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Neutrophil function in young and old caregivers

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Abstract

Objective: The present study examined the effects of caregiving stress and ageing on neutrophil function in young and older individuals. **Design:** As a model of caregiving, young parents (aged 38.3 ± 4.78) of children with developmental disabilities were recruited and compared to older caregivers (aged 70 ± 6.03), full time carers of a spouse with dementia. Age- and sex-matched controls were also assessed. **Methods:** Participants completed a questionnaire pack assessing health behaviours, psychosocial status, and caregiving characteristics, and provided a blood sample for assay of neutrophil function (phagocytosis of *E.coli* and generation of reactive oxygen species (ROS) to *E.coli*). **Results:** Despite scoring poorly on the majority of psychological and caregiving variables, neutrophil function in caregivers was comparable to that in controls and was unexpectedly higher in older adults when compared to younger adults overall. However, those caregivers who reported higher psychological morbidity (depression, perceived stress, poor sleep quality), and more burdensome caregiving showed some evidence of poorer neutrophil phagocytic function. **Conclusion:** To our knowledge, this is the first study to examine the effect of caregiving stress on neutrophil function in young and older participants simultaneously. Overall, neutrophil function was preserved in caregivers with neutrophil phagocytosis compromised only in those with the highest levels of distress. This suggests that, in future studies, more attention should be paid to individual differences among caregivers rather than caregiving status *per se*.

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1. Introduction

There is now substantial evidence that chronic psychological stress can impair immunity ((Vedhara & Irwin, 2005); for example, the experience of a large number of negative life events has been associated with reduced antibody responses to vaccination (Phillips, Burns, Carroll, Ring, & Drayson, 2005) and lower levels of secretory immunoglobulin A (sIgA) (Phillips, Carroll, Evans, et al., 2006). Specific chronic stress exposures, such as bereavement, have been related to poorer Natural Killer (NK) cell function (Irwin, Daniels, Smith, Bloom, & Weiner, 1987), reduced neutrophil superoxide generation (Khanfer, Lord, & Phillips, 2011), and a reduced response to the influenza vaccination (Phillips, Carroll, Bums, et al., 2006).

Caregiving for a sick or disabled relative has frequently been employed as a model for examining the effects of chronic stress on immune function (Vedhara & Irwin, 2005). Caregiving has been chosen as a model of chronic stress due to the increasing number of caregivers and the large amount of evidence for an influence on psychological and physical well being and quality of life among caregivers (Pinquart & Sorensen, 2003). In this group, dementia caregivers present a particularly vulnerable sub-group, affected both by ageing (the majority are older than 65 years), and the chronic stress of dealing with their partner's progressive deterioration (Potkin, 2002). Studies have assessed the impact of such caregiving on the antibody response to vaccination, rate of wound healing, and aspects of cellular immunity (Gallagher et al., 2008; Vedhara et al., 1999). However, the majority of these studies have been conducted in older adults. This raises the issue as to whether effects of caregiving stress are only observed in the context of immune ageing. For

example, sIgA secretion rates were found to be lower in caregivers relative to controls, but only for the oldest of three age cohorts, mean age 63 (Gallagher et al., 2008). Further, Vedhara et al. (2002) found that young caregivers (mean age 46) of patients with multiple sclerosis had similar antibody responses to influenza vaccination as control participants. In contrast, more recently, Gallagher et al. (2009a, b) found that parents caregiving for children with developmental disabilities (mean age 42.8) showed lower antibody responses to influenza and pneumococcal vaccinations relative to age- and sex-matched control parents. In addition, within the parental caregiving group, parents reporting more child conduct problems mounted a poorer antibody response (Gallagher, Phillips, Drayson, & Carroll, 2009a, 2009b). This suggests that the behavioural characteristics of the care-recipients may be a key determinant of whether or not immunity is compromised, and that an ageing immune system is not a pre-requisite for a poor response to vaccination or other immune responses in caregivers. Nevertheless, older caregivers tended to have a poorer antibody response to one of the influenza vaccine strains (Gallagher et al., 2009a), suggesting that we cannot dismiss the hypothesis that chronic stress and immune system ageing may have additive effects.

Neutrophils are an important part of the innate immunity, being the first line of defence against rapidly dividing pathogens such as bacteria and fungi. On the other hand, related to the way they exert their function (e.g. by releasing reactive oxygen species from their granules), neutrophils are also important contributors to the tissue damage during inflammatory reactions, as well as different autoimmune diseases such as atherosclerosis and diabetes (Mocsai, 2013; Nathan, 2006). This would suggest the need for carefully controlled

mechanisms that will, under physiological conditions, enable neutrophils to protect the organism from pathogens, but at the same time prevent causing further damage themselves during the exacerbated inflammatory reactions. This is partly achieved through the action of hypothalamic-pituitary-adrenal (HPA) axis, known to be activated during the stress response. Cortisol, one of the end products of this activation is known to be immunosuppressive (Charmandari, Tsigos, & Chrousos, 2005), decreasing adhesion and increasing mobility of neutrophils (Zak-Nejmark et al., 1998). On the other hand, dehydroepiandrosterone (DHEA), another steroid hormone produced by adrenal gland, and its sulphated form (DHEAS) is accepted as being immune-enhancing and with anti-glucocorticoid effects (Hazeldine, Arlt, & Lord, 2010) and an ability to increase neutrophil function *in vitro* (Radford et al., 2010). However, the *in vivo* effects of these hormones are likely to be more complex. For example, previous studies showing a higher cortisol:DHEAS ratio and reduced neutrophil function among sub-groups of older adults (Khanfer et al., 2011), did not find significant associations between this ratio and neutrophil function.

The effects of ageing per se on the immune system, termed immunosenescence, are well established (Gruver, Hudson, & Sennpowski, 2007). For neutrophils, many aspects of their bactericidal function, including phagocytosis and Reactive Oxygen Species (ROS) generation have been shown to be affected by increasing age (Shaw, Joshi, Greenwood, Panda, & Lord, 2010). Interestingly decreased neutrophil ROS production has been reported in older bereaved adults when compared to their age-matched controls (Khanfer et al., 2011) and in older hip fracture patients who reported depressive

symptomatology but not in those who did not display depressive symptoms (Duggal, Upton, Phillips, Hampson, & Lord, 2013). Overall, these data suggest that the way ageing affects neutrophil functioning depends, at least in part, on the presence or absence of, as well as the type of chronic stressor.

To date, the effects of caregiving stress have not been examined in younger and older participants in the same study with the exception of a previous study from our group showing that bereavement in older adults reduced neutrophil superoxide production relative to age- and sex-matched non-bereaved controls but had no effect in younger adults (Vitlic, Khanfer, Lord, Carroll, & Phillips, 2014). Further, this study is novel in considering neutrophil function in the context of chronic stress, which receives scant attention in the literature despite its relevance to health and disease.

The present study aimed to shed light on the associations between stress, ageing, and immunity through studying four categories of participant: parents of children with developmental disabilities; age- and sex-matched parents of typically developing children; older spousal dementia caregivers; and age- and sex-matched healthy older adults with immunosenescence but without caregiving stress. Innate immunity, specifically neutrophil function, was assessed. It was hypothesised that ageing and chronic stress of caregiving would act in synergy and relate to the poorest immune function in older dementia caregivers. It was also thought that the caregiving stress alone, present in younger parental caregivers would negatively influence immunity, while healthy older controls affected only by ageing, and younger controls without caregiving stress would have the most robust neutrophil function.

Finally, by measuring psychological and caregiving-specific characteristics in

greater detail than previous studies, the present study also aimed to elucidate the particular psychosocial variables that may be responsible for any caregiving stress effect on neutrophil function.

2. Methods

2.1. Participants

57 young parental caregivers and 34 matched parental controls, and 40 older caregivers and 42 matched older controls were recruited for the study between October 2010 and August 2013. Young caregivers had at least one child with a developmental disability (for definition, see Eunice Kennedy Shriver National Institute of Child Health and Human Development); older caregivers were aged 60+ years and full time spousal dementia carers. Parental caregivers were from different areas of United Kingdom, mainly recruited at national syndrome support group conferences; their children were aged between 3 and 18 years and living at home during the school term. Through this inclusion criterion we aimed to avoid the influence of the recent diagnostic process on parents' caregiving experience (Hastings, Daley, Burns, & Beck, 2006). The caregiver group consisted of 27 parents of a child with Smith-Magenis syndrome (47%), 22 parents with at least one child with Fragile X syndrome (39%), 7 parents of a child with Cornelia de Lange syndrome (12%), and 1 parent of a child with autism spectrum disorder (2%). Older caregivers were recruited from NHS trusts throughout England (namely, Birmingham and Solihull Mental Health NHS Foundation Trust, Lincolnshire NHS Trust, North Staffordshire NHS Trust and Bradford District Care Trust). All participants provided written informed consent

and the study had ethical approval from the local NHS research ethics committees.

Recruitment and withdrawal data are presented in Figure 1. Exclusion criteria were: being currently acutely ill or pregnant, taking medication known to alter immune function and/or steroid synthesis and metabolism or an ongoing chronic immune-related disease, for control group being a full-time carer, and for older control group not living with spouse/partner. Control parents of typically developing children were recruited via local advertisements. Older healthy controls were recruited through the Birmingham 1000 Elders group. The participation in the study was voluntary and no compensation was offered to the participants with the exclusion of travel expenses to those that attended testing session at the University. The aim was to recruit 30-35 participants in the control groups to meet the criteria of medium effect size (f = 0.29), and the attempt was made to match the groups as closely as possible on socio-demographic characteristics (Table 1). Due to older caregivers being more likely to be female, and most of older female controls who expressed their interest to participate were not married/living with a partner and therefore excluded from the study, there is a trend towards significant difference in gender between the groups in the older cohort. For that reason, where appropriate, the analyses were re-run controlling for age and gender.

[Insert Figure 1 about here]

2.2. Study design and procedure

This was a cross-sectional single session study with participants completing a questionnaire pack, and providing a fasted blood sample, taken between 9-11

a.m. by trained phlebotomists, in order to determine neutrophil function.

Participants were given an option to complete questionnaire packs and return them during the testing session, or to do it in their homes and post them back, in which case they were provided with Freepost envelopes.

2.3. Questionnaires

2.3.1. Health behaviours

A questionnaire adapted from the Whitehall II study (Marmot et al., 1991) was used to assess health behaviours. This questionnaire has been consistently used in previous stress and immunity research (Phillips et al., 2005). Participants were asked about their sleep duration, smoking status, how much alcohol they drank, levels of exercise, and consumption of various food items, and simple categorical scoring system was used in all cases. Details of this questionnaire have been given previously (Phillips et al., 2005; Vitlic, Phillips, et al., 2014).

Subjective sleep disturbances were reported by participants through the Pittsburgh Sleep Quality Index (Buysse, Reynolds, Monk, Berman, & Kupfer, 1989), where a higher overall score indicates poorer sleep quality. Adequate internal consistency was demonstrated in the present sample, $\alpha = .68$.

2.3.2. Depression and anxiety

The Hospital Anxiety and Depression Scale (HADS) (Zigmond & Snaith, 1983) was used to measure current anxiety and depressive symptomatology in participants. The HADS has acceptable internal consistency (.80 to .93 for anxiety and .81 to .90 for depression) (White & Hastings, 2004), and in the present study,.86 and .81, respectively.

2.3.3. Perceived stress

This 14-item stress scale was used to assess how unpredictable and overwhelming daily life was during the previous month (Cohen, Kamarck, & Mermelstein, 1983). The scale shows good internal reliability (Cronbach's α = .75); in the present study.86.

2.3.4. Caregiver burden

In order to assess the stress caused by a caregiving role, a short form version of the Zarit Burden Interview (BI) was used (Bédard et al., 2001). This version has a Cronbach's alpha of .88. In the younger group the questions were amended replacing 'your relative' with 'your child'. Internal consistency in the current sample was .91.

2.3.5. Time spent caregiving

The amount of time spent caregiving was assessed using a modified version of the Caregiver Activity Survey (Davis et al., 1997). Hours for each caring role (e.g., dressing, eating, transport) were summed together to yield a total daily score for time spent caregiving.

2.3.6. Social Support

The 12-item Support Functions Scale (Dunst, Trivette, & Deal, 1988) was used to assess types of support available to participants. This scale has been shown to be reliable (Cronbach's α = .86) and has been used previously in developmental disability research (White & Hastings, 2004). In the present study the internal consistency was .89. The type of questions allowed administration of this questionnaire in younger adults and older caregivers only. In the older group, perceived social support was measured using The Medical

Outcomes Study Social Support Survey (Sherbourne & Stewart, 1991). High internal consistency of .91 has been reported previously (Sherbourne & Stewart, 1991). The scale has been used previously in chronic stress research (Phillips et al., 2005; Phillips, Carroll, Bums, et al., 2006). Internal consistency in the current sample was .97.

2.3.7. Care-recipient problem behaviour

The Strengths and Difficulties Questionnaire (Goodman, 1997) was used to screen for child behaviour difficulties. The scale has been shown to be reliable (Cronbach's α = .76) and effective at identifying behavioural problems in children (Goodman, 1997). In the current sample the internal consistency for problem behaviour was .86. In the older adults, the Pearlin Problematic Behaviour subscale was used which is a part of longer model developed to assess the stress of the caregivers of people with dementia (Pearlin, Mullan, Semple, & Skaff, 1990). The subscale focuses on frequency certain behaviours occur in a patient and demand caregiver's attention. Participants were asked to mark the number of days they had to deal with certain situations weekly. Internal consistency in the current sample was .78.

2.4. Blood sampling and assays

Venous preprandial blood samples were collected in the morning (9-11a.m.), into one heparin containing tube (BD Vacutainer). This was used to isolate neutrophils for assessment of function the same day.

2.4.1. Neutrophil functional assays

Phagocytosis of *E.coli* was measured using a fluorescence-based kit (Phagotest, Orpegen Pharma GmvH, Heilderberg, Germany) following the

manufacturer's protocol. The assays were analysed using a three-laser Dako Cyan High Performance flow cytometer (Dako, Carpinteria, California), with Summit v 4.3 software. Neutrophils were distinguished from other leukocytes by gating on forward scatter and side scatter and any remaining bacterial aggregates were excluded using red fluorescence staining to identify cells having diploid DNA content. 10-15,000 leucocytes per sample were collected and the phagocytosis was presented as phagocytic index (PI), the amount of bacteria ingested per cell (calculated as mean fluorescence intensity) multiplied by the percentage of neutrophils that had phagocytosed *E.coli*.

Neutrophil oxidative burst in response to *E.coli* was measured using a commercial kit (Phagotest, Orpegen Pharma GmbH), the assays were performed according to the manufacturer's protocol. ROS generation was evaluated using flow cytometry, with mean fluorescent intensity used as a measure of the oxidative burst activity of neutrophils. Absolute numbers of neutrophils data were also available but did not differ between the groups, so analyses are not presented below.

2.5. Statistical analyses

Comparisons between the caregivers and control groups on socio-demographics and questionnaire scores were conducted by ANOVA and chi-square. Tests of the main effects of age and caregiving status as well as caregiving * age interaction effects were examined using 2 x 2 ANOVA; with effect sizes reported as η^2. Significantly different demographic or health behaviour variables between the groups were controlled for as potential confounding variables in further ANCOVAs. Using the full sample size (young and old, caregivers and controls) in the first instance, then split into the

separate age cohorts, potential effects on neutrophil function were further analysed in hierarchical regression models in relation to depression and anxiety symptomatology, burden index, perceived stress and sleep quality, while controlling for age cohort (only in the full sample analysis) and caregiving status. Correlations were used within the caregiver participants to determine whether or not psychosocial and caregiving variables were associated with neutrophil function. Where they were, hierarchical linear regression was conducted to further examine this association with age entered at step 1 and any significantly associated caregiving/psychosocial variable individually at step 2.

3. Results

3.1. Demographic characteristics and health behaviours

The demographic and health behaviour variables for each group are summarized in Table 1. In the younger sample, caregiver and control parents did not differ in their demographic characteristics. In terms of family size, 38 (67%) caregivers reported having only one child, 11 (19%) had two, 6 (11%) had three, and 2 (3%) had four children. The respective numbers in the control group were 14 (41%), 17 (50%), and 3 (9%). For health behaviours caregivers were more likely to drink alcohol daily (p=.005). In the older group, caregivers and controls were comparable on all of the demographic and health behaviour variables except for exercise scores where controls were more active than caregivers (p = .04).

[Insert Table 1 about here]

3.2. Caregiving and psychosocial characteristics of each group

Caregiving parents reported spending more time caring for their child than control parents (Table 2). Other psychosocial characteristics were significantly different between the groups and in the expected direction. Table 2 shows that depression, anxiety, perceived stress scores, sleep quality, and general social support were significantly worse in the older caregiver group compared to older controls.

[Insert Table 2 about here]

Caregiving burden reported by older caregivers was in the high range, according to the previously reported cut off of 17 (Bedard et al., 2001). Problematic behaviour on the Pearlin scale was higher than previously reported for samples of caregivers (Roepke et al., 2008). Interestingly, younger caregivers reported higher depression and anxiety symptoms, perceived stress and caregiving burden than the older caregivers (Table 2).

3.3. Neutrophil function across all groups

For neutrophil phagocytic ability, there was a main effect of age, $F(1,167) = 49.81$, $p < .001$, $\eta^2 = .230$, such that older adults had a higher PI , but no main effect of caregiving, $F(1,167) = 3.62$, $p = .06$, $\eta^2 = .021$, or caregiving * age interaction effect, $F(1,167) = .53$, $p = .47$, $\eta^2 = .003$. These effects are shown in Table 2 and Figure 2A. Repeated analyses with adjustment for alcohol intake and exercise level revealed the same significant main effect of age, $p < .001$.

For neutrophil superoxide production, there was a main effect of age, $F(1,164) = 12.23$, $p = .001$, $\eta^2 = .069$, and caregiving, $F(1,164) = 4.98$, $p = .03$, $\eta^2 = .029$, such that older participants and caregivers had significantly higher

superoxide production. However, there was no caregiving * age interaction effect, $F(1,164) = 0.18$, $p = .67$, $\eta^2 = .001$, see Table 2 and Figure 2B. Repeated analyses with adjustment for covariates (alcohol intake and exercise level) revealed only a main effect of age, $p = .001$; the caregiving main effect was no longer significant ($p = .13$). Correlations between all predictor variables, covariates and outcome variables are shown in Table 3.

[Insert Figure 2 and Table 3 about here]

3.4. Sensitivity analyses within the whole sample

In order to determine the importance of the severity of different psychological factors in respect to neutrophil parameters overall, rather than caregiving status *per se*, a series of hierarchical regressions were conducted. Models predicting immune parameters from perceived stress, depression and anxiety symptoms, burden levels and sleep quality scores were run, with adjustment for age cohort and caregiving status, as appropriate. It was observed that higher anxiety scores predicted lower neutrophil phagocytosis, $\beta = -.16$, $p = .039$, $\Delta R^2 = .019$, and poor sleep quality predicted lower neutrophil ROS production, $\beta = -.19$, $p = .022$, $\Delta R^2 = .033$. However, adjustment for multiple comparisons (α/N comparisons) showed that these associations would need to be significant at the 0.001 level to be truly reliable. When these models were rerun additionally entering the interaction term between caregiver status and each psychological variable in turn, there were no significant interactions between caregiver status and these variables.

3.5. Sensitivity analyses by age cohort

In the younger group only, depression, β = .26, p = .044, ΔR^2 = .048, and perceived stress scores, β = .30, p = .016, ΔR^2 = .068, predicted higher neutrophil ROS production, after controlling for caregiving status. In the older group, a higher burden index predicted worse neutrophil phagocytic ability, β = -.33, p = .009, ΔR^2 = .174; and poorer sleep quality was related to lower ROS production, β = -.24, p = .004, ΔR^2 = .104.

3.6. Sensitivity analyses within the caregiver groups

Regression analyses of caregivers only, predicting neutrophil function from psychological variables with adjustment for age cohort showed that high anxiety, β = -.23, p = .024, ΔR^2 = .046, and caregiver burden, β = -.25, p = .011, ΔR^2 = .058, significantly predicted poor phagocytic ability among caregivers overall. For oxidative burst activity, poor sleep quality adjusted for age cohort significantly predicted poorer neutrophil ROS production, β = -.23, p = .033, ΔR^2 = .051.

Within the younger caregivers' group only, similar to the younger participants overall, perceived stress predicted higher neutrophil ROS production, β = .31, p = .027, ΔR^2 = .098. Further, similar to the older group overall, among older caregivers a higher burden index was a significant predictor of poorer neutrophil phagocytosis, β = -.42, p = .009, ΔR^2 = .174, and poor sleep quality related to lower neutrophil ROS production, β = -.46, p = .003, ΔR^2 = .214. However, again, adjustment for multiple comparisons showed that these associations would need to be significant at the 0.001 level to be truly reliable.

4. Discussion

In the present study, caregivers and controls differed, as expected, on the majority of psychosocial and caregiving variables. Despite this, the neutrophil function of caregivers was comparable to that of the controls. The significant effects observed were for age; older adults had higher phagocytosis and oxidative burst activity than the participants from the younger groups. This ageing effect was contrary to what might be expected, as previous research including our own mainly reports a decrease in neutrophil function due to immunosenescence (Shaw et al., 2010). However, it is consistent with the previously reported increase in oxidative burst activity associated with ageing in some studies (Cannizzo, Clement, Sahu, Follo, & Santambrogio, 2011). The present results could also be interpreted as a support for the hypothesis that the innate immune system dominates in older age, and that immunosenescence is more evident in adaptive immunity (Franceschi, Bonafe, & Valensin, 2000). Alternatively, a robustness of neutrophil function with ageing we are observing in the present older sample, in particular older caregivers, could indicate the resistance of this particular aspect of immune response to chronic psychological stress. This selective effect of chronic caregiving stress has been observed previously, for example the antibody response to some influenza vaccine strains is more susceptible to the effect of psychological stress than others (Gallagher et al., 2009a; Phillips et al., 2005; Vedhara et al., 1999). Another possible interpretation of the data is that we are observing a robustness of neutrophil function in the current older sample, which is accompanied by a similar psychological resilience, as the overall scores for perceived stress, depression and anxiety reported by the older adults overall

were lower than those of the younger sample overall (see Table 2). Such psychological robustness in individuals aged over 60 years compared to younger groups has previously been reported (Gooding, Hurst, Johnson, & Tarrier, 2012).

Surprisingly, caregiving stress did not seem to diminish neutrophil function, in fact, if anything, caregivers showed higher neutrophil oxidative burst and a trend towards higher neutrophil phagocytosis, which both disappeared after covariate adjustment, i.e. alcohol intake and exercise level. This might suggest that higher neutrophil function in caregivers is primarily driven by those reporting higher alcohol consumption and lower exercise levels, potentially indicative of poorer health behaviours overall. This might seem surprising, considering the initial hypothesis that chronic stress will decrease neutrophil function, but could perhaps be explained by the underlying inflammatory state in these individuals characterised by the higher baseline neutrophil function, and that such inflammation is, in part, driven by the poorer health behaviours adopted by these groups – increased alcohol intake and reduced physical exercise. It is difficult to draw firm conclusions here, given that, on the whole, alcohol consumption and exercise scores did not significantly predict neutrophil function, also there are currently no agreed cut-offs for determining healthy versus unhealthy neutrophil function; previous studies define this by comparing stressed groups with control healthy participants and examining the correlates of impaired neutrophil function, i.e. increased infection rates, reduced physical function (Butcher et al., 2003; Duggal et al., 2013). Alternatively, it is possible that this aspect of innate immunity, unlike adaptive immune function such as the response to vaccination (Gallagher et al., 2009a, 2009b), is not diminished

by the chronic stress of caregiving. It is also possible that the general immune integrity observed in the current caregiving sample is due to the method of assessing immunity. Previous studies showing caregiving effects have used *in vivo* challenge of the immune response, e.g., vaccination (Gallagher et al., 2009b), and wound healing (Kiecolt-Glaser, Marucha, Malarkey, Mercado, & Glaser, 1995). These *in vivo* methods demand an integrated immune response. The present study, on the other hand, examined *in vitro* stimulation, without a systemic pathogenic challenge. The negative impact of caregiving may be more readily observed in models where the immune system is challenged with pathogens *in vivo*. Finally, it could be argued that the high scores in some of the psychosocial variables, such as sleep quality and perceived stress reported by the control group in both age cohorts could be responsible for the lack of differences in immune and endocrine parameters measured between the groups. However, significantly higher psychological morbidity in caregivers overall when compared to the controls suggest that this is unlikely to be the case.

Interestingly, sensitivity analysis indicated that those individuals with higher anxiety and caregiver burden had poorer neutrophil phagocytosis, which was characteristic of the older group. For neutrophil superoxide production, poorer sleep quality predicted poorer function. Although analyses within caregivers alone were conducted on somewhat decreased sample size (N=89), the effect sizes obtained were in the range of medium to large for multiple regression (f^2= 0.15 - 0.43), providing the power of > 0.90, suggesting some impact of psychological factors on immune parameters in the present study, although it should be noted that these associations were attenuated when adjusted for

multiple comparisons, so should be replicated in future research. Further, there were no significant interactions between psychological factors and caregiving status in predicting neutrophil function, suggesting that negative psychological characteristics can influence neutrophil function regardless of caregiving status.

A negative association between chronic stress and neutrophil superoxide generation has been reported previously in the recently bereaved (Khanfer et al., 2011), who also showed high levels of depression and anxiety. Also, among older hip fracture patients, only those who reported high levels of depression showed poorer neutrophil function, though again this was restricted to superoxide generation (Duggal et al., 2013). Similarly, poor sleep has been associated with alterations in immunity in a number of studies (Besedovsky, Lange, & Born, 2012). This suggests that while we observed no overall detrimental effect of caregiving, individuals reporting higher psychological distress and suffering poorer sleep have poorer innate immune function. On the other hand, higher neutrophil ROS production was observed with higher perceived stress scores in the younger group. This increase, rather than the expected decrease in this aspect of neutrophil function could be a consequence of the ability of the younger participants' immune systems to meet the challenge of stress in the absence of immunosenescence. Support for this notion can be found in the study where young medical students demonstrated an increase in DNA repair in response to psychological stress, unlike older psychiatric patients who exhibited a decrease in this activity (Yang & Glaser, 2003).

The study has a number of limitations. First, sample size in each group can be regarded as small, but is of a similar magnitude to other caregiving studies (Gallagher et al., 2009a, 2009b; Vedhara et al., 1999; Vedhara et al., 2002).

Second, it could be argued that due to the high burden of the caregiving role, those worst affected by this type of chronic stress would be less likely to participate in the study, making the current sample biased towards those that are healthier and cope better. The attempt to minimise such bias was through organising home visits and accommodating the testing session to best suit individual caregivers' needs even when they had high caregiver burden. Third, the scores on the psychological measures suggested that our sample did have high caregiver stress and burden similar to previous caregiver studies where immune effects are seen. Fourth, it could be argued that years spent caregiving might relate to immunity, which we did not measure in the older sample. However, previous caregiving studies have shown this not to relate to the level of distress in caregivers (e.g., Kiecolt-Glaser et al., 1991). Fifth, it could be argued that a one-off sample is not very informative, however, in this vulnerable group it was not practical to increase the number of blood samples taken. Further, others have reported that neutrophils, despite their short lifespan, are relatively stable over time in terms of number (van Rood et al., 1991) and tests of neutrophil function on whole blood are deemed to be low in susceptibility to artefact due to the ability to obtain and test the cells in their resting state (Lemke & Ward, 1994). Finally, the number of tests might have increased the likelihood of a Type I error, however, the sample size used was substantially bigger than what is practise for this type of caregiving research, and we have also included effect sizes, power, (and corrections for multiple comparisons where there were no a priori hypotheses) for neutrophil function, rather than relying on p values alone.

In conclusion, the neutrophil function in caregivers compared to controls overall was preserved, with the main differences emerging between young and old

participants. Nevertheless, there was an indication that caregivers who reported higher perceived stress, anxiety and caregiving burden levels, as well as poorer sleep quality, demonstrated poorer neutrophil function (phagocytosis). Implications for future research are that studies should focus less on caregiving status in general, and more on individual differences among caregivers, specifically those with high levels of psychological morbidity.

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Table 1. Demographic characteristics and health behaviours of young and old, caregivers and controls

	Young			Older		
	Caregivers (N = 53)	Controls (N = 33)		Caregivers (N =40)	Controls (N =42)	
	N (%) / Mean (SD)		*p*	N (%) / Mean (SD)		*p*
Age (years)	38.3 (4.78)[a]	40.1 (5.44)	.10	69.3 (5.81)	72.4 (5.42)	.06
Age of child/spouse (years)	7.4 (3.70)	7.2 (4.54)	.85	72.3 (8.06)	73.1 (6.04)	.63
Gender (Female)	38 (69)	21 (62)	.55	26 (65)	19 (45)	.07
Marital status (Partnered)	47 (89)[a]	30 (88)	.95	40(100)	42(100)	-
Ethnicity (Caucasian)	50 (93)	27 (79)	.07	38 (97)	39 (93)	.33
Occupational status (non-manual)	40 (85)[a]	31 (94)	.22	22(63)	32 (80)	.10
In full time work	21 (47)[a]	19 (63)	.16	3 (8)	1 (4)	.53
Taking medications	5 (9)[a]	7 (21)	.14	33 (80)	29 (74)	.51
Alcohol intake (daily or more)	14 (26)	1 (3)	.005	10 (28)	12 (30)	.83

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Smokers	10 (19)[a]	2 (6)	.10	1 (3)	3 (7)	.36
Body Mass Index	26.4 (4.89)	24.5 (3.58)	.06	26.3 (3.08)	26.1 (4.37)	.82
Exercise score	5.5 (4.99)[a]	6.4 (5.44)	.45	3.5 (3.16)	5.2 (3.71)	.04
Fruit and vegetable consumption score	8.2 (2.83)[a]	8.8 (2.36)	.25	9.7 (2.46)	10.3 (2.85)	.31
Fat consumption score	11.1 (3.31)[a]	10.9 (3.72)	.76	9.5 (3.62)	10.2 (3.91)	.46

[a] $p < .05$ between younger caregivers and older caregivers

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Table 2. Caregiving and psychosocial characteristics of young and old, caregivers and controls

	Young			Older		
	Caregivers (N = 53)	Controls (N = 33)		Caregivers (N = 40)	Controls (N = 42)	
	Mean (SD)		*p*	Mean (SD)		*p*
Hours spent caregiving per day	4.8 (4.15)	2.9 (3.23)	.03	3.6 (3.28)	-	-
Poor sleep quality score	8.4 (3.13)	6.7 (3.15)	.02	8.1 (3.47)	6.2 (3.80)	.02
HADS anxiety score	10.4 (3.52)[a]	6.4 (2.85)	<.001	7.7 (4.96)	4.1 (3.93)	<.001
HADS depression score	8.6 (3.04)[a]	4.2 (3.67)	<.001	6.0 (3.94)	2.8 (2.61)	<.001
Perceived stress score	30.8 (5.56)[a]	23.5 (6.59)	<.001	24.0 (8.21)	16.4 (7.83)	<.001
Social support score (SFS)	31.9 (7.99)	38.6 (9.62)	.001	33.6 (11.18)	-	-
Caregiver burden score (BI)	26.3 (7.67)[a]	13.7 (6.69)	<.001	21.0 (8.62)	-	-
Child behaviour problems (SDQ)	18.8 (4.64)	7.2 (3.99)	<.001	-	-	-
Pearlin problematic behaviour	-	-	-	22.9 (5.89)	-	-
Social support score (MOS)	-	-	-	63.1 (18.66)	86.0 (11.34)	<.001
Phagocytic Index	218.6 (62.26)	191.9 (61.74)	.05	283.1 (64.48)	271.1 (74.88)	.44

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Superoxide Production (MFI)	61.3 (33.13)	48.9 (20.56)	.06	75.6 (31.15)	67.2 (30.24)	.22

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Table 3: Correlations between independent and dependent variables and covariates

	Age	Phagocytic Index	Superoxide production	Anxiety	Depression	Sleep quality	Perceived Stress	Burden Index	Exercise score	Alcohol intake (≥daily/less = 1/0)
Caregiver (yes/no = 1/0)	-.26**	.07	.14	.44**	.49**	.30**	.47**	.48**	-.06	.13
Age		.47**	.28**	-.35**	-.33**	-.13	-.43**	-.08	-.13	.10
Phagocytic Index			.24**	-.22**	-.15	-.02	-.21**	-.07	-.06	.14
Superoxide production				-.07	.04	-.13	.01	.17	-.10	.18*
Anxiety					.74**	.47**	.73**	.50**	-.05	.03
Depression						.47**	.69**	.54**	-.05	.07
Sleep quality							.44**	.38**	-.08	-.03
Perceived Stress								.64**	-.02	.07
Burden Index									-.07	.22*
Exercise score										.20**

*p < .05, ** p < .01

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Legends to Figures

Figure 1. Diagram presenting recruitment, attrition and missing data in the study.

Figure 2. Neutrophil function in response to bacteria *E.coli.* A. Neutrophil phagocytic index (PI) which represents number of bacteria ingested per cell, in young and old, caregivers and controls. B. Neutrophil reactive oxygen species (ROS) production in response to bacteria *E.coli*, presented as mean fluorescence intensity (MFI). Error bars are standard errors of the mean (SEM) and * indicates $p < .05$.

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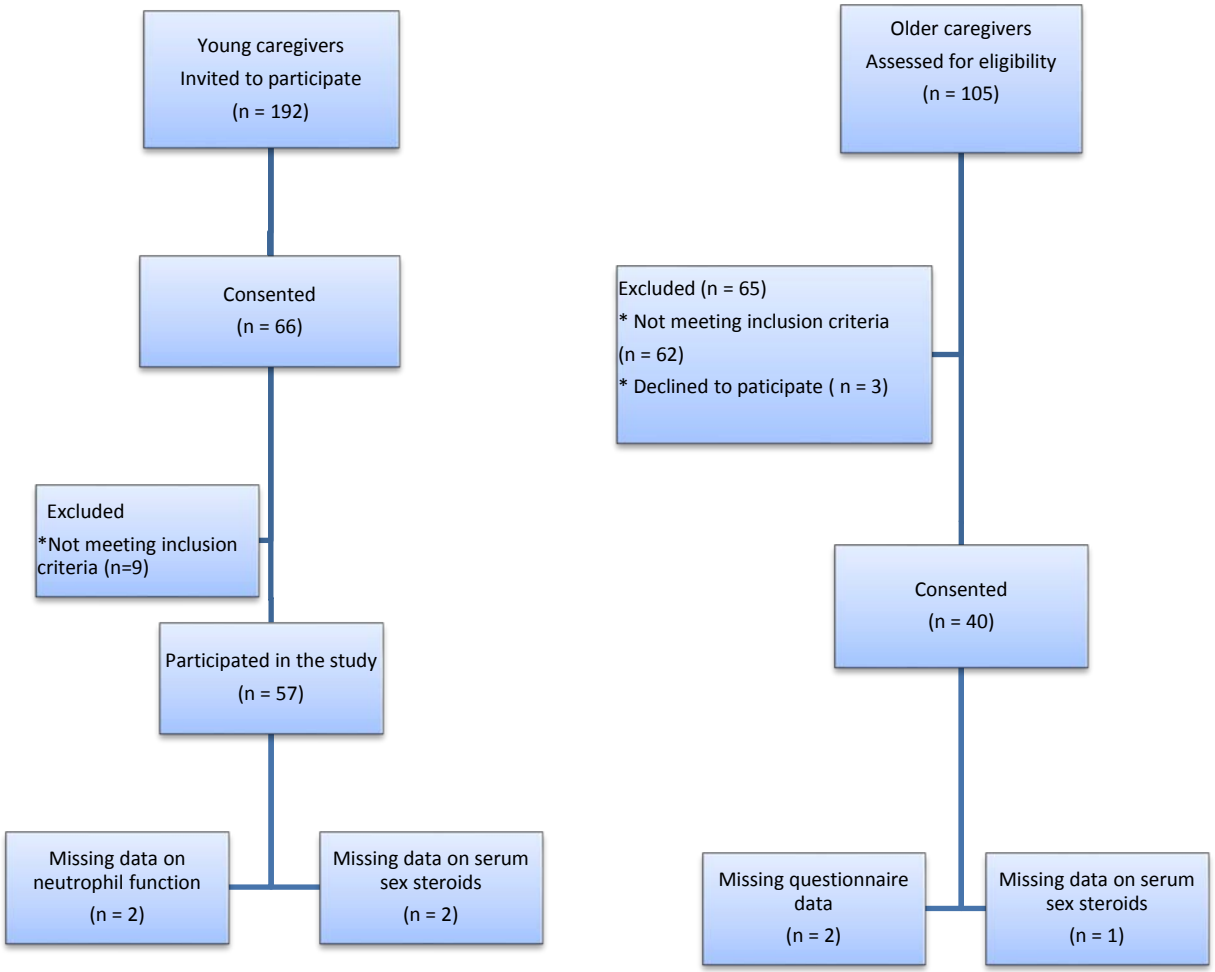
Figure 1.



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