1 Q192R, Nerve Agent and Gulf War Illness:
The Power of Gene-Environment Interaction to Establish Causation



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