Role of Persistent Pathogens, Total Pathogen Burden and Inflammation in Determining Risk for All-Cause and Cardiovascular Disease-related Mortality in the United States

by

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Dedication

To Adam, Addison and Alistair.

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Abstract

Although life expectancy has steadily increased in the United States (U.S.), socioeconomic disparities in risk for mortality persist, indicating the need to identify novel risk factors linking socioeconomic position (SEP) and mortality in the U.S. Several persistent pathogens pattern by SEP in early life and have been implicated in cardiovascular disease (CVD) and other chronic diseases later in life via pathogen-specific effects and non-specific inflammatory pathways. Therefore, individual persistent pathogens and increased total pathogen burden may serve as novel risk factors for mortality.

Using data from a U.S. representative sample of persons 25 years of age and older from the National Health and Nutrition Examination Survey III (1988-1994), we examined the association between cytomegalovirus (CMV) seropositivity, levels of C-reactive protein (CRP), a marker of systemic inflammation, and risk for all-cause/CVD-related mortality. In addition, we examined the association between summed total pathogen burden with CMV, herpes simplex virus type 1 (HSV-1) and -2 (HSV-2) and/or *Helicobacter pylori* (*H. pylori*) and all-cause/CVD-related mortality. Last, we assessed the extent to which there is variability in risk for all-cause/CVD-related mortality, *within* and *across* pathogen burden levels, according to the specific combination of pathogens for which individuals are seropositive.

The main findings from this work indicate that (1) CMV seropositivity is associated with increased risk for all-cause mortality, but not CVD-related mortality. However, the greatest risk for all-cause/CVD-related mortality is for the combined effect of CMV seropositivity and high CRP level, (2) there is an absence of a graded relationship between increased summed pathogen burden with CMV, HSV-1, HSV-2 and/or *H. pylori* and all-cause/CVD-related mortality and (3) there is variability in risk for all-cause/CVD-related mortality both *within* and *across* pathogen burden levels, according to pathogen combination, not accounted for by summed pathogen burden variables and the combined effect of seropositivity for specific combinations of pathogens are statistically significantly associated with all-cause (CMV and HSV-2 as well as CMV, HSV-1 and HSV-2) and CVD-related (CMV and HSV-2 as well as HSV-2 and *H. pylori*) mortality. Implications of these findings for future interventions targeting the prevention or immune control of infections related to mortality are discussed.

Chapter 1

Introduction

1.1 Introduction

There are vast disparities in mortality according to socioeconomic position (SEP).[1-11] Furthermore, although life expectancy has increased steadily over the past 50 years, it is increasing at a greater rate among those of high SEP and the gap in life expectancy between those of low and high SEP is ever widening.[12-15] Adler et al., describe a pathway in which SEP (education, occupation and/or income level) influences health via a combination of exposure to environments that impact health and one's ability to adapt to these environments.[16] (see Figure 1.1 on page 4) Persons living in poverty are disproportionately exposed to physical environments such as overcrowding, inadequate housing, exposure to toxins, pollution, carcinogens and pathogens as well as social environments such as unemployment, crime, discrimination and lack of job control.[16-22] In addition, persons of low SEP have access to fewer resources to cope with these environmental stressors such as lack of access to health care and healthy food choices, safe exercise environments and social support networks.[16-22] Adler et al. propose that increased levels of environmental stressors combined with fewer resources impacts health in three ways: 1) exposures (toxins, carcinogens and pathogens), 2) adoption of detrimental health behaviors, and 3) the physiological response to stress.[17]

The majority of research attempting to explain SEP disparities in health has focused on SEP patterning of health behavior and physiologic response to stress.[23-25] Persons living in poverty often adopt detrimental health behaviors including smoking, alcohol and drug use, decreased physical activity and poor diet as coping mechanisms in response to stress and few social resources.[18,21,26-28] These behaviors in turn have been shown to increase one's risk for chronic diseases such as cardiovascular disease (CVD), diabetes and physical and cognitive impairment and consequently mortality.[29-33] In addition to behavioral changes, persons of low SEP undergo physiologic changes when exposed to stressful physical and social environments. The stress response can become dysfunctional during exposure to chronic stressors in which the adaptation response is frequently initiated and may be ineffectively terminated, causing wear and tear on the body due to failure of the body's physiologic systems to properly adapt to stress.[21,25,27,34-36] As a result, levels of blood pressure, stress hormones and inflammatory markers are elevated over time, leading to organ damage and immune dysfunction.[35,36] Although, health behaviors and the physiologic response to stress have been typically studied in relation to

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risk for mortality, these factors do not fully explain SEP disparities in mortality[23-25,37], thus it remains essential to investigate novel pathways linking SEP and mortality.

Over the past forty years, numerous persistent pathogens which pattern by SEP early in life, have been implicated in later-life CVD and more recently, other chronic diseases of aging with inflammation-related etiology.[38-44] Persistent pathogens are linked to chronic disease via direct, pathogen-specific effects such as in the case of CVD, in which certain pathogens invade vascular tissues and trigger atherogenesis.[45-49] In addition, initial infection as well as reactivation with persistent pathogens triggers the release of acute phase proteins and increases levels of inflammatory markers such as C-reactive protein (CRP), which have been linked to CVD and other chronic diseases.[50-56] Thus, persistent pathogens have been hypothesized as a novel mediator in the pathway between SEP and chronic disease as well as mortality, especially in conjunction with increased levels of inflammatory markers.[57-62] Furthermore, increased total pathogen burden is thought to increase risk for chronic disease and mortality due to an accumulation of pathogen-specific effects and increased levels of systemic inflammation.

A measure of total pathogen burden often utilized by researchers interested in examining the association between total pathogen burden and mortality is 'summed pathogen burden'. Summed pathogen burden is defined as the total number of pathogens an individual tests seropositive for, based on antibody response, and allows researchers to examine whether there is a graded relationship between the total number of pathogens an individual is seropositive for (i.e., summed pathogen burden level) and mortality, regardless of pathogen type. Unfortunately, there are some shortcomings related to the use of summed pathogen burden variables for examining the relationship between total pathogen burden and risk for mortality because variables representing summed pathogen burden have not permitted an assessment of the impact of specific types or combinations of pathogens on health/mortality outcomes.

The objectives of this dissertation are: 1) to examine the relationship between seropositivity for cytomegalovirus (CMV), a persistent pathogen linked to several chronic disease outcomes, elevated CRP and all-cause/CVD-related mortality, 2) to examine the relationship between summed pathogen burden with four pathogens previously implicated in the etiology of several chronic diseases (CMV, herpes simplex virus -1(HSV-1) and 2 (HSV-2), and *Helicobacter pylori* (*H. pylori*) in all-cause/CVD-related mortality, and 3) to examine the association between the specific combinations of these four pathogens and risk for all-cause/CVD-related mortality and assess the extent to which use of summed pathogen burden variables in models examining the association between total pathogen burden and mortality limit the identification of the specific combinations of pathogens that most increase risk for mortality, using data from a U.S. representative sample of adults from the National Health and Nutrition Examination Survey III.

1.2 Specific Aims and Hypotheses

Aim 1: Examine the association between seropositivity for CMV, CRP level and risk for CVD-related/all-cause mortality.

Hypothesis 1a: Seropositivity for CMV at baseline will predict an increased risk for all-cause and CVD-related mortality.

Hypothesis 1b: The observed association between CMV and risk for all-cause and CVD-related mortality will be augmented among participants with high CRP level.

Aim 2: Examine the association between summed total pathogen burden with CMV, HSV-1, HSV-2 and/or *H. pylori* and risk for all-cause and CVD-related mortality.

Hypothesis 2a: Summed total pathogen burden will have a graded relationship with risk for all-cause mortality.

Hypothesis 2b: Summed total pathogen burden will have a graded relationship with risk for CVD-related mortality.

Aim 3: To estimate the combined effect of seropositivity to each possible combination of four pathogens (CMV, HSV-1, HSV-2 and *H. pylori*) on risk for all-cause/CVD-related mortality and to assess the extent to which, use of summed pathogen burden variables in models examining the association between total pathogen burden and mortality, limit our ability to make inferences about the specific pathogens which alone, or in combination most increase risk for death.

Hypothesis 3a: The combined effect of each combination of pathogens on risk for all-cause/CVD-related mortality will not be uniform *within* each pathogen burden level.

Hypothesis 3b: The combined effect of different combinations of pathogens on risk for all-cause/CVD-related mortality will vary *across* pathogen burden levels such that the risk for mortality associated with each specific combination of pathogens will not necessarily be higher than the risk for mortality for all combinations of fewer pathogens.

1.3 Background

Socioeconomic Position, Chronic Disease and Mortality in the United States

The relationship between chronic disease and mortality is well-established.[63] Although death rates (per 100,000 per year) have decreased overall in the past 30 years from 1242 in 1970 to 845 in 2002, the absolute number of deaths from the six leading causes of death; heart disease, cancer, stroke, chronic obstructive pulmonary disease, accidents and diabetes, the majority of which are chronic diseases, continues to increase.[64] Furthermore, gains in life expectancy have been primarily among those of higher socioeconomic position.[12-15] For example, Meara et al., examined changes in mortality according to education level in non-Hispanic blacks and whites from 1981-2000, finding that the educational gaps in life expectancy increased by about 30% during this time period and that the widening gap cannot be attributed to increased racial or gender differences in mortality.[14] Similar results were found recently by Cristia, who examined the relationship between lifetime earnings and mortality from 1983-2003.[16] The

difference in life expectancy for those in the top lifetime earning quintile versus the bottom quintile increased by 30 percent for men and almost doubled for women over this time period.[15]

Psychosocial Model Linking Socioeconomic Position and Health

Research over the past 30 years has pointed to both structural and physiologic mechanisms by which living in poverty may impact health.[16-19,21,22,65] Adler et al., describe a pathway in which SEP (education, occupation and/or income level) influences health via a combination of exposure to environments that impact health and one's ability to adapt to these environments (see Figure 1.1 below).[17] Persons living in poverty are disproportionately exposed to physical environments such as overcrowding, inadequate housing, exposure to toxins, pollution, carcinogens and pathogens as well as social environments such as unemployment, crime, discrimination and lack of job control.[16-19,21,22,65] In addition, persons of low SEP have access to fewer resources to cope with these environmental stressors such as lack of access to health care and healthy food choices, safe exercise environments and social support networks.[16-19,21,22,65] Adler et al. propose that increased levels of environmental stressors combined with fewer resources impacts health in three ways; 1) exposures (toxins, carcinogens and pathogens), 2) adoption of detrimental health behaviors, and 3) the physiological response to stress.[17]
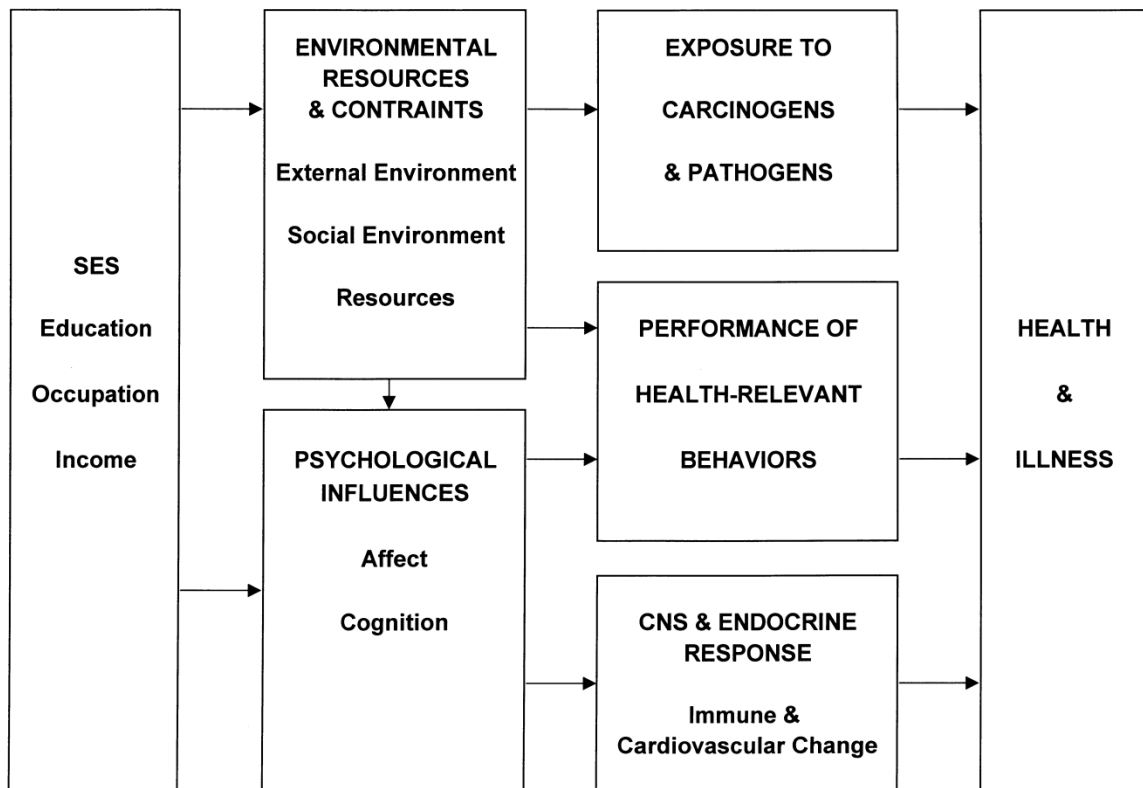
Figure 1.1 Model of the Pathways by which Socioeconomic Status Influences Health[17]

The majority of research attempting to explain SEP disparities in health has focused on SEP patterning of health behavior and physiologic response to stress.[23,25,66] Persons living in

poverty often adopt detrimental health behaviors including smoking, alcohol and drug use, decreased physical activity and poor diet as coping mechanisms in response to stress and have few social resources.[18,21,26-28] These behaviors in turn have been shown to increase one's risk for chronic diseases such as cardiovascular disease (CVD), diabetes and physical and cognitive impairment and consequently mortality.[29-33] In addition to behavioral changes, persons of low SEP undergo physiologic changes when exposed to stressful physical and social environments. The stress response can become dysfunctional during exposure to chronic stressors in which the adaptation response is frequently initiated and may be ineffectively terminated, causing wear and tear on the body due to failure of the body's physiologic systems to properly adapt to stress.[21, 25, 27, 34-36] As a result, levels of blood pressure, stress hormones and inflammatory markers are increased over time, leading to organ damage and immune dysfunction.[35, 36]

However, neither traditional health behaviors nor the physiologic response to stress have been found to fully explain SEP disparities in mortality.[23,25,37,66] A recent study by Rask et al., examined the relationship between SEP, health behavior and mortality in subjects 18 years and older in NHANES III.[23] The authors found that the relationship between income (relative to the poverty level) and mortality was not significantly attenuated after controlling for age, gender, self-reported health status, behavioral risk factors such as smoking status, waist circumference and physical activity as well as clinical risk factors.[23] Likewise, Seeman et al., examined whether a cumulative index of biologic risk (i.e., allostatic load) mediated the relationship between education level and mortality among subjects aged 70-79 from a 7-year follow-up study from the MacArthur Study of Successful Aging (1988-1995), finding that it explained only 35.4% of the difference in mortality risk between those with less than high school education versus high school or greater.[25]

Although life expectancy is increasing overall in the United States the absolute number of deaths from chronic diseases continues to increase, the gap in life expectancy between those of low and high SEP is ever widening and traditional risk factors hypothesized to lie in the pathway between SEP and disease don't completely explain the SEP patterning of disease observed in the United States. Thus, it remains important to explore novel risk factors for chronic disease and mortality. Over the past several decades, a growing body of evidence has implicated persistent pathogens which pattern according to SEP in early life in the etiology of cardiovascular disease (CVD) as well as several other chronic diseases of inflammatory etiology and mortality later in life.[38-44]

Persistent pathogens are linked to chronic disease via direct, pathogen-specific effects such as in the case of CVD, in which certain pathogens invade vascular tissues and trigger atherogenesis.[45-49] In addition, initial infection as well as reactivation with persistent pathogens triggers the release of acute phase proteins and increases levels of inflammatory markers such as CRP, which have been linked to CVD and other chronic diseases.[50-56] Therefore, persistent pathogens have been hypothesized as a novel mediator in the pathway between SEP and

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chronic disease as well as mortality, especially in conjunction with increased levels of inflammatory markers.[57-62] Research examining the relationship between persistent pathogens, inflammation and mortality is needed to better understand the role of these factors in determining risk for death in the general U.S. population.

1.4 Cytomegalovirus, Inflammation, Chronic Disease and Mortality

CMV is a highly transmissible and prevalent beta herpesvirus.[67,68] This pathogen is never cleared from the body, persisting in a number of tissues via hypothesized mechanisms including chronic productive infection and/or latent infection with periodic subclinical reactivation.[68,69] Recently, CMV has been linked to a variety of chronic diseases with an inflammatory component including cardiovascular disease (CVD) [40,42,70,71], cancer [72,73], cognitive decline including vascular dementia[38,74] and functional impairment.[39,57,75]

Cytomegalovirus, Inflammation and Chronic Disease

The specific mechanisms responsible for the association between CMV and various chronic diseases are related to pathogen-specific effects and to have an inflammatory component. For example, in terms of increasing risk for CVD, CMV exerts several pathogen-specific effects that increase risk for CVD. CMV is also capable of invading vascular tissues by either directly infecting the vessels of the heart where it replicates at low levels, or when it is delivered to the vessel wall by infected circulating monocytes arriving at sites of existing cardiovascular injury or inflammation.[76] Both CMV antigen and DNA previously identified in atherosclerotic vessels of the human cardiovascular system [45,77,78] The presence of CMV in the vessel walls may induce smooth muscle cell proliferation and migration, increased uptake of oxidized low-density lipoprotein and expression of cytokines and chemokines as well as increased procoagulant activity by endothelial cells.[79-82] CMV may also cause vascular damage without direct invasion as a result of molecular mimicry, whereby viral antigens trigger an immune response cross-reacting on self-peptides expressed on uninfected host tissues.[79,83,84] For example, two CMV-derived proteins, UL122 and US28, are homologous to an amino acid sequence at position 153-160 of heat shock protein (HSP) 60.[85,86] Thus, there are several pathogen-specific effects by which CMV directly influences the development of atherosclerosis and subsequent CVD outcomes.

In addition, to exerting specific effects, CMV has been linked to several other chronic diseases with inflammation-related etiology such as physical impairment, cognitive decline and cancer.[38,39,57,72-75] Reactivations or superinfections may result in higher titers of CMV immunoglobulin G (IgG) antibodies and increased levels of pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α).[50,87,88] CRP levels also increase as a consequence of reactivation or leakage of the virus from host cells, via the action of IL-6 produced in the liver.[80,89,90] Elevated levels of these inflammatory markers have been linked to CVD as well as a myriad of other chronic disease conditions.[50-56] Thus, CMV may impact both

CVD-related and all-cause mortality through pathogen-specific effects for CVD and via inflammation-related pathways.

Epidemiologic Studies Examining the Association between Cytomegalovirus and Mortality

Few studies have examined the association between CMV, inflammation and all-cause/CVD-related mortality. A recent study by Roberts et al., examined the relationship between CMV antibody titer and all-cause/CVD mortality among elderly Latinos finding that subjects with CMV IgG antibody titers in the highest quartile had 1.43 (95% CI: 1.14, 1.79) times higher risk for all-cause mortality and 1.35 (95% CI: 1.01, 1.80) times higher risk for CVD mortality in models adjusted for age, gender, education and comorbidity index.[58] The authors found the effect of CMV on mortality was partially mediated by a composite measure of two inflammatory markers, TNF-α and IL-6, but not CRP since it was unrelated to mortality in their study population.[58] In contrast, Blankenberg et al. found CMV to be associated with cardiac mortality among persons with existing coronary artery disease (CAD) only in subjects with elevated IL-6 levels (hazard ratio (HR) 3.2, 95% confidence interval (CI): 1.4, 7.3) and not in those without IL-6 elevation, suggesting effect modification by IL-6 in the association between CMV and CVD-related mortality.[91]

Also examining the effect of CMV antibody titers on mortality, Strandberg et al. assessed 7-year risk for mortality in a small cohort of community-dwelling 75-90 year-olds with underlying CVD in Finland.[59] The authors found that mortality was significantly greater among subjects in the highest CMV IgG quartile, compared with the lowest quartile and that this association remained significant after controlling for several covariates including CRP, suggesting that CRP did not mediate this pathway.[59] Furthermore, Muhlestein et al. examined the association between seropositivity to CMV, *H. pylori* and *Chlamydia pneumoniae (C. pneumoniae)* infections, as well as high CRP level and mortality among patients with a mean age of 65 years that were predominantly male and had angiographically demonstrated CAD.[92] Of the three pathogens assessed, only seropositivity to CMV was significantly predictive of mortality. The authors also found that risk for mortality was greatest among CMV seropositive subjects with CRP levels in the highest tertile, suggesting a joint effect of CMV seropositivity and high CRP level on mortality among their sample of individuals with underlying CAD.[92]

Although these studies suggest an association between CMV and mortality, they were conducted in predominantly older age populations, of specific gender or race/ethnic groups and/or among participants who have underlying CAD or CVD health conditions.[57-59,91,92] Moreover, the role of inflammatory markers such as IL-6, TNF-α and CRP as mediators or effect modifiers of the relationship between CMV and mortality is unclear.[57-59,91,92] Therefore, the first aim of this dissertation examines whether seropositivity for CMV predicts all-cause as well as CVD-related mortality and whether CRP mediated or modified these relationships in a nationally representative U.S. population of individuals, ≥ 25 years of age, from NHANES III.

1.5 Pathogen Burden, Chronic Disease and Mortality

In addition to CMV, over the past forty years, numerous other persistent pathogens which pattern by SEP early in life, have been implicated in later-life CVD and more recently, other chronic diseases of aging with inflammation-related etiology.[41,44,72,73,93] Other persistent pathogens, such as herpes simplex -1 (HSV-1) and 2 (HSV-2), Epstein Barr Virus (EBV), and various bacterial pathogens such as *C. pneumoniae*, *H. pylori*, *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans* have also been linked to chronic disease through direct pathological effects on organs and tissue as well as through more non-specific inflammatory pathways. For example, regarding CVD, some of these pathogens are capable of invading vascular tissues, causing vascular damage and triggering atherosclerotic processes such as smooth muscle cell proliferation.[45-49,94-106] Pathogens such as CMV and *H. pylori*, can also induce vascular damage without infecting the vasculature by triggering an immune response via cross-reacting on self-peptides expressed on uninfected host tissues, known as molecular mimicry.[83,84,107,108] In addition to these pathogen-specific effects on CVD, pathogens such as *H. pylori*, Epstein Barr virus and recently, CMV, have been linked directly to various cancers through cellular processes such as cell migration, apoptosis and cell proliferation.[72,73,109-111] Thus, increased pathogen burden is thought to increase risk for chronic disease, in part, via the accumulation of pathogen-specific effects on chronic diseases.

Increased pathogen burden is also thought to impact the development of chronic diseases via increasing systemic inflammation.[50-56] Once acquired, persistent viral pathogens such as herpesviruses undergo periodic reactivation during times of physical or psychosocial stress, immunosupression, and during the aging process and chronic bacterial infections such as *H. pylori* and *C. pneumoniae* develop persistent antibodies, which in turn, stimulate an immune response and increased levels of inflammatory markers.[112-117] Increased levels of inflammatory markers, such as CRP have in turn, been associated with increased risk for CVD as well as other chronic disease outcomes including physical and cognitive impairment, metabolic disorders and cancers [38,39,41,44,52,53,55,72,73,75,93,118] For example, Figure 1.2 on the next page, illustrates the contribution of pathogen-specific effects as well as non-specific inflammatory effects of selected persistent pathogens on risk for CVD.

Non-Specific Inflammatory Effects		Pathogen-Specific Effects on CVD
•Initial infection stimulates production of acute-phase proteins •Replication/reactivation and persistence of antibodies stimulates release of pro-inflammatory cytokines •Inflammatory markers attracted to sites of both vascular and non-vascular tissue invasion	CMV HSV-1 HSV-2 *H. Pylori* *C. pneumoniae* Periodontal Pathogens	•Invasion of vascular tissues/plaques •Direct damage/indirect damage via molecular mimicry •Endothelial dysfunction •Platelet aggregation •Foam cell formation •Smooth muscle cell proliferation •Increased pro-coagulant activity

Increased Systemic Inflammation → Increased Risk for CVD

Figure 1.2 Illustration of the Accumulation of Both Pathogen-Specific Effects and Non-Specific Inflammatory Effects of Various Pathogens For Increasing Risk for Cardiovascular Disease.

Thus, persistent pathogens have been linked to both CVD and other chronic diseases through a combination of pathogen-specific effects as well as through non-specific effects of increased inflammation. For these reasons, increased pathogen burden may also be associated with increased risk for mortality as well.

Summed Pathogen Burden

Due to an accumulation of both pathogen-specific effects as well as overall increased levels of inflammation, it has been hypothesized that the more pathogens an individual is seropositive for, the greater their risk for chronic disease and mortality (see Figure 1.3 on next page).[94] In other words, it is thought that seropositivity for any two pathogens increases risk beyond that of seropositivity to a single pathogen, seropositivity for any three pathogens increases risk for disease beyond seropositivity for one or two pathogens, and so forth, regardless of which pathogens an individual is seropositive for (see Figure 1.4 on next page).

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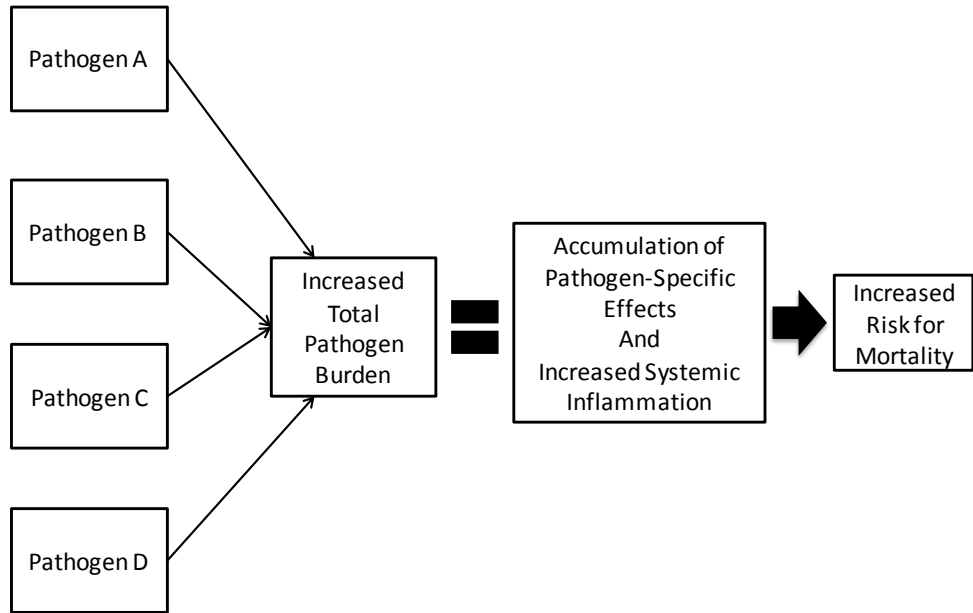
Figure 1.3 Pathway between Increased Total Pathogen Burden and Mortality

Number of Pathogens Individuals Are Seropositive For-Summed Pathogen Burden
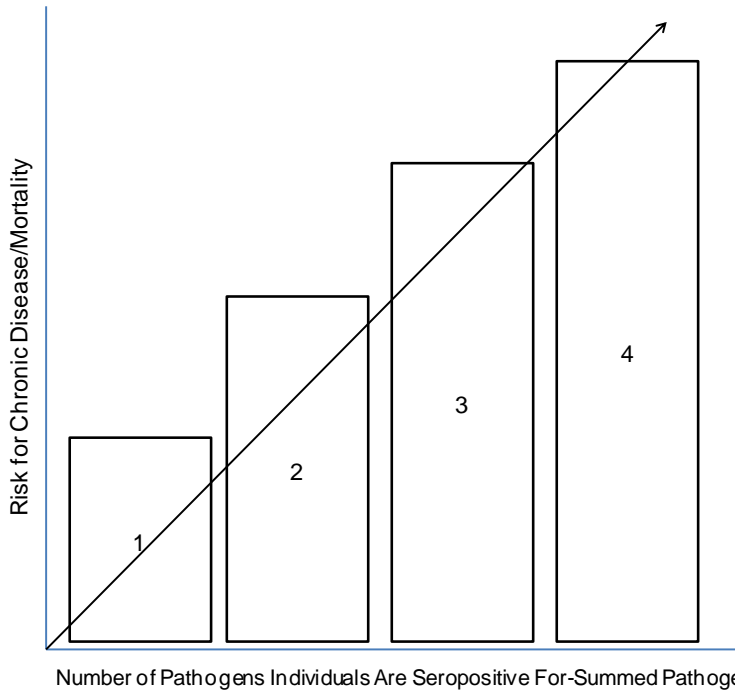
Figure 1.4 Illustration of Hypothesized Monotonic Relationship between Increasing Summed Pathogen Burden Level and Risk for Chronic Disease/Mortality.

As a result, studies typically construct summed pathogen burden variables when modeling the relationship between total pathogen burden and mortality. Summed pathogen burden is defined as the total number of pathogens an individual tests seropositive for, based on antibody response, and allows researchers to examine whether there is a graded relationship between the total number of pathogens an individual is seropositive for (i.e., summed pathogen burden level) and mortality, regardless of pathogen type. Despite the hypothesized relationship between increased summed pathogen burden and numerous chronic diseases, very few studies have examined the association between summed pathogen burden and mortality. The few studies that exist were primarily conducted among persons with existing CVD, of older age and from specific race/ethnic groups limiting their generalizability to the general population.[60-62,119] Thus, there is a need to examine whether there is a graded relationship between summed pathogen burden and mortality in the U.S. general population.

Epidemiologic Studies Examining the Association between Summed Pathogen Burden and Mortality

Although several studies have examined the association between summed pathogen burden, inflammation and various chronic disease few studies have examined the relationship between summed pathogen burden and all-cause/CVD-related mortality and results across studies are conflicting.[60-62,119] Most recently, a study by Elkind et al., measured the association between a constructed infectious burden index (sum of the beta coefficients for the effect of each pathogen for which individuals were seropositive on risk for stroke) and vascular, non-vascular and all-cause deaths among subjects 40 years of age and older (mean age 68.4) from Manhattan, New York, without history of stroke.[119] The authors found that infectious burden index (including *C. pneumoniae*, CMV, HSV-1, HSV-2 and *H. pylori*), was associated with an increased risk for nonvascular deaths (HR per standard deviation change in IB 1.23, 95% CI 1.04, 1.45) in the fully adjusted model.[119] However, after confounder adjustment, the index was no longer statistically significantly associated with risk for vascular deaths or deaths from all causes.[119] In contrast, Zhu et al., examined the relationship between summed pathogen burden with CMV, Hepatitis A, HSV-1 and 2 and all-cause death among a mostly male cohort of Utah residents, with a mean age of 65.3 years and existing CAD.[60] The authors found a significant trend (p-value = 0.020) in risk for death from all-causes with increasing pathogen burden (although individual effects were not statistically significant).[60]

Two other studies by Rupprecht et al. and Espinola-Klein et al., examined the association between summed pathogen burden with eight pathogens (HSV-1, HSV-2, CMV, Epstein-Barr virus, *Haemophilus influenzae, C. pneumoniae Mycoplasma pneumoniae* and *H. pylori)* and CVD-related mortality among German subjects with existing coronary artery disease (CAD).[61,62] In both studies the authors found a significant trend in risk for CVD mortality with increasing

pathogen burden of 4-5 and 6-8 pathogens compared to seropositivity for 0-3 pathogens.[61,62] Rupprecht et al. further examined the association between increased pathogen burden with bacterial and viral pathogens and CVD-related mortality, separately (comparing seropositivity for 2 vs. 0-1 pathogens and for 3-4 vs. 0-1 pathogens) finding that the association between increased pathogen burden with herpesviridae and CVD-related mortality was stronger than that with bacterial burden.[61] However, the groupings were not mutually exclusive, thus it is not possible to completely discern the effects of viral pathogen burden alone from co-infection with viral and bacterial pathogens in terms of risk for CVD-related mortality from the analysis.[61]

These studies suggest a graded association between summed pathogen burden and all-cause/CVD-related mortality among persons with existing CVD but not among those without existing CVD. However, these previous studies were conducted among older populations, mostly consisted of persons with existing CVD and from specific geographic regions (i.e. Utah, New York and Germany).[60-62,119] Therefore, further research is needed to determine the association between summed pathogen burden, inflammation and all-cause/CVD-related mortality among the U.S. general population. The second aim of this dissertation will examine whether there is a graded relationship between increasing pathogen burden with four persistent pathogens (CMV, HSV-1, HSV-2 and/or *H. pylori*, previously implicated in CVD and other chronic diseases, and CVD-related/all-cause mortality in a U.S. representative population of persons ≥ 25 years of age from NHANES III.

1.6 Summed Pathogen Burden- Issues in Measurement

Summed pathogen burden variables may be useful for examining whether there is a graded relationship between the total number of pathogens an individual is seropositive for (i.e., summed pathogen burden level) and mortality, regardless of pathogen type. However, there are several measurement issues that limit the use of this variable for assessing other factors that my play an important role in determining risk for mortality such as seropositivity for specific combinations of pathogens.

There are two main reasons why use of the summed pathogen burden variable may not provide a complete assessment of risk for mortality related to persistent pathogens. First, in models examining the association between summed pathogen burden and mortality, all persons seropositive to a given number of pathogens (i.e., classified as having the same summed pathogen burden level) are estimated as having the same risk for disease/mortality by the model, regardless of the specific combination of pathogens for which an individual is seropositive (see Table 1.1 on next page). For example, when two individuals are categorized as having a summed pathogen burden level of two, one of these individuals may be seropositive for pathogens A and C and the other may be seropositive for pathogens B and D. Thus, these two individuals would be categorized as having a summed pathogen burden level of two and therefore be estimated as having the same risk for mortality, despite being seropositive for two entirely different sets of

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pathogens. Second, assuming a monotonic relationship between summed pathogen burden level and risk for mortality whereby an increase in summed pathogen burden level leads to an increased risk for mortality (see Figure 1.2, page 9) implies that the risk for mortality associated with any specific combination of pathogens is greater than risk for mortality associated with all combinations of fewer pathogens, regardless of pathogen type.

Table 1.1 An Illustration of the Potential Heterogeneity of Pathogen Combinations Within Pathogen Burden Level

	Pathogen Types				
	A	B	C	D	
Pathogen Combinations	Serostatus				Pathogen Burden Level
1	+	-	-	-	
2	-	+	-	-	
3	-	-	+	-	1
4	-	-	-	+	
5	+	+	-	-	
6	+	-	+	-	
7	+	-	-	+	
8	-	+	+	-	2
9	-	+	-	+	
10	-	-	+	+	
11	+	+	+	-	
12	+	-	+	+	
13	+	+	-	+	3
14	-	+	+	+	
15	+	+	+	+	4

Although a graded relationship between total pathogen burden and mortality may exist in certain populations, there is also evidence that pathogens may biologically interact such that combined seropositivity could increase or decrease risk for mortality.[120,121,122] While summed pathogen burden variables may be useful for examining the hypothesis that "more is worse" when it comes to total pathogen burden and risk for mortality, limiting the examination of the relationship between total pathogen burden and mortality to this variable, hinders an assessment of other factors that might play an important role in the association between total pathogen burden and mortality such as the specific combinations of pathogens, for which individuals are seropositive. Thus, the use of summed pathogen burden variables for examining the association between total pathogen burden and mortality, disregards potentially important variability in risk

associated with different combinations of pathogens both *within* pathogen burden levels and *across* pathogen burden levels.

While measures of summed pathogen burden may be useful for examining the hypothesis that "more is worse", further research that also incorporates an assessment of the relationship between specific types of persistent pathogens alone or in combination and risk for mortality is needed. To our knowledge, no study has explicitly examined the extent to which the use of summed pathogen burden variables, limits the ability of researchers to make inferences regarding the contribution of specific types of pathogens and interactions among pathogens to mortality outcomes. The third aim of this dissertation addresses these issues by 1) estimating the combined effects of seropositivity to four persistent pathogens (CMV, HSV-1, HSV-2 and *H. pylori*) on all-cause/CVD-related mortality in a U.S. representative population of individuals ≥ 25 years of age and older, and 2) examining the variability in risk for mortality associated with specific combinations of pathogens *within* and *across* pathogen burden levels.

1.7 Public health significance

Although life expectancy is increasing overall in the United States the absolute number of deaths from chronic diseases continues to increase, the gap in life expectancy between those of low and high SEP is ever widening and traditional risk factors hypothesized to lie in the pathway between SEP and disease don't completely explain the SEP patterning of disease observed in the United States. It remains important to explore novel risk factors for chronic disease and mortality. Over the past several decades, a growing body of evidence has implicated persistent pathogens, many of which pattern according to SEP in early life, in the etiology of cardiovascular disease (CVD) and other chronic diseases.[50-56] Persistent pathogens are thought to impact risk for chronic disease and mortality in part via pathogen-specific effects and in part, because infection increases inflammatory levels. In addition, elevated inflammation is potentially a marker of "reactive" infection which may lead to exacerbated damage by persistent pathogens. Elevated inflammatory markers are in turn, associated with risk for chronic diseases such as CVD, physical impairment and metabolic disorders.[50-56]

Thus, it has been hypothesized that individual pathogens impact risk for chronic disease and consequently mortality via both pathogen-specific effects and by contributing to elevated inflammation. However, the role of particular pathogens, in conjunction with elevated inflammation in determining risk for mortality has primarily been examined in older populations of specific race/ethnicity and gender, thus the association between these factors in the general U.S. adult population is not clear. Furthermore, increased total pathogen burden is thought to also play an important role in determining risk for mortality than seropositivity due to the accumulation of pathogen-specific effects and increased inflammation. However, it is also not clear whether there is a graded relationship between summed pathogen burden and all-cause/CVD-related mortality in the general population. In addition, the use of summed pathogen burden variables, while

useful for examining whether there is a monotonic relationship between summed pathogen burden and mortality, may limit the ability of researchers to make inferences regarding the contribution of specific types of pathogens and interactions among pathogens for risk for mortality. Research is needed to determine whether seropositivity for specific combinations of pathogens plays an important role in determining risk in addition to the role of increased numbers of pathogens and to assess the extent to which the use of summed pathogen burden variables in models assessing the relationship between total pathogen burden and mortality limit the ability for researchers to make inferences about the pathogens that are most detrimental to health in the general population.

The first aim of this dissertation examines whether seropositivity for CMV, a persistent pathogen implicated in several chronic disease outcomes, in conjunction with elevated CRP level increases risk for all-cause/CVD-related mortality. The second aim examines whether there is a graded relationship between summed pathogen burden with CMV, HSV-1, HSV-2 and/or *H. pylori* and all-cause/CVD-related mortality. The third aim will examine the association between seropositivity for specific combinations of pathogens and risk for all-cause/CVD-related mortality and assess the extent to which use of summed pathogen burden variables impact the ability of researchers to identify which particular pathogens, alone or in combination are most detrimental for health.

The results of this research will benefit public health by clarifying the role of CMV, a persistent pathogen implicated in the etiology of numerous chronic diseases, inflammation and all-cause/CVD-related mortality. If CMV infection plays a strong role in the etiology of all-cause/CVD-related mortality, particularly in conjunction with elevated CRP level, the elimination of CMV infection via the development and administration of treatments or vaccines and/or targeting the interaction of infection and CRP levels may reduce mortality rates in the United States. Furthermore, better understanding of the association between summed pathogen burden level and risk for all-cause/CVD-related mortality as well as the role of seropositivty for specific combinations of pathogens on determining risk for death will inform whether infection-related interventions should focus solely on reducing the crude number of pathogens individuals are exposed to or whether interventions focusing on the specific combinations of pathogens that are most detrimental to health would be more effective in reducing mortality in the U.S. general population. Thus, this work has important implications for future public health efforts designed to reduce mortality via infection and inflammation-related pathways.

Seropositivity for Cytomegalovirus, Inflammation, All-cause and Cardiovascular Disease-related Mortality in the United States

2.1 Background

Although a strong and consistent relation between psychological stress and Cytomegalovirus (CMV) is a highly transmissible and prevalent beta herpesvirus.[68,123] This pathogen is never cleared from the body, persisting in a number of tissues via hypothesized mechanisms including chronic productive infection and/or latent infection with periodic subclinical reactivation.[68,69] Recently, CMV has been linked to a variety of chronic diseases with an inflammatory component including cardiovascular disease (CVD) [40,42,70,71], cancer [72,73], cognitive decline including vascular dementia [38,74] and functional impairment.[39,57,75]

Several mechanisms have been hypothesized to link CMV infection and CVD in both human and animal studies.[79,80,94,124-126] CMV antigen and DNA have been identified in atherosclerotic vessels of the human cardiovascular system [45,77,127] and murine models suggest an inflammatory link with CVD progression.[125,126] It has been hypothesized that CMV either directly infects the vessels of the heart and replicates at low levels, or is delivered to the vessel wall by infected circulating monocytes arriving at sites of cardiovascular injury or inflammation.[76] The presence of CMV in the vessel walls may induce smooth muscle cell proliferation and migration, increased uptake of oxidized low-density lipoprotein and expression of cytokines and chemokines as well as increased procoagulant activity by endothelial cells.[79-82] CMV may also cause vascular damage without direct invasion as a result of molecular mimicry, whereby viral antigens trigger an immune response cross-reacting on self-peptides expressed on uninfected host tissues.[79,83,84] For example, two CMV-derived proteins, UL122 and US28, are homologous to an amino acid sequence at position 153-160 of heat shock protein (HSP) 60.[85,86] Thus, infection with CMV may contribute to progression of atherosclerosis and other CVD health outcomes via several mechanisms.

CMV has also been associated with other chronic diseases of aging, including physical impairment, cognitive decline and cancer.[38,39,57,72-75] The specific mechanisms responsible for these associations have not been fully elucidated, but are likely to have an immune and inflammatory component. Indeed, CMV seropositivity belongs to a cluster of immune factors constituting an "immune risk profile" associated with all-cause mortality at 2, 4 and 6-year follow-up in elderly Swedes in the OCTO/NONA longitudinal studies.[128-131] CMV is a driver of age-associated immune changes in elderly populations which lead to a reduction in the number of

naïve T cells available for fighting new infections.[131-134] Reactivations or superinfections may result in higher titers of CMV immunoglobulin G (IgG) antibodies and increased levels of pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α).[50,87,88] C-reactive protein (CRP) levels also increase as a consequence of reactivation or leakage of the virus from host cells, via the action of IL-6 produced in the liver.[80,89,90,135] These inflammatory markers have been linked to both all-cause and CVD-related mortality.[118,136,137] Thus, CMV may impact both CVD-related and all-cause mortality through its affects on chronic inflammatory and immune-related changes seen with aging.

Although not all studies have supported a relationship between CMV and chronic disease outcomes,[138,139] a majority of published studies have reported a significant relationship between CMV and mortality, in conjunction with markers of inflammation.[57-59,91,92] A recent study by Roberts et al., examined the relationship between CMV antibody titer and all-cause/CVD mortality among elderly Latinos finding that subjects with CMV IgG antibody titers in the highest quartile had 1.43 (95% CI: 1.14, 1.79) times higher risk for all-cause mortality and 1.35 (95% CI: 1.01, 1.80) times higher risk for CVD mortality in models adjusted for age, gender, education and comorbidity index. [58] The authors found the effect of CMV on mortality was partially mediated by a composite measure of two inflammatory markers, TNF-α and IL-6, but not CRP since it was unrelated to mortality in their study population.[58] In contrast, Blankenberg et al. found CMV to be associated with cardiac mortality among persons with existing coronary artery disease (CAD) only in subjects with elevated IL-6 levels (hazard ratio (HR) 3.2, 95% confidence interval (CI): 1.4, 7.3) and not in those without IL-6 elevation, suggesting effect modification by IL-6 in the association between CMV and CVD-related mortality.[91] Also examining the effect of CMV antibody titers on mortality, Strandberg et al. assessed 7-year risk for mortality in a small cohort of community-dwelling 75-90 year-olds with underlying CVD in Finland.[59] The authors found that mortality was significantly greater among subjects in the highest CMV IgG quartile, compared with the lowest quartile and that this association remained significant after controlling for several covariates including CRP, suggesting that CRP did not mediate this pathway.[59] Furthermore, Muhlestein et al. examined the association between seropositivity to CMV, *H. pylori* and *Chlamydia pneumoniae* infections, as well as high CRP level and mortality among patients with a mean age of 65 years that were predominantly male and had angiographically demonstrated CAD.[92] Of the three pathogens assessed, only seropositivity to CMV was significantly predictive of mortality. The authors also found that risk for mortality was greatest among CMV seropositive subjects with CRP levels in the highest tertile, suggesting a joint effect of CMV seropositivity and high CRP level on mortality among their sample of individuals with underlying CAD.[92]

Even though these studies suggest an association between CMV, inflammation and mortality, they were conducted in predominantly older age populations, of specific gender or race/ethnic groups and/or among participants who have underlying CAD or CVD health

conditions. While one cross-sectional study conducted among a U.S. population-based cohort of individuals aged 45 and older found that CMV was associated with reported history of CVD, it remains unclear whether a temporal relationship exists between CMV and mortality in the U.S. population.[40] Moreover, the role of inflammatory markers such as IL-6, TNF-α and CRP as mediators or effect modifiers of the relationship between CMV and mortality is unclear.[57-59,91,92] Some studies support mediation by these markers, while others suggest effect modification.[57-59,91,92] Therefore, research examining whether inflammatory markers mediate or modify the relationship between CMV and mortality in the U.S. is needed.

The purpose of this study was to examine whether seropositivity for CMV predicts all-cause as well as CVD-related mortality and whether CRP mediated or modified these relationships in a nationally representative U.S. population of individuals aged 25 and older.

2.2 Methods

Study population

Data come from the National Health and Nutrition Examination Survey (NHANES) III (1988–1994), a population-based, multistage stratified probability survey which collects information on the health and nutrition of the United States civilian non-institutionalized population. The survey was carried out by the National Center for Health Statistics (NCHS), Centers for Disease Control and Prevention and is meant to be representative of the U.S. population. In addition, we used data from the NHANES III-Linked Mortality file in which the mortality status of NHANES III participants was determined by probabilistic matching between NHANES III participants and the U.S. National Death Index (NDI).[140]

A total of 33994 subjects were interviewed in NHANES III. Our study sample is limited to subjects that were 25 years of age and older (range 25-90 years of age) at time of examination (N=15242 (48.7%)), were tested for CMV serostatus and CRP level (N=14164 (92.9%)) and eligible for mortality follow-up on December 31st, 2006 (N=14153 (99.9%). Forty-eight subjects (0.3%) were excluded from the analyses of CVD-related mortality because their cause of death could not be ascertained.

Laboratory Analyses

CMV-specific IgG was measured by a commercially available Enzyme Linked Immunosorbent Assay (ELISA) (Quest International, Inc., Miami, FL). Sera with values near the ELISA cutoff were confirmed with a second ELISA assay (bioMerieux, Inc., Durham, NC). If the results from the first two tests disagreed, an Immunofluorescence Assay (IFA) (Bion International, Inc., Park Ridge, IL), was used and results from this test were provided as the final seropositivity test result. The sensitivity and specificity of these tests have been estimated to be 98% and 99%, respectively.[67, 141] Although CMV IgG and IgM antibody titer levels are available in NHANES III, values for subjects over age 49 were top-coded, making them unusable as a predictor of mortality.

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Sera collected for the purpose of CRP testing were stored at -70 C and analyzed within 2 months using a modification of the Behring Latex-Enhanced CRP assay on the Behring Nephelometer Analyzer system™ (Behring Diagnostics, Westwood, MA). Both within and between-assay quality control procedures were used and the coefficient of variation was 3.2%-16.1% through the period of data collection. The limit of detection for CRP was 0.3 mg/dL.[142] Sera collected for the purpose of cholesterol testing were frozen and stored at -20 C, then shipped on dry ice within four weeks to the Johns Hopkins University Lipid Research Clinic Laboratory which participates in the Lipid Standardization Program of the Centers for Disease Control and Prevention. Total cholesterol was measured enzymatically on a Hitachi 717 analyzer using a commercially available reagent mixture (Boehringer Manheim Diagnostics, Indianapolis, IN).[142]

Measures

Results from the CMV IgG antibody tests were dichotomized as seronegative or seropositive based on the ELISA results. Results from the CRP tests were dichotomized as low: < 0.3 mg/dL and high: ≥ 0.3 mg/dL according to commonly used cut-off values thought to have clinical significance for prediction of heart attack or stroke.[143] Combined CMV serostatus and CRP level was categorized as CMV seronegative/low CRP, CMV seronegative/high CRP, CMV seropositive/low CRP and CMV seropositive/high CRP.

Mortality status was obtained primarily from the NDI; however, other sources of mortality status included indication of deceased status from the Social Security Administration, the Centers for Medicare and Medicaid Services, or death certificate review.[140] Cause of death was coded using the International Classification of Diseases, Ninth Revision (ICD-9) up until 1998 and ICD-10 for 1999-2006. All deaths before 1999 were recoded by the NCHS into comparable ICD-10 codes.[144] ICD-10 codes I00-I99 were classified as CVD-related deaths and included, causes of death such as hypertensive heart disease, atherosclerosis including coronary and cerebrovascular disease, heart failure, and aortic aneurysms. Persons who survived the entire follow-up period were administratively censored on December, 31st 2006. Follow-up time for each person was calculated as the difference between the NHANES III examination date and the last known date alive or censored.[144] Persons who died of non-CVD causes were considered censored at the date of death for CVD mortality analysis.

Covariates hypothesized to be potential confounders of the relationship between CMV serostatus and mortality included age, gender, race/ethnicity, country of origin, body mass index (BMI) (kg/m^2), smoking status, diabetes status and education level, because these factors have been shown to predict both risk for infection and mortality.[23, 67,145-148] Age in years at examination was self-reported and treated as a continuous variable. Gender was dichotomized as female and male. Race/ethnicity was self-reported as non-Hispanic white, non-Hispanic black, Mexican-American or Other. Country of origin was reported as the state or foreign country in which

subjects were born and was categorized as U.S. or Other. Education level was chosen as a marker of life course socioeconomic position most likely to precede CMV infection and self-reported as years of education and treated as continuous.[149,150] BMI (kg/m^2) was computed from weight and standing height and categorized as BMI < 25, 25 ≤ BMI < 30 and BMI ≥ 30. Smoking status was self-reported and categorized into never (did not smoke 100+ cigarettes in one's lifetime), past (smoked 100+ cigarettes in one's lifetime but do not currently smoke) and current smoker (smoked 100+ cigarettes in one's lifetime and currently smoke). Diabetes was self-reported as whether a doctor ever informed subjects they had diabetes or not and dichotomized as reported or not reported. In addition, use of non-steroidal anti-inflammatory drugs (NSAID) was hypothesized as a confounder in the association between the combined effect of CMV serostatus/CRP level and mortality.[151,152] Subjects were considered to use NSAIDs if they reported use of prescription drugs in the past month with a primary classification of NSAID according to the Product Information Branch, Center for Drug Evaluation and Research at the U.S. DHHS Food and Drug Administration or if subjects self-reported taking any Advil, Nuprin, Medipren or Ibuprofen in the past month.[153]

Total serum cholesterol and hypertension were hypothesized as mediators in the pathway between the combined effect of CMV serostatus/CRP level and CVD-related mortality. Serum total cholesterol level was dichotomized as low (< 240 mg/dL) and high (≥ 240 mg/dL) according to the National Cholesterol Education Program Expert Panel [154] and hypertension was reported as ever having been told by a doctor or health professional that you had hypertension, also called high blood pressure.

Statistical analyses

Statistical analyses were performed using SAS, version 9.2, with SAS-callable SUDAAN, version 10.0.1 (SAS Institute, Inc., Cary, NC).[155] All analyses used appropriate weights and adjustments for strata and clustering used in the complex study design used in NHANES III. Bivariate relationships between CMV serostatus, CRP level, all-cause and CVD-related mortality and potential confounders including age, gender, race/ethnicity, country of origin, education level, BMI (kg/m^2), smoking status, diabetes status and NSAID use were assessed. Covariates were considered confounders based on a priori hypotheses and if they were associated with the exposure and associated with the outcome among the unexposed.[156] T-tests (two-tailed) for difference in means and Pearson chi-square tests of independence for proportions and test for trend among demographic groupings were calculated.

Kaplan-Meier survival curves were plotted for all-cause and CVD-related mortality by CMV serostatus to examine the unadjusted association between CMV serostatus and all-cause/CV-related mortality. Survival time was measured in months since mobile or home examination. Cox proportional hazard models were used to estimate the confounder adjusted HR and 95% CI for the association between CMV serostatus and all-cause/CVD-related mortality first

in models adjusted for sociodemographic factors (age, gender, race/ethnicity, country of origin and education level), then, in models additionally adjusted for clinical factors (BMI (kg/m^2), smoking status and diabetes status and last in models also controlling for CRP level. In order to assess whether CRP level mediated the relationship between CMV and mortality we compared the HR for all-cause and CVD-related mortality, before and after controlling for CRP in the fully adjusted model. Adjusted Wald F statistics were estimated to compare follow-up time in months from exam to death between those CMV seronegative versus CMV seropositive in fully adjusted models.

To assess whether CRP modified the relationship between CMV and all-cause/CVD-related mortality we first examined the crude association between combined CMV serostatus and CRP level by plotting unadjusted Kaplan-Meier survival curves for all-cause and CVD-related mortality by combined CMV serostatus and CRP level over the follow-up period. Survival time was measured in months since mobile or home examination. Next we used Cox proportional hazard models to estimate the confounder adjusted HR and 95% CI for the association between CMV serostatus/CRP level and all-cause/CVD-related mortality first in models adjusted for sociodemographic factors (age, gender, race/ethnicity, country of origin and education level) and second, in models adjusted for clinical factors (BMI (kg/m^2), smoking status, diabetes status and NSAID use). Last, adjusted Wald F statistics were estimated to compare follow-up time in months from exam to death between each combination of CMV serostatus and CRP level, adjusting for covariates.

To examine whether more proximal risk factors for CVD, including total cholesterol level and hypertension, lie in the pathway between the combined effect of CMV seropositivity/high CRP level and CVD-related mortality, we compared the HR for CVD-related mortality for those CMV seropositive with high CRP level to individuals that were CMV seronegative with low CRP level, before and after controlling for these factors.

2.3 Results

Weighted estimates of the bivariate relationships between covariates of interest and CMV serostatus are shown in Table 2.1. The weighted proportion of those seropositive to CMV was 66.7%. Higher mean age, female gender, non-white race/ethnicity, country of origin outside the U.S., lower mean education level, low BMI (< 25 kg/m2) and high BMI (≥ 30 kg/m^2) compared to medium BMI (25 ≤ BMI < 30), reported diabetes and high CRP level (≥ 0.3 mg/dL) were associated with CMV seropositivity. Although hypothesized as a potential confounder in the association between CMV serostatus and mortality, smoking status was not associated with CMV serostatus in our study. During the mean 13.7 years of follow-up from exam, the population estimate for the proportion dying from all causes was 18.9% and the proportion dying from CVD-related mortality was 8.0%.

Table 2.1 Demographic and Clinical Characteristics (Weighted) by Cytomegalovirus Serostatus Among Subjects Aged 25 and Older in NHANES III.

	CMV Serostatus (N=14153)		
	Seronegative	Seropositive	
	33.3%	66.7%	
Covariate			p-value
Age (Years at Examination) (Mean ± SE)	41.3 ± 0.43	51.1 ± 0.51	<.0001*
Gender			
Female	44.3%	56.1%	<.0001*
Male	55.7%	43.9%	
Race/Ethnicity			
Non-Hispanic White	91.7%	71.0%	<.0001*
Non-Hispanic Black	4.1%	13.1%	
Mexican-American	1.6%	6.2%	
Other	2.6%	9.7%	
Country of Origin[†]			
United States	95.7%	82.1%	<.0001*
Other	4.3%	17.9%	
Education Level (Years)[‡] (Mean ± SE)	13.5 ± 0.09	11.8 ± 0.09	<.0001*
Body Mass Index (kg/m^2)[±]			
≤ 24.9	45.4%	40.8%	.0317*
25-29.9	32.6%	34.9%	
≥30	22.0%	24.3%	
Smoking Category			
Never smoker	44.8%	44.1%	.9221
Former smoker	26.8%	28.4%	
Current smoker	28.4%	27.5%	
Diabetes[§]			
No	96.0%	93.0%	<.0001*
Yes	4.0%	7.0%	
C-reactive Protein Level			
Low (< 0.3 mg/dL)	76.1%	67.0%	<.0001*
High (≥ 0.3 mg/dL)	23.9%	33.0%	

*Significant at p<0.05, t-tests for difference in means and Pearson chi-square tests for differences in proportions or test for trend among demographic groupings were calculated.
[†] N=14114 due to 39 subjects missing data on country of origin.
[‡] N=14059 due to 94 subjects missing data on education level.
[±] N=14118 due to 35 subjects missing data on body mass index (kg/m^2).
[§] N=14139 due to 14 subjects missing data on diabetes status.

The unadjusted Kaplan-Meier survival curves for all-cause mortality by CMV serostatus are shown in Figures 2.1 and 2.2 below. Overall mean survival duration since time of exam for those CMV seropositive was 13.4 years (160.4 ± 2.40 months) whereas mean survival duration was 14.5 years (173.5 ± 3.15 months) for CMV seronegative subjects.
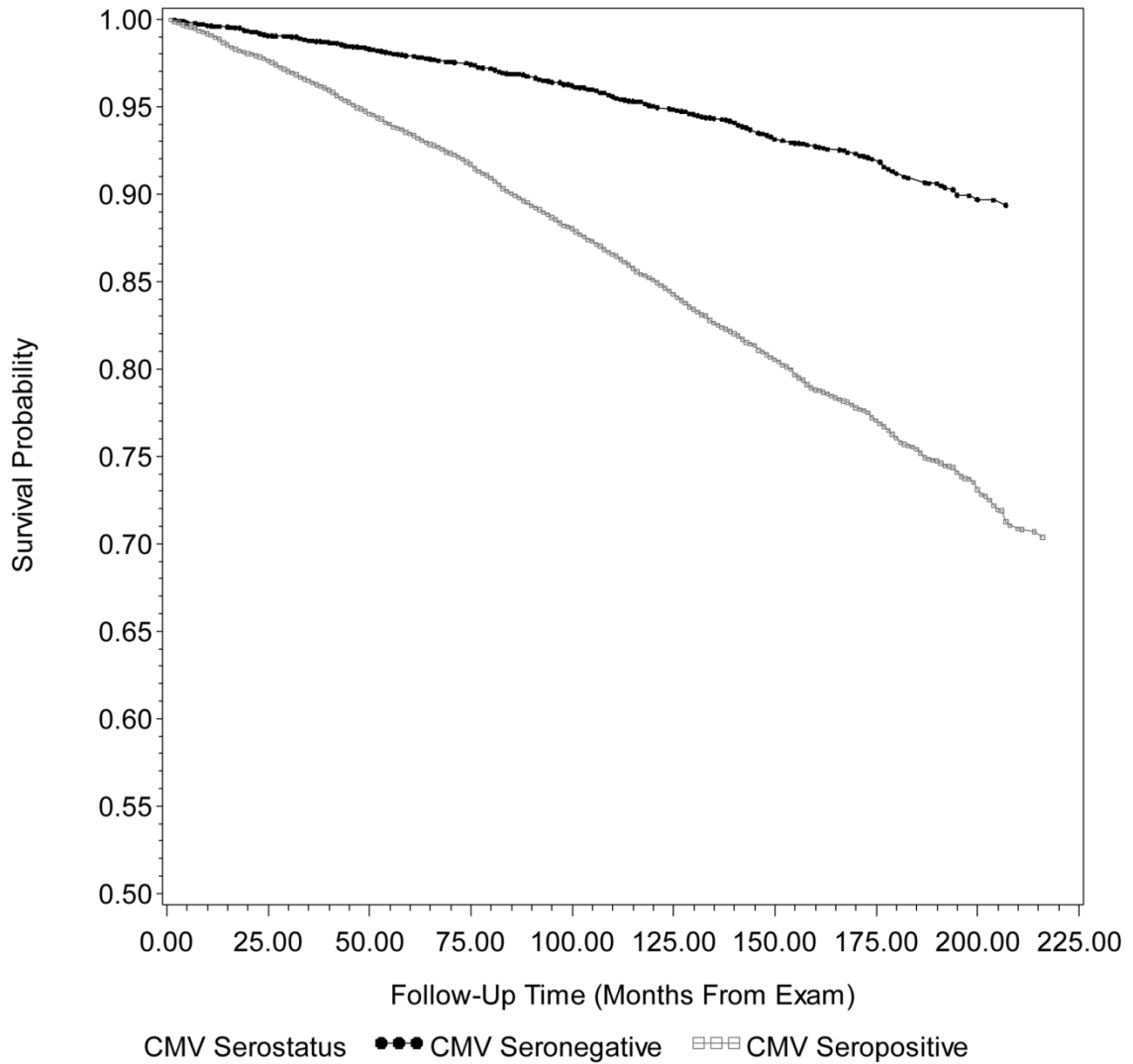
Figure 2.1 Kaplan-Meier Survival Curve for All-Cause Mortality by Cytomegalovirus Serostatus. Unadjusted Kaplan-Meier survival curves for all-cause mortality by cytomegalovirus (CMV) serostatus for 14153 subjects, $\geq$ 25 years of age, in the National Health and Nutrition Examination Survey (NHANES) III from 1988-2006. After adjusting for age, gender, race/ethnicity, country of origin, education level, body mass index (kg/m^2), smoking status and diabetes status, follow-up time from exam to death from all causes was significantly different between CMV seronegative and CMV seropositive subjects (Adjusted Wald F = 4.66, p-value = 0.0358). CMV=cytomegalovirus.
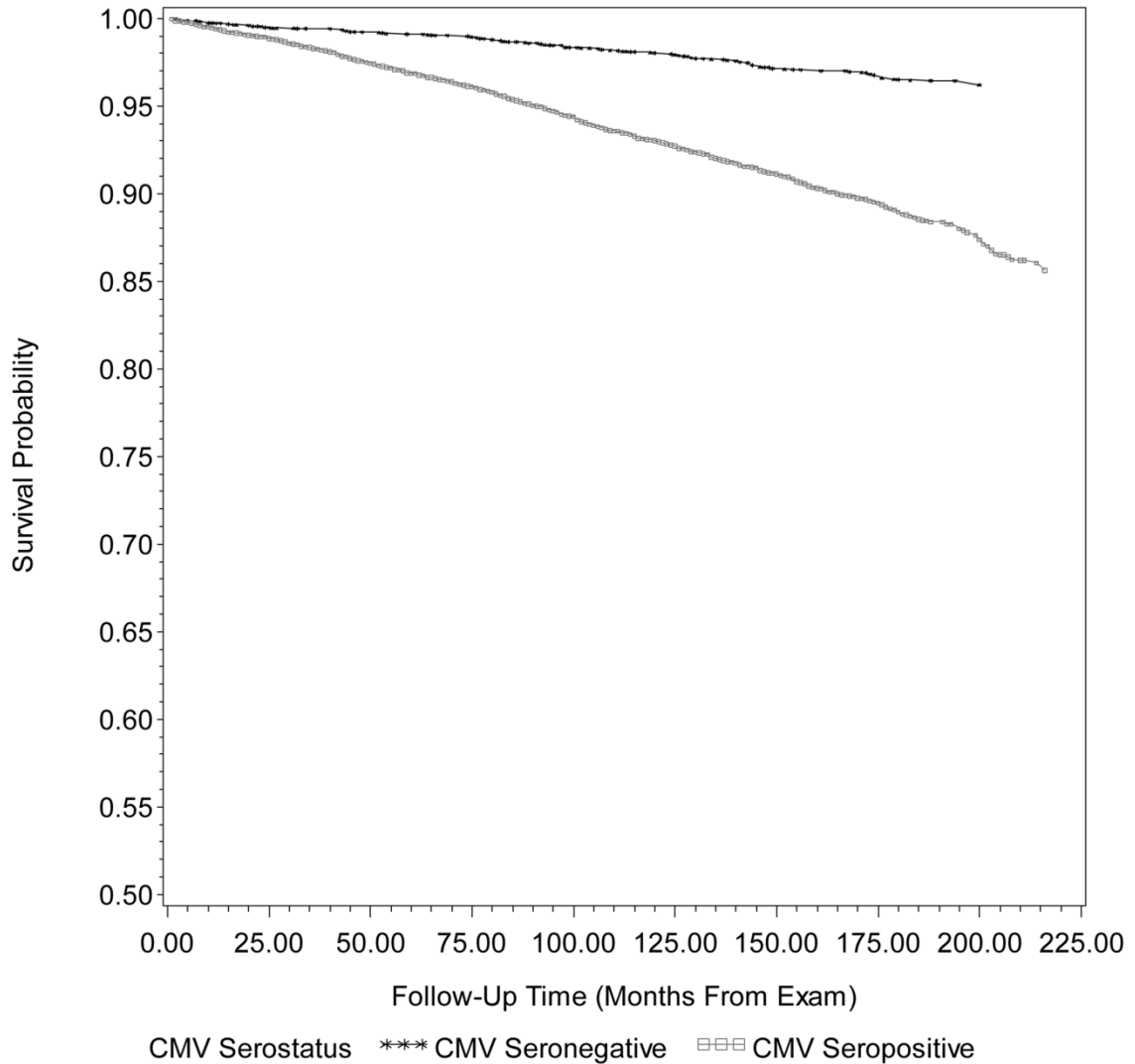
Figure 2.2 Kaplan Meier Survival Curve for Cardiovascular Disease-related Mortality by Cytomegalovirus Serostatus. Unadjusted Kaplan-Meier survival curves for cardiovascular disease (CVD)-related mortality by cytomegalovirus (CMV) serostatus for 14105 subjects, ≥ 25 years of age, in the National Health and Nutrition Examination Survey (NHANES) III from 1988-2006. After adjusting for age, gender, race/ethnicity, country of origin, education level, body mass index (kg/m^2) , smoking status and diabetes status, follow-up time from exam to death from CVD was not significantly different between CMV seronegative and CMV seropositive subjects (Adjusted Wald F = 2.66, p-value = 0.1092. CMV=cytomegalovirus and MEC=mobile examination center.

Table 2.2 shows the HR and 95% CI from Cox proportional hazard models examining the association between CMV and all-cause/CVD-related mortality, mutually adjusted for hypothesized confounders. After adjusting for age, gender, race/ethnicity, country of origin, education level, BMI (kg/m^2), smoking status and diabetes status, CMV seropositivity remained statistically significantly associated with all-cause mortality (HR 1.19, 95% CI: 1.01, 1.41) and follow-up time from exam to death from all causes was significantly different between CMV seronegative and CMV seropositive subjects (Adjusted Wald F = 4.66, p-value = 0.0358) in model 2. The effect of CMV on all-cause mortality was not further attenuated after controlling for CRP level (HR 1.19, 95% CI: 1.01, 1.40 versus HR 1.19, 95% CI: 1.01, 1.41). Although the magnitude of effect for CVD-related mortality was similar to that of all-cause mortality, the association between CMV and CVD-related mortality was not statistically significant in the fully adjusted model and follow-up time from exam to death from CVD-related mortality for those CMV seropositive versus seronegative was not statistically significantly different (Adjusted Wald F = 2.66, p-value = 0.1092). Similarly, adjustment for CRP level did not further attenuate the association between CMV and CVD-related mortality (HR 1.19, 95% CI: 0.95, 1.49 versus HR 1.19, 95% CI: 0.96, 1.49).

Table 2.2 The Relationship Between Cytomegalovirus Serostatus, C-reactive Protein Level and All-Cause/Cardiovascular Disease-related Mortality in Subjects 25 Years of Age and Older in NHANES III

	Hazard Ratio (95% Confidence Interval)					
	All-Cause Mortality			Cardiovascular Disease-Related Mortality		
	Model 1[†]	Model 2[‡]	Model 3[¶]	Model 1[†]	Model 2[‡]	Model 3[¶]
CMV						
Seronegative	1.0	1.0	1.0	1.0	1.0	1.0
Seropositive	1.20 (1.03, 1.41)*	1.19 (1.01, 1.41)*	1.19 (1.01, 1.40)*	1.20 (0.96, 1.50)	1.19 (0.96, 1.49)	1.19 (0.95, 1.49)
Age (years since exam)	1.09 (1.09, 1.10)*	1.10 (1.09, 1.11)*	1.10 (1.09, 1.10)*	1.11 (1.11, 1.12)*	1.12 (1.11, 1.13)*	1.12 (1.11, 1.13)*
Gender						
Female	1.0	1.0	1.0	1.0	1.0	1.0
Male	1.49 (1.35, 1.64)*	1.40 (1.27, 1.54)*	1.42 (1.29, 1.57)*	1.58 (1.39, 1.80)*	1.54 (1.34, 1.77)*	1.57 (1.36, 1.80)*
Race/Ethnicity						
Non-Hispanic	1.0	1.0	1.0	1.0	1.0	1.0
Non-Hispanic	1.27 (1.12, 1.45)*	1.20 (1.05, 1.37)*	1.17 (1.03, 1.33)*	1.20 (1.00, 1.44)	1.11 (0.92, 1.35)	1.09 (0.90, 1.30)
Mexican-American	0.88 (0.74, 1.05)	0.88 (0.74, 1.05)	0.87 (0.73, 1.03)	0.89 (0.67, 1.17)	0.86 (0.66, 1.13)	0.85 (0.65, 1.12)
Other	0.83 (0.64, 1.07)	0.76 (0.59, 0.99)*	0.78 (0.60, 1.01)	0.88 (0.53, 1.47)	0.78 (0.45, 1.35)	0.80 (0.47, 1.37)
Country of Origin						
United States	1.0	1.0	1.0	1.0	1.0	1.0
Other	0.69 (0.57, 0.84)*	0.77 (0.64, 0.93)*	0.78 (0.65, 0.93)*	0.64 (0.48, 0.85)*	0.70 (0.53, 0.91)*	0.70 (0.54, 0.91)*
Education Level (years)	0.95 (0.94, 0.97)*	0.96 (0.95, 0.98)*	0.97 (0.95, 0.98)*	0.96 (0.95, 0.97)*	0.97 (0.96, 0.98)*	0.97 (0.96, 0.99)*
Body Mass Index (kg/m^2)						
< 25	----	1.18 (1.04, 1.33)*	1.22 (1.07, 1.38)*	----	1.14 (0.97, 1.34)	1.18 (1.00, 1.39)

25 ≤ BMI < 30	----	1.0	1.0	----	1.0	1.0
≥ 30	----	1.21 (1.05, 1.40)*	1.16 (1.00, 1.33)	----	1.34 (1.08, 1.66)*	1.28 (1.03, 1.58)*
Smoking						
Never	----	1.0	1.0		1.0	1.0
Former	----	1.28 (1.14, 1.44)*	1.27 (1.13, 1.42)*		1.16 (1.00, 1.35)	1.15 (1.00, 1.33)
Current	----	2.27 (2.00, 2.58)*	2.20 (1.93, 2.50)*		1.96 (1.62, 2.37)*	1.90 (1.58, 2.29)*
Diabetes						
No	----	1.0	1.0	----	1.0	1.0
Yes	----	1.90 (1.65, 2.19)*	1.87 (1.63, 2.15)*	----	1.99 (1.62, 2.46)*	1.96 (1.60, 2.42)*
C-reactive Protein Level						
Low (< 0.3	----	----	1.0	----	----	1.0
High (≥ 0.3	----	----	1.34 (1.23, 1.45)*	----	----	1.36 (1.17, 1.59)*

*Significant at p<0.05

[†]Model 1 for all-cause mortality (N=14029) and for cardiovascular disease-related mortality (N=13981) adjusted for age, gender, race/ethnicity, country of origin and education level and reduced by 124 subjects due to missing data on country of origin and/or education level.

[‡] Model 2 for all-cause mortality (N=13981) and for cardiovascular disease-related mortality (N=13934) adjusted for age, gender, race/ethnicity, country of origin, education level, body mass index (kg/m^2), smoking status and/or diabetes status. Model 2 for all-cause mortality reduced by an additional 48 subjects and for cardiovascular disease-related mortality reduced by an additional 47 subjects due to missing data on body mass index, smoking status and/or diabetes status.

[¶] Model 3 for all-cause mortality (N=13981) and for cardiovascular disease-related mortality (N=13934) adjusted for age, gender, race/ethnicity, country of origin, education level, body mass index (kg/m^2), smoking status, diabetes status and C-reactive protein level.

Figures 2.3 and 2.4 show the unadjusted Kaplan-Meier survival curves for all-cause and CVD-related mortality by the four different permutations of CMV serostatus and CRP level. The overall mean survival times from exam to mortality for each combination of CMV serostatus and CRP level were 14.7 years (176.0 ± 3.20 months) for CMV seronegative individuals with low CRP, 13.8 years (165.6 ± 3.49 months) for CMV seronegative individuals with high CRP, 13.8 years (165.5 ± 2.46 months) for CMV seropositive individuals with low CRP and 12.5 years (150.0 ± 3.49 months) for CMV seropositive individuals with high CRP.
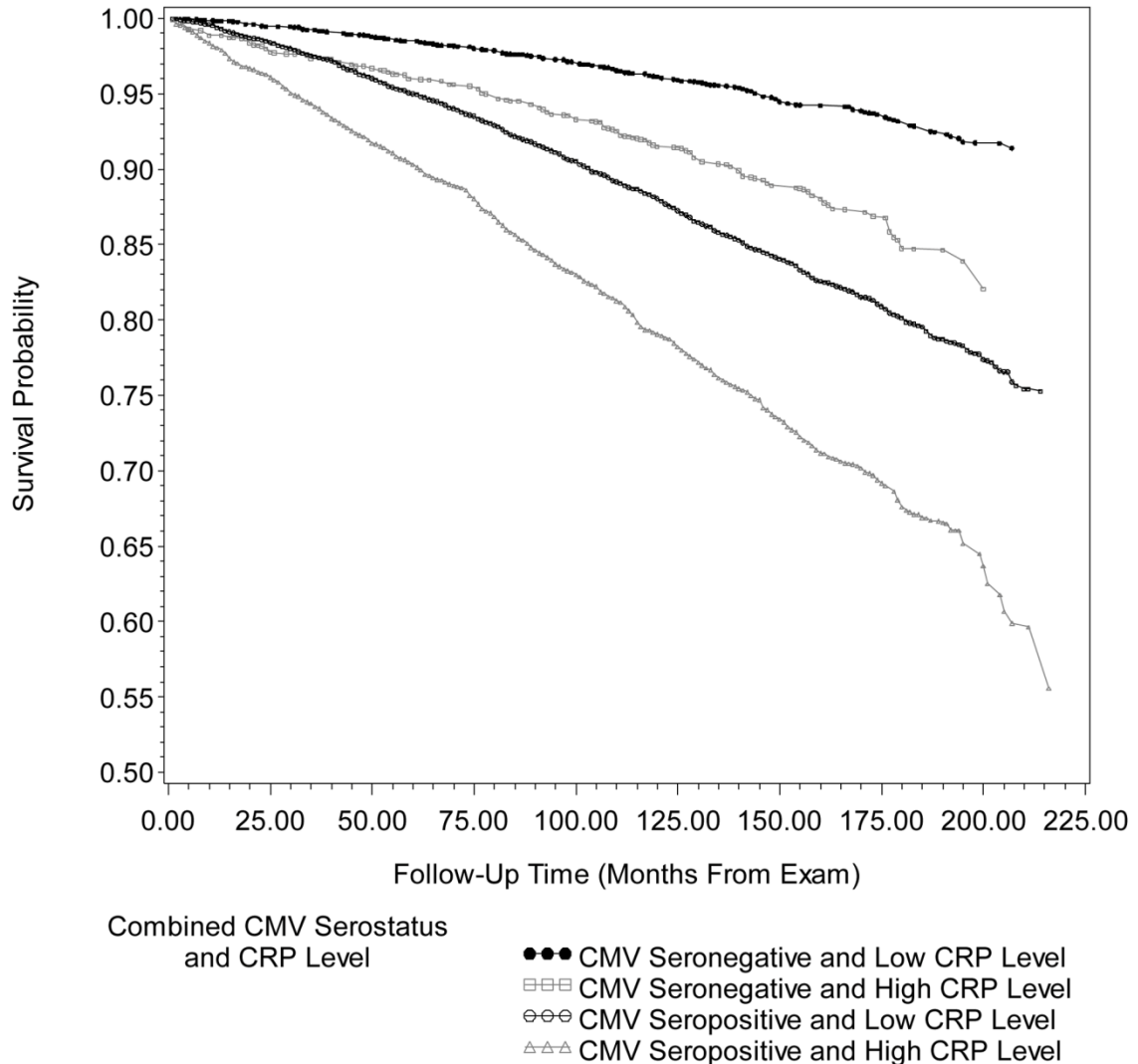
Figure 2.3 Kaplan-Meier Survival Curve for All-Cause Mortality by Combined Cytomegalovirus Serostatus and C - reactive protein Level. Unadjusted Kaplan-Meier survival curves for all-cause mortality by combined cytomegalovirus (CMV) serostatus and C-reactive Protein (CRP) level for 14011 subjects, ≥ 25 years of age, in the National Health and Nutrition Examination Survey (NHANES) III from 1988-2006. After adjusting for age, gender, race/ethnicity, country of origin, education level, BMI (kg/m^2), smoking status, diabetes status and non-steroidal anti-inflammatory drug use, follow-up time from exam to death from all-causes for CMV seropositive individuals with high CRP level was significantly different from CMV

seropositive individuals with low CRP level (Adjusted Wald F = 36.19, p<0.0001). CMV=cytomegalovirus, CRP=C - reactive protein. High CRP level: ≥ 0.3 mg/dL.
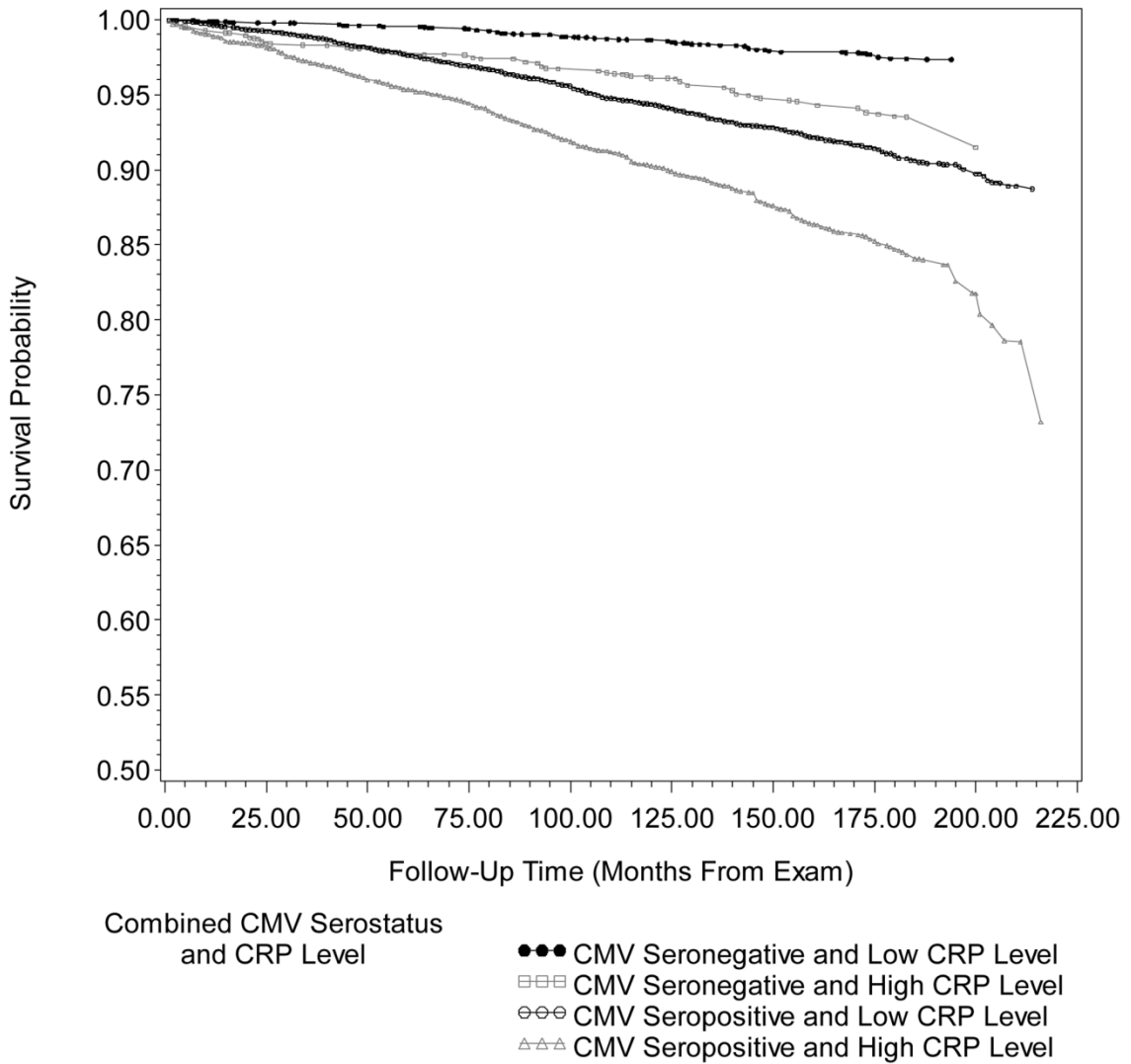
Figure 2.4 Kaplan Kaplan-Meier Survival Curve for Cardiovascular Disease-Related Mortality by Combined Cytomegalovirus Serostatus and C - reactive protein Level. Unadjusted Kaplan-Meier survival curves for cardiovascular disease (CVD)-related mortality by combined cytomegalovirus (CMV) serostatus and C-reactive Protein (CRP) level for 13963 subjects, ≥ 25 years of age, in the National Health and Nutrition Examination Survey (NHANES) III from 1988-2006. After adjusting for age, gender, race/ethnicity, country of origin, education level, BMI (kg/m^2), smoking status, diabetes status and non-steroidal anti-inflammatory drug use, follow-up time from exam to CVD-related death for CMV seropositive individuals with high CRP level was significantly different from CMV seropositive individuals with low CRP level (Adjusted Wald F = 9.10, p = 0.0040). CMV=cytomegalovirus, CRP=C - reactive protein. High CRP level: ≥ 0.3 mg/dL.

Table 2.3 shows the HR and 95% CI from Cox proportional hazard models examining the association between different permutations of CMV serostatus and CRP level and all-cause or CVD-related mortality, adjusted for confounders. After adjustment for age, gender, race/ethnicity, country of origin, education level, BMI (kg/m^2), smoking status, diabetes status and NSAID use, the highest HR for all-cause and CVD-related mortality was found among people who were both CMV seropositive and had high CRP levels (HR 1.60, 95% CI: 1.31, 1.94, and HR 1.71, 95% CI: 1.21, 2.42, respectively), compared to CMV seronegative subjects with low CRP. Thus, even after confounder adjustment, subjects that were CMV seropositive and had high CRP had a 30.1% higher risk for all-cause mortality and 29.5% higher risk for CVD-related mortality compared to CMV seropositive subjects who had low CRP levels. The follow-up time from exam to death from all-causes for CMV seropositive individuals with high CRP was significantly different from CMV seropositive individuals with low CRP (Adjusted Wald F = 36.19, p<0.0001) in fully adjusted models. In addition, the follow-up time from exam to CVD-related death for CMV seropositive individuals with high CRP was significantly different from CMV seropositive individuals with low CRP (Adjusted Wald F = 9.10, p = 0.0040) in fully adjusted models. After adjustment for total cholesterol level and hypertension, the HR for CVD-related mortality among those CMV seropositive with high CRP level compared to individuals that were CMV seronegative with low CRP, was attenuated by 3.5% (HR 1.65 95% CI: 1.16, 2.35, compared to HR 1.71, 95% CI: 1.21, 2.42).

Table 2.3 The Combined effect of Cytomegalovirus (CMV) Serostatus and C-reactive Protein (CRP) Level on All-Cause/Cardiovascular Disease (CVD) -related Mortality in Subjects 25 Years of Age and Older in NHANES III

| | Hazard Ratio (95% Confidence Interval) | | | |
| | All-Cause Mortality | | CVD-related Mortality | |
Combined Factors	**Model 1[†]**	**Model 2[‡]**	**Model 1[†]**	**Model 2[‡]**
CMV Seronegative and Low CRP Level	1.0	1.0	1.0	1.0
CMV Seronegative and High CRP Level	1.56 (1.23, 1.97)*	1.46 (1.16, 1.83)*	1.81 (1.17, 2.78)*	1.67 (1.08, 2.57)*
CMV Seropositive and Low CRP Level	1.25 (1.04, 1.49)*	1.23 (1.03, 1.47)*	1.33 (0.97, 1.81)	1.32 (0.97, 1.78)
CMV Seropositive and High CRP Level	1.73 (1.41, 2.11)*	1.60 (1.31, 1.94)*	1.85 (1.31, 2.60)*	1.71 (1.21, 2.42)*

*Significant at $p < 0.05$
[†]Model 1 for all-cause mortality (N=14029) and for CVD-related mortality (N=136981) adjusted for age, gender, race/ethnicity, country of origin and education level and reduced by 124 subjects due to missing data on country of origin and/or education level.
[‡]Model 2 for all-cause mortality (N=13611) and for CVD-related mortality (N=13569) adjusted for age, gender, race/ethnicity, country of origin, education level, body mass index (kg/m^2), smoking status, diabetes status and non-steroidal anti-inflammatory drug use. Model 2 for all-cause mortality reduced by an additional 418 subjects and model 2 for CVD-related mortality reduced by an additional 322 subjects due to missing data on body mass index, smoking status, diabetes status and/or non-steroidal anti-inflammatory drugs.

2.4 DISCUSSION

This study examined whether seropositivity for CMV, an indicator of prior infection with this persistent herpesvirus, predicts all-cause as well as CVD-related mortality and whether CRP mediates or modifies this relationship in a nationally representative U.S. population of individuals 25 years of age and older. The results of our study suggest that CMV seropositivity is independently associated with all-cause mortality, after controlling for age, gender, race/ethnicity, country of origin, education level, BMI (kg/m^2), smoking status and diabetes status. Furthermore, adjustment for CRP level did not attenuate this relationship.

After confounder adjustment, CMV serostatus was no longer significantly associated with CVD mortality. It is possible that the strong relationship between CMV and all-cause mortality observed in this study indicates that CMV seropositive subjects are dying of competing causes before they have the chance to develop CVD, attenuating the relationship between CMV and CVD-related mortality. High levels of CRP, however, augmented the effect of CMV seropositivity on both all-cause and CVD-related mortality. Among CMV seropositive subjects, those with high CRP levels showed approximately a 30% higher risk for all-cause mortality and for CVD-related mortality, compared to those with low CRP levels. Adjusting for CVD risk factors such as high total cholesterol and hypertension resulted in a moderate attenuation (decrease of 3.5%) of the association between combined CMV seropositivity and high CRP level and CVD-related mortality, supporting the hypothesis that CMV seropositivity along with subclinical inflammation impacts risk for mortality in part through their combined contribution to other more proximal CVD risk factors. Nonetheless, these markers of CVD did not completely attenuate the association, indicating that other factors may also be on the pathway or that there is a direct relationship between CMV infection, CRP levels and CVD-related mortality. Thus, our research suggests that efforts identifying the mechanisms of these additive effects and targeted CVD intervention studies among subpopulations with these biomarker risk profiles are warranted.

It has been suggested that individuals who are CMV seropositive and also have a subclinical inflammatory profile are more susceptible to the atherogenic effects of CMV infection, whereas those without a subclinical response are not as susceptible.[92,135] Studies support a correlation between increased CRP and increased CMV antibody titers- a marker of reactivation - but the directionality among these two biological markers is unclear.[58,59,157] If elevated CRP is a marker of "reactivating" CMV infection, whereas low CRP indicates resolved or "latent" CMV infection, we would expect CRP levels to be increased during times of CMV reactivation and for CMV infection to be most detrimental under these circumstances. Since CMV has been found to directly invade cardiovascular tissues[45,77,127] periods of CMV "reactivation" may accelerate the atherogenic process by increasing the detrimental effects of tissue invasion such as smooth muscle cell proliferation and migration.[79-82] On the other hand, if, as hypothesized, molecular mimicry plays a role in the relationship between CMV and CVD-mortality, the immune system

may become hyper-stimulated during periods of CMV "reactivation" leading to an exacerbated attack against host tissues presenting cross-reacting human peptides.[83-86] For these reasons, the greatest physiological harm caused by CMV is likely to occur during "reactivation" events, possibly at a subclinical level. If subclinical "reactive" CMV infection is most important in the etiology of mortality, then situations that cause CMV reactivation over the lifecourse, such as stress, inflammation caused by co-infections, immunosuppression and aging may play important key roles in determining the extent to which CMV infection is detrimental to health.

Although CMV IgG and IgM antibody titer levels are available in NHANES III, values for subjects over age 49 were top-coded, making them unusable as a predictor of mortality. It was also not possible to correlate antibody levels as an indicator of "reactivating" versus "latent" CMV infection in our study population. In addition, approximately 64% of our study population had CRP levels under the limit of detection (< 0.3 mg/dl), because more sensitive assays that are currently available for measurement of high-sensitivity CRP below this limit of detection were not used in NHANES III, making it difficult to examine CRP levels continuously. Therefore, one limitation of our study is that were unable to examine the relationship between CMV antibody titer and CRP level more closely, which might have allowed us to identify a more precise interaction between CMV infection and CRP levels and their effect on all-cause and CVD-related mortality. Another limitation associated with using CRP as a predictor of mortality is that very high values of CRP may represent acute infection or injury not necessarily associated with cardiovascular disease. In addition, the causal relations and therapeutic implications pertaining to CRP and CVD are currently unclear.[158-161] However, other inflammatory markers that have been shown to play a significant role in the pathway between CMV and mortality, such as IL-6, are not available in NHANES III. Thus, it was not possible to determine whether other inflammatory markers in addition to CRP mediate or modify the relationship between CMV and mortality. Nonetheless, our exposure of interest, CMV serostatus, was laboratory-confirmed and CRP was measured by standard methodologies.[67, 140,142]

Another limitation to our study is that we utilized the updated NHANES III Linked Mortality Public-use File which was subject to data perturbation in which synthetic data was substituted for the actual date of death and underlying cause of death for selected descendent records in order to reduce risk of respondent re-identification.[162] Despite the data perturbation of date of death for selected decedents in the public-use file, a comparison study between the public-use and restricted-use file (which does not contain perturbed data) conducted by the NCHS, found that analysis of all-cause and cause-specific mortality (heart disease, ischemic heart disease and cerebrovascular disease, in particular) by sociodemographic factors including as age, sex, race/ethnicity and education level to be comparable across files and concluded that the discrepancies between the public-use and restricted-use file were minor and that analysts should use the public-use file with confidence.[162] Importantly, mortality status was rigorously verified by

two sources (National Death Index (NDI) and/or death certificate review.[140] Furthermore, NDI is a validated method for matching deaths in the U.S. to population-based datasets and for obtaining cause of death.[163]

2.5 CONCLUSION

Several studies have examined the relationship between CMV and mortality.[57-59, 91, 92] However, these earlier studies have only examined the relationship among specific gender and racial/ethnic subgroups, often among subjects with existing CVD and primarily compared mortality by CMV antibody titer level (not CMV serostatus). To the best of our knowledge, our study is the first to utilize a large, nationally representative U.S. population aged 25 and older with up to 18.1 years of follow-up (mean of 13.7 years) to examine the relationship between CMV serostatus, CRP level and all-cause/CVD-related mortality. Therefore, the population studied here is much broader in age and on average younger than the study populations utilized in several earlier studies examining the impact of CMV infection on mortality. A moderate increase in risk among a large population with a wide age range in the US has important implications for potentially shifting the population burden of diseases.[164]

If CMV infection plays a strong role in the etiology of all-cause and CVD-related mortality in conjunction with high CRP level, the elimination of CMV infection via the development and administration of treatments or vaccines [164] and/or targeting the interaction of infection and CRP levels may reduce mortality rates in the United States. Colugnati et al. predicted that a vaccination against CMV would not need to have high efficacy nor wide-spread coverage to make a substantial impact on CMV transmission and elimination of CMV from the population has the potential to greatly reduce the incidence of disease attributable to CMV infection.[40, 165] Therefore, elimination of CMV infection is a potentially feasible and important avenue of study for preventing mortality from all-causes and CVD in the United States.

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Chapter 3

Summed Pathogen Burden and Risk for All-Cause and Cardiovascular Disease-related Mortality in the United States

3.1 Background

Over the past 30 years, a growing body of evidence has implicated numerous individual pathogens, both viral and bacterial species, in cardiovascular disease (CVD) [40,43,166] as well as several other chronic diseases with inflammation-related etiology including metabolic disorders [41], physical [39,75] and cognitive impairment [38,167] and cancer. [72,73,93,110] Persistent pathogens are linked to chronic disease via direct, pathogen-specific effects such as in the case of CVD, in which certain pathogens invade vascular tissues and trigger atherogenesis.[46,79,124,127] In addition, initial infection as well as reactivation with persistent pathogens triggers the release of acute phase proteins and increases levels of inflammatory markers such as C-reactive protein, which have been linked to CVD and other chronic diseases.[50-56] As a result, it has been hypothesized that due to an accumulation of both pathogen-specific effects, as well as overall increased levels of inflammation, the more pathogens an individual is seropositive for (i.e., increased summed total pathogen burden), the greater their risk for chronic disease and mortality.[52,54,84,168-171]

Despite the hypothesized relationship between summed pathogen burden, inflammation and chronic disease, very few studies have examined the association between summed pathogen burden and mortality.[60-62,119] The few studies that exist were primarily conducted among persons with existing CVD, of older age and from specific race/ethnic groups limiting their generalizability to the general population.[60-62,119] Therefore, the objective of this study is to examine whether there is a graded relationship between increasing pathogen burden with four persistent pathogens (cytomegalovirus (CMV), herpes simplex virus-1(HSV-1), herpes simplex virus-2 (HSV-2) and/or *Helicobacter pylori* (*H. pylori*)) implicated in CVD as well as other chronic diseases with inflammatory etiology[38,41,44,46,74,93,172-174] and all-cause/CVD-related mortality in a U.S. representative population of individuals ≥ 25 years of age.

3.2 METHODS
Study population

Data come from the National Health and Nutrition Examination Survey (NHANES) III (1988–1994); a population-based, multistage stratified probability survey which collected information on the health and nutrition of the United States civilian non-institutionalized population. The survey was carried out by the National Center for Health Statistics (NCHS), Centers for Disease Control and Prevention. A total of 33994 subjects were interviewed in

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NHANES III, but our study sample is limited to subjects who were 25 years of age and older at time of examination (N=15242 (48.7%)), had mortality follow-up (N=15229 (99.9%)) and were tested for serostatus for all four pathogens (N=6522 (42.8%)). Sera was selected randomly for seropositivity testing, however, only subjects in phase I were tested for *H. pylori*, therefore, our analysis was limited to subjects tested for seropositivity for the other three pathogens in phase I. In addition, we used data from the NHANES III-Linked Mortality file in which the mortality status of NHANES III participants was determined by probabilistic matching between NHANES III participants and the U.S. National Death Index (NDI).[140] Cause of death was unavailable for 18 subjects, thus, these subjects were excluded from analysis for CVD-related mortality.

Laboratory Analyses

CMV-specific IgG was measured by a commercially available Enzyme Linked Immunosorbent Assay (ELISA) (Quest International, Inc., Miami, FL). Sera with values near the ELISA cutoff were confirmed with a second ELISA assay (bioMerieux, Inc., Durham, NC). If the results from the first two tests disagreed, an Immunofluorescence Assay (IFA) (Bion International, Inc., Park Ridge, IL), was used and results from this test were provided as the final seropositivity test result. The sensitivity and specificity of these tests have been estimated to be 98 and 99%, respectively.[67,141] HSV-1 and HSV-2 seropositivity was assessed by solid-phase enzymatic immunodot assays using purified glycoprotein gG-1 of HSV-1 and gG-2 of HSV-2 as the antigen.[175] These immunodot assays have also been shown to have high sensitivity and specificity.[176] Subjects 20 years and older from phase 1 (1988-1991) were tested for *H. Pylori* antibody in 1996 using *H. Pylori* IgG ELISA (Wampole Laboratories, Cranbury, NJ). The cut points for seropositivity to each infection are based on commercially available immunoassay clinical diagnostics that provide information on IgG antibody seropositivity.[177]

Measures

Results from the IgG antibody tests for each pathogen were dichotomized as seronegative or seropositive based on the ELISA results. Summed pathogen burden was constructed as the total number of pathogens for which each subject was seropositive (range 0-4), producing 5 pathogen burden levels, with seropositivity for zero pathogens serving as the referent category.

Mortality status was obtained by NCHS primarily from the National Death Index (NDI); however, other sources of mortality status included indication of deceased status from the Social Security Administration, the Centers for Medicare and Medicaid Services, or death certificate review.[140] Cause of death was coded using the International Classification of Diseases, Ninth Revision (ICD-9) up until 1998 and ICD-10 for 1999-2006. All deaths before 1999 were recoded by the NCHS into comparable ICD-10 codes.[144] ICD-10 codes I00-I99 were classified as CVD-related deaths and included, causes of death such as hypertensive heart disease, atherosclerosis including coronary and cerebrovascular disease, heart failure, and aortic aneurysms. Persons

who survived the entire follow-up period were administratively censored on December, 31st 2006. Follow-up time for each person was calculated in months as the difference between the NHANES III examination date and the last known date alive or censored.[144] Persons who died of non-CVD causes were considered censored at the date of death for CVD mortality analysis.

Covariates hypothesized to be potential confounders of the relationship between summed pathogen burden and mortality included age, gender, race/ethnicity, country of origin, body mass index (BMI) (kg/m^2), smoking status, diabetes status and education level, because these factors have been shown to predict both risk for infection and mortality.[23, 67,145-148] Age in years at examination was self-reported and treated as a continuous variable. Gender was dichotomized as female and male. Race/ethnicity was self-reported as non-Hispanic white, non-Hispanic black, Mexican-American or Other. Country of origin was reported as the state or foreign country in which subjects were born and was categorized as U.S. or Other. Education level was chosen as a marker of life course socioeconomic position most likely to precede CMV infection and self-reported as years of education and treated as continuous.[149,150] BMI (kg/m^2) was computed from weight and standing height and categorized as BMI < 25, 25 ≤ BMI < 30 and BMI ≥ 30. Smoking status was self-reported and categorized into never (did not smoke 100+ cigarettes in one's lifetime), past (smoked 100+ cigarettes in one's lifetime but do not currently smoke) and current smoker (smoked 100+ cigarettes in one's lifetime and currently smoke). Diabetes was self-reported as whether a doctor ever informed subjects they had diabetes or not and dichotomized as reported or not reported.

Statistical analyses

Statistical analyses were performed using SAS, version 9.2, with SAS-callable SUDAAN, version 10.0.1 (SAS Institute, Inc., Cary, NC).[155] All analyses used appropriate weights and adjustments for strata and clustering used in the complex study design in NHANES III. Bivariate relationships between summed pathogen burden and all-cause/CVD-mortality and survival duration as well as between summed pathogen burden and potential confounders including age, gender, race/ethnicity, country of origin, education level, BMI (kg/m^2), smoking status and diabetes status were assessed. T-tests (two-tailed) for difference in means and Pearson chi-square tests of independence for proportions and test for trend among demographic groupings were estimated.

To examine whether there was a graded relationship between summed total pathogen burden and all-cause/CVD-related mortality, Kaplan-Meier survival curves were plotted for all-cause and CVD-related mortality by pathogen burden level to first examine the unadjusted association between summed pathogen burden and mortality. Survival time was measured in months since mobile or home examination. Cox proportional hazard models were used to estimate the confounder adjusted HR and 95% CI for the association between pathogen burden level and CVD-related/all-cause mortality first in models adjusted for sociodemographic factors

(age, gender, race/ethnicity, country of origin and education level), then, in models additionally adjusted for clinical factors (BMI (kg/m^2), smoking status and diabetes status.

3.3 RESULTS

Weighted estimates of the bivariate relationships between covariates of interest and summed total pathogen burden are shown in Table 3.1. The weighted proportion of individuals in each pathogen burden level ranging from 0 to 4 was 11.0%, 21.7%, 29.5%, 29.1% and 8.7%, respectively. Higher age, female gender, non-white race/ethnicity, country of origin outside the U.S., lower education level, low BMI (< 25 kg/m^2) and high BMI (≥ 30 kg/m^2) compared to medium BMI (25 ≤ BMI < 30) and reported diabetes were associated with increased pathogen burden. Although hypothesized as a potential confounder in the association between pathogen burden and mortality, smoking status was not associated with total pathogen burden in our study. There was a statistically significant trend for death from all-causes and CVD-related death with increasing pathogen burden. Overall mean survival duration since time of exam for those seropositive to zero pathogens was 16.1 years (193.5 ± 2.3 months), to one pathogen was 15.7 years (188.1 ± 2.1 months), to two pathogens was 14.9 years (179.3 ± 2.0 months), to three pathogens was 14.4 years (172.7 ± 1.9 months) and to all four pathogens was 13.8 years (165.2 ± 2.5). During the mean 15.0 years of follow-up from exam, among subjects in our sample, the population estimate for the proportion dying from all causes was 21.0% and from CVD-related causes was 8.7%.

Table 3.1. Demographic and Clinical Characteristics (Weighted) by Summed Pathogen Burden Level Among Subjects Aged 25 and Older in NHANES III

N=6522	Pathogen Burden Level					P-value
	0	1	2	3	4	
Covariates (%)						
All-Cause Mortality						
Survived	13.2	24.0	29.2	26.4	7.2	<0.0001*
Died	2.8	13.1	30.9	39.0	14.3	
Mean Survival Duration (months ± SE)	(193.5 ± 2.3)	(188.1 ± 2.1)	(179.3 ± 2.0)	(172.7 ± 1.9)	(165.2 ± 2.5)	0.0012[§]
CVD-Related Mortality						
Survived	11.9	22.6	29.3	27.9	8.3	<0.0001*
Died	1.9	12.4	31.7	40.9	13.1	
Age Category						
25-34	20.5	29.9	26.0	18.4	5.2	<0.0001
35-44	11.5	24.7	31.1	25.3	7.3	
45-54	10.8	18.5	31.1	31.1	8.5	
55-64	4.4	18.6	32.0	34.6	10.5	
65-74	1.8	13.2	30.3	41.1	13.4	
75+	1.0	6.2	28.2	47.8	16.8	
Gender						
Female	8.8	18.7	31.4	31.0	10.1	<0.0001*
Male	13.7	24.9	27.5	26.9	7.2	
Race/Ethnicity						
Non-Hispanic White	13.3	25.0	31.1	25.4	5.2	<0.0001*
Non-Hispanic Black	2.5	7.5	23.0	38.0	28.9	
Mexican-American	1.7	6.0	23.9	48.3	20.1	
Other	2.3	12.4	24.1	47.4	13.9	
Country of Origin[†]						
United States	12.3	23.5	30.1	26.5	7.6	<0.0001*
Other	2.0	9.4	25.3	47.1	16.3	
Education Level[‡]						
< High School	3.6	9.0	26.3	44.6	16.5	<0.0001*
High School	9.3	22.3	31.1	29.3	8.0	
> High School	17.4	29.4	30.3	18.7	4.2	
Body Mass Index (kg/m^2)[‡]						
< 25	13.7	21.5	31.5	26.2	7.2	<0.0001*

25 ≤ BMI < 30	9.3	23.1	27.9	30.1	9.5	
≥ 30	8.2	20.0	27.9	33.1	10.8	
Smoking Status						
Never	12.5	22.2	27.6	28.6	9.1	0.0639
Former	10.3	22.0	30.8	29.8	7.1	
Current	9.6	20.7	31.0	29.0	9.7	
Diabetes[§]						
No	11.4	22.3	29.2	28.7	8.5	<0.0001*
Yes	4.9	13.2	35.0	34.2	12.7	

*Significant at p<0.05, t-tests for difference in means and Pearson chi-square tests for differences in proportions or test for trend among demographic groupings were calculated.

[†]N=6516 due to 6 subjects missing data on country of origin.

[‡]N=6480 due to 42 subjects missing data on education level.

[±]N=6499 due to 23 subjects missing data on body mass index (kg/m^2).

[§]N=6511 due to 11 subjects missing data on diabetes status.

The Kaplan-Meier survival curves showing the unadjusted association between summed pathogen burden level and all-cause and CVD-related mortality are shown in Figures 3.1 and 3.2.
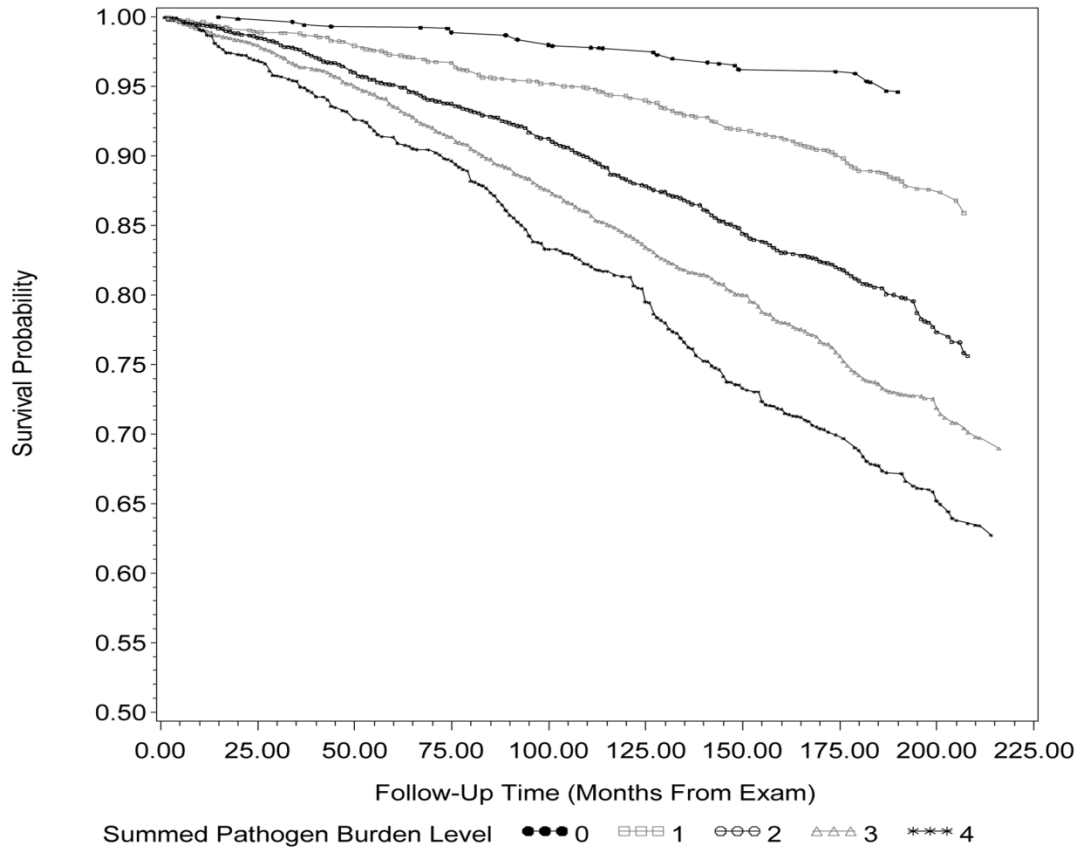
Figure 3.1 Kaplan-Meier Survival Curve for All-Cause Mortality by Pathogen Burden Level. Unadjusted Kaplan-Meier survival curves for all-cause mortality by pathogen burden level for 6522 subjects, ≥ 25 years of age, in the National Health and Nutrition Examination Survey (NHANES) III from 1988-2006.

Figure 3.2 Kaplan-Meier Survival Curve for Cardiovascular disease-related Mortality by Pathogen Burden Level. Unadjusted Kaplan-Meier survival curves for all-cause mortality by pathogen burden level for 6504 subjects, ≥ 25 years of age, in the National Health and Nutrition Examination Survey (NHANES) III from 1988-2006.
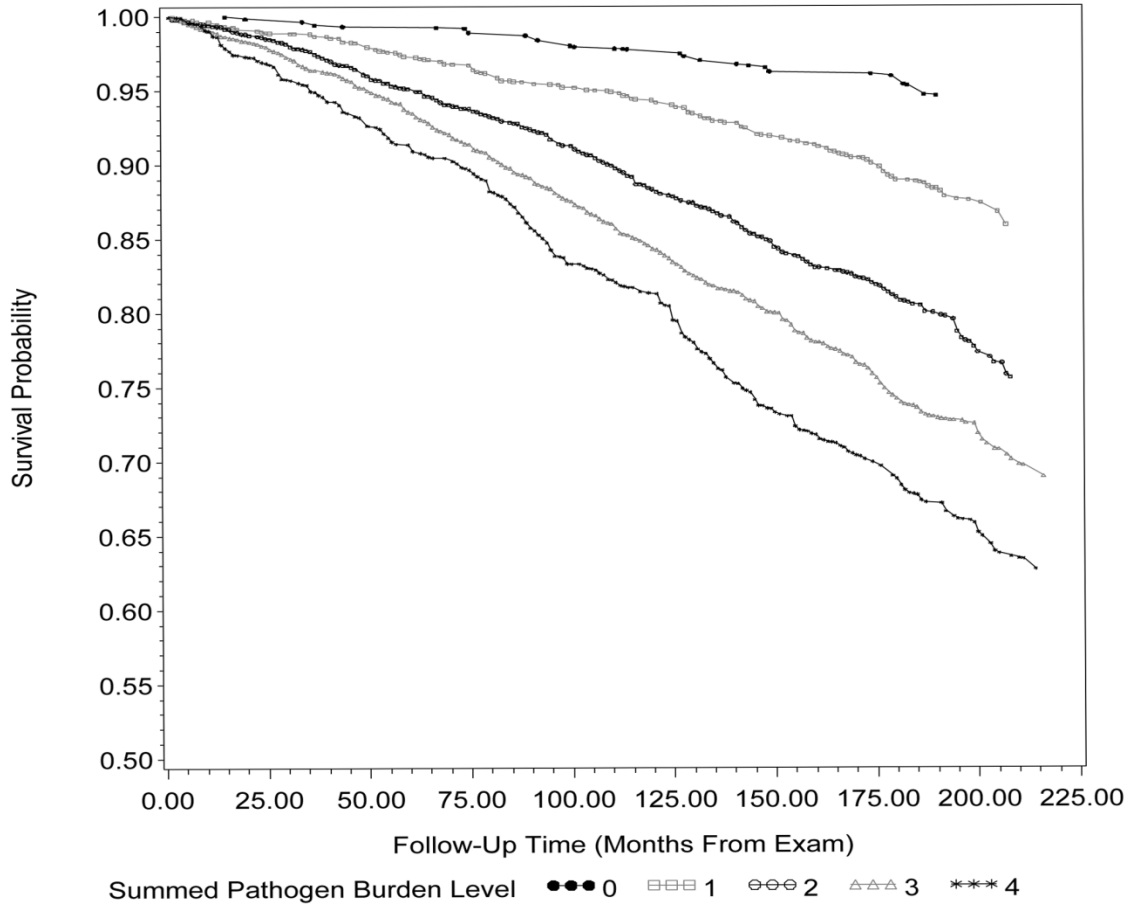
Table 3.2 shows the HR and 95% CI from Cox proportional hazard models examining the association between summed total pathogen burden and all-cause/CVD-related mortality. After adjustment for age, gender, race/ethnicity, country of origin and education level, seropositivity for 2, 3 or 4 pathogens were associated with all-cause mortality compared to seropositivity for 0 pathogens, but did not follow a graded pattern. After additional adjustment for BMI (kg/m^2), diabetes status and smoking status, these associations were no longer statistically significant (HR (95% CI) of 1.31 (0.87, 1.95), 1.46 (0.98, 2.18), 1.30 (0.92, 1.84) and 1.46 (0.96, 2.22), respectively) and did not follow a graded pattern. Similarly, the in fully adjusted models, the association between each pathogen burden level and CVD-related mortality was not statistically significant, nor was there a graded relationship between increased pathogen burden and CVD-related mortality.

Table 3.2 Association Between Summed Pathogen Burden and All-Cause/Cardiovascular Disease-related Mortality Among Subjects Aged 25 and Older in NHANES III

| Pathogen Burden Level | Hazard Ratio and 95% Confidence Interval | | | |
| | All-Cause Mortality | | CVD-Related Mortality | |
	Model 1[†]	Model 2[‡]	Model 1[†]	Model 2[‡]
0	1.0	1.0	1.0	1.0
1	1.37 (0.93, 2.00)	1.31 (0.87, 1.95)	1.58 (0.80, 3.15)	1.51 (0.72, 3.14)
2	1.56 (1.06, 2.30)*	1.46 (0.98, 2.18)	1.80 (1.01, 3.21)*	1.73 (0.91, 3.29)
3	1.45 (1.05, 2.01)*	1.30 (0.92, 1.84)	1.63 (0.89, 2.98)	1.48 (0.76, 2.88)
4	1.58 (1.06, 2.36)*	1.46 (0.96, 2.22)	1.49 (0.72, 3.09)	1.39 (0.62, 3.13)

*Significant at p<0.05. [†]Model 1 adjusted for age, gender, race/ethnicity, country of origin and education level (N=6474) for all-cause mortality and for CVD-related mortality (N=6456).
[‡]Model 2 additionally adjusted for BMI, diabetes status and smoking status for all-cause mortality (N=6441) and for CVD-related mortality (N= 6423).

3.4 DISCUSSION

To the best of our knowledge, our study is the first to utilize a large, nationally representative sample of U.S. adults aged 25 and older with up to 18.1 years of follow-up to examine whether there is a graded relationship between increasing pathogen burden with CMV, HSV-1 and -2 and *H. pylori* and all-cause as well as CVD-related mortality. The results of our study suggest that after confounder adjustment including age, gender, race/ethnicity, country of origin, education level, BMI (kg/m^2), diabetes status and smoking status, there is not a graded relationship between increasing pathogen burden with CMV, HSV-1, HSV-2 and/or *H. pylori* and all-cause or CVD-related mortality in the U.S. adult population.

Few studies have examined the relationship between total pathogen burden and all-cause/CVD-related mortality and results across studies are conflicting.[60-62,119] Most recently, a study by Elkind et al., measured the association between an infectious burden index (constructed by summing the beta coefficients for the effect of each pathogen for which individuals were seropositive on risk for stroke) and vascular, non-vascular and all-cause deaths among subjects 40 years of age and older (mean age 68.4) from Manhattan, New York, without history of stroke.[119] The authors found that the infectious burden index (including *Chlamydia pneumoniae*

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(*C. pneumoniae)*, CMV, HSV-1, HSV-2 and *H. pylori*), was associated with an increased risk for nonvascular deaths (HR per standard deviation change in IB 1.23, 95% CI 1.04, 1.45) in the fully adjusted model.[119] However, after confounder adjustment, the index was no longer statistically significantly associated with risk for vascular deaths or deaths from all causes.[119] In contrast, Zhu et al., examined the relationship between summed pathogen burden with CMV, Hepatitis A, HSV-1 and 2 and all-cause death among a mostly male cohort of Utah residents, with a mean age of 65.3 years and existing CAD.[60] The authors found a significant trend (p-value = 0.020) in risk for death from all-causes with increasing pathogen burden (although the risk for mortality associated with each pathogen burden level effects was not statistically significant).[60]

Two other studies by Rupprecht et al. and Espinola-Klein et al., examined the association between summed pathogen burden with eight pathogens (HSV-1, HSV-2, CMV, Epstein-Barr virus, *Haemophilus influenzae, C. pneumoniae Mycoplasma pneumoniae* and *H. pylori)* and CVD-related mortality among German subjects with existing coronary artery disease (CAD).[61,62] In both studies the authors found a significant trend in risk for CVD-related mortality with increasing pathogen burden of 4-5 and 6-8 pathogens compared to seropositivity for 0-3 pathogens.[61,62] Rupprecht et al. further examined the association between increased pathogen burden with bacterial and viral pathogens and CVD-related mortality, separately (comparing seropositivity for 2 vs. 0-1 pathogens and for 3-4 vs. 0-1 pathogens) finding that the association between increased pathogen burden with herpesviridae and CVD-related mortality was stronger than that with bacterial burden.[61] However, the pathogen groupings were not mutually exclusive, so it is not possible to completely discern the effects of viral pathogen burden alone from co-infection with viral and bacterial pathogens in terms of risk for CVD-related mortality from the analysis.[61]

The study populations from these previous reports were older, mostly consisted of persons with existing CVD and who were from specific geographic regions (i.e. Utah, New York and Germany), thus their results may not be generalizable to the U.S. general population.[60-62,119] However, our study suggests, similar to Elkind et al., a lack of association between increased pathogen burden and all-cause/CVD-related mortality in persons *without* existing CVD.[119] In contrast, the other previous studies suggest there is a significant association between increased pathogen burden and all-cause/CVD-related mortality among persons *with* existing CVD.[60-62] Because the study populations in several of the previous studies had existing CVD, it is possible that increased pathogen burden is particularly detrimental for CVD-related mortality among those with existing CVD. Espinola-Klein et al. for example, found that the effect of increased pathogen burden on CVD-related death was particular strong among those with advanced atherosclerosis.[62] This may explain why our study, in a population-representative sample of the U.S., we did not identify a graded relationship between increased pathogen burden and all-cause/CVD-related mortality.

It is also possible that the particular combination of pathogens included in measures of summed pathogen burden in the studies by Rupprecht et al. and Espinola-Klein et al. are particularly detrimental for CVD-related mortality.[61,62] For example, there is in vitro evidence suggesting that *Chlamydia pneumoniae* (*C. pneumoniae*), a persistent bacterial infection included in these previous studies, can stimulate the expression of CMV genes, thus these pathogens may biologically interact in such a way that co-infection with *C. pneumoniae* exacerbates the effect of CMV on atherosclerosis and mortality.[121] Therefore, in our study, persons seropositive for CMV whom also had undetected seropositivity for *C. pneumoniae* may have elevated risk for mortality. Thus, if *C. pneumoniae* been included in our measure of summed pathogen burden, those individuals, whose risk for mortality was potentially higher due to co-infection with *C. pneumoniae* would have actually been categorized at a higher pathogen burden level. Overall, unmeasured co-infection with *C. pneumoniae,* as well as other persistent pathogens that have been implicated in chronic diseases, could have impacted our ability to detect a graded relationship between summed pathogen burden and mortality. Unfortunately, serologic results for only one of the bacterial pathogens examined in earlier studies, *H. pylori*, was available in NHANES III. Had we been able to include other persistent pathogens in our measure of summed pathogen burden it is possible that we would have detected a similar relationship between increased pathogen burden and all-cause/CVD-related mortality in our study, as was detected in previous studies.

There are a few limitations to our study. We utilized the updated NHANES III Linked Mortality Public-use File which was subject to data perturbation in which synthetic data was substituted for the actual date of death and underlying cause of death for selected descendent records in order to reduce risk of respondent re-identification.[162] Despite the data perturbation of date of death for selected decedents in the public-use file, a comparison study between the public-use and restricted-use file (which does not contain perturbed data) conducted by the NCHS, found that analysis of all-cause by sociodemographic factors including as age, sex, race/ethnicity and education level to be comparable across files and concluded that the discrepancies between the public-use and restricted-use file were minor and that analysts should use the public-use file with confidence.[162] Importantly, mortality status was rigorously verified by two sources (National Death Index (NDI) and/or death certificate review).[140] Furthermore, NDI is a validated method for matching deaths in the U.S. to population-based datasets and for obtaining cause of death.[163]

Another limitation to our study is that we only had information on *seropositivity* and not immune response to infection. Because high antibody titer to persistent infections is likely a marker of "reactive" infection which may be associated both exacerbated damage to invaded host tissues and with increased levels of inflammation, it is likely that high antibody titer to an increased number of pathogens also plays an important role in determining risk for mortality.[178] Although no studies to date, have explored the relationship between the total number of

pathogens for which and individual has a high antibody titer and mortality, two recent studies demonstrated that elevated CMV antibody titer was statistically significantly associated with mortality among elderly populations.[57,58] Thus, it is possible that although we failed to detect a graded relationship between summed pathogen burden and all-cause/CVD-related mortality, we would have seen a trend for increasing risk for mortality with increasing number of pathogens for which individuals had a high antibody titer.

3.5 Conclusion

Overall, our findings indicate the absence of a graded relationship between increased summed pathogen burden with CMV, HSV-1, HSV-2 and/or *H. pylori*, and all-cause/CVD-related mortality in the general U.S. population aged 25 years and older. Taken together with evidence from other studies, it is possible that certain combinations of persistent pathogens may play a more important role in determining risk for mortality among persons with and without existing CVD. These results indicate that there is a need for future research that focuses not just on the relationship between the crude number of pathogens individuals are seropositive for, but which also explores other aspects of the relationship between total pathogen burden and risk for mortality such as the role of biological interactions between pathogens in determining risk for mortality. This study suggests that interventions focused solely on reducing the total number of persistent pathogens for which individuals are seropositive is unlikely to significantly impact mortality in the U.S.

Chapter 4

Seropositivity for Specific Combinations of Persistent Pathogens and Risk for All-Cause and Cardiovascular Disease-related Mortality in the United States

4.1 Background

      Over the past two decades there has been a growing interest in examining whether there is a monotonic relationship between seropositivity for an increased number of persistent pathogens and risk for chronic diseases and mortality.[44,60,62,179-181] Persistent pathogens are linked to chronic disease via direct, pathogen-specific effects such as in the case of cardiovascular disease (CVD), in which certain pathogens invade vascular tissues and trigger atherogenesis.[45-49] Initial infection as well as reactivation with persistent pathogens triggers the release of acute phase proteins and increases levels of inflammatory markers linked to CVD and other chronic diseases.[50-56] Thus, increased total pathogen burden is thought to increase risk for chronic disease and mortality due to an accumulation of pathogen-specific effects and increased levels of systemic inflammation.[94]

      Most studies utilize summed pathogen burden variables, defined as the total number of pathogens an individual tests seropositive for, based on antibody response, to examine whether there is a graded relationship between increased total pathogen burden and chronic disease/mortality. Three previous studies conducted among populations with existing CVD found a statistically significantly graded relationship between summed pathogen burden including variable pathogens across studies and all-cause/CVD-related mortality. These studies suggest that among persons with existing CVD, there is a monotonic relationship between total pathogen burden and mortality, regardless of pathogen type. However, in our study described in chapter 3 of this dissertation, we did not detect a graded relationship between summed pathogen burden with cytomegalovirus (CMV), herpes simplex virus-1 (HSV-1), herpes simplex virus-2 (HSV-2) and/or *Helicobacter pylori* (*H. pylori*) and all-cause/CVD-related mortality among a nationally representative population of persons 25 years of age and older. In contrast, we found that the risk for mortality associated with summed pathogen burden level 1 and 3 was similar, as was the risk for mortality associated with summed pathogen burden level 2 and 4 (see Table 3.2, Chapter 3). It is possible that discrepancies between our study and previous studies are due to population differences. However, it is also possible that other factors, such as the specific combinations of pathogens for which individuals are seropositive plays a more important role than the crude

number of pathogens for which individuals are seropositive, in terms of determining risk for mortality in the general population.

While summed pathogen burden variables may be useful for assessing whether there is a graded association between total pathogen burden and mortality, regardless of pathogen type, use of this variable limits the ability for researchers to identify whether seropositivity for specific combinations of pathogens, in addition to the crude number of pathogens individuals are seropositive for, plays an important role in determining risk for mortality. There are two main reasons why use of the summed pathogen burden variable may not have provided a complete assessment of risk for mortality related to persistent pathogens in our earlier study, warranting further exploration of the relationship between specific combinations of pathogens and risk for mortality among the general U.S. population. First, in models examining the association between summed pathogen burden and mortality, all persons seropositive to a given number of pathogens (i.e., classified as having the same summed pathogen burden level) are estimated as having the same risk for disease/mortality by the model, regardless of the specific combination of pathogens for which an individual is seropositive. For example, when two individuals are categorized as having a summed pathogen burden level of two, one of these individuals may be seropositive for HSV-1 and CMV and the other may be seropositive for *H. pylori* and *Chlamydia pneumoniae* (*C. pneumoniae*). Thus, these two individuals would be categorized as having a summed pathogen burden level of two and therefore be estimated as having the same risk for mortality, despite being seropositive for two entirely different sets of pathogens. Second, assuming a monotonic relationship between summed pathogen burden level and risk for mortality whereby an increase in summed pathogen burden level leads to an increased risk for mortality implies that the risk for mortality associated with any specific combination of pathogens is greater than risk for mortality associated with all combinations of fewer pathogens, regardless of pathogen type.

Although a graded relationship between total pathogen burden and mortality may exist in certain populations, there is also evidence that pathogens may biologically interact such that combined seropositivity could increase or decrease risk for mortality.[120,121,122] The use of summed pathogen burden variables for examining the association between total pathogen burden and mortality, disregards potentially important variability in risk associated with different combinations of pathogens both *within* pathogen burden levels and *across* pathogen burden levels. Thus, while summed pathogen burden variables may be useful for examining the hypothesis that "more is worse" when it comes to total pathogen burden and risk for mortality, limiting the examination of the relationship between total pathogen burden and mortality to this variable, hinders an assessment of other factors that might play an important role in the association between total pathogen burden and mortality such as the specific combinations of pathogens for which individuals are seropositive.

To our knowledge, no study has explicitly examined the extent to which the specific combinations of pathogens for which individuals are seropositive predicts risk for mortality, beyond that of the use of summed pathogen burden variables in the general U.S. population. Therefore, the purpose of this study is to 1) estimate the combined effects of seropositivity to four persistent pathogens (CMV, HSV-1, HSV-2 and *H. pylori*) on all-cause/CVD-related mortality in a U.S. representative population of individuals ≥ 25 years of age and older, and 2) to compare the risk for mortality associated with seropositivity for each specific combination of pathogens among persons *within* the same pathogen burden level as well as among persons *across* different pathogen burden levels to assess the extent to which use of summed pathogen burden variables in models examining the association between total pathogen burden and mortality limit our ability to make inferences about the specific pathogens which alone, or in combination most increase risk for death.

4.2 Methods

Study population

Data come from the National Health and Nutrition Examination Survey (NHANES) III (1988–1994); a population-based, multistage stratified probability survey which collected information on the health and nutrition of the United States civilian non-institutionalized population. The survey was carried out by the National Center for Health Statistics (NCHS), Centers for Disease Control and Prevention. A total of 33994 subjects were interviewed in NHANES III, but our study sample is limited to subjects who were 25 years of age and older at time of examination (N=15242 (48.7%)), had mortality follow-up (N=15229 (99.9%)) and were tested for serostatus for all four pathogens (N=6522 (42.8%)). Sera was selected randomly for seropositivity testing, however, only subjects in phase I were tested for *H. pylori*, therefore, our analysis was limited to subjects tested for seropositivity for the other three pathogens in phase I. In addition, we used data from the NHANES III-Linked Mortality file in which the mortality status of NHANES III participants was determined by probabilistic matching between NHANES III participants and the U.S. National Death Index (NDI).[140] Cause of death was unavailable for 18 subjects, thus, these subjects were excluded from analysis for CVD-related mortality.

Laboratory Analyses

CMV-specific IgG was measured by a commercially available Enzyme Linked Immunosorbent Assay (ELISA) (Quest International, Inc., Miami, FL). Sera with values near the ELISA cutoff were confirmed with a second ELISA assay (bioMerieux, Inc., Durham, NC). If the results from the first two tests disagreed, an Immunofluorescence Assay (IFA) (Bion International, Inc., Park Ridge, IL), was used and results from this test were provided as the final seropositivity test result. The sensitivity and specificity of these tests have been estimated to be 98 and 99%, respectively.[67,141] HSV-1 and HSV-2 seropositivity was assessed by solid-phase enzymatic immunodot assays using purified glycoprotein gG-1 of HSV-1 and gG-2 of HSV-2 as the

antigen.[175] These immunodot assays have also been shown to have high sensitivity and specificity.[176] Subjects 20 years and older from phase 1 (1988-1991) were tested for *H. pylori* antibody in 1996 using *H. pylori* IgG ELISA (Wampole Laboratories, Cranbury, NJ). The cut points for seropositivity to each infection are based on commercially available immunoassay clinical diagnostics that provide information on IgG antibody seropositivity.[177]

Measures

Results from the IgG antibody tests for each pathogen were dichotomized as seronegative or seropositive based on the ELISA results. The summed pathogen burden level (i.e., the total number of pathogens for which subjects were seropositive) of subjects ranged from 0- 4. A categorical variable was constructed in which subjects were classified according to the specific combination of pathogens for which they were seropositive (1 of 16 possible pathogen combinations). Specifically, subjects with a pathogen burden level of 0 were seronegative to all four pathogens (referent group), subjects with a pathogen burden level of 1 were seropositive to one of the four pathogens alone, subjects with a pathogen burden level of 2 were seropositive for one of six combinations of two pathogens (seropositivity to CMV and HSV-1 or CMV and HSV-2 or CMV and *H. pylori* or HSV-1 and HSV-2 or HSV-1 and *H. pylori* or HSV-2 and *H. pylori*) , subjects with a pathogen burden level of 3 were seropositive for one of four pathogen combinations of three pathogens (seropositivity to CMV, HSV-1 and HSV-2, or CMV, HSV-1 and *H. pylori*, or CMV, HSV-2 and *H. pylori*, or HSV-1, HSV-2 and *H. pylori)* and subjects with a pathogen burden level of 4 were seropositive to all four pathogens.

Mortality status was obtained by NCHS primarily from the National Death Index (NDI); however, other sources of mortality status included indication of deceased status from the Social Security Administration, the Centers for Medicare and Medicaid Services, or death certificate review.[140] Cause of death was coded using the International Classification of Diseases, Ninth Revision (ICD-9) up until 1998 and ICD-10 for 1999-2006. All deaths before 1999 were recoded by the NCHS into comparable ICD-10 codes.[144] ICD-10 codes I00-I99 were classified as CVD-related deaths and included, causes of death such as hypertensive heart disease, atherosclerosis including coronary and cerebrovascular disease, heart failure, and aortic aneurysms. Persons who survived the entire follow-up period were administratively censored on December, 31st 2006. Follow-up time for each person was calculated in months as the difference between the NHANES III examination date and the last known date alive or censored.[144] Persons who died of non-CVD causes were considered censored at the date of death for CVD mortality analysis.

Weighted estimates of the bivariate relationships between covariates of interest and summed total pathogen burden were estimated in our previous study (see Table 3.1 in Chapter 3). Covariates associated with summed pathogen burden level and hypothesized to be predictors of mortality including age, gender, race/ethnicity, country of origin, body mass index (BMI) (kg/m^2), smoking status, diabetes status and education level (see Table 3.1 in Chapter 3). Age in

years at examination was self-reported and treated as a continuous variable. Gender was dichotomized as female and male. Race/ethnicity was self-reported as non-Hispanic white, non-Hispanic black, Mexican-American or Other. Country of origin was reported as the state or foreign country in which subjects were born and was categorized as U.S. or Other. Years of education was self-reported and treated as continuous. BMI (kg/m^2) was computed from weight and standing height and categorized as BMI < 25, 25 ≤ BMI < 30 and BMI ≥ 30. Smoking status was self-reported and categorized into never (did not smoke 100+ cigarettes in one's lifetime), past (smoked 100+ cigarettes in one's lifetime but do not currently smoke) and current smoker (smoked 100+ cigarettes in one's lifetime and currently smoke). Diabetes was self-reported as whether a doctor ever informed subjects they had diabetes or not and dichotomized as reported or not reported.

Statistical analyses

Statistical analyses were performed using SAS, version 9.2, with SAS-callable SUDAAN, version 10.0.1 (SAS Institute, Inc., Cary, NC).[155] All analyses used appropriate weights and adjustments for strata and clustering used in the complex study design in NHANES III. Bivariate relationships between summed pathogen burden and all-cause/CVD-mortality and survival duration as well as between summed pathogen burden and potential confounders including age, gender, race/ethnicity, country of origin, education level, BMI (kg/m^2), smoking status and diabetes status were assessed previously (see Table 3.1, Chapter 3). We estimated the distribution of the specific combination of pathogens for which subjects were seropositive within each pathogen burden level, in order to better understand which specific combination of pathogens were most prevalent among persons within each pathogen burden level.

To estimate the combined effects of seropositivity to four persistent pathogens (CMV, HSV-1, HSV-2 and *H. pylori*) on all-cause/CVD-related mortality we used Cox proportional hazard models to estimate the confounder adjusted HR and 95% CI for the association between each of the 15 pathogen burden combinations and all-cause/CVD-related mortality, compared to the referent category of seronegativity to all four pathogens. Models were adjusted for sociodemographic factors (age, gender, race/ethnicity, country of origin and education level), for clinical factors (BMI (kg/m^2), smoking status and diabetes status. Next, we compared the HR for all-cause/CVD-related mortality yielded by each combination of pathogens *within* each pathogen burden level as well as *across* all pathogen burden levels to assess whether measuring pathogen specific estimates provides information above and beyond summed pathogen burden for all-cause/CVD-related mortality outcomes.

4.3 RESULTS

Weighted estimates of the bivariate relationships between covariates of interest and summed total pathogen burden were estimated in our previous study (see Table 3.1, Chapter 3). Covariates associated with summed pathogen burden level were higher age, female gender, non-

white race/ethnicity, country of origin outside the U.S., lower education level, low BMI (< 25 kg/m^2) and high BMI (≥ 30 kg/m^2) compared to medium BMI (25 ≤ BMI < 30) and reported diabetes were associated with increased pathogen burden. Although hypothesized as a potential confounder in the association between pathogen burden and mortality, smoking status was not associated with total pathogen burden in our study. There was a significant trend for death from all-causes and CVD-related death with increasing pathogen burden. Overall mean survival duration since time of exam for those seropositive to zero pathogens was 16.1 years (193.5 ± 2.3 months), to one pathogen was 15.7 years (188.1 ± 2.1 months), to two pathogens was 14.9 years (179.3 ± 2.0 months), to three pathogens was 14.4 years (172.7 ± 1.9 months) and to all four pathogens was 13.8 years (165.2 ± 2.5). During the mean 15.0 years of follow-up from exam, among subjects in our sample, the population estimate for the proportion dying from all causes was 21.0% and from CVD-related causes was 8.7%.

The weighted proportion of individuals within each pathogen burden level and the weighted proportion of individuals with each possible combination of pathogens are shown in Table 4.1 below.

Table 4.1 Weighted Distribution of Seropositivity for Persistent Pathogens Across Pathogen Burden Level in Subjects Aged 25 and Older in NHANES III

Pathogen Combinations (N=6522)	Pathogen Burden Level				
	0	**1**	**2**	**3**	**4**
N (%)	349 (11.0%)	857 (21.7%)	1706 (29.5%)	2472 (29.1%)	1138 (8.7%)
HSV-1	---	53.1%	---	---	---
CMV	---	30.8%	---	---	---
HSV-2	---	10.5%	---	---	---
H. pylori	---	5.6%	---	---	---
CMV and HSV-1	---	---	62.6%	---	---
HSV-1 and *H. pylori*	---	---	12.3%	---	---
HSV-1 and HSV-2	---	---	10.0%	---	---
CMV and HSV-2	---	---	7.4%	---	---
CMV and *H. pylori*	---	---	7.0%	---	---
HSV-2 and *H. pylori*	---	---	0.7%	---	---
CMV, HSV-1 and *H. pylori*	---	---	---	64.8%	---
CMV, HSV-1 and HSV-2	---	---	---	27.6%	---
CMV, HSV-2 and *H. pylori*	---	---	---	3.8%	---
HSV-1, HSV-2 and *H. pylori*	---	---	---	3.8%	---
CMV, HSV-1, HSV-2 and *H. pylori*	---	---	---	---	100%

The weighted proportion of individuals at each pathogen burden level were 11.0%, 21.7%, 29.5%, 29.1% and 8.7%, for those with the pathogen burden level of 0, 1, 2, 3, and 4, respectively. Among those seropositive for one pathogen, the majority were seropositive to HSV-1 or CMV (53.1% and 30.8%, respectively). Among those seropositive for two pathogens, the greatest proportion, were seropositive for CMV and HSV-1 combined (62.6%) and the lowest proportion of subjects were seropositive for HSV-2 and *H. pylori* (0.7%). Among those seropositive for three pathogens, the greatest proportion of subjects, were seropositive for CMV,

HSV-1 and *H. pylori* combined (64.8%) followed by CMV, HSV-1 and HSV-2 combined (27.6%) and 3.8% were seropositive for CMV, HSV-2 and *H. pylori* as well as for HSV-1, HSV-2 and *H. pylori.*

Tables 4.2 and 4.3 show the HR (95% CI) from Cox proportional hazard models estimating the combined effect of seropositivity to each of the 15 pathogen burden combinations (compared to seropositivity to zero pathogens) on risk for all-cause/CVD-related mortality, respectively. After adjustment for age, gender, race/ethnicity, country of origin, education level, BMI (kg/m^2), smoking status and diabetes status, the effect of each individual pathogen as well as the combined effect of all pathogen burden combinations were positively (although not all statistically significantly) associated with all-cause mortality/CVD-related except for the combined effect of seropositivity for HSV-1 and *H. pylori* on risk for CVD-related mortality. Overall, the combined effect of seropositivity to CMV and HSV-2 (HR 2.06, 95% CI 1.22, 3.47) as well as the combined effect of seropositivity for CMV, HSV-1 and HSV-2 (HR 1.52, 95% CI 1.01, 2.31) were statistically significantly associated with all-cause mortality, even after adjusting for age, gender, race/ethnicity, country of origin, education level, BMI (kg/m^2), diabetes status and smoking status. For CVD-related mortality, combined seropositivity for CMV and HSV-2 (HR 4.05, 95% CI 2.07, 7.91) and combined seropositivity for HSV-2 and *H. pylori* (HR 6.27, 95% CI 1.56-25.20) were statistically significantly associated with CVD-related mortality in the fully adjusted model.

Table 4.2 Combined Effect of Each Pathogen Combination on All-Cause Mortality By Pathogen Burden Level Among Subjects Aged 25 Years and Older in NHANES III

Pathogen Combinations	Pathogen Burden Level				
	0	**1**	**2**	**3**	**4**
	Hazard Ratio and 95% Confidence Interval				
Seronegative for All Pathogens	1.00				
HSV-1	---	1.08 (0.69, 1.70)	---	---	---
HSV-2	---	1.35 (0.73, 2.48)	---	---	---
CMV	---	1.51 (0.93, 2.46)	---	---	---
H. pylori	---	1.95 (0.89, 4.28)	---	---	---
CMV and *H. pylori*	---	---	1.18 (0.66, 2.12)	---	---
CMV and HSV-1	---	---	1.40 (0.95, 2.06)	---	---
HSV-1 and HSV-2	---	---	1.43 (0.70, 2.91)	---	---
HSV-1 and *H. pylori*	---	---	1.76 (0.96, 3.25)	---	---
CMV and HSV-2	---	---	2.06 (1.22, 3.47)*	---	---
HSV-2 and *H. pylori*	---	---	2.22 (0.60, 8.15)	---	---
CMV, HSV-1 and *H. pylori*	---	---	---	1.24 (0.88, 1.74)	---
HSV-1, HSV-2 and *H. pylori*	---	---	---	1.25 (0.50, 2.96)	---
CMV, HSV-2 and *H. pylori*	---	---	---	1.28 (0.62, 2.65)	---
CMV, HSV-1 and HSV-2	---	---	---	1.52 (1.01, 2.31)*	---
CMV, HSV-1, HSV-2 and *H. pylori*	---	---	---	---	1.47 (0.96, 2.24)

*Significant at p<0.05. Model adjusted for age, gender, race/ethnicity, country of origin, education level, body mass index kg/m^2, diabetes status and smoking status (N=6456). CMV=cytomegalovirus, HSV-1=herpes simplex virus-1, HSV-2=herpes simplex virus-2 and *H. pylori=Helicobacter pylori*

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Table 4.3 Combined Effect of Each Pathogen Combination on Cardiovascular Disease-Related Mortality By Pathogen Burden Level Among Subjects Aged 25 Years and Older in NHANES III

	Pathogen Burden Level				
	0	**1**	**2**	**3**	**4**
Pathogen Combinations		Hazard Ratio and 95% Confidence Interval			
Seronegative to all pathogens	1.00				
HSV-1	---	1.45 (0.64, 3.27)	---	---	---
HSV-2	---	2.56 (0.66, 9.94)	---	---	---
CMV	---	1.36 (0.54, 3.41)	---	---	---
H. pylori	---	2.27 (0.47, 10.90)	---	---	---
CMV and *H. pylori*	---	---	1.24 (0.37, 4.17)	---	---
CMV and HSV-1	---	---	1.71 (0.87, 3.36)	---	---
HSV-1 and HSV-2	---	---	1.43 (0.70, 2.91)	---	---
HSV-1 and *H. pylori*	---	---	1.65 (0.68, 3.98)	---	---
CMV and HSV-2	---	---	4.05 (2.07, 7.91)*	---	---
HSV-2 and *H. pylori*	---	---	6.27 (1.56, 25.20)*	---	---
CMV, HSV-1 and *H. pylori*	---	---	---	1.41 (0.70, 2.84)	---
HSV-1, HSV-2 and *H. pylori*	---	---	---	0.53 (0.10, 2.84)	---
CMV, HSV-2 and *H. pylori*	---	---	---	1.82 (0.73, 4.50)	---
CMV, HSV-1 and HSV-2	---	---	---	1.83 (0.94, 3.57)	---
CMV, HSV-1, HSV-2 and *H. pylori*	---	---	---	---	1.43 (0.63, 3.25)

*Significant at p<0.05. Model adjusted for age, gender, race/ethnicity, country of origin, education level, body mass index kg/m^2, diabetes status and smoking status (N=6423). CMV=cytomegalovirus, HSV-1=herpes simplex virus-1, HSV-2=herpes simplex virus-2 and *H. pylori=Helicobacter pylori*

Next we compared the risk for mortality associated with each possible pathogen combination within the same pathogen burden level, finding that there was variability in the risk for all-cause/CVD-related mortality associated with each combination of pathogens within each pathogen burden level. For example, among those with a pathogen burden level of two, the HR (95% CI) for all-cause mortality for those seropositive for CMV and *H. pylori* was 1.18 (0.66, 2.12) whereas the HR (95% CI) for those seropositive for CMV and HSV-2 was 2.06 (1.22, 3.47), although differences were not statistically significant. For CVD-related mortality, among those seropositive for two pathogens, the HR (95% CI) for CVD-related mortality, among those with a pathogen burden level of 2, the HR (95% CI) for seropositivity for CMV and HSV-2 (4.05 (2.07, 7.91)) was statistically significantly different from the HR (95% CI) for combined seropositivity for CMV and HSV-1 (1.71 (0.87, 3.36)) (Adjusted Wald F = 8.95, p-value = 0.0065) as well as from the HR (95% CI) for the combined effect for seropositivity for HSV-1 and HSV-2 (1.43 (0.70, 2.91)) (Adjusted Wald F= 5.39, p-value=0.0294) and from the HR (95% CI) for combined seropositivity for HSV-1 and *H. pylori* (1.65 (0.68, 3.98)) (Adjusted Wald F= 4.88, p-value = 0.0375). Thus, all pathogen burden combinations within the same pathogen burden level did not necessarily yield the same risk for all-cause/CVD-related mortality.

Next, we compared the HRs and 95% CIs for all-cause/CVD-related mortality yielded by each combination of pathogens *across* all pathogen burden levels finding that the combined effect of seropositivity for all pathogen combinations on all-cause/CVD-related mortality was not, in all cases, greater than the risk for mortality associated with the combined effect of fewer pathogens. For example, combined seropositivity for CMV and HSV-2 (HR 2.06, 95% CI 1.22, 3.47), yielded a higher risk for all-cause mortality than combined seropositivity for all but one combination of three pathogens and a higher risk for all-cause mortality than for seropositivity for all four pathogens (p<0.05). Similarly, for CVD-related mortality, combined seropositivity for CMV and HSV-2 (HR 4.05, 95% CI 2.07, 7.91) and combined seropositivity for HSV-2 and *H. pylori* (HR 6.27, 95% CI 1.56, 25.20), yielded higher risks for mortality than nearly all combinations of seropositivity for three pathogens as well as combined seropositivity for all four pathogens (P<0.05). Thus, in our study, we found that the combined effect of the specific combinations of pathogens individuals could be seropositive for did not always yield a risk for all-cause/CVD-related mortality that was greater than combinations of fewer pathogens at lower pathogen burden levels.

4.4 DISCUSSION

To the best of our knowledge, our study is the first to utilize a large, nationally representative sample of U.S. adults aged 25 and older with up to 18.1 years of follow-up (mean of 15.0 years) to estimate the combined effect of seropositivity for each combination of four persistent pathogens previously implicated in the etiology of several chronic diseases and all-cause/CVD-related mortality. After adjustment for age, gender, race/ethnicity, country of origin,

education level, BMI (kg/m^2), diabetes and smoking status, the combined effect for seropositivity to CMV and HSV-2 (HR 2.06, 95% CI 1.22, 3.47)) as well as the combined seropositivity to CMV, HSV-1 and HSV-2 (HR 1.52, 95% CI 1.01, 2.31) were statistically significantly associated with all-cause mortality and the combined effect for seropositivity for CMV and HSV-2 (HR 4.05, 95% CI 2.07, 7.91) and the combined effect for seropositivity for HSV-2 and *H. pylori* (HR 6.27, 95% CI 1.56-25.20) were statistically significantly associated with CVD-related mortality.

We also examined the extent to which use of summed pathogen burden variables for modeling the relationship between total pathogen burden and mortality may limit the ability of researchers to identify the specific combinations of pathogens for which the combined effects are most detrimental to health. First, we assessed whether individuals *within* the same pathogen burden level have the same risk for mortality, regardless of pathogen combination. We found that the risk for all-cause/CVD-related mortality associated with combined effect of all specific combinations of pathogens individuals could be seropositive for *within* each pathogen burden level was not uniform. For example, for CVD-related mortality, among those seropositive for two pathogens, the HR (95% CI) among those with a pathogen burden level of 2, that were seropositive for CMV and HSV-2 was 4.05 (2.07, 7.91) whereas the HR (95% CI) for those seropositive for CMV and HSV-1 was 1.71 (0.87, 3.36) (Adjusted Wald F = 8.95, p-value = 0.0065. In other words, all persons seropositive for two pathogens in our study did not have the same risk for mortality, regardless of pathogen type.

Second, our study demonstrated that the risk for all-cause/CVD-related mortality for each pathogen burden combination within a given pathogen burden level, was not necessarily greater than that of all pathogen burden combinations at each lower pathogen burden level. For example, combined seropositivity for CMV and HSV-2, yielded a higher risk for all-cause and CVD-related mortality than combined seropositivity for all but one combination of three pathogens and a higher risk for all-cause mortality than for seropositivity for all four pathogens (p<0.05). Thus, in our study, we did not find that the risk for all-cause/CVD-related mortality for individuals with a given summed pathogen burden level was uniform, regardless of which combination of pathogens individuals were seropositive for, nor that increased summed pathogen burden level guaranteed an increased risk for all-cause/CVD-related mortality, regardless of pathogen combination. These results suggest that the use of summed pathogen burden variables to assess whether there is a monotonic relationship between total pathogen burden and mortality likely limits our ability to identify potentially significant variability in risk attributable to the specific combination of pathogens individuals are seropositive for. In addition, the risk associated with mortality for those categorized as having a particular pathogen burden level may be less a reflection of the total number of pathogens individuals are seropositive for than the result of particular combinations of pathogens that in combination, strongly increase or decrease risk for mortality.

To our knowledge no other studies have examined the association between the combined effect of specific combinations of pathogens and all-cause/CVD-related mortality. However, a recent study by Miggins et al., examined the association between non-specific viral and bacterial infection and mortality in a population based retrospective cohort study in which the infection and mortality status of immunocompetent but critically ill individuals admitted for hospital stay to the intensive care unit was obtained.[120] The authors divided patients into four groups: 1) individuals without any documented infection at admission or during hospitalization, 2) individuals diagnosed with a viral infection during their hospitalization, 3) individuals diagnosed with a bacterial infection during their hospitalization and 4) individuals diagnosed with both viral and bacterial infections during their hospitalization (co-incident infection). Next, they compared the relative risk for those with co-incident infection to those with only viral or only bacterial infection, finding that those with bacterial infection alone, had a higher relative risk for death (RR 4.13, 95% CI 3.76, 4.53) than those with viral infection alone (RR 2.36, 95% 1.73, 3.24) and that there was significant additive interaction suggestive of biologic interaction between viral and bacterial pathogens in relation to risk for mortality.[120]

Unlike our study, Miggins et al. did not examine the association between every specific combination of viral and/or bacterial infection individuals were seropositive for and mortality nor did they identify which particular viral or bacterial pathogens individuals were diagnosed with during hospitalization. However, the authors did estimate the risk for death for a few specific pathogens with and without concurrent bacterial infection, finding that the combined effect of diagnosis with CMV infection and bacterial infections was RR 6.66 (95% CI 4.35, 10.19) compared to an RR (95% CI) of 4.21 (2.07, 8.55) for those diagnosed with CMV infection but no bacterial infections (increased RR of 54%).[120] Similarly, the RR (95% CI) for death for those diagnosed with HSV infection and bacterial infections was doubled compared to those diagnosed with HSV and no bacterial infection.[120] The study by Miggins et al, similar to ours, implicates the herpesviruses, CMV and HSV, in increasing risk for death, particularly among those co-infected with bacterial infections. Although this study was conducted among a very specific cohort of critically ill patients, it adds evidence supporting the hypothesis that pathogens likely biologically interact and that certain combinations of pathogens garner greater risk for mortality than others.[120]

Identifying which persistent pathogens, individually, or in combination, are most strongly associated with mortality and identifying how pathogens interact to increase risk for chronic disease and death remains important for focusing interventions that reduce exposure to and/or transmission of infections most detrimental to health. Our findings as well as those by Miggins et al., suggest that herpesviruses may play an important role in determining risk for all-cause/CVD-related mortality. Furthermore, Miggins et al., demonstrated that the effect of herpesviruses on risk for death was augmented by bacterial co-infection.[120] We similarly found that individuals

seropositive for HSV-2 as well as the bacterial infection *H. pylori* had a statistically significantly increased risk for CVD-related mortality and that there was evidence of biological interaction between the two pathogens (relative risk due to biologic interaction > 0, data not shown). [156] However, we were unable to include other persistent pathogens that have been associated with chronic disease and/or mortality in previous studies such as *C. pneumonaie*[101, 156,182-184] because they were not serologically tested for in NHANES III. Therefore, other bacterial pathogens that may also play an important role in determining risk for mortality and/or interact with pathogens included in this study in such a way that their inclusion would have altered the risk for all-cause/CVD-related mortality associated with certain combinations of pathogens. Therefore, we cannot rule out that the associations between specific combinations of pathogens and all-cause/CVD-related mortality observed in our study are not due to unmeasured co-infection with other persistent pathogens. Further research examining the risk for mortality associated with a comprehensive variety of pathogen combinations is needed to fully understand which particular combinations of pathogens are most strongly associated with death in the U.S. general population.

To our knowledge, only one other recent study by Elkind et al., measured the association between pathogen burden and vascular, non-vascular and all-cause deaths utilizing a measure of pathogen burden other than summed pathogen burden level.[119] Specifically, Elkind et al. constructed an infectious burden index, by summing the beta coefficients for the effect of each pathogen on risk for stroke for which a subject was seropositive (including *Chlamydia pneumoniae* (*C. pneumoniae)*, CMV, HSV-1, HSV-2 and *H. pylori*).[119] They found that the infectious burden index was associated with an increased risk for non-vascular deaths (HR per standard deviation change in IB 1.23, 95% CI 1.04, 1.45) in the fully adjusted model. However, after confounder adjustment, the infectious burden index was no longer statistically significantly associated with risk for vascular deaths or deaths from all causes among a cohort of subjects 40 years of age and older from Manhattan, New York, without history of stroke.[119]

In constructing an infectious burden index, which summed the beta coefficients for the association between each pathogen and risk for stroke that an individual was seropositive for, Elkind et al., utilized a measure of total pathogen burden which attempted to overcome the methodologic limitations associated with using summed pathogen burden measures to model the association between total pathogen burden and mortality.[119] Namely, the index modeled the association between total pathogen burden and mortality, allowing for the risk for death associated with different combinations of pathogens to vary according to the specific combination of pathogens individuals are seropositive for.[119] However, the weighting approach applied to this measure of total pathogen burden assumes the combined effect of seropositivity to multiple pathogens is strictly additive, i.e., there is not biologic interaction between pathogens which could cause the combined effect of seropositivity to multiple pathogens to be greater or less than that of

the sum of the effect of the individual pathogens. Although it was not part of our main analysis, we did observe evidence of both positive and negative interaction on the additive scale (suggestive of biologic interaction) for certain combinations of pathogens in determining risk for mortality in our study. Taken together with the study by Miggins et al., the assumption that the effect of pathogens on risk for mortality is strictly additive may be problematic.[120] For these reasons, studies that have utilized summed pathogen burden variables or other infectious burden indexes in modeling the association between total pathogen burden and mortality do so at the expense of identifying which persistent pathogens alone or in combination are most detrimental to health.

There are a few limitations to our study. We utilized the updated NHANES III Linked Mortality Public-use File which was subject to data perturbation in which synthetic data was substituted for the actual date of death and underlying cause of death for selected descendent records in order to reduce risk of respondent re-identification.[162] Despite the data perturbation of date of death for selected decedents in the public-use file, a comparison study between the public-use and restricted-use file (which does not contain perturbed data) conducted by the NCHS, found that analysis of all-cause by sociodemographic factors including as age, sex, race/ethnicity and education level to be comparable across files and concluded that the discrepancies between the public-use and restricted-use file were minor and that analysts should use the public-use file with confidence.[162] Importantly, mortality status was rigorously verified by two sources (National Death Index (NDI) and/or death certificate review).[140] Furthermore, NDI is a validated method for matching deaths in the U.S. to population-based datasets and for obtaining cause of death.[163]

4.5 Conclusion

In conclusion, the potential for specific combinations of pathogens to strongly influence risk for mortality, calls into question the use of summed pathogen variables alone in studies examining the association between total pathogen burden and mortality. Our results suggest that due to potentially important variability in risk for mortality associated with specific combinations of pathogens *within* and *across* pathogen burden level, researchers should also consider the types of pathogens that individuals are exposed to at each pathogen burden level in order to identify which particular pathogens seropositivity most increases risk for mortality. While measures of summed pathogen burden may be useful for examining the hypothesis that "more is worse" when it comes to the relationship between total pathogen burden and mortality, use of this variable is done so at the expense of identifying which persistent pathogens alone or in combination are most detrimental to health. We found that seropositivity for specific combinations of pathogens (herpesviruses in particular) may play an important role in determining risk for all-cause/CVD-related mortality in the general U.S. population. Thus, interventions focused solely on reducing the number of pathogens individuals are seropositive for are unlikely to significant impact

mortality in the U.S., whereas interventions that focus on the elimination of specific combinations of pathogens such as CMV and HSV-2 may provide a better target for reducing the burden of mortality among infected populations. If herpesviruses such as CMV, HSV-1 and HSV-2 as well as the bacterial pathogen, *H. pylori*, are potentially driving the association between pathogen burden and risk for mortality in studies of summed pathogen burden, emphasis should be made on determining the specific mechanisms by which these particular pathogens act together to increase risk for death and whether delayed acquisition and/or improved immune control of these infections over the life course could serve to decrease risk for mortality.

Chapter 5

Conclusion

5.1 Conclusion

This dissertation examined the association between persistent pathogens, inflammation and risk for all-cause/cardiovascular disease (CVD)-related mortality in the general U.S. population 25 years of age and older. This work demonstrated that seropositivity for CMV is associated with all-cause mortality but was not statistically significantly associated with CVD-related mortality. However, the combined effect of cytomegalovirus (CMV) seropositivity and high C-reactive protein (CRP) level was associated with a greater risk for all-cause/CVD-related mortality than either CMV seropositivity or high CRP level alone suggesting that both CMV infection and CRP levels should be considered interventions focused on reducing mortality in the United States general population. In addition, we did not detect a graded relationship between summed pathogen burden with CMV, herpes simplex virus-1 (HSV-1), herpes simplex virus-2 (HSV-2) and/or *Helicobacter pylori* (*H. pylori*) and all-cause/CVD-related mortality. Last, we estimated the risk for mortality associated with the combined effect of seropositivty for each combination of seropositivity with CMV, HSV-1, HSV-2 and/or *H. pylori* and assessed the extent to which use of summed pathogen burden variables for modeling the association between total pathogen burden and mortality limits the ability of researchers to identify the specific combinations of pathogens most strongly associated with risk for mortality. We found that the risk for all-cause/CVD-related mortality was not uniform within pathogen burden levels as is assumed in models using measures of summed pathogen burden. Furthermore, the risk for mortality associated with specific combinations of pathogens was not in all cases greater than that yielded by seropositivity for fewer pathogens. Thus, increased pathogen burden did not increase risk for all-cause/CVD-related mortality, regardless of pathogen type as hypothesized in models using measures of summed pathogen burden to examine whether there is a graded relationship between total pathogen burden and mortality. Importantly, we identified particular combinations of pathogens that were statistically significantly associated with increased risk for all-cause mortality (combined seropositivity for CMV and HSV-2 and combined seropositivity for CMV, HSV-1 and HSV-2) and with CVD-related mortality (combined seropositivity for CMV and HSV-2 and combined seropositivity for HSV-2 and *H. pylori*).

These findings provide a major contribution to the current understanding of the relationship between persistent pathogens, inflammation and risk for all-cause/CVD-related mortality in the U.S. general population. This dissertation confirms the role of CMV seropositivity,

especially in conjunction with elevated CRP level in predicting all-cause and CVD-related mortality and supports interventions aimed at the elimination of CMV infection via the development and administration of treatments or vaccines and/or targeting the interaction of infection and CRP levels for reducing mortality rates in the United States adult population.

Further, this work indicates that research examining the association between total pathogen burden and mortality should shift away from measures of summed pathogen burden and instead focus on examining the role of seropositivity for specific combinations of pathogens in determining risk for mortality in order to focus interventions on the elimination of pathogens that are most detrimental to health in the general U.S. population.

5.2 Aim 1

The first aim of this dissertation examined whether seropositivity for CMV predicted all-cause as well as CVD-related mortality in the U.S. general population and whether CRP mediated or modified the association in a U.S representative population 25 years of age and older. CMV seropositivity was statistically significantly associated with all-cause mortality (HR 1.19, 95% CI 1.01, 1.40), but not CVD-related mortality (HR 1.19, 95% CI 0.95, 1.49) in models adjusted for age, gender, race/ethnicity, country of origin, education level, body mass index, diabetes status and smoking status. Further adjustment for CRP level did not attenuate this relationship. When we examined the combined effect of CMV seropositivity and high CRP level, we found the combined effect of these factors to be statistically significantly associated with both all-cause and CVD-related mortality and to be greater than risk for mortality associated with either factor alone.

Aim 1 fills several gaps in the current literature examining the relationship between CMV, inflammation and mortality. First, it establishes a significant relationship between CMV seropositivity and all-cause mortality in the general U.S. population. This relationship has been described earlier in studies conducted among specialized populations of older age individuals and specific race/ethnic or gender groups. CMV seropositivity was not statistically significantly associated with CVD-related mortality, however, it is possible that the strong relationship between CMV and all-cause mortality observed in this study indicates that CMV seropositive subjects are dying of competing causes before they have the chance to develop CVD, attenuating the relationship between CMV and CVD-related mortality. We did find that the combined effect of CMV seropositivity and high CRP level was significantly associated with both all-cause and CVD-related mortality and the combined effect of these factors was greater than the effect of CMV seropositivity alone or high CRP level alone. These findings lend support for interventions that focus on both preventing CMV infection and decreasing CRP levels for decreasing risk for mortality in the general U.S. population.

CMV has been previously linked to a variety of chronic diseases including CVD, physical and cognitive impairment and cancer and this study establishes that CMV seropositivity is also associated with all-cause mortality in the general U.S. population.[38, 39, 57, 72-75] CMV is thought to

impact risk for chronic disease in part via direct effects such as the ability to invade vascular tissues and cause vascular damage or the ability to impact the regulation of cellular process such as cell migration and apoptosis which influence the development of cell proliferation and cancer.[51,94] In addition, CMV is thought to impact risk for chronic diseases of inflammation-related etiology because this infection is subject to "reactivation" during times of stress, immunosupression and ageing and viral replication may lead to the release of acute phase proteins and inflammatory markers.[50,80,87-90] Furthermore, the pathogen-specific effects of persistent infections that are "reactive" are likely to be exacerbated.[79-86] It is possible that, elevated CRP could be marker of "reactivating" CMV infection, whereas low CRP level may indicate resolved or "latent" CMV infection. However, elevated CRP levels may also represent acute infection or injury not necessarily related to CMV infection and thus, CRP levels may be elevated in persons seropositive for CMV for other reasons. Because elevated CRP levels have been implicated in CVD and mortality independent of CMV infection, CMV infection and elevated CRP levels may in some cases, compete to increase risk for mortality whereas in other cases, CMV infection in conjunction with elevated CRP may be indicative of "reactive" CMV infection which is associated with exacerbated effects on mortality.

Overall, if CMV infection plays a strong role in the etiology of all-cause and CVD-related mortality in conjunction with high CRP level, the elimination of CMV infection via the development and administration of treatments or vaccines and/or targeting the interaction of infection and CRP levels may reduce mortality rates in the United States. Furthermore, if subclinical "reactive" CMV infection is most important in the etiology of mortality, then situations that cause CMV reactivation over the lifecourse, such as stress, inflammation caused by co-infections, immunosuppression and aging may play important key roles in determining the extent to which CMV infection is detrimental to health. Since seropositivity as well as immune response for CMV patterns strongly by SEP, interventions that focus on the prevention of primary CMV infection as well as CMV "reactivation" could also serve as a novel point of intervention for reducing social disparities in mortality observed in the United States.

5.3 Aim 2

The second aim of this dissertation examined whether there is a graded relationship between increasing pathogen burden with CMV, HSV-1 and -2 and *H. pylori* and all-cause as well as CVD-related mortality. In addition, the second aim assessed the combined effect of pathogen burden and CRP on all-cause/CVD-related mortality in a U.S representative population 25 years of age and older. There was not a graded relationship between summed pathogen burden with these four pathogens and risk for all-cause/CVD-related mortality in models adjusted for age, gender, race/ethnicity, country of origin, education level, body mass index, diabetes status and smoking status.

Aim 2 fills several gaps in the current literature on summed pathogen burden, inflammation and mortality. First, previous studies examining the relationship between summed pathogen burden and all-cause or CVD-related mortality, were conducted among older study populations from specific geographic regions and primarily among persons with existing CVD. Thus, this work highlights for the first time the relationship between summed pathogen burden and mortality in the general U.S. population and shows that there is no significant graded relationship between summed pathogen burden with these particular pathogens and mortality.

It has been hypothesized that "more is worse" in terms of the number of pathogens to which individuals are seropositive in predicting risk for mortality and that increased pathogen burden impacts risk for mortality in part through increasing systemic inflammation. However, the results of our study suggest that other factors, such as the specific combinations of pathogens individuals are seropositive for, may also be important for determining risk for mortality in the US general population.

Given that seropositivity for specific combinations of persistent pathogens may impact risk for all-cause and CVD-related mortality differently in populations with and without existing CVD, future research examining the role of specific combinations of pathogens and inflammation in determining risk for all-cause/CVD-related mortality is warranted. Identifying the specific pathogens that alone, or in the presence of co-infection are most detrimental to mortality would inform future interventions and/or treatments focused on infection-related prevention of mortality. Therefore, this dissertation assesses whether specific combinations of pathogens influences mortality in the US population in Aim 3.

5.4 Aim 3

The third aim of this dissertation estimated the combined effect for each combination of four persistent pathogens (CMV, HSV-1, HSV-2 and/or *H. pylori*) previously implicated in the etiology of several chronic diseases, and all-cause/CVD-related mortality in a U.S representative population 25 years of age and older. We found that after adjustment for age, gender, race/ethnicity, country of origin, education level, BMI (kg/m^2), diabetes and smoking status, combined seropositivity for CMV and HSV-2 (HR 2.06, 95% CI 1.22, 3.47)) and combined seropositivity for CMV, HSV-1 and HSV-2 (HR 1.52, 95% CI 1.01, 2.31) were statistically significantly associated with all-cause mortality and combined seropositivity for CMV and HSV-2 (HR 4.05, 95% CI 2.07, 7.91) and combined seropositivity for HSV-2 and *H. pylori* (HR 6.27, 95% CI 1.56-25.20) were statistically significantly associated with CVD-related mortality.

We also examined the extent to which use of summed pathogen burden variables for modeling the relationship between total pathogen burden and mortality may limit the ability of researchers to identify the specific combinations of pathogens for which the combined effects are most detrimental to health. We found that the risk for all-cause/CVD-related mortality for individuals with a given summed pathogen burden level was not uniform, regardless of which

combination of pathogens individuals were seropositive for, nor that increased summed pathogen burden level guaranteed an increased risk for all-cause/CVD-related mortality, regardless of pathogen combination. These results suggest that the use of summed pathogen burden variables to assess whether there is a monotonic relationship between total pathogen burden and mortality likely limits our ability to identify potentially significant variability in risk attributable to the specific combination of pathogens individuals are seropositive for. In addition, the risk associated with mortality for those categorized as having a particular pathogen burden level may be less a reflection of the total number of pathogens individuals are seropositive for than the result of particular combinations of pathogens that in combination, strongly increase or decrease risk for mortality.

In our case, our earlier study in chapter 3 (Aim 2) did not find an association between summed pathogen burden and mortally. Other studies have identified such a relationship but primarily among older study populations, from specific geographic regions and with existing CVD. It is possible that regardless of the results whether null or significant for the association between summed pathogen burden and mortality, these earlier studies are not capturing important variability attributed to the types and combinations of pathogens that may contribute to mortality risk. Aim 3, therefore, fills several gaps in the current literature examining the relationship between total pathogen burden and mortality. First, the majority of previous studies examining the relationship between total pathogen burden and mortality have utilized summed pathogen burden variables for modeling the association and to our knowledge no previous studies have estimated the risk for mortality for the combined effect of specific combinations of persistent pathogens. This study is also the first to assess the extent to which the use of summed pathogen burden variables limits the ability of researchers to identify which particular pathogens alone, or in combination are most detrimental to health. Importantly, this study identifies several combinations of pathogens that are associated with increased risk for all-cause and CVD-related mortality among the general U.S. population aged 25 and older.

Identifying which persistent pathogens, individually, or in combination, are most strongly associated with mortality and identifying how pathogens interact to increase risk for chronic disease and death remains important for focusing interventions that reduce exposure to and/or transmission of infections most detrimental to health. Our findings suggest that herpesviruses may play an important role in determining risk for all-cause/CVD-related mortality. Interventions focused solely on reducing the number of pathogens individuals are seropositive for may not eliminate the pathogens most detrimental for mortality In contrast, interventions that focus instead on the elimination of specific combinations of pathogens that are most strongly associated with increased mortality in specific at-risk populations are more likely to be effective at reducing infection-related mortality. If herpesviruses such as CMV, HSV-1 and HSV-2 as well as the bacterial pathogen, *H. pylori*, are potentially driving the association between pathogen burden

and risk for mortality, emphasis should be made on determining the specific mechanisms by which these particular pathogens act together to increase risk for death and whether delayed acquisition and/or improved immune control of these infections over the life course could serve to decrease risk for mortality.

These findings indicate that future research should examine both the role of summed pathogen burden as well as the role of specific combinations of pathogens in determining risk for mortality in order to better understand how pathogen burden acts to increase risk for mortality. Although measures of summed pathogen burden are useful for examining whether there is a graded relationship between total pathogen burden and risk for mortality, regardless of pathogen type, limiting the examination of the relationship between total pathogen burden and mortality to testing the "more is worse" hypothesis limits the ability of researchers to identify which persistent pathogens alone or in combination are most detrimental to health.

5.5 Dissertation summary

Using population-based data on persons 25 years of age and older from NHANES III, this dissertation demonstrated that seropositivity for CMV increased risk for all-cause mortality, independent of CRP level, but not CVD-related mortality. However, the combined effect of these factors on risk for all-cause/CVD-related mortality was greater than risk for both all-cause/CVD-related mortality associated with either factor alone. These findings support the hypothesis that CMV infection, especially in conjunction with elevated inflammation, which may be a marker of "reactive" infection may play an important role in predicting risk for mortality and supports future interventions that focus on the elimination of CMV infection via the development and administration of treatments or vaccines and/or targeting the interaction of infection and CRP levels may reduce mortality rates in the United States.

In addition, we found that there was not a graded relationship between summed pathogen burden with CMV, HSV-1, HSV-2 and/or *H. pylori* and all-cause/CVD-related mortality and the combined effect of pathogen burden level and CRP level on mortality also did not follow a graded pattern. These findings indicate that other factors, such as the specific combinations of pathogens individuals are seropositive for, may play a more important role in determining risk for mortality in the general U.S. population. Last, we examined the association between seropositivity for specific combinations of pathogens and risk for all-cause/CVD-related mortality finding the risk for all-cause/CVD-related death was not uniform within pathogen burden levels and risk for mortality associated with specific combinations of pathogens was not in all cases greater than that yielded by seropositivity for fewer pathogens. In addition, particular combinations of pathogens were statistically significantly associated with increased risk for all-cause mortality (seropositivity for CMV and HSV-2 and seropositivity for CMV, HSV-1 and HSV-2) and with CVD-related mortality (seropositivity for CMV and HSV-2 and seropositivity for HSV-2 and *H. pylori*). These findings suggest that interventions focused solely on reducing the number

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of pathogens individuals are seropositive for are unlikely to eliminate the pathogens most detrimental for mortality in specific populations in the U.S and that interventions should focus instead on the elimination of specific combinations of pathogens most detrimental to health to have the greatest impact on decreasing mortality in the U.S.

Taken together, this dissertation benefits the public health by clarifying the role of CMV, a persistent pathogen implicated in the etiology of numerous chronic diseases, inflammation in determining risk for all-cause/CVD-related mortality. Furthermore, this work betters our understanding of the association between summed pathogen burden level and risk for all-cause/CVD-related mortality compared to that of seropositivty for specific combinations of pathogens for determining risk for mortality. These results have important implications for future public health efforts designed to reduce mortality via infection and inflammation-related pathways.

5.6 Strengths and limitations

This dissertation is the first to examine the relationship between persistent pathogens, inflammation and all-cause as well as CVD-related mortality in a U.S. nationally representative population of individuals 25 years of age and older allowing generalizability of our results to the general U.S. population. Furthermore, this was the first study to explicitly examine the extent to which the use of summed pathogen burden variables for modeling the relationship between total pathogen burden and mortality limit the ability of researchers to identify the specific combinations of pathogens that most increase risk for mortality. In addition, measures of pathogen serostatus and CRP level were laboratory confirmed and measures by standard methodologies. We were also able to control for several covariates of interest including age, gender, race/ethnicity, body mass index, diabetes status, smoking status and NSAID use in our statistical analysis. Last, mortality status was rigorously verified by the National Death Index (NDI), a validate method for matching deaths in the U.S. to population-based datasets and for obtaining cause of death, as well as by death certificate review.

Several limitations are also noteworthy. First, we utilized the updated NHANES III Linked Mortality Public-use File which was subject to data perturbation in which synthetic data was substituted for the actual date of death and underlying cause of death for selected descendent records in order to reduce risk of respondent re-identification. However, a comparison study between the public-use and restricted-use file (which does not contain perturbed data) conducted by the National Center for Health Statistics, found that analysis of all-cause and cause-specific mortality by various sociodemographic were comparable across files.

Another limitation to our study is that we only had information on *seropositivity* for persistent pathogens and not immune response to infection and data for only one marker of systemic inflammation, CRP. Because high antibody titer to persistent infections is likely a marker of "reactive" infection which may be associated both exacerbated damage to invaded host tissues and with increased levels of inflammation, it is likely that high antibody titer persistent pathogens

plays an important role in determining risk for mortality. In addition, approximately 64% of our study population had CRP levels under the limit of detection (< 0.3 mg/dl), because more sensitive assays that are currently available for measurement of high-sensitivity CRP below this limit of detection were not used in NHANES III, making it difficult to examine CRP levels continuously. Therefore, another limitation of our study is that were unable to examine the relationship between pathogen antibody titer and CRP level more closely, which might have allowed us to identify a more precise interaction between CMV and CRP levels and their effect on all-cause and CVD-related mortality. Last, other inflammatory markers that have been shown to be association with individual pathogens as well as increased pathogen burden and might also have a significant role in the pathway between and mortality, such as IL-6, are not available in NHANES III. Thus, it was not possible to determine whether other inflammatory markers in addition to CRP, play a role in the relationship between persistent pathogens and mortality.

Last, we were unable to include other persistent pathogens that have been associated with chronic disease and/or mortality in previous studies because they were not serologically tested for in NHANES III. Therefore, other viral or bacterial pathogens that may also play an important role in determining risk for mortality and/or interact with pathogens included in this study in such a way that their inclusion would have altered the risk for all-cause/CVD-related mortality associated with certain combinations of pathogens. Therefore, we cannot rule out that the lack of graded relationship between summed pathogen burden and all-cause/CVD-related mortality observed in our study nor the associations between specific combinations of pathogens and all-cause/CVD-related mortality observed in our study are not due to unmeasured co-infection with other persistent pathogens. Further research examining the risk for mortality associated with a comprehensive variety of pathogen combinations is needed to fully understand which particular combinations of pathogens are most strongly associated with death in the U.S. general population.

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