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Association between antibodies to multiple infectious and food antigens and new onset schizophrenia among US military personnel

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ABSTRACT

Introduction: Multiple studies have documented immune activation in many individuals with schizophrenia suggesting that antigens capable of generating a prolonged immune response may be important environmental factors in many cases of this disorder. While existing studies have found single-agent associations of antibodies to food and neurotropic infectious agents with schizophrenia, a simultaneous examination of multiple agents may shed light on agent interactions or possible etiopathogenic pathways.
Methods: We used traditional regression and novel statistical techniques to examine associations of single and combined infectious and food antigens with schizophrenia. We tested 6106 serum samples from 855 cases and 1165 matched controls.
Results: Higher antibody levels to casein were borderline significant in the prediction of schizophrenia (HR = 1.08, p = 0.06). Study participants with higher cytomegalovirus (CMV) IgG antibody levels had a reduced risk of developing schizophrenia (HR = 0.90; p = 0.02). While IgG antibodies to gliadin, *Toxoplasma gondii*, vaccinia, measles, and human herpesvirus-6 (HHV-6) showed no significant independent associations with schizophrenia, the increase in antibody levels to several combinations of agents, to include casein, measles, CMV, *T. gondii* and vaccinia, was predictive of an 18–34% increase in the risk of developing schizophrenia.
Conclusion: Certain patterns of antibodies, involving some agents, were predictive of developing schizophrenia, with the magnitude of association rising when the level of antibodies increased to two or more agents. A heightened antibody response to a combination of several infectious/food antigens might be an indicator of an altered immune response to antigenic stimuli.

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1. Introduction

Schizophrenia is a pervasive neuropsychiatric disorder with uncertain etiology and pathogenesis, variable clinical presentations and outcomes. The etiology of schizophrenia is complex and is likely to involve both genetic and environmental components, which are very challenging to disentangle (O'Donovan et al., 2003; Maki et al., 2005; Tandon et al., 2008). Multiple studies have documented immune activation in many individuals with schizophrenia, suggesting that antigens capable of generating a prolonged immune response may be important environmental factors in this disorder (Moscavitch et al., 2009). These antigens are likely to interact with human leukocyte antigens (HLA) and the products of other immune response genes that have recently been found to increase the risk of schizophrenia in several different populations (Carter, 2009; Stefansson et al., 2009).

Existing studies examining single-agent associations of antibodies to food and neurotropic infectious agents with schizophrenia have yielded noteworthy but modest associations (Leweke et al., 2004; Yolken, 2004; Wang et al., 2006; Niebuhr et al., 2008a, 2008b; Dickerson et al., 2010a; Severance et al., 2010; Niebuhr et al., 2011; Jin et al., 2012). Problems analyzing single-agent associations are compounded by the heterogeneity of IgG levels depending on the temporal proximity of the specimen collection to the onset of schizophrenia and subjects' demographic characteristics. One limitation in the analysis of environmental factors in a complex disorder such as schizophrenia is the lack of existing statistical techniques to evaluate the possible direct or indirect involvement of multiple environmental agents at different time points before and after symptom onset.

The use of regression techniques with multiple biomarkers is particularly difficult when the sample size is limited. Stepwise regression is a well-known method for dimension reduction that is widely applied and available in most commonly used statistical software packages. One of the major limitations of this algorithm is its inability to address

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multicollinearity. In practice, multiple independent variables used in a regression may have a high degree of correlation, making it difficult, if not impossible, to distinguish the effect of each agent on the dependant variable. As a result, the estimation and the test of statistical significance become unreliable due to a violation of the assumption of independence required for these tests. In the past several decades, a small number of regression methods that adopt regularization have been introduced: ridge regression (Hastie et al., 2001), subset selection, and principle component analysis. Recently, there has been an increasing interest in replacing the sample covariance with some sparse estimates of the true covariance or its inverse for high-dimensional regression problems (Witten and Tibshirani, 2009). Li and Niebuhr (2011) have recently developed a gradient noise orthogonal (GNO) method based on finding the most significant linear combination of the biomarkers in order to reduce the dimension.

We used traditional regression and novel GNO statistical techniques (Li and Niebuhr, 2011) to examine associations of single and combined infectious and food antigens with schizophrenia. Based on the biological plausibility and previously reported associations, four neurotropic viruses [cytomegalovirus (CMV), human herpesvirus 6 (HHV-6), measles virus, and vaccinia virus], an apicomplexan protozoa (*Toxoplasma gondii*) and two food antigens (casein and gliadin) were selected for this study (Albrecht et al., 1980; Leweke et al., 2004; Yolken, 2004; Wang et al., 2006; Dickerson et al., 2010a; Prasad et al., 2011; Jin et al., 2012; Severance et al., 2012).

2. Methods

2.1. Data collection

Data for US military service members who received medical discharges from the military with a diagnosis of schizophrenia from 1992 to 2005 were obtained from the US Army Physical Disability Agency, the Secretary of the Navy Council of Review Boards, and the Air Force Personnel Center/US Air Force Physical Disability Division. The rest of the data were obtained using the Defense Medical Surveillance System (DMSS). The diagnostic process leading to medical discharge from military service and validity of the psychiatric diagnosis has been detailed elsewhere (Millikan et al., 2007; Niebuhr et al., 2008a).

Those aged 18 and older who were on active duty at the time of their schizophrenia diagnosis and who had at least one serum sample of 0.5 ml or greater in the Department of Defense Serum Repository (DoDSR) obtained prior to diagnosis were selected as potential study cases. Hospitalized cases were preferentially selected so virtually all (99%) study subjects were hospitalized with a psychiatric disorder before their discharge from military service. The time of onset was estimated as the earliest date of either the first hospitalization with psychiatric disorder International Classification of Disease 9th Revision (ICD-9-CM) codes (290–319) or the date the medical or physical evaluation boards were initiated.

Control subjects, who were over the age of 18 with no inpatient or outpatient psychiatric disorder diagnoses, were selected from the active duty US military service population. One control per case was selected for 700 males. To increase statistical power for comparison among females in a predominantly male population, three controls were selected for each of the 155 female cases. All control subjects were matched to their cases on sex, race, branch of military service, date of birth (+/−12 months), military entrance (+/−12 months), and serum specimen collection time (+/−90 days). Serum specimens, stored at −30 °F, were obtained from the DoDSR. At least one, and up to four, matched (+/−90 days) specimens were selected for each study subject. The enzyme-linked immunosorbent assay (ELISA) was used to measure antibody levels to *T. gondii*, CMV, HHV-6, vaccinia and measles, as well as to the food antigens casein and gliadin using previously described methods. This study was approved by the Walter Reed Army Institute of Research Institutional Review Board and the Institutional Review Board of the Johns Hopkins School of Medicine.

3. Statistical analyses

The samples from each case–control group were analyzed on the same microplate making within group comparisons more valid. In order to control any systematic bias between plates, all agent measurements were normalized. Robust random effect normalization considering both standard errors between and within plates was performed. To minimize the differences in the ranges of antibody levels to the examined agents, their values were transformed into normal Z scores. The standard deviation was chosen as a unit of measurement for antibody levels.

To study the relationship between multiple agent levels and the risk of schizophrenia at different time points before and after its clinical presentation, we grouped all specimens into several time periods. For controls, the date of "diagnosis" was assigned from its matched case keeping the serum collection order (i.e., the first serum sample of controls was matched with the first serum sample of the matched case, and the 2nd sample of controls was matched with 2nd sample of matched case). While we controlled for all of the matched characteristics in the models, the number of serum specimens differed between cases and controls. However, because the number of specimens for each group had to be the same to be included in the models, we used imputation. If controls had fewer specimens than their matched cases, we randomly selected existing control samples with replacement to complete the match and assigned weights to the entire set of specimens for these controls. The weight was calculated by dividing the number of serum samples of a control by the number of samples of its matched case and assigned to each control sample. The entire set of specimens which included imputed samples was arranged by sample collection time. All IgG values for existing and imputed serum specimens were included in the models adapted to incorporate multiple observations per person. The standard error was adjusted by the ratio of the generated sample size and actual sample size. Univariate and multivariate analyses were used to select agents. Conditional logistic models were used to examine the associations of agent-specific IgG antibody levels with schizophrenia. The associations were evaluated between schizophrenia and single/multiple agents as well as their interactions using the GNO method (Li and Niebuhr, 2011), which is composed of the following steps:

1. Finding the plane best separating cases and controls in a high-dimensional space. The normal vector of the plane is defined as gradient.
2. Finding the plane worst at separating cases and controls in a high-dimensional space, the normal vector of which is defined as noise.
3. Finding other vectors, which are orthogonal to each other as well as to the gradient and noise.
4. Usually, only the gradient reaches significance in distinguishing cases from controls.
5. Removing the agents with small absolute gradient coefficients, and therefore, contributing to a conversion from a high-dimensional to a low-dimensional regression.

We controlled for time from serum specimen collection to diagnosis either continuously or categorically. The overall effect of IgG immune response to selected infectious and food antigens on consequential development or manifestation of schizophrenia was reported.

The proportional hazard model in SAS 9.3 was used to perform the conditional logistic regression. The measure of association between antibody levels and schizophrenia was the hazard ratio (HR) presented with the 95 percent confidence interval (95% CI).

4. Results

We tested a total of 6106 serum samples from 855 cases with schizophrenia and 1165 controls. As shown in (Table 1), the majority of cases were men, white, younger than 25 and had less than 3 years of service. Fewer than 3% of the cases had only one serum specimen available, about 30% had two or three, and approximately 40% had four or more specimens. About 90% of the pre-onset specimens were collected within 4 years before diagnosis and 96% of the post-onset specimens were collected within 2 years after diagnosis.

Each of the seven agents was fit independently to a conditional logistic model controlling for gender, race, age and time to diagnosis (see Table 2). Independently, no individual agent except for CMV had major influence on the risk of schizophrenia. Our data show that study participants with higher cytomegalovirus (CMV) IgG antibody levels had a reduced risk of developing schizophrenia (HR = 0.90; p = 0.02). Higher antibody levels to casein were borderline significant in the prediction of schizophrenia with a hazard ratio of 1.08 (p = 0.06). The other agents selected for this study, gliadin, *T. gondii*, vaccinia, measles and HHV-6, showed no significant independent associations with schizophrenia.

Upon further exploration, it was determined that several agents had significant interactions capable of altering the risk of schizophrenia. Though, independently, an increase in casein antibody levels resulted in just a minor elevation in the risk of schizophrenia and vaccinia antibody levels did not differ between cases and controls, a simultaneous increase in the levels of antibodies to both casein and vaccinia was associated with a corresponding and dramatic increase in the risk of developing schizophrenia (see Fig. 1A). Similarly, Fig. 1B shows the joint effect of measles and CMV. Table 3 details the parameter estimates and p-values for these and other interactions that were included in the final model. Though no four-way interaction terms were significant a three-way interaction of measles, CMV and vaccinia had a p-value of 0.04. Thus, all subsets of two-way interaction terms were included in the final model. Additionally, the two-way interaction of casein and *T. gondii* had a p-value of 0.15 which was deemed low enough for acceptance into the final model. Thus, five agents casein, measles, CMV, vaccinia and *T. gondii* were kept in the final model.

By using the 3-way interaction model, we estimated an effect of several combinations of the 2 standard deviation increase in antibody levels of selected infectious and food antigens on the risk of schizophrenia (Table 4). The subjects with 2 standard deviation simultaneous increases in antibody levels to casein, CMV, *T. gondii* and vaccinia had almost 34% elevated risk of developing schizophrenia. A 2 standard deviation increase of antibody levels to casein, measles, CMV and vaccinia was associated with a corresponding 30% increase in the risk of developing schizophrenia (HR = 1.30). The individual associations of antibody levels to these infectious and food antigens and schizophrenia were nonexistent or barely significant.

Table 1
Description of schizophrenia study cases and controls.

Characteristic	Level	Cases		Controls	
		n	%	n	%
Gender	Female	155	18.1	465	39.9
	Male	700	81.9	700	60.1
Race	Black	310	36.3	462	39.7
	White	471	55.1	619	53.1
	Other	74	8.7	84	7.2
Age categories	18–21	255	29.8	333	28.6
	22–26	320	37.4	422	36.2
	≥27	280	32.8	410	35.2
Time in service[a]	≤1	171	20.0	166	14.2
	>1 to ≤3	306	35.8	419	36.0
	>3 to ≤5	133	15.6	200	17.2
	>5 to ≤10	146	17.1	234	20.1
	>10	99	11.6	146	12.5
Number of serum draws per subject	1	22	2.6	106	9.1
	2	252	29.5	414	35.5
	3	225	26.3	327	28.1
	4	174	20.4	318	27.3
	≥5	182	21.3	0	0.0

[a] Time in years to case diagnosis.

Table 2
Single-agent antibody level associations with schizophrenia.

Agent modeled[a]	Hazard ratio	95% CI		p-Value
		Lower	Upper	
Casein	1.08	1.00	1.17	0.06
Gliadin	1.05	0.97	1.13	0.26
T. gondii	1.04	0.97	1.12	0.27
CMV	0.90	0.83	0.99	0.02
Vaccinia[b]	0.98	0.90	1.07	0.63
Measles	1.05	0.97	1.14	0.25
HHV6	1.03	0.95	1.11	0.54

Abbreviations: CMV = cytomegalovirus, HHV6 = human herpesvirus type 6.
[a] Conditional logistic model was used for each individual agent antibody level controlling for gender, race, age and time to diagnosis.
[b] 378 out of a total of 751 vaccinia IgG positive subjects had documentation of smallpox vaccination (http://www.vaccines.mil/Smallpox). Presumably the rest were either vaccinated outside of the military or exposed to a wild type.

5. Discussion

In this study, we found that schizophrenia cases had significantly lower CMV IgG antibody levels compared to matched controls. A combination of genetic and environmental factors resulting in some immune dysregulation in general and the lack of the CMV IgG antibodies in particular could be the most plausible speculations. While there are no specific research results supporting this finding in existing literature, the activation of the immune system and prolonged neuroinflammation are well established phenomena implicated in the etiopathogenesis and symptomatology of schizophrenia (Lin et al., 1998; Nikkila et al., 1999; Kim et al., 2000; Theodoropoulou et al., 2001; Ebrinc et al., 2002; Patterson, 2002; Zhang et al., 2002; Garver et al., 2003; Zhang et al., 2004; Coelho et al., 2008; Potvin et al., 2008; Doorduin et al., 2009; Kim et al., 2009; Song et al., 2009; Drexhage et al., 2010; Romero et al., 2010; Drexhage et al., 2011; Weigelt et al., 2011; Beumer et al., 2012; Brito-Melo et al., 2012). Other mechanisms could also be considered. A protective effect of CMV was previously reported in patients with multiple sclerosis (Zivadinov et al., 2006; Pirko et al., 2012). Although an autoimmune nature of schizophrenia has not been determined (Schattner et al., 1996; Goldsmith and Rogers, 2008), results of some studies are suggestive of the possibility that autoimmune components play a role in etiopathogenic pathways leading to schizophrenia, at least in some subgroups of subjects (Eaton et al., 2006; Goldsmith and Rogers, 2008; Eaton et al., 2010; Chen et al., 2012). Because CMV has an immunosuppressive effect (Rubin, 1989; Zivadinov et al., 2006; Varani et al., 2009), it might ameliorate the autoimmune damage and consequently decrease the likelihood of developing schizophrenia in vulnerable individuals with autoimmune reactions, which are potentially implicated in the etiopathophysiology of schizophrenia. Thus those who have the same vulnerabilities for developing schizophrenia and did not encounter CMV are potentially more prone to become cases. Whether or not autoimmune components are present in schizophrenia, CMV was also reported to contribute to a decrease in CD4+ cells and/or their activation (Sedmak et al., 1995; Heise et al., 1998; Essa et al., 2002; Zivadinov et al., 2006; Pirko et al., 2012), which produce proinflammatory cytokines to include TNF-α. The TNF-α can signal through the endothelial cells to alter the tight junction structure, leading to the increased permeability of the blood–brain barrier and changed brain structure and function (Sharief and Hentges, 1991; Zeni et al., 2007).

No other changes in IgG levels to a single antigen were significantly associated with the subsequent onset of schizophrenia. Although

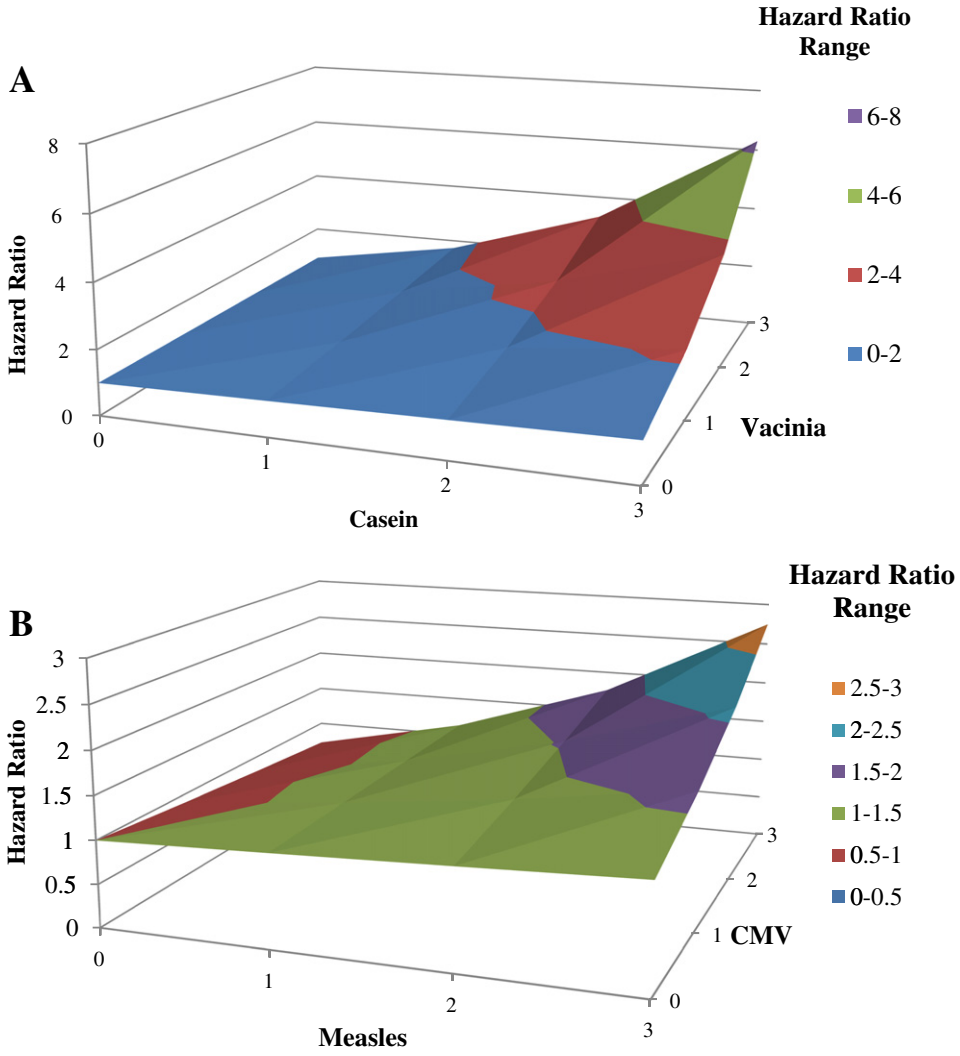

Fig. 1. Hazard ratio as a bivariate function of standard value in casein and vaccinia antibody levels (A) and in CMV and measles antibody levels (B).

numerous research reports, including our pilot study, found an association between *T. gondii* and schizophrenia (Mortensen et al., 2007; Torrey et al., 2007; Niebuhr et al., 2008a; Pedersen et al., 2011; Wang et al., 2013), a few others lack positive findings (Buka et al., 2001; Conejero-Goldberg et al., 2003; Leweke et al., 2004; Xiao et al., 2009; Yolken et al., 2011). Similar inconsistency is observed with other agents under consideration (Albrecht et al., 1980; Shrikhande et al., 1985; Gordon et al., 1996; Fukuda et al., 1999; Niebuhr et al., 2008b; Dickerson et al., 2010a, 2010b; Niebuhr et al., 2011; Jin et al., 2012).

Table 3
The multiple agent antibody level association with schizophrenia including interaction terms (from the GNO analysis).

Agents	Parameter estimate	Standard error	p-Value
Casein	0.07	0.04	0.11
Measles	0.06	0.04	0.20
CMV	−0.08	0.04	0.06
Measles * CMV	0.12	0.07	0.11
Vaccinia	−0.05	0.05	0.28
Measles * vaccinia	0.05	0.06	0.49
CMV * vaccinia	0.15	0.09	0.08
Measles * CMV * vaccinia	−0.27	0.13	0.04
Casein * vaccinia	0.17	0.07	0.01
T. gondii	0.03	0.04	0.40
Casein * *T. gondii*	−0.12	0.08	0.15

Abbreviations: CMV = cytomegalovirus, GNO = gradient noise orthogonal.

Such various findings in the literature might be due to the multifactorial genetic and environmental impacts leading to schizophrenia, its etiopathogenic and clinical heterogeneity, as well as the absence of laboratory methods that can be used to confirm a categorically defined diagnosis.

We also determined that agent selection by the gradient vector can be a method of choice in high-dimensional regressions. The gradient vector identifies the most predictive combination of the individual agents. In fact, the increase in IgG antibody levels to several combinations of agents to include casein, measles, CMV, *T. gondii* and vaccinia was predictive of an 18–34% increase in the risk of developing schizophrenia. Including multiple agents in the model shows that even when a single agent may have a weak association with schizophrenia, multiple agents may interact to increase the risk. In practice, however, it would be very challenging to find a substantial number of subjects with a simultaneous change in IgG antibody levels to all aforementioned agents as the likelihood of this event is quite low. In fact, only 1% of subjects had simultaneously above-average antibody levels to casein, vaccinia, *T. gondii* and CMV. Because antibody levels with a normal distribution typically have a range covering six standard deviations, it might be useful to categorize the population into three groups using the tertiles of their IgG levels towards the selected agents. While bottom and middle tertiles would include subjects with low to moderate IgG antibody levels to these agents, the top tertile could contain those with high IgG levels to multiple agents, which in turn might be

Table 4
Hazard ratios (HRs) for risk of schizophrenia per two standard deviation increase in combined agents antibody levels with different permutations[a].

Casein	Measles	CMV	*T. gondii*	Vaccinia	Parameter	Standard error	Hazard ratio	95% CI	
								Lower	Upper
1	0	1	1	1	0.29	0.13	1.34	1.03	1.75
1	1	1	0	1	0.26	0.06	1.30	1.15	1.47
1	1	1	0	0	0.24	0.04	1.27	1.17	1.38
0	1	1	1	1	0.19	0.21	1.21	0.80	1.83
0	1	1	1	1	0.19	0.21	1.21	0.80	1.83
0	1	0	1	1	0.17	0.09	1.19	1.00	1.40
0	0	0	1	1	0.17	0.08	1.18	1.02	1.38
1	0	0	1	1	0.16	0.06	1.18	1.04	1.33

[a] 1 = agent included in the model, 0 = agent excluded from the model.

indicative of a higher risk of developing schizophrenia. Although modest, the increased risk of schizophrenia associated with exposure to these antigens is relatively large compared with other risk factors such as common genetic polymorphisms.

In our study, antibodies to a single infectious or food antigen were not associated with an increased risk of developing schizophrenia. Certain patterns of antibodies involving some but not other agents were predictive of schizophrenia, with the magnitude of association increasing with the level of antibodies to two or more agents. A heightened antibody response to a combination of several infectious and food antigens might be an indicator of altered immune reaction rather than an implication of any particular agent.

There are several possible ways that immune response as measured by antibody levels could be involved in the etiopathogenesis of schizophrenia. First, a persistent low grade inflammation accompanying a response to a certain combination of infectious and food antigens in individuals with genetic predisposition to schizophrenia might contribute to the risk of developing schizophrenia. In this situation, the chronic inflammation would be considered an independent environmental risk factor. Alternatively, the same genetic variation(s) could be independently responsible for both schizophrenia and alterations of immune regulation, in which case accompanying inflammation might not be directly associated with schizophrenia etiopathogenesis but rather a comorbid condition. Yet another possible scenario is when the same and/or different genetic variations might contribute to both schizophrenia and heightened immune reaction resulting in chronic inflammation. In this case, the chronic inflammation would be considered a consequence of gene–environmental interaction and could have an additive or multiplicative effect to other genetically determined mechanisms leading to schizophrenia. It is of note that the antibody levels we measured could not be attributed to anti-psychotic medications or other epiphenomena related to the diagnosis and treatment of schizophrenia since they were present prior to disease diagnosis.

This study has some notable strength as well as some limitations. Strengths of this study include the follow-up of an initially disease-free population with subsequent complete and reliable case/control ascertainment (Millikan et al., 2007). Due to the large available control population, we also were successful in matching on a number of variables to control for potential confounding. Collecting multiple serum specimens years before disease onset also provides a more reliable and longitudinal assessment of a subject's IgG levels. Because we did not detect inverse correlations between specimens' age and IgG antibody levels, the length of the specimen storage should not be a source of non-differential bias. In addition, case and control serum specimens were matched on the time of collection and the length of storage, so we do not expect our results to be differentially biased or confounded.

This study population was composed mostly of young adults (18–40) who were screened medically and cognitively before entering military service and, therefore, may not be generalizable to other populations. In addition, this study considered only seven infectious and food antigens preselected based on prior studies for possible association with schizophrenia. Additional agents should be the focus of future studies. Also, some cases had more serum specimens retrieved from the DoDSR than their controls. It was financially prohibitive to have as many specimens per control as per case and from a study power perspective, not prudent to have as few specimens per case as per control, so this limitation was addressed statistically. Furthermore, no genetic information was available on the subjects. A future study could benefit from this information by attempting to interconnect various environmental and genetic factors.

It is of note that the associations we found between antibodies were complex, requiring multiple samplings for identification. Our findings reinforce the notion that the etiology and pathogenesis of schizophrenia are very complex and depend on multiple environmental and genetic factors which may be mediated by non-specific changes in immune system reactions. Our results are consistent with the growing body of literature indicating that schizophrenia is likely to involve multiple etiologies and biological pathways. The importance of performing longitudinal investigations of biologically relevant markers in complex brain disorders such as schizophrenia is hard to overestimate.

Future studies should focus on the potential gene–environment interaction as well as nonlinear effects. Such studies should also be longitudinal and allow for analysis of genetic markers as well as pre-onset serum infectious, inflammatory and environmental biomarkers independently and interactively. The successful conclusion of such studies will contribute to the understanding of the causal pathway and potentially the early diagnosis, treatment and prevention of this devastating disease.

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Contributors

Drs. Niebuhr, Cowan, Yolken, Li and Weber designed the study and wrote the protocol. Drs. Yolken, Weber, Mr. Fisher and Ms. Larsen managed the literature searches. Drs. Weber, Yolken, Niebuhr, Li, Cowan, Mr. Fisher and Ms. Larsen developed introduction and discussion. Dr. Li undertook the statistical analysis and wrote statistical analysis and result sections of the manuscript. All authors contributed to and have approved the final manuscript.

Conflicts of interest

The views expressed are those of the authors and should not be construed to represent the positions of the Department of the Army or Department of Defense. No authors have any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three (3) years of beginning the work submitted that could inappropriately influence, or be perceived to influence, their work.

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