Associations of Immune Genetic Variability with Gulf War Illness in 1990-1991 Gulf War Veterans from the Gulf War Illness Consortium (GWIC) Multisite Case-Control Study

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GWIC

Gulf War Illness Consortium

16 collaborators from 9 study sites including US and Australia

Designed to bring preclinical (cell and animal) and clinical (human) researchers together to speed development of understanding pathobiology of Gulf War Illness (GWI), identify diagnostic markers of GWI and to develop treatments.

Our focus - study brain-immune pathways and chronic release of chemical messengers and excitatory neurotransmitters from the immune cells of the brain that lead to chronic inflammation. These messengers included cytokines and chemokines.

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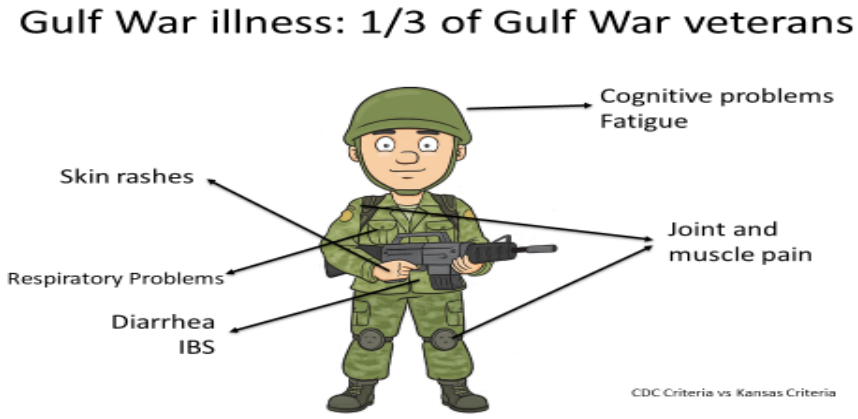
https://sites.bu.edu/gwic

Introduction



Gulf War Illness (GWI):

- Suffered by 1/3 of nearly 700,000 U.S. soldiers who served in the 1990–1991 Gulf War (GW)[1]
- Symptoms: persistent fatigue, cognitive difficulties and musculoskeletal pain; significant changes in central nervous system (CNS) and immune functioning[2-5]
- CNS inflammatory markers and immune system activation key in the chronic symptoms in veterans with GWI[6]
- CNS neuro-immune signaling via glial cells: important role in the development, and sustained symptoms / cognitive decrements associated with GWI[7]

[1]RAC-GWVI (2008) Gulf War Illness and the Health of Gulf War Veterans: Scientific Findings and Recommendations; [2]White et al. (2016) *Cortex* 74, 449–475; [3]Cheng et al. (2020) *Brain Behav. Immun*. 89, 281–290; [4]Guan et al. (2020) *Brain Sci.* 10, 884; [5]Lacagnina et al. (2021) *Brain Behav. Immun.* 91, 418–428; [6]Alshelh et al. (2020) *Brain Behav. Immun.* 87, 498–507; [7]Jeffrey et al. (2019) *Front. Psychol.* 10, 2088.

Introduction

Glial cell activation in GWI:

- Occurs via myelin and neuronal breakdown products in the extracellular spaces sending endogenous danger signals and activating microglia through toll-like receptor 4 (TLR4)[1,2,5,6] TLR4 – ‘not right, not OK, not me receptor’
- Once activated, glial cells release pro-inflammatory cytokines (e.g., interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)). When in pain pathways this results in chronic pain.
- CNS inflammatory response[3] = sickness response symptoms similar to GWI; exacerbated with multiple exposures (e.g. neurotoxicants and mild TBI)[4]
- Neuroimmune pathway activation is a mechanism contributing to symptoms of GWI

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[1] Abou-Donia et al. (2017) *Neurotoxicol. Teratol.* 61, 36–46; [2] Abou-Donia et al. (2020) *Brain Sci.* 10, 610; [3] Takeda et al. (2015) *Curr. Protoc Immunol.* 109, 1–14; [4] Janulewicz et al. (2018) *Brain Sci.* 8, 198 5 Cheng et al. (2021) *Brain, Behav, Immun. 18, 100364 6 Garza-Lombo et al. (2021)* Transl Psychiatry. 11(1):390.

Introduction

GWI & Genetics:

- But only some GW veterans have chronic illness while others with similar exposures do not
- Variability in genetic susceptibility for chronic inflammation or risk susceptibility of GWI?

- No validated biomarker of risk for GWI but some genetics related to GWI:
  - major histocompatibility class (MHC) II family of human leukocyte antigens (HLA)[1]
  - enzymes butyrylcholinesterase (BChE) and paraoxonase-1 (PON1)[2,3]

[1]James et al. (2016) *EBioMedicine* 13, 72–79; [2] Steele et al. (2015) *Environ. Health* 14, 4; [3] Haley et al. (1999) *Toxicol. Appl. Pharmacol.* 157, 227–233.

Hypothesis & aims

Given impact of immune responses on GWI symptomology & variability in immune response between individuals

Hypothesis:

GWI biomarkers reside in genes that control or enhance inflammation, e.g. TGF-beta (TGF-β, TGF)

Aim:

To investigate the associations between a number of immune and pain genetic loci and GWI in GW veterans with and without the disorder

Methods

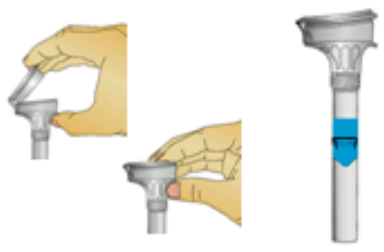
GWIC Study participants:

- GW veterans recruited during Boston University-based Gulf War Illness Consortium (GWIC) study[1]:
  - 223 GWI cases; 46 GW veteran controls
- Study protocol & informed consent documents were approved by institutional review boards; participants provided written informed consent in accordance with the Declaration of Helsinki
- GWI case / control status: Kansas GWI case definition criteria[1]
  - Exclusion: previous diagnosis of predetermined list of chronic medical conditions
- Participant information: deployment and medical history, physical characteristics, demographic characteristics (e.g. sex, age, race, ethnicity)

[1]Steele et al. (2021) *Brain Sci.* 11, 1132.

Methods

GWIC



Genetic analysis:

- Genomic DNA isolated from saliva samples
- 21 single nucleotide polymorphisms (SNPs) in following genes, customized Agena Mass Array assay (Australian Genome Research Facility, Brisbane, Australia)[1]:
  - Cytokines, cytokine receptors & cytokine enzymes: *IL1B, IL2, IL6, IL10, IL6R, TNF, TGF, interleukin converting enzyme (ICE, CASP1)*
  - TLR & accessory proteins: *TLR2, TLR4, MD2, MYD88*
  - Other factors (neurotrophic, inflammation): *BDNF, CRP*
  - Mu opioid receptor: *OPRM1* (pain severity, opioid requirement)
- 1 SNP in *COMT* (pain severity) commercially available TaqMan® SNP genotyping assay kit (ThermoFisher Scientific, Scoresby, Australia)

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[1]Coller et al. (2015) *Support Care Cancer.* 23:1233-1236.

Methods

Statistical analysis:

- Caucasian only, avoid race obscuring associations: 170 cases, 34 controls, SNP Hardy Weinberg Equilibrium ($p > 0.77$)
- Differences in demographic, deployment and military characteristics and genotypes between cases & controls:
  - Chi-square, Fisher's exact & Mann–Whitney U tests
- Logistic regression modeling - strongest predictive genetic risk GWI model: step-wise approach of model building; factors added & removed based on Akaike information criterion (AIC)
- % AUC of the ROC curve[1]: assessed ability of the models to predict GWI, value indicating the percentage of veterans who were correctly classified by the model as having GWI
- All analysis in R and RStudio[2,3]

[1]Hosmer et al. (2013) Applied Logistic Regression, 3rd ed; pp. 173–182; [2]R Core Team (2018) R: A Language and Environment for Statistical Computing; R Foundation for Statistical Computing: Vienna, Austria; [3]RStudio. Available online: https://www.rstudio.com/

Results

Cases vs controls baseline information:

- No differences in:
  - Sex $p = 0.43$
  - Age $p = 0.56$
  - Highest education level $p = 0.93$
  - Branch of service in 1990 $p = 0.59$
  - Service component in 1990 $p = 0.96$
  - Gulf War Deployment: Service period in theater $p = 0.25$
  - Median number of months in theater $p = 0.94$
- Difference in:
  - Rank in 1990 $p = 0.006$; 89% cases enlisted vs 68% controls; Odds ratio (95% confidence interval) = 3.8 (1.6–8.8) previously reported[1-4], unlikely to impact genetic associations

[1]Steele et al. (2021) *Brain Sci.* 11, 1132; [2]Nisenbaum et al. (2000) *J. Nerv. Ment. Dis.* 188, 259–266; [3] Steele (2000) *Am. J. Epidemiol.* 152, 992–1002; [4]Cherry et al. (2001) *Occup. Environ. Med.* 58, 299–306.

Results

Cases vs controls genetic differences, univariate analysis:

Table 2. Percentage *TGF* (rs1800469), *IL6R* (rs8192284) and *TLR4* (rs4986791) genotype frequencies in Caucasian GWI cases and controls. *p*-values were obtained by Chi-square tests comparing the two groups.

Genotype	Cases (%)	Controls (%)	Chi-Square Value	*p*
TGF				
C/C	41.8	70.6	9.5	0.009
C/T	45.9	23.5		
T/T	12.3	5.9		
IL6R				
A/A	36.1	64.7	10.3	0.006
A/C	50.9	23.5		
C/C	13.0	11.8		
TLR4				
C/C	88.8	70.6	7.63	0.006
C/T	11.2	29.4		
T/T	0	0		

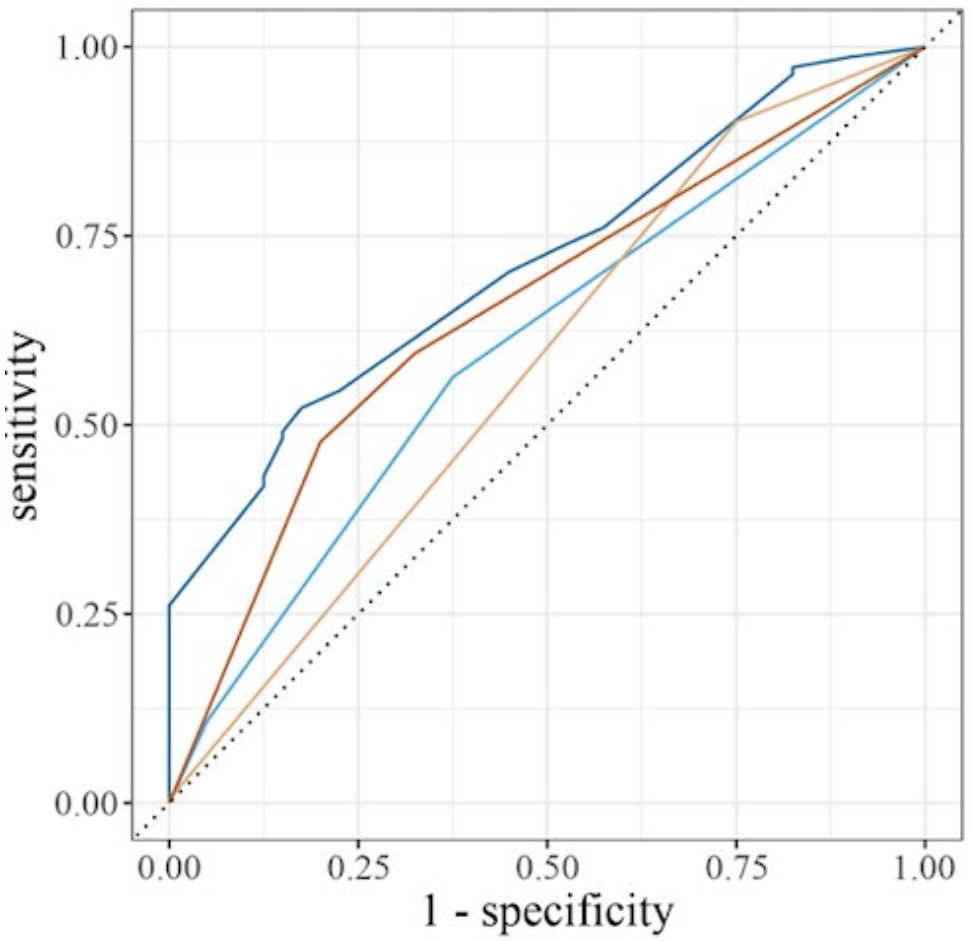
Results

Cases vs controls genetic differences, predictiveGWI logistic regression model:

- Most specific & sensitive model = *TGF* rs1800469 (p = 0.009) + *IL6R* rs8192284 (p = 0.004) +*TLR4* rs4986791 (p = 0.013)

- % AUC ROC curves:

Model	% AUC
TGF rs1800469	60
IL6R rs8192284	65
TLR4 rs4986791	58
All 3 SNPs	71



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Discussion

- Study first to model the impact of genetic variability in pro-inflammatory / anti-inflammatory cytokines and receptors, innate immune response pathways and pain signaling pathways in determining risk of GWI:
  - Final model: 71% chance of correctly classifying a veteran with GWI, considered acceptable in the statistical literature[1]
- *TGF* SNP resides in the promoter region of the gene:
  - variant has been linked to higher serum TGF-β concentrations[2]
- *IL6R* SNP resides in the coding region of the gene:
  - variant has been linked to higher levels of soluble IL-6 receptor[3], potential for increased IL-6 inflammatory signaling

[1]Hosmer et al. (2013) Applied Logistic Regression, 3rd ed; pp. 173–182; [2]Grainger et al. (1999) *Hum. Mol. Genet.* 8, 93–97 ; [3]Galicia et al. (2004) *Genes Immun.* 5, 513–516.

Discussion

How can variant *TGFB* and *IL6* lead to GWI?

- Presence of both TGF-β and IL-6 signaling in inflammatory conditions, differentiation of Th17 cells occurs; this can further drive inflammation[1]
  - presence of variant SNPs = increased TGF-β and IL-6 after immune challenges, e.g. deployment exposure to neurotoxicants causes heightened and sustained inflammatory responses
  - hence the higher frequency of *TGF* and *IL6R* variant genotype carriers in GWI cases vs controls
- Supported by previous links:
  - increased IL-6 expression with other chronic inflammatory diseases e.g. rheumatoid arthritis[2], inflammatory bowel disease[3]

[1]Korn et al. (2009) *Annu. Rev. Immunol.* 27, 485–517; [2]Narazaki et al. (2017) *Expert Rev. Clin. Immunol.* 13, 535–551 ; [3] Rose-John et al. (2009) *Curr. Pharm. Des*. 15, 2095–2103 .

Discussion

- *TLR4* SNP resides in the coding region of the gene:
  - variant causes an amino acid change (Thr399Ile) in TLR4 in the extracellular domain, lessened response to TLR4 agonist (lipopolysaccharide) binding & reduced nuclear factor kappa B signaling[1]

How can variant *TLR4* protect against GWI?

- this variant decreases inflammatory signaling
- hence lower frequency of variant genotype carriers in GWI cases vs controls, e.g. protected

[1]Rallabhandi et al. (2006) *J. Immunol.* 177, 322–332.

Conclusions

- First GWI genetic risk model to implicate immune genetic variability of the *TGF*, *IL6R* and *TLR4* genes being markers of risk that is both specific and sensitive
- Could assist in identifying therapies to improve the daily life of GW veterans living with GWI by personalizing treatment based on genetics
- But some limitations & next steps to address these:
  - Small sample size of control group; needs validation study in larger independent veteran group
  - Impact of additional deployment and health factors (e.g. immune / CNS function & comorbidities) currently being determined in further analysis to see if model can be improved to predict a higher % of veterans with GWI or identify additional subgroups within GWI

[1]Korn et al. (2009) *Annu. Rev. Immunol.* 27, 485–517; [2]Narazaki et al. (2017) *Expert Rev. Clin. Immunol.* 13, 535–551 ; [3] Rose-John et al. (2009) *Curr. Pharm. Des.* 15, 2095–2103 .

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