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Publication Date

DOI

Peer reviewed

Divergent Gene Expression Responses to Complicated Grief and Non-complicated Grief

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Abstract

The "widowhood effect" (i.e., morbidity/mortality in recently bereaved spouses) may be related to changes in immune function, but little is known about the impact of bereavement on gene transcription in immune cells. This study examined how Complicated Grief and Non-complicated Grief responses to bereavement differentially affect leukocyte gene expression. Genome-wide transcriptional profiling and bioinformatic analyses were completed on 63 older adults. Thirty-six of them had lost their spouse/partner on average 2 years ago, and 27 were nonbereaved, married controls. Twelve of the bereaved participants met criteria for Complicated Grief. Compared to nonbereaved controls, bereavement (both Complicated Grief and Non-complicated Grief) was associated with upregulated expression of genes involved in general immunologic activation and a selective downregulation of genes involved in B lymphocyte responses. However, Complicated Grief and Non-complicated Grief differed markedly in their expression of Type I interferon-related transcripts, with Non-complicated Grief subjects showing substantial upregulation relative to nonbereaved controls and Complicated Grief subjects showing substantial downregulation. Bereavement significantly modulates immune function gene expression. The magnitude of bereavement-related distress (i.e., Complicated Grief vs. Non-complicated Grief) is linked to differential patterns of transcription factor activation and gene expression involved in innate antiviral responses. These findings provide a molecular framework for understanding the health effects of bereavement, as well as new insights into the particular gene modules that are most sensitive to the individual's psychological response to loss.

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1. Introduction

An extensive body of literature shows an increased risk for morbidity and mortality following bereavement (Boyle et al., 2011), and distress-related changes in immune cell function have been hypothesized as key mediator for these effects (Glaser and Kiecolt-Glaser, 2005). A long history of research has identified bereavement-related changes in lymphocyte function and their relative proportions in circulation (Bartrop et al., 1977; Gerra et al., 2003; Irwin et al., 1988). Bereaved widows also show reduced antibody titers to vaccination (Phillips et al., 2006). Physical health effects may be particularly pronounced in bereaved persons with specific inflammatory genetic polymorphisms (Schultze-Florey et al., 2012). Given that bereavement has effects on physical health beyond one year (Martikainen and Valkonen, 1996), immunological alterations may persist long after the death event.

In response to bereavement, the majority of persons cope resiliently with this potentially traumatic event (Bonanno et al., 2002). However, a disorder termed Complicated Grief (CG) affects about 7% of bereaved persons and about 20% of conjugally bereaved (Kersting et al., 2011). CG is characterized by persistent intense grief with ongoing separation distress (Prigerson et al., 2009; Shear et al., 2011). Bereaved individuals who do not have CG may still experience intermittent distress (e.g., sadness), but such Non-complicated Grief (Non-CG) reactions do not significantly impair their interpersonal or emotional functioning.

To clarify how bereavement influences immune system function more broadly, and to determine how CG might differ from Non-CG in its immunologic effects, we assessed leukocyte genome-wide transcriptional profiles in relationship to empirically-validated diagnostic criteria for CG (Prigerson et al., 1995a; Shear et al., 2011). Previous social genomics studies have linked other types of social adversity (e.g., isolation, stress, low SES) with a Conserved Transcriptional Response to Adversity (CTRA) characterized by downregulation of antiviral genes and upregulation of inflammation-related genes (Irwin and Cole, 2011). Similar effects were seen in caregiving spouses of brain cancer patients (Miller et al., 2008).

In the present preliminary study we sought to determine: 1) to what extent one of the most intense social adversities (i.e., bereavement) can modulate leucocyte gene expression, and 2) whether these dynamics are more pronounced for those reacting to bereavement with greater distress (i.e., with CG). Consistent with patterns of gene transcriptional responses to other major life adversities, we hypothesized that bereavement would show the CTRA pattern of increased expression of immune activation-related genes (CG more than Non-CG), and decreased expression of antiviral genes (CG more than Non-CG). Monocytes and dendritic cells are implicated as primary mediators of the CTRA (Cole et al., 2011). However, because of altered function of natural killer (NK) cells in bereavement (Gerra et al., 2003; Irwin et al., 1988) we hypothesized that NK cell-expressed genes might also be affected (CG more than Non-CG).

2. Methods and materials

2.1. Participants

We recruited 63 older adults (age 61–83) from the Los Angeles community through advertisement at senior centers and direct mailing to age-appropriate citizens. Interested participants were then categorized as two groups. Thirty-six participants had experienced the death of their spouse or partner on average in the past 2 years (mean: 23.56 months, SD 16.10), with 12 of the subjects meeting criteria for CG (Prigerson et al., 1995b). The other 27 participants were nonbereaved married/partnered control subjects who had not lost a first-degree relative or spouse within the prior 36 months. The UCLA Human Research Protection Program approved the study and all participants gave written informed consent after complete description of the study. As described in our previous report on genetic effects (Schultze-Florey et al., 2012), exclusion criteria included: (a) presence of a current major psychiatric disorder (e.g., Major Depressive Disorder, Post-Traumatic Stress Disorder, alcohol dependence) as assessed with the Structured Clinical Interview for DSM-IV (SCID-I; Spitzer et al., 1994); (b) psychotropic medication use initiated after the death event; (c) immunosuppressive medication (d) major medical illnesses (e.g., cancer); (e) current smokers.

2.2. Psychological Measures

All participants received the Perceived Stress Scale (PSS) (Cohen et al., 1983) ($\alpha = 0.81$), the UCLA Loneliness Scale (Russell, 1996) ($\alpha = 0.89$), and the revised Social Readjustment Rating Scale (SRRS-R) (Hobson and Delunas, 2001).

Bereaved participants were given the Inventory of Complicated Grief (ICG) (Prigerson et al., 1995b), to assess symptoms and behavior that define CG. Consistent with prior studies, the cut-off for CG was 30 (Shear et al., 2005) ($\alpha = 0.90$). The Impact of Events Scale (IES) (Horowitz et al., 1979) ($\alpha = 0.85$) and the Yearning in Situations of Loss (YSL) Scale (O'Connor et al., in press) ($\alpha = 0.94$) were also administered to all bereaved participants.

2.3. Gene expression profiling

Five million peripheral blood mononuclear cells (PBMCs) were isolated and total RNA was extracted (RNeasy; Qiagen, Valencia, CA), tested for suitable mass (Nanodrop ND1000; Thermo Scientific, Rockford, IL) and integrity (Bioanalyzer 2100; Agilent, Santa Clara, CA), and subjected to genome-wide transcriptional profiling using Illumina Human HT-12 v3 Expression BeadChips (Illumina Inc., San Diego, CA), following the manufacturer's standard protocol in the UCLA Southern California Genotyping Consortium Core Laboratory, described previously (Cole et al., 2010, 2007). Gene expression values were quantile normalized (Bolstad et al., 2003) and transformed to $\log_2$ for genome-wide general linear model analyses that controlled for age, sex, race (Caucasian vs. non-Caucasian), education (years of schooling), current employment status, BMI, and alcohol consumption. Differentially expressed genes were identified by adjusted parameter estimates exceeding a 1.25-fold difference between groups. No statistical testing was applied at the level of individual genes because this study had no single-gene hypotheses and this study was not designed to detect statistically significant associations between single gene transcripts and

bereavement status. Differentially expressed genes were identified only to serve as intermediate inputs into higher-order bioinformatics analyses that maintain their own false positive statistical control in analyses of Gene Ontology (GO) functional characteristics, transcription control pathways, and originating cell types, as previously detailed (Fredrickson et al., 2013).

Functional characteristics of differentially expressed genes were identified by GOstat (http://gostat.wehi. edu.au/) Gene Ontology (GO) analysis with False Discovery Rate-adjusted p-values (Beissbarth and Speed, 2004). To assess *a priori* hypotheses regarding specific transcription control pathways that might contribute to observed effects, we used TELiS (http://www.telis.ucla.edu/) bioinformatic analysis (Cole et al., 2005) of transcription factor-binding motifs in promoters of differentially expressed genes (NF-κB motifs assessed by TRANSFAC matrix V$CREL_01, Type I interferon response factors (IRFs) V$ISRE_01 and IRF1_01, cAMP response element-binding protein (CREB) V$CREB_02, and GATA-binding protein 1 (GATA1) V$GATA1_04). To identify specific leukocyte subsets predominately mediating the observed differences in gene expression, Transcript Origin Analysis (TOA) (Cole et al., 2011) was carried out. TELiS and TOA analyses were carried out in an *a priori* hypothesis testing format (i.e., only specifically hypothesized effects were subject to statistical testing and reporting in primary results; comprehensive exploratory/discovery findings are reported separately as such in supplemental data files).

2.4. Statistical Analysis

SPSS 19 (SPSS, Chicago, IL, USA) was used for statistical analyses. ANOVA or chi-square analyses and t-tests were used for planned *post hoc* group comparisons. To analyze relationships while controlling for demographic, medical, or biobehavioral confounds regression analyses were used. Significance was defined as p-values <0.05.

3. Results

3.1 Demographic and psychological characteristics

Demographic characteristics of the three groups are shown in Table 1. ANOVA analysis and *post hoc* t-test showed that the Non-CG group had been married fewer years than the Control group (t = 2.35, *p* = 0.02). Importantly, unexpected deaths (defined in the present study as knowing that the spouse would die for less than one week) were not significantly different between CG and Non-CG[1].

Psychological differences between groups are displayed in Supplemental Figure S1. Stressful life events (including death of a spouse) were only different between the Control and CG groups in a *post hoc* t-test (t = 2.55, p < .02) and not between the Non-CG and CG groups. Perceived stress did not differ between the bereaved groups, but CG had more intrusive thoughts and avoidance (e.g., IES).

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[1]Debate in the literature exists as to whether unexpectedness causes greater bereavement distress and whether it is predictive of CG (for a review, see Currier et al., 2006).

3.2 Gene expression profiling and bioinformatics analyses

Because CG and Non-CG are behaviorally distinct, and show different endocrine profiles (O'Connor et al., 2012), our primary analyses contrasted each of those groups with the common reference group of Nonbereaved individuals (rather than pooling CG and Non-CG for comparison to Nonbereaved controls).

3.2.1. Non-CG vs. Nonbereaved Control—The list of genes upregulated by 25% or more in Non-CG vs. Nonbereaved (Supplemental Table S1a) included multiple transcripts involved in Type I interferon responses (CXCL10, IFI44, IFI44L, IFIT1, IFIT2, IFIT3, MX1, OAS3, OASL). Interferons fight infectious disease, named for their ability to "interfere" with viruses. For a complete list of up- and downregulated gene names see Table S1a.

GO analyses characterized the function of these upregulated genes as being involved in immune activation (inflammatory response, $p < 0.0001$; humoral immune response, $p < 0.0001$; defense response to bacterium, $p < 0.0001$; defense response to fungus, $p = 0.0001$; response to virus, $p = 0.0002$; innate immune response, $p = 0.0004$; complement activation, $p = 0.003$; cytokine activity, $p = 0.004$; adaptive immune response, $p = 0.01$; and B cell costimulation, $p = 0.02$). Downregulated genes were involved in general aspects of the immune response (immune response, $p = 0.01$; immune system process, $p = 0.003$) and B lymphocyte function (B cell activation $p < 0.0001$; B cell proliferation $p = 0.04$).

Using TELiS analysis (Cole et al., 2005) we tested the hypothesis that several specific transcription factors, previously implicated in gene expression alterations during social adversity (Cole et al., 2011, 2007), might play a role in the bereavement response. Results of this *a priori* hypothesis-based TELiS analysis are shown in Figure 1b, and suggest a relative upregulation of GATA1 (+ 31.91%, $p = 0.004$) and IRFs (+56.11%, $p = 0.01$), a downregulation of CREB (− 47.98%, $p = 0.005$), and no significant difference in activity of NF-κB (+16.54%, $p = 0.35$). Ancillary results from discovery-based TELiS analyses (i.e., not *a priori* hypothesis-based), can be found in the Supplemental Table S2a.

We also conducted TOA analyses to identify cell types that might contribute to the general up- and downregulation profile of genes within PBMCs. Results are shown in Supplemental Figure S2a. Genes upregulated in Non-CG compared to Controls were identified as originating predominately from monocytes and NK cells, whereas downregulated genes were identified predominately with B-cells.

3.2.2. CG vs. Nonbereaved Control—In contrast to the relative upregulation of Type I interferon-related transcripts observed above in Non-CG, CG participants showed downregulation of multiple interferon-related transcripts compared to Nonbereaved controls (Table S1b: CXCL10, IFI44, IFI44L, IFIT1, IFIT2, IFIT3, MX1, OAS3, OASL). Figure 1a portrays the divergence in interferon-related gene expression across CG and Non-CG bereaved groups. For a complete list of regulated genes see Table S1b. GO analysis again indicated increased expression of immune activation genes in general (defense response to bacterium, $p < 0.0001$; response to xenobiotic stimulus, $p < 0.0001$; defense response to fungus, $p < 0.0001$; response to virus, $p = 0.04$; innate immune response, $p = 0.04$)

accompanied by a selective decrease in B lymphocyte-related transcripts (B cell activation, *p* = 0.04). Analyses also indicated increased activity of genes involved in dopamine receptor binding (*p* = 0.04) as well as regulation of circadian sleep/wake cycle (*p* = 0.01).

Results of the *a priori* hypothesis-based TELiS analysis is shown in Figure 1b, indicating that the CG group has upregulated activity of CREB (+123.12%, *p* = 0.002), downregulated activity of GATA1 (−27.91%, *p* = 0.0001) and IRFs (−70.97%, *p* = 0.01), and no significant difference in activity of NF-κB (−29.86%, *p* = 0.096). Results from discovery-based (i.e., not *a priori* hypothesis based) TELiS analysis are in Table S2b.

TOA results (Figure S2b), like the Non-CG vs. Nonbereaved comparison, implicated monocytes and NK cells in CG-related transcriptional upregulation, and B-cells in CG-related downregulation of gene expression (Figure S2a, b).

3.2.3. CG vs. Non-CG—Consistent with the results shown in Figure 1, direct comparison of CG with Non-CG participants showed CG downregulation of multiple interferon-related transcripts (Table S1c: CXCL9, CXCL10, GBP1, GBP4, GBP5, IFI27, IFI30, IFI35, IFI44, IFI44L, IFIT1, IFIT2, IFIT3, IFITM3, IRF7, ISG15, MX1, OAS1, OAS2, OAS3, OASL). GO analysis confirmed reduced expression of genes involved in antiviral responses (*p* < 0.0001), and also linked downregulated genes to chemotaxis and chemokine activity, wound healing, and cell development and myeloid differentiation (all p < .0001).

Results of the *a priori* hypothesis-based TELiS indicated that the CG group showed downregulated activity of GATA1 (−23.74%, *p* = 0.002), IRFs (−76.2%, *p* = 0.013), and NF-κB (−27.34%, *p* = 0.048), and no significant difference in activity of CREB (+7.42%, *p* = 0.59). Results from discovery-based (i.e., not *a priori* hypothesis based) TELiS analysis are in Table S2c.

Consistent with the results shown in Figure S2a,b TOA implicated B cells and NK cells in CG-related transcriptional upregulation, and monocytes and plasmacytoid dendritic cells in CG-related downregulation of gene expression.

4. Discussion

These preliminary analyses indicate that bereavement is associated with systematic alterations in leukocyte gene expression profiles that may potentially contribute to the increased health risks associated with bereavement in previous studies. However, the specific nature of the grief response (i.e., CG vs. Non-CG) appears to differentially affect one key feature of transcriptional alteration involving innate antiviral responses. Both CG and Non-CG are associated with upregulated expression of genes involved in general immunologic activation and defense responses against diverse pathogen classes, accompanied by downregulation of genes involved specifically in B lymphocyte responses. Bioinformatic analyses implicate upregulated monocyte and NK cell function and B cell downregulation in both types of grief. However, CG and Non-CG differed markedly in their expression of Type I interferon-related transcripts, with Non-CG subjects showing substantial upregulation of such genes relative to nonbereaved controls and CG subjects showing substantial downregulation of the same genes. Paralleling that divergent expression

of antiviral genes in CG and Non-CG, promoter-based bioinformatic analyses also indicated differential patterns of transcription factor activation with Non-CG bereavement related to increased activity of GATA1 and IRFs and decreased activity of CREB. In contrast, CG was linked to the opposite profile for each factor and, additionally, had a trend towards reduced NF-κB activity (which reached statistical significance in direct comparison to Non-CG bereavement). These results suggest that bereavement may have both general effects on immune function (i.e., general immunologic activation accompanied by selectively decreased B cell function) and specific effects that depend on the magnitude of bereavement-related distress (i.e., upregulated innate antiviral responses in Non-CG and downregulated antiviral and pro-inflammatory responses in CG). Such findings provide a molecular framework for understanding the health effects of bereavement, as well as new insights into the particular gene modules that are most sensitive to the individual's psychological response to loss.

Interestingly, TOA analyses implicated the same set of cells in structuring both CG and Non-CG transcriptional responses (i.e., upregulated monocyte- and NK cell-expressed genes and downregulated B cell genes). However, TELiS analyses of transcription factor activity suggested divergent patterns of activity for GATA1, IRFs, and CREB factors in CG vs. Non-CG. One parsimonious mechanistic explanation for these findings would be that bereavement generally affects a fixed set of cell types, but that a subset of transcription factors are differentially activated by CG and mediate different patterns of transcriptional response (e.g., differential interferon responses). To more directly test this hypothesis, future studies should directly assess transcription factor activity within isolated populations of the candidate cell types identified here.

The decreased expression of antiviral response genes among individuals with CG has clinical implications for their resistance to infectious disease. Research has shown that older widows have significantly lower influenza vaccine response, compared to nonbereaved controls (Phillips et al., 2006), although they did not assess CG status in their widow group. In addition, the odds of dying of infections, sepsis, influenza or pneumonia are increased for men and women (Elwert and Christakis, 2008), although no data on CG was provided in this study either. The present results suggest a mechanism bridging the CG bereavement response to increased mortality from infections, namely, the downregulation of Type I interferon gene transcription. This may help to explain the well-established widowhood effect on morbidity/mortality, and improve the ability to predict mortality risk by assessing for CG to identify widow(er)s at the highest risk.

It remains unclear from the present data whether the observed difference in Type I interferon-related gene expression constitute 1) a direct consequence of bereavement-related neural or endocrine alterations (Irwin and Cole, 2011), or 2) an indirect consequence of the reactivation of latent viral infections in bereaved individuals (Glaser and Kiecolt-Glaser, 2005; Kiecolt-Glaser et al., 2003). It is conceivable that both dynamics occur. Neural- or endocrine-mediated viral reactivation may occur in both CG and Non-CG (Glaser and Kiecolt-Glaser, 2005), but CG may also induce a more profound inhibition of the innate antiviral response (as previously observed in animal models of social stress (Sloan et al., 2007). In contrast, antiviral responses may emerge relatively unopposed by distress-related

neural/endocrine inhibition in Non-CG. Acute increases in catecholamines, which can suppress antiviral response, are found during the initial months of bereavement (Buckley et al., 2012), and those with CG and with the highest levels of epinephrine are least likely to be responsive to psychotherapy (O'Connor et al., 2012).

Limitations of this study include the cross-sectional design and the relatively small sample size. The limited sample size and attending low statistical power may explain this study's failure to find statistically significant differences in indicated NF-κB activity between bereaved groups and the non-bereaved controls. Therefore the results of this pilot study need to be replicated. However, there is a good record of hypothesis-driven bioinformatic results replicating, even when derived from small samples. Also, in this study we do not focus at the level of individual genes, but rather investigate the regulatory upstream level of gene expression, increasing replicability. Associations between bereavement status and individual transcripts (Table S1) should not be interpreted as individually significant or replicable; only higher-order inferences about general categories of gene function, transcription control pathways, and cellular origins should be regarded as statistically reliable. Future studies should also include direct measures of transcription factor activity and functional assays of immune cell biology (e.g., induction of the Type I interferon "antiviral state") and pathogen biology (e.g., viral reactivation). Moreover, sex differences in gene expression are an important topic for future research. Studies that link leukocyte gene regulation and biological function to clinical disease endpoints (e.g., incidence of infectious diseases or other illnesses) would also help define the clinical translational significance of the present findings.

This study identified bereavement as significant modulator of immune cell gene expression, and linked individual psychological responses (i.e., CG vs. Non-CG) to divergent patterns of transcription factor activation and expression of genes involved in innate antiviral responses. This striking suppression in anti-viral gene transcription in CG is supported by the same type of downregulation seen in other kinds of social adversity. The downregulation of these genes is consistent with the increased vulnerability to infectious diseases in bereaved older adults, and may suggest that those diagnosed with CG should be monitored more closely for health effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.
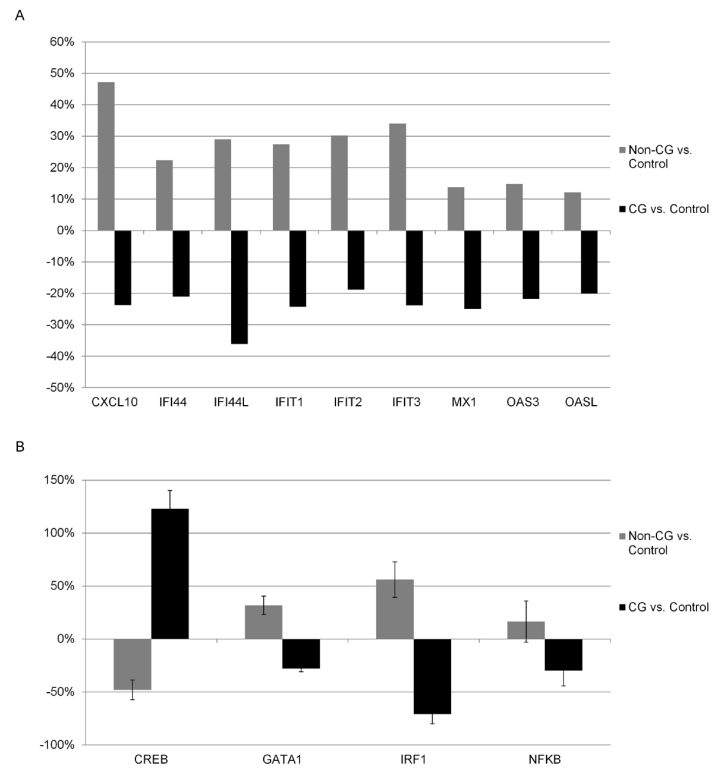
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Research Highlight

Different psychological responses to bereavement are associated with divergent gene expression patterns in immune cells.

Figure 1a,b.
Differences in gene expression in non-complicated grief (Non-CG) vs. Control and complicated grief (CG) vs. Control comparisons in Type I interferon-related genes (A) and in transcription control pathways activation (B).

Table 1

Demographic Characteristics

	Control (N=27)	Non-CG (N=24)	CG (N=12)	Control vs. Non-CG		Control vs. CG		Non-CG vs. CG	
	Mean/N (SD/%)	Mean/N (SD/%)	Mean/N (SD/%)	t (df) / X^2	P-value	t (df) / X^2	P-value	t (df) / X^2	P-value
Age	72.3 (4.2)	72.9 (5.9)	72.5 (5.3)	−0.05 (49)	0.71	−0.11 (37)	0.92	0.19 (34)	0.85
Gender (Female)	14 (52%)	13 (54%)	10 (83%)	0.03	0.87	3.48	0.06	2.95	0.09
Ethnicity (non-Caucasian)	4 (15%)	6 (25%)	5 (42%)	0.84	0.36	3.37	0.07	1.05	0.31
Employment (Retired)	14 (52%)	16 (67%)	8 (67%)	1.15	0.28	0.74	0.39	0.00	1.00
Education (Post graduate)	13 (48%)	9 (38%)	5 (42%)	0.59	0.44	0.14	0.71	0.06	0.81
Years married/partnered	42.1 (10.3)	32.5 (18.2)	41.3 (14.3)	2.26 (33.5)	0.31	0.21 (37)	0.83	−1.45 (33)	0.16
Body mass index	27.1 (5.5)	27.3 (5.7)	26.0 (4.9)	−0.17 (48)	0.87	0.59 (37)	0.56	0.70 (33)	0.49
Months from death	–	24.4 (17.2)	21.8 (14.2)	–	–	–	–	0.45 (34)	0.66
Unexpectedness of the death	–	13 (54%)	5 (42%)	–	–	–	–	0.50	0.48

Continuous variables: mean (±SD), t value (independent samples t-test); categorical variables: n (%), X^2 value (Chi Square Test). CG = complicated grief.