



Spousal bereavement is associated with more pronounced ex vivo cytokine production and lower heart rate variability: Mechanisms underlying cardiovascular risk?



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ABSTRACT

The loss of a spouse is a highly stressful event that puts people at excess risk of mortality. Excess mortality among those who are widowed is highest in the first six months after the death of a spouse and decreases over time. Heart disease accounts for the largest proportion of these deaths. The psychological stress associated with stressful life events can enhance inflammation and lower heart rate variability (HRV). Both lower HRV and higher inflammation are risk factors for cardiovascular morbidity and mortality. Thirty-two recently bereaved individuals (Mean = 89.68 days since death, SD = 17.09) and 33 age-matched comparisons completed a blood draw, EKG, and self-report questionnaires. In both adjusted and unadjusted models, spousal bereavement was associated with enhanced pro-inflammatory cytokine production by in vitro lipopolysaccharide-stimulated peripheral blood leukocytes. Moreover, spousal bereavement was associated with lower HRV in comparison to the comparison group. This study is the first to demonstrate that bereavement is associated with a more pronounced ex vivo cytokine production and lower HRV in a population that exclusively consisted of widows and widowers. These findings add to the growing literature revealing the mechanisms that underlie bereavement-related cardiovascular problems. Future longitudinal studies are needed to determine the temporal relation between these risks. Understanding the biological mechanisms that underlie this stressful life event could allow researchers to create therapeutic targets for interventions to reduce or prevent the toll of a “broken heart.”

1. Introduction

The loss of a spouse is a highly stressful event. Indeed, the death of a spouse ranks *first* on the social readjustment rating-scale (Moon et al., 2013). The period surrounding spousal bereavement puts people at considerable increased risk for morbidity and mortality (Moon et al., 2013). Excess mortality among those who are widowed is highest in the first three to six months after bereavement and decreases over time (Stahl et al., 2016). Heart disease accounts for the largest proportion of these deaths attributed to spousal bereavement (Stahl et al., 2016). Depression, anxiety, and somatic complaints such as fatigue and sleep disturbances are hallmark characteristics of bereavement (Assareh et al., 2015).

Autonomic nervous system functioning is likely dysregulated when one is bereaved. Higher parasympathetic activity facilitates energy conservation (Thayer and Sternberg, 2006). Parasympathetic underactivity has been linked with a number of adverse mental and physical health outcomes. The variability in heart rate in the high frequency range is directly mediated by the vagus nerve and serves as a marker for vagally regulated heart rate variability (described as HRV for the remainder of this manuscript). Lower tonic HRV is a marker of all-cause mortality (Thayer and Sternberg, 2006); importantly, lower HRV is a well-established risk factor for cardiovascular disease, as well as diabetes, even after controlling for other cardiovascular risk factors (Haensel et al., 2008). Stress and depression can lower HRV.

A few studies have examined the association between HRV and

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bereavement with conflicting results. One study which consisted of 10 bereaved individuals and two other comparison groups (one depressed and one healthy comparison group), did not show differences between spousal bereavement and HRV (O'Connor et al., 2002). However, in this study, bereaved individuals who exhibited more depressive symptoms had lower HRV than those bereaved who reported less depressive symptoms (O'Connor et al., 2002). Yet in this study, widowers participated up to 24 months after the death of their spouse (O'Connor et al., 2002). Given cardiovascular risk is highest in the initial months after the loss (Moon et al., 2013), those who lost their spouse more recently may be most at risk for lower HRV. Another study showed that bereavement was associated with reduced HRV (Buckley et al., 2012); however, participants in this study consisted of bereaved spouses, bereaved significant others (i.e. romantic partner, unmarried), and bereaved parents. Given the psychological processes one experiences when losing a child are different from losing a spouse (i.e., more intense grief) (Sanders, 1980), it is possible that the data of those who lost a child disproportionately contributed to this finding (Stanley et al., 2004).

Psychological stress can enhance inflammation. Inflammation of the vessel wall promotes both the initiation and progression of atherosclerosis (Ellenbogen et al., 2002; Lai and Linden, 1992; Ridker et al., 2000; Roy et al., 2001). Given that the stress-response system boosts inflammation, which is a major factor underlying cardiovascular disease, researchers have begun investigating links between bereavement and inflammation. In a study of 64 older adults (36 widows), bereaved participants had higher circulating plasma levels of IL-1RA and IL-6 if they also carried the IL-6 174 SNP, a single-nucleotide polymorphism (SNP) in the promoter region of the IL-6 gene (Schultze-Florey et al., 2012). Secondary data analysis from Midlife in the United States (MIDUS) II biomarkers project revealed that those who experienced the death of a person close to them 5–63 months prior to assessment had higher levels of inflammatory circulating biomarkers IL-6 and E-selectin, but not of ICAM-1 or C-reactive protein (CPR) (Cohen et al., 2015). Although partially impacted by third variable influences, these studies provide some evidence that bereavement is associated with increased levels of circulating pro-inflammatory cytokines.

A major limitation of the use of circulating (serum or plasma) cytokines as biomarkers of inflammation is that their levels are often close to, and more often below, the limit of detection of the assay and exhibit extreme variability as result of a number of factors including diurnal variation, changes in plasma volume, and enlargement of the cell pool (Stephoe et al., 2007). A more complete cytokine signature can be obtained by analyzing the capacity of immune cells to produce inflammatory mediators after *ex vivo* stimulation. This method more likely represents the *in vivo* situation where cytokines are produced by the immune system in response to stress or infection (Korenromp et al., 2011; Mommersteeg et al., 2008). Moreover, the *ex vivo* stimulation method allows to discriminate between production of cytokines by monocytes/macrophages and by T cells, depending on the stimulus applied in the cultures (Korenromp et al., 2011; Mommersteeg et al., 2008).

In the current study, we examined individuals shortly after the loss of a spouse who had been married for at least three years (most of whom were married for much longer). We examined whether or not (a) bereaved individuals had lower HRV than age-matched non-bereaved individuals (b) bereaved individuals showed more pro-inflammatory cytokine production by peripheral blood leukocytes stimulated with lipopolysaccharide than age-matched non-bereaved individuals. We hypothesized that bereaved individuals would exhibit lower HRV than non-bereaved individuals (Hypothesis 1). We also hypothesized that bereaved individuals would have higher levels of *ex vivo* cytokine production than age-matched non-bereaved comparisons (Hypothesis 2). We also explored whether those who were bereaved reported more depressive symptoms than non-bereaved individuals. We expected that, on average, bereaved individuals would meet the clinical cut-off score for major depressive disorder (MDD) based on the extant literature

suggesting that symptoms in the early stages of bereavement mirror those of patients with MDD (Fried et al., 2015).

2. Methods

2.1. Study sample

Thirty-two recently bereaved individuals (Mean = 89.68 days since death, SD = 17.09) and 33 age-matched comparisons completed a blood draw, EKG, and self-report questionnaires. The primary aim of this study was to determine the mechanisms that underlie the increased CVD risk among bereaved adults. Individuals who recently experienced the loss of their spouse were contacted and recruited from obituaries, support groups, flyer distribution, online postings, and community events. Comparison participants who had not experienced the loss of a loved one in the past year were also recruited through flyers, community events, and online advertisements. Exclusion criteria included significant visual or auditory impairment, being pregnant or nursing (women), autoimmune and inflammatory diseases, having experienced bereavement due to loss of another loved one in the last year, divorced within the past year, and previously widowed. All participants were English-speakers to ensure understanding of the questionnaires. We contacted 346 bereaved individuals and 149 comparisons. Of those, 54 bereaved individuals agreed to be screened along with 85 comparisons. A total of 38 bereaved individuals were eligible to participate, along with 64 comparisons. Of those, 33 bereaved and 32 comparisons participated in the visit. Bereaved participants must have recently lost their spouse no later than 14 weeks before the visit. They must have been married to their partner for at least 3 years before the loss. Then, the comparison group was matched based on gender, age, BMI, and education.

Research assistants administered assessments at the participants' home or in the Bioscience Research Collaborative Community Research Center in the Texas Medical Center. During these visits, participants completed a questionnaire packet, which included self-report demographic questionnaires and clinical questionnaires. Anthropometric measurements including weight, height, and waist circumference and non-fasting blood samples were collected during the early hours of the morning. All samples were collected between 7:30 and 11:00 AM to control for diurnal variation. The laboratory personnel who analyzed blood samples were blind to whether or not the participant was bereaved.

The day before the visit, a research assistant called the participants and reminded them of the next day visit. Given that inflammatory markers may be elevated during acute illnesses (e.g., upper respiratory infections), we asked them if they were experiencing any illness symptoms (e.g., fever, congestion, sore throat, or acute infections due to injury). Participants were asked to avoid any strenuous physical activity 48 h before all visits. Participants were rescheduled for a different time if they were ill or did not follow the exercise restriction. All participants provided informed consent and procedures were approved by the Rice University Institutional Review Board (IRB).

2.2. Measures

2.2.1. Heart rate variability

HRV was continuously measured (5 min) non-invasively with the Polar s810 wristwatch and Wearlink 31 belt band; the 1000 Hz sampling rate provides valid and reliable ECG data (Gamelin et al., 2006; Nunan et al., 2009). All participants were in a sitting position. Before analyzing HRV, we preprocessed the raw interbeat intervals for artifacts using KUBIOS HRV analysis software (Tarvainen et al., 2009). For every phase of the experiment, the KUBIOS software produced values for vagally-mediated (parasympathetic) HRV using the time-domain method, square root of mean successive differences (RMSSD) between R-Waves. Higher scores indicate higher vagally-mediated HRV. RMSSD

is determined by calculating the differences between consecutive interbeat (RR) intervals before squaring and summing them; the values are then averaged and the square root obtained (Malik et al., 1996; Stein et al., 1994). RMSSD is highly correlated with spectral derived measures of HRV and is less affected by respiration and other artifacts than spectral indices of HRV (Penttila et al., 2001). All procedures followed the recommendations of the Task Force of the European Society of Cardiology and the North American Society of Pacing Electrophysiology (Malik et al., 1996).

2.2.2. Cytokines

To measure the reactivity of the leukocytes to challenge we treated whole blood to induce cytokine/chemokine production. The pro-inflammatory cytokines included IL1 β , IL-6, TNF α , CCL2, CCL4, IL-6R, and TNFRI. The array we used was selected based our earlier research and was custom-made (van Zuiden et al., 2011). We did not measure any other pro-inflammatory cytokines/chemokines. Supernatants were collected after 24 h of culture and stored at -80°C until they were analyzed using multiplex assays according to the manufacturer's instructions (R&D Biosystems). Lipopolysaccharide (LPS)-induced cytokine/chemokine production was evaluated by heparinized whole blood, diluted 1:10 with RPMI-1640 (Gibco), and was stimulated with 1 ng/mL LPS (Sigma) at 37°C and 5% CO $_2$ for 24 h. Mean intra- and inter-assay CV (%) values for the different cytokines are as follows: IL-6: intra-assay: 7.4%; inter-assay: 7.8%; IL-6R: intra-assay: 4.5%, inter-assay: 5.1%; TNF- α : intra-assay: 2%, inter-assay: 6.5%; TNFRI: intra-assay: 3.5%; inter-assay: 4%; IL-1Ra: intra-assay: 5.3%, inter-assay: 8.6%.

2.2.3. Sleep disturbance

The Pittsburgh Sleep Quality Index was used as a measure of sleep disturbance. The PSQI is a widely used instrument for the evaluation of sleep disturbances which consists of seven component scores that are aggregated in a global score with a range of 0–21 (Buysse et al., 1989). Individual subscales included Subjective Sleep Quality, Sleep Latency, Sleep Duration, Sleep Efficiency, Sleep Disturbances, Use of Sleep Medication and Daytime Dysfunction. Higher scores on each subscale of the global score are indicative of greater sleep disturbance.

2.2.4. Depression

The Center for Epidemiologic Studies Depression Scale (CES-D) (Radloff, 1977) was used as a measure to assess prevalence of depression and also included in regression models as a control variable due to its close association with inflammation. The CES-D is a widely utilized measure of depression and has been validated across populations. Higher scores on this scale indicate greater depressive symptomatology. The clinical depression cut-score for MDD is 16.

2.2.5. The Charlson index (Charlson et al., 1994)

The most widely used comorbidity index for predicting mortality, was used to assess comorbidities. The measure assigns weights to 19 comorbid conditions based on their potential influence on one-year mortality. This was used as a covariate in the analysis.

2.2.6. The community healthy activities model program for seniors (CHAMPS)

Questionnaire assessed the weekly frequency and duration of various physical activities. Excellent for middle-aged and older populations (Demark-Wahnefried et al., 2003), we used the moderate exercise index as defined by the CHAMPS as a covariate in our analysis.

2.2.7. Other covariates

Demographic factors, health behaviors, comorbid conditions and body mass index (BMI) were also included in models as covariates. Participants provided self-reports of their age, gender, race/ethnicity and education, smoking status, and alcohol use. BMI was computed as

weight in kilograms divided by height in meters squared. We also assessed all medications and examined their impact in ancillary analyses.

2.3. Statistical analysis

Preliminary statistical analysis included descriptive statistics and assessment of normality of distributions. We checked the scores to ensure they were biologically possible. After careful inspection, we had no need to remove outliers. We also examined for skewness and kurtosis. HRV was relatively normally distributed; however, the inflammatory markers were skewed as would be expected. Accordingly, (base 10) log transformed values were calculated for each marker. After running each analyses, we examined the residuals to ensure they distributed normally. The pro-inflammatory markers included IL1 β ($M = 743.98$ pg/mL, $SD = 424.99$), IL-6 ($M = 2268.76$ pg/mL, $SD = 2284.17$), TNF α ($M = 425.02$ pg/mL, $SD = 351.23$), CCL2 ($M = 6255.24$ pg/mL, $SD = 4503.33$), CCL4 ($M = 6255.23$ pg/mL, $SD = 9804.80$), IL-6R ($M = 997.30$ pg/mL, $SD = 281.55$), and TNFRI ($M = 287.78$ pg/mL, $SD = 119.95$). In order to prevent type I error we created a composite index of all proinflammatory markers. The proinflammatory markers had a Cronbach of 0.80 (using log-transformed values). Zero-order correlations revealed that most combinations of inflammatory markers were highly positively associated. TNF α was associated with IL-6 ($r = 0.77$, $p < 0.001$), CCL2 ($r = 0.47$, $p < 0.001$), IL-6R ($r = -0.20$, $p = \text{ns}$), CCL4 ($r = 0.61$, $p < 0.001$), IL1 β ($r = 0.47$, $p < 0.001$) and TNFRI ($r = 0.31$, $p < 0.05$). IL-6 correlated with CCL2 ($r = 0.55$, $p < 0.001$), IL-6R ($r = 0.02$, $p = \text{ns}$), IL1 β ($r = 0.31$, $p < 0.05$), CCL4 ($r = 0.61$, $p < 0.001$), and TNFRI ($r = 0.38$, $p < 0.01$). CCL2 correlated with IL-6R ($r = 0.25$, $p < 0.05$), IL1 β ($r = 0.27$, $p < 0.005$), CCL4 ($r = 0.49$, $p < 0.001$), and TNFRI ($r = 0.65$, $p < 0.001$). IL-6R was correlated with IL1 β ($r = 0.14$, $p = \text{ns}$), CCL4 ($r = 0.01$, $p = \text{ns}$), and TNFRI ($r = 0.50$, $p < 0.001$). IL1 β was associated with CCL4 ($r = 0.35$, $p = .01$), and TNFRI ($r = 0.17$, $p = \text{ns}$). CCL4 was associated with TNFRI ($r = 0.40$, $p < 0.001$). For each cytokine, the z scores derived from the (base 10) log transformed values were calculated, and averaged to produce the summary construct for each participant. This is an established method to analyze multiple correlated dependent variable and previously used to examine individual immune markers that function similarly (Dattalo, 2013; Fagundes et al., 2012; Huberty and Morris, 1989; Rosnow and Rosenthal, 1989). These pro-inflammatory markers operate together in vivo; this combined index reflects a coordinated pro-inflammatory immune response. We used this mean inflammatory z score as a primary outcome of interest.

Multiple imputation was employed to impute missing data (which was less than 5% of the sample and did not include any immune data nor data on bereavement status). Multiple imputation produces unbiased parameter estimates that appropriately reflect the true variability of the missing data and has been shown through simulation studies to be a more valid and less biased analytical approach than listwise deletion. Multiple imputation has been shown to perform well when data are missing at random and even acceptable under some cases of nonrandom missingness. It is robust to departures from normality assumptions and performs well even with low sample size. Following standard practice, the imputation procedure was repeated five times in order to approximate the true measurement variance represented in real data. All analyses were completed with each of the full imputed data sets, and the coefficients generated by each separate data set were averaged to produce final estimates (Graham, 2009).

We ran three regression models. In the first model, HRV was the dependent variable. In the second model, the cytokine profile was the dependent variable. In the third model, depressive symptoms were the dependent variable. All analyses were run using a mixed models regression program. We adjusted for key potential confounds including age, sex, BMI, physical activity, comorbidities, sleep disturbance, alcohol use, and age as a continuous variable. Smoking status (1 = current smoker, 0 = nonsmoker) was coded as a categorical variable.

Table 1
Descriptive Statistics and Mean Differences Between Groups.

Characteristic	Overall (n = 65)		Bereaved (n = 32)		Control (n = 33)		P-Value
	N	Mean (SD)	N	Mean (SD)	No	Mean (SD)	
RMSSD							
Mean (SD)		34.52 (5.67)		26.39 (5.29)		42.40 (10.30)	0.04
Age, years							
Mean (SD)		67.87 (1.00)		67.12 (1.43)		68.6 (1.42)	0.45
Smoking Status	4	6.0%	1	3.1%	3	9.1%	0.33
BMI							
Mean (SD)		28.54 (0.78)		26.7 (0.90)		30.3 (1.24)	0.03
Activity, days per week							
Mean (SD)		2.64 (.36)		1.92 (.39)		3.30 (0.58)	0.06
Sleep Problems							
Mean (SD)		6.63 (0.56)		7.58 (0.83)		5.71 (0.70)	0.12
Ethnicity							0.15
Other	4	6.2%	1	3.1%	3	9.1%	
Black	11	16.9%	1	3.1%	10	30.3	
Latinx	6	9.2%	4	12.5%	2	6.1%	
White	44	67.7%	26	81.2%	18	54.5%	
Education level							0.68
High school or less	11	16.9	6	18.7	5	15.1	
Some College	8	12.3	4	12.5	6	18.1	
College or University Graduate	19	29.2	8	25	11	33.3	
Master's	13	20	7	21.9	2	6	
Doctorate	4	6.1	2	6.2	2	6.1	
Professional (e.g. MD, JD)	4	6.1	4	13	0	0	
Do not choose to specify	6	9.2	1	3.1	5	15.2	
Alcoholic Drinks Per Week							0.33
Mean (SD)		3.39 (.64)		2.8(.70)		4.01 (1.07)	
Charlson Comorbidity Index							0.47
Mean (SD)		.35(0.19)		.31 (.19)		.39 (6.864)	
Sex (Female)							0.63
Proinflammatory Composite		.17 (.67)				−0.16(.61)	0.04
CESD (Depressive Symptoms)		14.54 (12.10)		17.75 (13.03)		11.24 (10.62)	0.04

Importantly, we removed sleep disturbance from the model when predicting CES-D scores given that the CES-D has a sleep disturbance component.

We were careful not to adjust for extraneous factors to reduce risk of overfitting the models. For example, 41.5% of our sample reported taking a low dose Aspirin. We chose not to exclude or adjust for this medication because Aspirin use (325 mg/day) did not reduce IL-6 and CRP inflammation in a prospective cross-over design with 37 healthy volunteers (Azar et al., 2003). Epidemiological studies have not found that individuals taking antidepressants have significantly lower levels on key markers of inflammation than individuals who are not taking antidepressants, and the data showing relationships between HRV and anti-depressants is mixed (Penninx et al., 2003). We elected not to adjust for anti-depressants a priori. We examined residuals from all analyses to confirm that they were distributed normally.

3. Results

Important characteristics of the study sample are presented in Table 1. There was no significant difference between bereaved and age-matched comparisons in regard to age, smoking status, sleep disturbance, ethnicity, education level, alcoholic drinks per week, and comorbidities. Sex was distributed equally among both groups. Interestingly, BMI was lower among bereaved individuals than age-matched comparisons. The pro-inflammatory cytokine composite index (IL1 β , IL-6, TNF α , CCL2, CCL4, IL-6R, and TNFR1) was higher among bereaved individuals compared with age-matched comparisons (Table 2). Furthermore, HRV (as indexed by RMSSD) was lower among bereaved individuals than age-matched comparisons. Accordingly, in the unadjusted analyses of variance, hypothesis 1 and hypothesis 2 were confirmed. Depressive symptoms were also higher among bereaved individuals than age-matched comparisons. Of note, on average, bereaved individuals were above the clinical cut-off for depression,

Table 2

Point estimates, adjusted means, effect size, and *p*-values for differences between bereaved and not bereaved individuals for HRV, proinflammatory composite, and depressive symptoms.

Variable	Mean bereaved	Mean control	Partial Eta Squared	P
HRV (RMSSD)	26.39	42.40	0.04	0.05
Proinflammatory Composite	0.222	−0.215	0.09	0.03
Depressive Symptoms	18.47	10.73	0.11	0.01

while matched comparisons were not. In the bereaved group, days since the death of the spouse was not associated with the cytokine composite ($r = -0.10$, $p = ns$), HRV ($r = 0.04$, $p = ns$), depressive symptoms ($r = -0.02$, $p = ns$) nor any of the individual inflammatory markers.

Unadjusted zero-order correlations revealed that HRV was not associated with age, comorbidities, smoking, alcoholic drinks consumed, BMI, sex, exercise, or sleep ($p = ns$). Those that had higher levels of the stimulated profile did not have significantly lower HRV ($B = -0.11$, $p = ns$), although the coefficient was in the expected inverse direction. The stimulated cytokine profile was not associated with age, comorbidities, smoking, alcoholic drinks consumed, BMI, sex, exercise, or sleep. Those that had more depressive symptoms had more sleep disturbances ($B = 0.68 < 0.001$), and were younger ($B = -0.32$, $p < 0.001$). Importantly, those who were bereaved had more depressive symptoms and higher levels of cytokines in the unadjusted zero-order model ($p < 0.05$). HRV was not associated with the cytokines.

We adjusted for key confounds in Table 2. As can be seen, when adjusting for key a priori confounds, those who were bereaved had lower HRV than age-matched comparisons (displayed in Fig. 1). Furthermore, those who were bereaved had significantly higher levels of the composite stimulated cytokine profile compared with those who

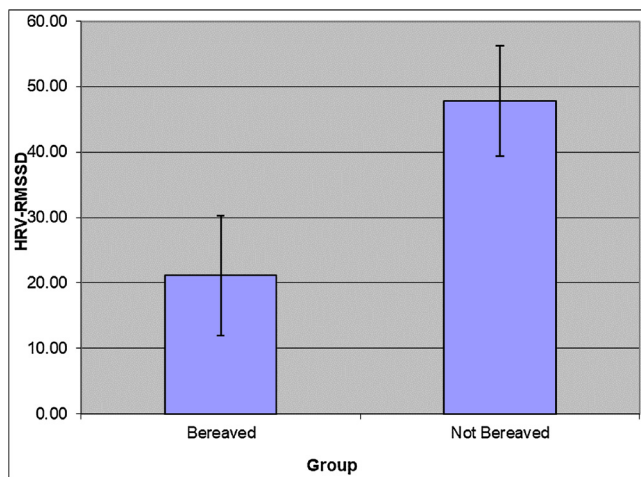


Fig. 1. Bar graph displaying adjusted means and corresponding standard errors between bereaved and not bereaved individuals for the RMSSD calculation of HRV.

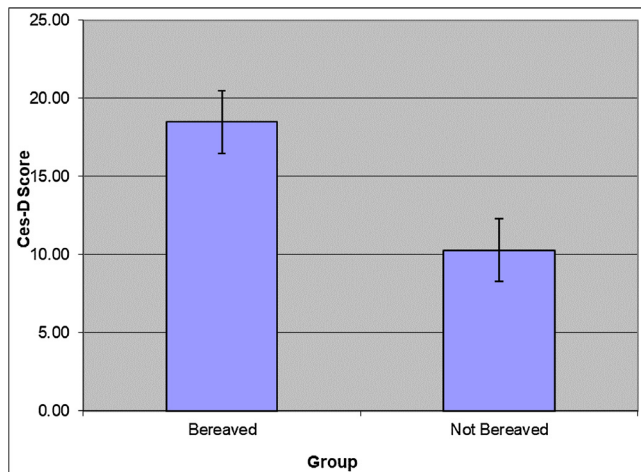


Fig. 2. Bar graph displaying adjusted means and corresponding standard errors between bereaved and not bereaved individuals for the CES-D measure of depressive symptoms.

were not bereaved as can be seen in Table 2. Accordingly, in both the adjusted and unadjusted models, bereaved individuals had lower HRV, and higher levels of cytokines. Finally, bereaved individuals reported more depressive symptoms than those who were not bereaved (displayed in Fig. 2). In ancillary analyses, we also adjusted for depression, antidepressants and statin use, which did not alter the aforementioned associations. As previously mentioned, HRV was not significantly associated with depressive symptoms in this sample.

We then conducted post-hoc tests to evaluate each individual inflammatory marker (logged) individually. Each marker was higher among bereaved than age matched comparisons: IL1 β (Bereaved M = 2.86, Comparison M = 2.73, $p = ns$), IL-6 (Bereaved M = 3.32, Comparison M = 3.17, $p < 0.05$), TNF α (Bereaved M = 2.65, Comparison M = 2.48, $p < 0.05$), CCL2 (Bereaved M = 3.45, Comparison M = 3.22, $p = ns$), CCL4 (Bereaved M = 3.73, Comparison M = 3.60, $p < 0.10$), IL-6R (Bereaved M = 2.99, Comparison M = 2.97, $p = ns$), and TNFRI (M = 287.78, SD = 119.95, $p = ns$). TNF-alpha and IL-6 clearly were significantly higher among bereaved than the age matched comparison group. Furthermore, CCL4 was higher among bereaved than the comparison, albeit at trend level using a two-tailed test. CCL2, IL1 β , IL-6R and TNFRI did not significantly differ between groups.

In further ancillary analyses, we also adjusted for depression,

antidepressants and statin use, which did not alter any of the aforementioned associations.

4. Discussion

Spousal bereavement was associated with lower HRV and a more pronounced ex vivo cytokine production in both unadjusted and adjusted models. Indeed, even after adjusting for confounding factors including age, sex, BMI, physical activity, comorbidities, sleep disturbance, alcohol use, and depressive symptoms—spousally bereaved individuals demonstrated an enhanced pro-inflammatory response and lower HRV. Our findings add to the growing literature regarding the mechanisms that may underlie bereavement-related cardiovascular problems.

To our knowledge, this is the first study to demonstrate that spousally bereaved individuals have lower HRV than non-bereaved individuals in a population that exclusively consisted of widows and widowers. As described above, lower HRV is prognostic for cardiovascular disease (Thayer and Sternberg, 2006). Lower HRV was also significantly lower among women with Takotsubo cardiomyopathy compared with women in a comparison group (Norcliffe-Kaufmann et al., 2016). Given Takotsubo is characterized by considerable emotional distress and even colloquially called “broken heart syndrome” these findings may play an etiological role in Takotsubo cardiomyopathy (Norcliffe-Kaufmann et al., 2016). The substantial relationship between HRV and bereavement is notable. Aging is associated with a decreased autonomic regulation and HRV parameters may actually underlie a portrayal of potential longevity and energy (Almeida-Santos et al., 2016). Indeed, based on research that demonstrated age-related HRV (RMSSD) reductions of 3.6 milliseconds per decade, our findings indicate that bereavement may substantially accelerate the aging process (Antelmi et al., 2004).

This study is also the first to demonstrate that spousal bereavement is associated with more pronounced ex vivo cytokine production. Atherosclerosis is an inflammatory process; inflammation (especially monocyte/macrophage activation) plays a key role in destabilization and rupture of atherosclerotic plaques, leading to cardiovascular events. Under normal conditions, adhesion of leukocytes to the endothelial cells of the vessel wall is low (Libby, 2006). However, when the system is activated by the stress-response (or other factors), the endothelial cells increase their adhesion molecules and thereby selectively recruit (pro-inflammatory) leukocytes. Once adhered to the arterial endothelium, monocytes differentiate into macrophages and will be activated, while storing fat particles. These ‘foamy macrophages’ in the vessel wall promote the formation of atherosclerotic lesions. T cells also contribute to lesion evolution and secrete pro-inflammatory cytokines, which stimulate the passage and propagation of smooth muscle cells. Finally, lymphocytes produce cytokines that inhibit the production of collagen by smooth muscle cells, which weakens the fibrous cap. When the fibrous plaque ultimately breaks, a thrombus forms that leads to most heart attacks (Libby, 2006).

We performed a post-hoc analysis that revealed IL-6 and TNF-alpha showed the largest and only statistically significant group differences. A recent meta-analysis revealed a moderate increase in both stimulated IL-6 and TNF-a production that peaked between 10 and 30 min after acute stress; thus, it is possible that these two markers are more sensitive to the stress associated with bereavement than the others measured in the current study (Marsland et al., 2017). There are clearly bidirectional interactions between the HPA axis and IL-6 and TNF-alpha, which may further explain this link (Marsland et al., 2017).

We used LPS as the stimulus. The pro-inflammatory stimulus LPS polarizes monocytes from M0 to M1 mostly secreting pro-inflammatory mediators and releasing soluble cytokine receptors. When monocytes are polarized to the anti-inflammatory state using, for example, the T cell cytokines IL4 or IL13, the pattern of activation would have been more skewed to the anti-inflammatory phenotype (Moghaddam et al.,

2018). Inflammation is associated with changes in adhesive determinants allowing changes in the adhesion of NK cells and other cell types that are released from the vessel wall. In the case of NK cells, this increases the number of circulating NK cells. Moreover, the pro-inflammatory milieu is associated with an activation of various other cell types including macrophages (Korenromp et al., 2011; Mommersteeg et al., 2008).

The average depression score for bereaved individuals was one point above the clinical threshold for major depressive disorder (MDD). Although a formal diagnosis of MDD can only occur after a clinical interview, this score is notable. It is relatively normative to experience significant depressive symptoms immediately after the loss of a spouse, but these symptoms are typically alleviated over time. It would be interesting to determine if initial levels of inflammation and HRV are prognostic for sustained high levels of depressive symptoms in a longitudinal design.

Early work in the field of psychoneuroimmunology revealed links between bereavement and other markers of immunity. In a classic study, T and B cell numbers and function were examined approximately 2 weeks after bereavement and at a 6 week follow-up. The response to phytohaemagglutinin was significantly lower in the bereaved group on the second occasion, as was the response to concanavalin A at 6 weeks. Yet there was no difference in T and B cell numbers, an important finding showing that cell count may be less impacted by stress than cell proliferation (Bartrop et al., 1977). Indeed, bereavement has been linked to lower natural killer cell cytotoxicity; furthermore, lymphocyte proliferative response to phytohemagglutinin was decreased among the bereaved (Goodkin et al., 1996; Irwin et al., 1988). In an early study that is closely related to the present investigation, results indicated that bereavement is associated with persistent activation of T cells and the production of IFN- γ , an important activator of macrophages and inducer of Class II major histocompatibility complex (MHC) molecule expression (Pettingale et al., 1994).

Interventions that target inflammation and HRV are needed to reduce cardiovascular risk in this vulnerable population. Although exercise is not generally prescribed after the death of a spouse, it could be beneficial given the strong evidence showing that it promotes higher HRV, lowers inflammation, and reduces depressive symptoms (Kiecolt-Glaser et al., 2015). Given the age of most bereaved individuals, low impact exercise routines (e.g. yoga, water aerobics, etc.) may be more feasible than intense cardiovascular training. Mind-body interventions have been demonstrated to be feasible and effective in other distressed older populations (Patel et al., 2012). Recent work suggests that non-pharmacological interventions such as HRV biofeedback may be another promising therapeutic intervention (Karavidas, 2008; Zucker et al., 2009). HRV biofeedback enhances baroreflex gain and measures of vagus nerve activity. Indeed, it has been shown to decrease symptoms and physiological signs of inflammation, in addition to symptoms of depression and post-traumatic stress (Karavidas, 2008; Zucker et al., 2009).

Important strengths of our study include its rigorous methodology, including careful consideration of time of day of blood draws, age-matching, and homogeneity in type of loss. Due to the cross-sectional design, we cannot say with certainty that lower HRV and inflammation are causally related to bereavement, which is a limitation of the study. Yet reverse causality seems extremely implausible. The sample is predominantly white, another limitation that should be addressed in future work. Future work should examine inflammation and HRV over time to examine the time course of bereavement-related changes in HRV and inflammation. The bereaved participants lost their spouse rather recently; accordingly, we did not assess for complicated grief. Future work should consider evaluating complicated grief in relation to the biological outcomes presented.

A possible limitation of the study is that we did not assess the culture without stimulus; however, given there is very little production of cytokines at baseline levels, we do not think this additional information

would have been informative or changed the conclusions of this study. Stimulated cytokine profiles are ideal to determine the capacity of the circulating leukocytes to produce cytokines. However, stimulated cytokine profiles reflect a general pattern of the activation state of the immune system since the subsets of T cells and monocytes (and thus the capacity to secrete certain cytokines) can differ between samples. However, the potential difference in the number of leukocytes in the samples do not dictate the level of cytokine responses because there is no correlation between absolute number of leukocytes and the various cytokine responses (see supplemental Fig. 1a–1e). We used receptor values with ligand values as a reflection of a pro-inflammatory condition based on previously established literature showing both soluble receptors as well as the ligands are secreted because of a pro-inflammatory response (Bower et al., 2011; Collado-Hidalgo et al., 2006). This was an a priori decision that was supported by a high alpha level. For some cytokines, the soluble receptor facilitates the action of the cytokine, whereas for others it can be inhibitory. It is possible that the reason why receptor values did not differ between groups in post-hoc analyses was because they were inhibitory.

Although LPS is known to mostly stimulate monocytes, we cannot rule out that LPS also induces cytokines in other cell types such as granulocytes. Although we did not determine cytokine levels in unstimulated cultures, we do know that levels of cytokines measured in plasma are much lower than what we detect here even though in the cultures the blood was diluted 1:10. Therefore, it seems unlikely that there is a significant contribution of spontaneously secreted cytokines to the levels detected in the LPS stimulated cultures.

5. Conclusions

In sum, this study adds to our growing understanding of the mechanisms that underlie bereavement-related cardiovascular risk. Future longitudinal studies are needed to determine the temporal relation between the risks. Understanding the biological mechanisms that underlie bereavement could allow researchers to create therapeutic targets for interventions to reduce or prevent the toll of a “broken heart.”

Conflicts of interest

The authors have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.psyneuen.2018.04.010>.

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