Neuropsychiatric Aspects of Healthy Aging:
an Epidemiological Approach

Jelena Milic

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The work presented in this thesis was conducted within the Psychiatric Epidemiology and ErasmusAGE group at the Department of Epidemiology and the Department of Public Health, Erasmus Medical Center, Rotterdam. The Psychiatric Epidemiology Group investigates common psychiatric problems of the elderly. The focus has been on determinants and consequences of depressive disorders, but anxiety disorders, sleep disturbances and complicated grief are also being studied. The ErasmusAGE group investigates the role of lifestyle on health and nutrition across the life-course, funded by Nestlé Nutrition (Nested Ltd.) and Metagenics Inc. No funding source had the ability to veto publication or study results. Original studies in this thesis were performed within the Rotterdam Study. Rotterdam study is supported by Erasmus Medical Center and Erasmus University, Rotterdam, the Netherlands; the Netherlands Organisation for scientific research (NWO); and the Netherlands Organization for Health Research and Development (ZonMw).

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"It can be very dangerous to see things from somebody else's point of view without the proper training."

Douglas Adams

MANUSCRIPTS THAT FORM THE BASIS OF THIS THESIS

Chapter 2

Wen, K.-x.*, **Milic, J***., El-Khodor, B., Dhana K., Nano, J., Pulido, T., Kraja, B., Zaciragic, A., Bramer, W.M.,Troup, J., Chowdhury, R., Ikram, M.A., Dehghan, A., Muka, T., Franco, O.H. (2016) The Role of DNA Methylation and Histone Modifications in Neurodegenerative Diseases: A Systematic Review. *PLoS ONE* 11(12): e0167201. https://doi.org/10.1371/journal.pone.0167201

Chapter 3

Vargas, K. G.*, **Milic, J.***, Zaciragic, A., Wen, K.-x., Jaspers, L., Nano, J., Franco, O. H. (2016). The functions of estrogen receptor beta in the female brain: A systematic review. *Maturitas*, 93(Supplement C), 41-57. doi:https://doi.org/10.1016/j.maturitas.2016.05.014

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Chapter 4

Saavedra Perez, H. C., Direk, N., **Milic, J**., Ikram, M. A., Hofman, A., & Tiemeier, H. (2017). The Impact of Complicated Grief on Diurnal Cortisol Levels Two Years After Loss: A Population-Based Study. *Psychosomatic Medicine*, 79(4), 426-433. DOI: 10.1097/PSY.0000000000000422

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Chapter 5

Milic, J., Muka, T., Ikram, M. A., Franco, O. H., & Tiemeier, H. (2017). Determinants and Predictors of Grief Severity and Persistence: The Rotterdam Study. *Journal of Aging and Health*, 0898264317720715. doi:10.1177/0898264317720715

* Denotes equal contribution

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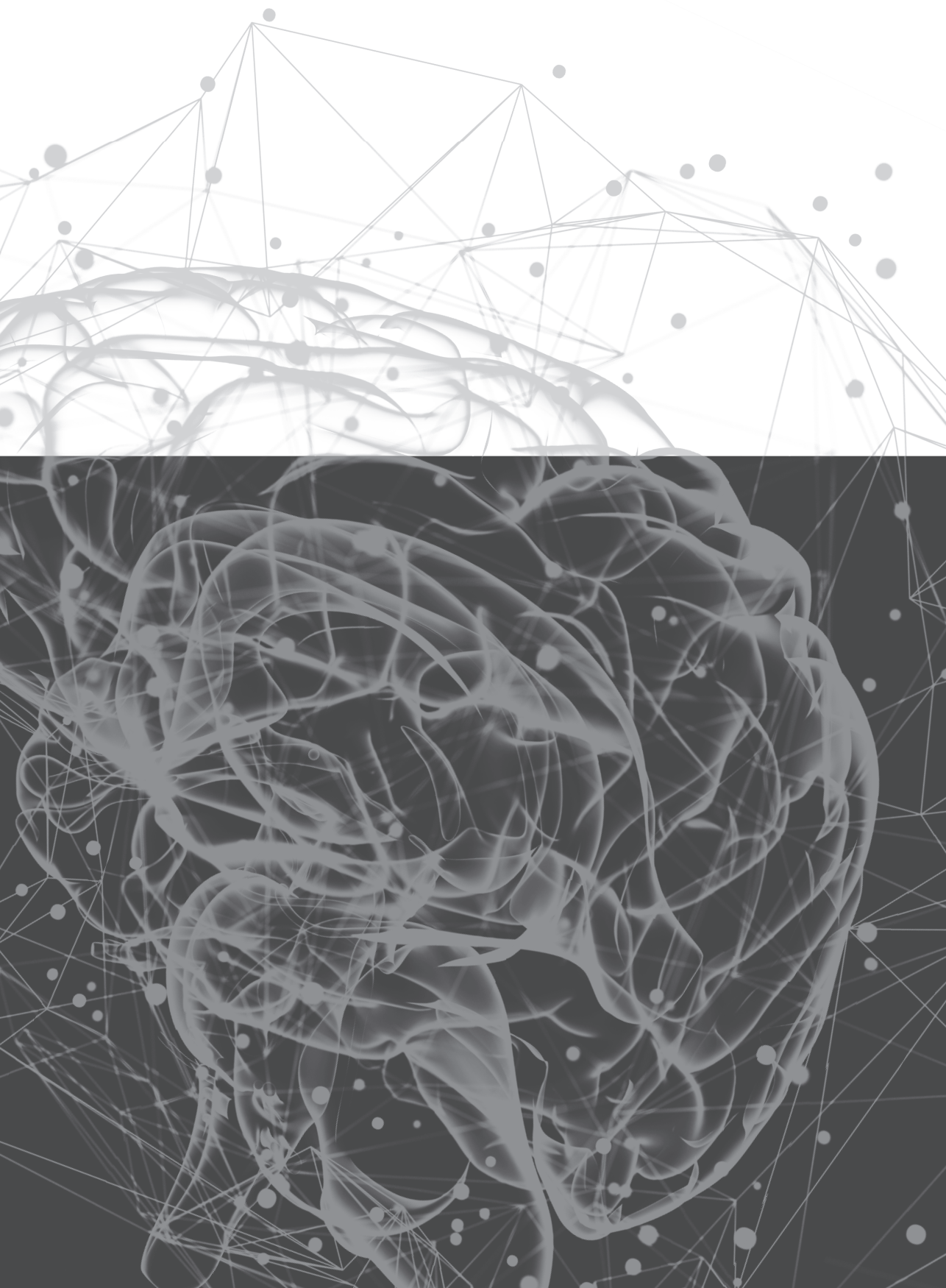
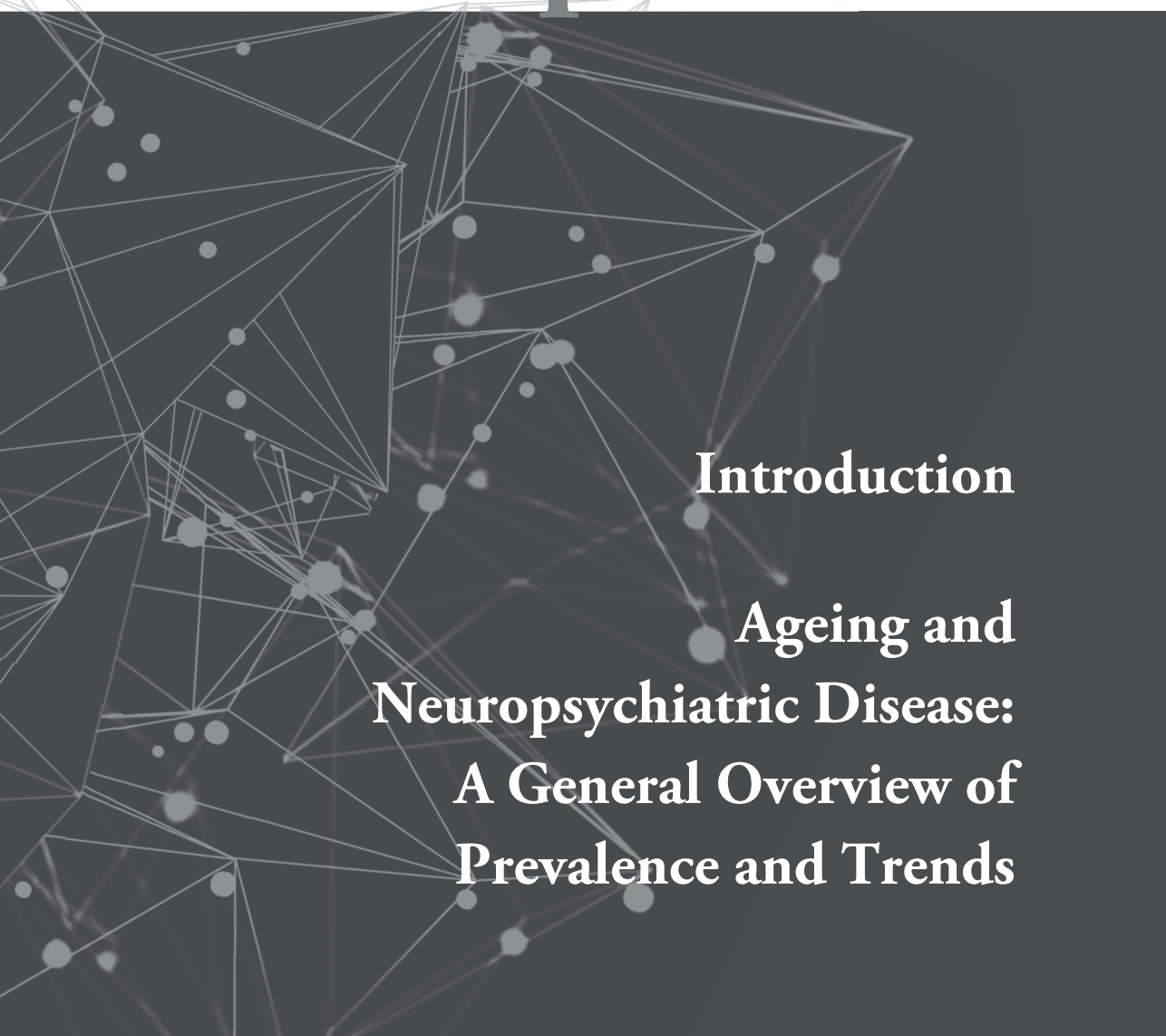


Chapter 1



Introduction

Ageing and Neuropsychiatric Disease: A General Overview of Prevalence and Trends

Aging of the population is a global phenomenon: it is accelerating and becoming a significant demographic, societal and economic challenge (Oeppen & Vaupel, 2002). Many societies, especially in Western countries, already have a population that is older than has ever been seen in the past and low, and middle-income countries are also progressing toward a similar population structure (Salomon et al., 2012). Globally, there are more than 900 million people aged 60 or more years, and their number is projected to grow to 1.4 billion by 2030, and to 2.1 billion by 2050 (United Nations Population Fund (UNFPA), 2015). Further, within elderly populations, there is a large subgroup of "very old aged people" comprising of individuals between the age of 80-85 years (Forman, Berman, McCabe, Baim, & Wei, 1992). Far-reaching economic and social adjustments will be required in most countries, as well as understanding the impact of this transformation on health and everyday living (Colchero et al., 2016).

The brain undergoes morphological changes associated with aging so many elderly people are affected by neuropsychiatric diseases (Taneri et al., 2016). Depression is amongst the most common psychiatric disorders, affecting elderly people, while Alzheimer's Disease (AD) and Parkinson's disease (PD) are the most common neurodegenerative diseases among the elderly (Djernes, 2006; United Nations Population Fund (UNFPA), 2015). Recent systematic reviews have shown that the prevalence of major depression in late life ranged from 4.6% to 9.3%, and that of depressive disorders ranged from 4.5% to 37.4%. Pooled prevalence was 7.2% (95% CI 4.4–10.6%) for major depression and 17.1% (95% CI 9.7–26.1%) for depressive disorders (Luppa et al., 2012). Further, worldwide, the global prevalence of dementia is estimated to be 3.9 % in people aged 60+ years, most of whom suffer from AD (Qiu, Kivipelto, & von Strauss, 2009). PD is the second most common neurodegenerative disorder after AD and is expected to impose an increasing social and economic burden on societies as populations age (de Lau & Breteler, 2006). The prevalence of PD in industrialised countries is generally estimated at 0.3% of the entire population and about 1% in people over 60 years of age (Nussbaum & Ellis, 2003). Due to ageing population, this number is expected to double every 20 years (Qiu et al., 2009). Neuropsychiatric diseases pose a high economic burden to societies. Currently they account for 6.6% of all disability adjusted life years (DALYs) and 17.4% of Years Lived with Disability (YLDs), and these numbers will increase in the future (WHO, 2016).

The impact of neuropsychiatric diseases in the elderly can further increase as a result of alcohol misuse and the consequences of traumatic life events. Emerging evidence indicates that in the coming years, there will be an increase in the absolute number of elderly people with alcohol misuse disorders and, thus, with notable impairment in physiological and cognitive health (Briggs, Magnus, Lassiter, Patterson, & Smith, 2011; Connell, Chin, Cunningham, & Lawlor, 2003; Han, Gfroerer, Colliver, & Penne, 2009). Moreover, death of someone close, like family or friends, is generally accepted as among the most common and traumatic life event in late life. In late life most frequent loss event is of a spouse (Rozenzweig, Prigerson, Miller, & Reynolds, 1997) Depression (along with suicide), anxiety, substance abuse, and symptoms of "complicated" grief are among the most important psychiatric conditions associated with spousal bereavement and the challenges of adaption to becoming widowed. (Rozenzweig et al., 1997) Widowhood occurs more frequently in older women than older men, with estimates nearing 42.2% for widows and 13.1% for widowers among U.S. community dwelling older adults (Federal Interagency Forum on Age-Related Statistics, 2008).

Epigenetic, an Emerging Factor in Development of Neurodegenerative Diseases

The last decade broadened our knowledge about the etiology of AD and PD (Bertram, McQueen, Mullin, Blacker, & Tanzi, 2007; Farrer, 2006; Gusella & MacDonald 1993, Karch & Goate, 2015). It is now widely accepted that there is a strong genetic component in the development of AD and PD, including chromosomal aberrations and gene mutations (Ghani et al., 2015; Karch & Goate, 2015). One of the factors that has enhanced the understanding of genetic modifications is epigenetics. (Jakovcevski & Akbarian, 2012). Epigenetics literally means "above" or "on top of" genetics. It refers to external modifications to DNA that turn the expression of genes "on" or "off." Epigenetic modifications do not change the DNA sequence, but instead, they affect how cells "read" genes by altering the physical structure of DNA. Epigenetic mechanisms are known to alter gene expression in a heritable manner mainly via modifications to DNA methylation and histone proteins (Henikoff & Matzke, 1997). Several epidemiological studies suggest an epigenetic contribution to the etiology of AD and PD (Bollati et al., 2011; Mogi et al., 1996; Urdinguio, Sanchez-Mut, & Esteller, 2009). Furthermore, DNA methylation and histone acetylation have recently been implicated in the development of depression (Sun, Kennedy, & Nestler, 2013). Depression is an important correlate of neuropsychiatric diseases and it includes some neurodegenerative processes (Rickards, 2005). Despite this evidence, there has not yet been a comprehensive assessment and understanding of the role of epigenetic mechanisms, such as DNA methylation or histone modifications, in the development of neurodegenerative disorders.

Neuropsychiatric Disorders in Women: Role of Menopause Transition and Estrogen

The majority of the common neurological diagnoses, including depression, AD, and PD, are more common in women than in men, and among women, more prevalent in postmenopausal women (Mary, 1997). Furthermore, some other neurological diseases such as stroke tend to present more severely in women than in men (Hofman, de Jong, van Duijn, & Breteler, 2006). Females have longer lifespan predisposing them to psycho-neurological disease (O'Neal, 2013). Furthermore, women are more prone to neuropsychiatric disorders due to unique risk factors such as hypercoagulable states caused by pregnancy and hormonal contraceptives (Katz & Beilin, 2015; Thornton & Douglas, 2010; Wolski, 2014). Once women reach menopause, hormonal changes cause women to lose the protective anti-inflammatory effects previously conferred by estrogen, and hormonal replacement therapy replenishment seems to be insufficient to compensate for the state of hypoestrogenia (O'Neal, 2013).

Estrogen has many physiological roles in the body and brain, all of which are mediated by its main receptors α and β (ERβ). The newly discovered ERβ is widely distributed in the brain. It is unclear whether ERβ has favourable functions in the female brain and whether it could be considered as a novel target therapy for prevention and treatment of neuropsychiatric diseases in menopausal women.

Also, data indicate that estrogenic signalling can change with menopausal status and age. As women age and live through menopause, alcohol misuse and dependence also become more prevalent (Epstein, Fischer-Elber, & Al-Otaiba, 2007). Due to sex-differences in metabolism of alcohol, women are more vulnerable to alcohol's harmful effects, and tend to develop alcohol-related diseases and other consequences of drinking earlier in life than men (Epstein et al., 2007). Moreover, elderly

people, especially older women, are particularly vulnerable to the adverse effects of alcohol, and alcohol use disorders in this subgroup are often overlooked or misdiagnosed (Bratberg et al., 2016). Also, women have more difficulty gaining access to treatment and recovering from alcohol dependence (Epstein et al., 2007).

Bereavement in the Elderly Population: Impact on Health and Quality of Life

Experiencing the loss of a close person is a traumatic event that is likely to be experienced during a person's life span (Boelen & Hoijtink, 2009; Monk, Germain, & Reynolds, 2008; Shear, 2015). With aging, death and loss might occur once or several times. Even though it is a traumatic event, most people recover within six to twelve months after the loss (Kacel, Gao, & Prigerson, 2011). However, an estimated 10-20% of bereaved people continue to grieve for a prolonged period, imprisoned by memories, regrets and a sense of guilt (Prigerson et al., 1997; Prigerson et al., 2009). This complex condition is termed as Prolonged Grief Disorder (PGD), also referred to as 'complicated grief'(Prigerson et al., 1997; Prigerson et al., 2009). Noticeably, the grieving process is distinct from depression, anxiety, and can lead to impairment in social and interpersonal daily functioning, leading to impairment of overall well-being (Newson, Boelen, Hek, Hofman, & Tiemeier, 2011). Emerging evidence shows that acute grief and complicated grief can induce changes of the circadian rhythm (Monk, Begley, et al., 2008), eating patterns (Hall et al., 2014), cognition (Hall et al., 2014) and sleep patterns (Milic et al., 2017). Since all these aspects of daily functioning are subject to change, grief can severely influence the quality of life.

As the loss of a loved one is a highly upsetting event, intense emotional stress might be triggered. Under intense emotional stress, the hypothalamic-pituitary-adrenocortical (HPA) axis is stimulated and activates the secretion of cortisol into the bloodstream as an adaptation to the stressor. Dysregulation of cortisol is associated with problematic alcohol use and dependence, memory loss, physical and psychological impairment and, thus, reduced quality of life and higher risk of mortality (Kumari, Shipley, Stafford, & Kivimaki, 2011; Mura et al., 2014; Stephens & Wand, 2012). Few studies have focused on the effect of grief on the neuroendocrine system, in particular on cortisol secretion patterns (Saavedra Perez et al., 2017). Further, previous studies suggest that grief and complicated grief are associated with significant sleep impairment (Hall et al., 1997; Kowalski & Bondmass, 2008; Monk, Begley, et al., 2008) Schwartz and Sprangers have explored how changes in general health status affect the quality of life of a grieving person (QoL) (Schwartz & Sprangers, 1999). Other authors have found that in the phase of grief, an individual's diminished capacity to preserve desirable physical, psychological and social responses can reduce an individual's level of satisfaction and sense of self-worth (Cousson-Gelie, de Chalvron, Zozaya, & Lafaye, 2013; Ozer, Firat, & Bektas, 2009; Schwartz & Sprangers, 1999).

Despite on-going research, our collective knowledge of the associations of grief and health outcomes is limited. Research in this field tends to include studies with a small sample size and cross-sectional design (Boelen & Lancee, 2013; Germain, Caroff, Buysse, & Shear, 2005; Maytal et al., 2007; Monk, Begley, et al., 2008; Purebl, Pilling, Konkoly, Bodizs, & Kopp, 2012; Spira, Stone, Beaudreau, Ancoli-Israel, & Yaffe, 2009). Likewise, the association between grief, complicated grief, and the domains of QoL remains unclear. The available evidence examining the association between grief and

quality of life has yet to be rigorously reviewed in order to help us to understand how bereavement might impair quality of life and everyday life.

Grief Cessation and its Determinants

For some bereaved individuals, the adaptation to life without their loved one might be complicated, slowed, or halted, leading to incessant grief (Boelen & van den Bout, 2008; Shear et al., 2007). Lasting grief impairs daily functioning and sleep, and may increase the risk of cancer and cardiovascular disease (Simon et al., 2005; Simon et al., 2007). The severity of grief relates to severity of impairment. Therefore, identifying the determinants and predictors of grief severity and its persistence is of crucial importance in identifying bereaved individuals at high risk for long-term dysfunction. After individuals at high risk of prolonged grief are identified, novel interventions for the disorder may be developed and implemented (Currier, Neimeyer, & Berman, 2008).

Several factors have been suggested to influence the duration and severity of grief, including gender. After loss of a spouse, males suffer more severe health consequences and decrease in overall quality of life (Stroebe & Schut, 2001). Mortality rates of persons are higher for both males and females compared to non-bereaved people, but the relative increase in mortality is higher for males (Stroebe & Schut, 2001). Previous studies have suggested that the explanation for this discrepancy might be related to the weaker social support common among bereaved men. Also, woman have better overall coping abilities and capacities for self-empowerment (Neimeyer, 2006). In addition, age may also play a significant role in how well people can recover during the bereavement period. According to Stroebe and Schut, younger bereaved persons encounter more complications after a loss, including more serious health consequences, both psychological and physical (Stroebe & Schut, 2001). Younger grievers might experience more unexpected and sudden losses, which could lead to more severe grief. Older grievers may have better coping strategies due to life experiences or because as people age they become less susceptible to dramatic emotional shifts (Onrust, Cuijpers, Smit, & Bohlmeijer, 2007). Even though younger grievers may experience more health-related complications during the acute phase of grieving, previous studies suggest that they recover more quickly. They may recover more quickly because they have greater access to various types of social support (Stroebe & Schut, 2001).

Beyond the role of age and gender in grief severity, studies have aimed to understand other determinants and predictors of grief severity and persistence such as ethnicity (Fitzpatrick & Tran, 2002), education level (Boelen, Van Den Bout, De Keijser, & Hoijtink, 2003) and previous depressive symptoms. (Tsai et al., 2016; Tsuboya et al., 2016) However, most studies on these topics have been cross-sectional and limited in sample size. The few longitudinal studies that investigated determinants of grief severity and persistence had a short follow-up period of less than 24 months (Bonanno et al., 2002; Prigerson et al., 2009; Tsai et al., 2016)). Furthermore, none of the previous studies have utilized a population-based cohort study with sufficient power to explore determinants of grief related to long-term bereavement and other types of loss. (Bonanno et al., 2002; Prigerson et al., 2009; Tsai et al.,2016; Zisook, Paulus, Shuchter, & Judd, 1997; Zisook & Shuchter, 1991; Zisook et al., 1994). Further studies are necessary to identify the factors associated with grief severity.

General Aim of the Thesis

The overall aim of the thesis was to identify factors associated with neuropsychiatric disorders among the elderly. The first objective of the thesis was to identify epigenetic and women-specific factors and that can play a role in the development of neuropsychiatric outcomes. A second objective was to identify the impact of grief and complicated grief on cortisol secretion, sleep pattern, and overall quality of life, as well as to identify factors associated with grief persistence.

Study Design

Systematic Reviews

To meet the aims of the thesis, some of the chapters are comprised of systematic reviews of the literature. The reviews were conducted using a predefined protocol in accordance with the PRISMA (Moher, Liberati, Tetzlaff, Altman, & The PRISMA Group, 2009) and MOOSE (Stroup et al., 2000) guidelines. By searching numerous electronic databases as specified in detail in each chapter, we found relevant citations for further screening. Our search was performed without any language or study design restriction, with the help of an experienced medical information specialist. Two independent reviewers screened the titles and abstracts of all studies initially identified, according to the selection criteria, and any disagreement was resolved through consensus or consultation with a third independent reviewer. Full texts were retrieved from studies that satisfied all selection criteria. Additionally, reference lists of the included studies were screened to identify further studies. Two independent reviewers extracted the data using a pre-designed data collection form. The Newcastle-Ottawa Scale was used to assess the risk of bias in observational studies.(Wells et al., 2011) Where possible, the inverse variance weighted method was used to combine the reported estimates from each study to produce a pooled estimate using random- effects meta-analysis models to allow for between study heterogeneity. Heterogeneity was assessed using the Cochrane $\chi 2$ statistic and the I^2 statistic. Publication bias was evaluated through a funnel plot and Egger's test. Further details on the methods can be found in the specific chapters.

Rotterdam Study

The studies presented in chapters 3 and 4 of the thesis were carried out within the framework of the Rotterdam Study, a population-based prospective cohort study started in 1990 in the Ommoord district in the city of Rotterdam, the Netherlands. Details regarding the design, objectives, and methods of the Rotterdam Study have been described in details elsewhere (Hofman et al., 2015). In brief, in 1990, all inhabitants of a well-defined district of Rotterdam were invited to participate in the study, of whom 7983 agreed (78,1%). In 2000, an additional 3011 participants were enrolled (RS-II), consisting of all persons living in the study district who had turned at least 55 years of age between 1991 and 2000). The third cohort was established in 2006 and included 3932 participants 45 years and older (RS-III). Follow up examinations were performed approximately every 3 to 5 years (Hofman et al., 2015). There were no eligibility criteria to enter the Rotterdam Study cohort except the minimum age requirement and that the individual resided in the Ommoord residential area, defined based on zip codes.

Grief and complicated grief as used in this thesis was assessed and diagnosed at the fourth (RS –I-4 and RS-II-2) round (2002-2005) and fifth round (2009-2012) of the Rotterdam Study using a Dutch version of the 17-item Inventory of Complicated Grief (ICG) originally constructed by Prigerson et al. (which contains 19 items) (Prigerson et al., 2009). First, participants were asked if they were currently grieving, if a positive answer was received they were classified as grievers and the ICG was administered, if not, they were categorized as non-grievers. The Dutch version of the Inventory of Complicated grief contains 17 items and has been previously validated (Boelen, 2003). These seventeen questions were asked and responses were provided on a 5-point scale to reflect an increase in severity (0-never, 1-seldom, 2-sometimes, 3-often, 4-always). We divided all interview participants into groups of non-grievers, normal grievers, and complicated grievers. Complicated grief symptoms were assessed in participants who scored equal to or greater than 22 on the ICG score and who indicated they had grieved for longer than 6 months (Newson et al., 2011; Saavedra Perez et al., 2015). Sleep duration and sleep quality were measured with the Pittsburgh Sleep Quality Index (PSQI), a self-reported questionnaire (Buysse, Reynolds, Monk, Berman, & Kupfer, 1989). The PSQI consists of 19 self-rated items. Questions are grouped into seven component scores, each weighted equally on a 0-3 scale. The seven component scores are then summed to yield a global PSQI score, which is used in all further analyses. This score has a range of 0-21. Higher scores indicate worse sleep quality. In the current study we use total sleep time in hours to indicate sleep duration, and total score of PSQI to indicate sleep quality. Salivary cortisol was obtained by saliva samples that were collected on awakening (T1), 30 min after awakening (T2), at 1700 h (T3), and at bedtime (T4). Salivary cortisol concentrations were measured using a commercial immunoassay with chemiluminescence detection (CLIA; IBL Hamburg, Hamburg, Germany).

Outline of this Thesis

Following this general introduction, the aims of this thesis will be addressed in six chapters. In the second chapter I use systematic reviews to explore the epigenetic mechanisms that play a role in neurodegenerative disorders. I searched the existing published literature on the topic of epigenetics, with a focus on the role of DNA methylation and histone modifications on AD and PD.

The third chapter is dedicated to the specific risk factors related to female gender in the process of neurodegeneration and neuropsychiatric disorders. In chapter 3.1 I present a systematic review in which we explore the functions of estrogen receptor beta in the female brain. In Chapter 3.2 I critically appraise the literature on the impact of the menopausal transition on alcohol misuse and dependence in women.

In chapter 4 I present the results of studies on the psychiatric aspects of healthy aging with a focus on bereavement. For chapter 4 I conducted original research within the population-based Rotterdam cohort, along with systematic reviews. In chapter 4.1 I explore the impact of complicated grief on diurnal cortisol levels. In chapter 4.2 I assess the prospective association between grief and complicated grief with sleep duration and in chapter 4.3 I investigate the association between grief and quality of life by conducting a systematic review.

In chapter 5 I prospectively examine the determinants and predictors of grief cessation.

Lastly, chapter 6 provides an overview of the main findings of this thesis, including methodological considerations, clinical implications and future directions for research.

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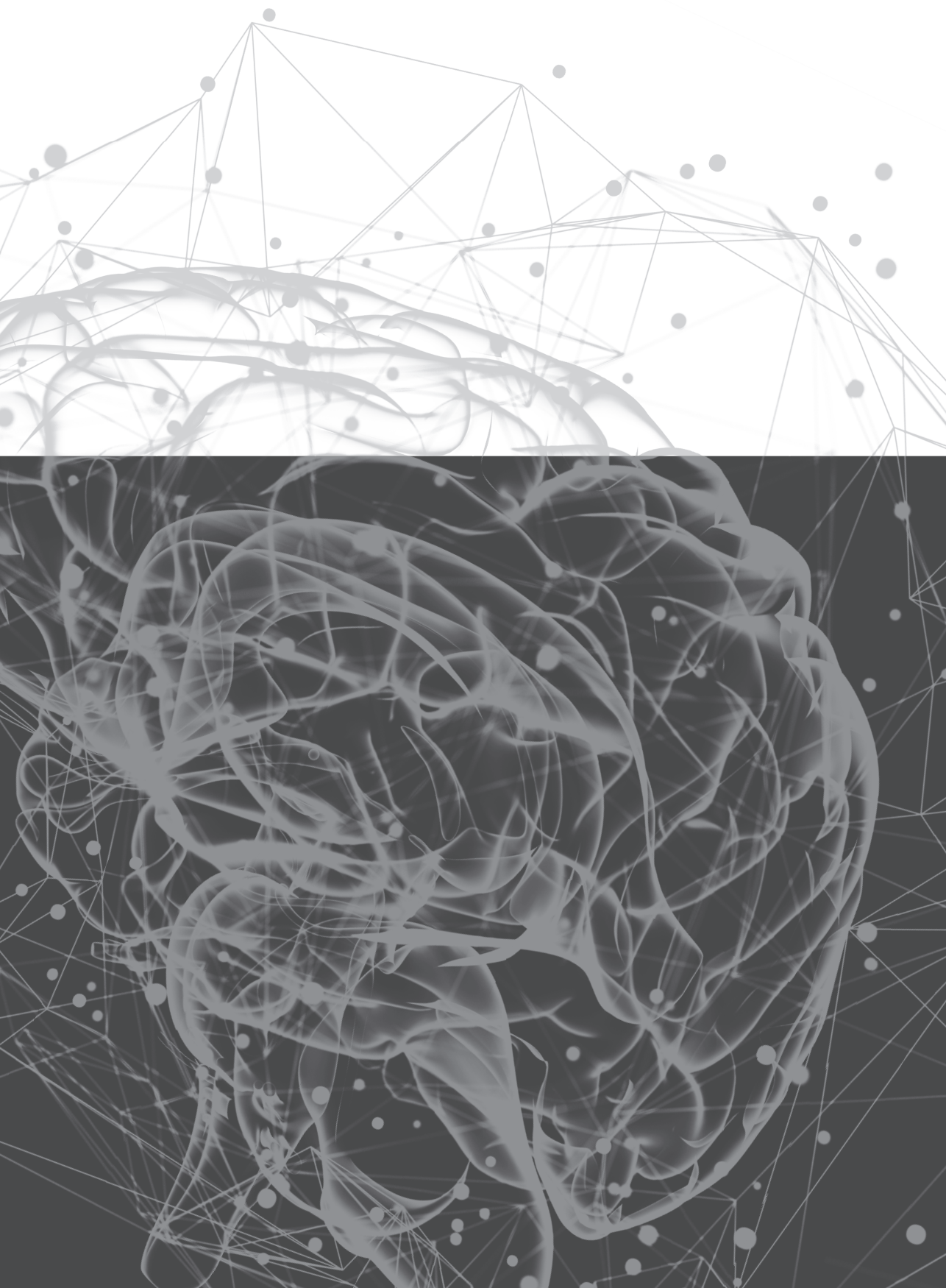
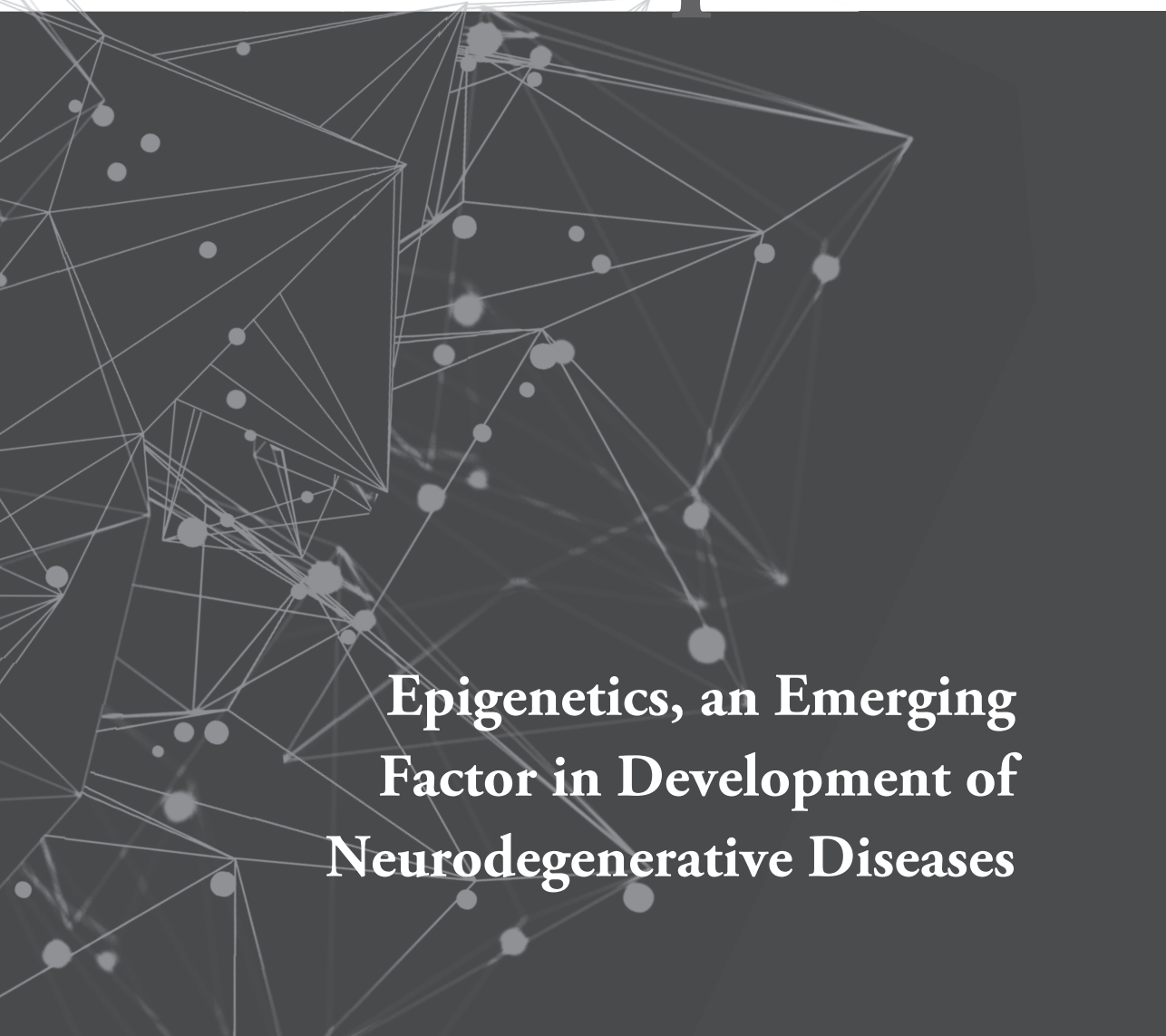


Chapter 2



Epigenetics, an Emerging Factor in Development of Neurodegenerative Diseases

2.1

The role of DNA Methylation and Histone Modifications in Neurodegenerative Diseases: A Systematic Review

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ABSTRACT

Objective: To systematically review studies investigating epigenetic marks in AD or PD.
Methods: Eleven bibliographic databases (Embase.com, Medline (Ovid), Web-of-Science, Scopus, PubMed, Cinahl (EBSCOhost), Cochrane Central, ProQuest, Lilacs, Scielo and Google Scholar) were searched until July 11th 2016 to identify relevant articles. We included all randomized controlled trials, cohort, case-control and cross-sectional studies in humans that examined associations between epigenetic marks and ND. Two independent reviewers, with a third reviewer available for disagreements, performed the abstract and full text selection. Data was extracted using a pre-designed data collection form.
Results: Of 6927 searched references, 73 unique case-control studies met our inclusion criteria. Overall, 11,453 individuals were included in this systematic review (2640 AD and 2368 PD outcomes). There was no consistent association between global DNA methylation pattern and any ND. Studies reported epigenetic regulation of 31 genes (including cell communication, apoptosis, and neurogenesis genes in blood and brain tissue) in relation to AD and PD. Methylation at the *BDNF*, *SORBS3* and *APP* genes in AD were the most consistently reported associations. Methylation of α-synuclein gene (*SNCA*) was also found to be associated with PD. Seven studies reported histone protein alterations in AD and PD.
Conclusion: Many studies have investigated epigenetics and ND. Further research should include larger cohort or longitudinal studies, in order to identify clinically significant epigenetic changes. Identifying relevant epigenetic changes could lead to interventional strategies in ND.

INTRODUCTION

Alzheimer's disease (AD) and Parkinson's disease (PD) are the most common neurodegenerative disorders and are a major cause of disability and premature death among older people worldwide (Bird et al., 2003; de Lau & Breteler, 2006; Savica et al., 2013). Due to global population ageing, prevalence of AD and PD is expected to increase, imposing a social and economic burden on society (Hebert, Scherr, Bienias, Bennett, & Evans, 2003; Kowal, Dall, Chakrabarti, Storm, & Jain, 2013). The causes of most cases of neurodegenerative diseases remain largely unknown. However, in the last decade great advances have been made in our understanding of the pathogenetic mechanisms that lead to AD and PD (Bertram, McQueen, Mullin, Blacker, & Tanzi, 2007; Farrer, 2006; Karch & Goate, 2015). It has been accepted that there are several genetic causes that play a role in the development of these disorders, including chromosome aberrations and gene mutations (Ghani et al., 2015; Karch & Goate, 2015). Additionally, environmental exposures have been suggested to play a crucial role in the etiological process of neurodegenerative diseases. Both AD and PD are thought to be caused by complicated interactions between genetic and environmental factors (Cannon & Greenamyre, 2011). Despite improvements in knowledge and understanding, there are currently no disease-modifying therapies for these diseases. A large amount of the variance in the risk of developing neurodegenerative diseases remains to be explained.

The epigenome is responsible for the molding and the three-dimensional structure of the genomic material in the cell nucleus. It provides a bridge between genes and environment and may help to improve our understanding on the etiology of complex diseases, AD, and PD (Jakovcevski & Akbarian, 2012). Epigenetic mechanisms are known to alter gene expression or cellular phenotype in a heritable manner (Henikoff & Matzke, 1997). DNA methylation and modifications of histone proteins are the most intensively studied among the major epigenetic modifications. DNA methylation occurs when a methyl group is added at a cytosine nucleotide that precede guanines (so-called CpG dinucleotides). It further influences the function of DNA by activating or repressing the transcriptional activity of a gene (Henikoff & Matzke, 1997). Posttranslational histone modifications, such as methylation and acetylation of lysine residues on histone tails, are another type of epigenetic modification. Histone modifcations affect gene expression mainly by altering chromatin structure (Henikoff & Matzke, 1997; Shen & Casaccia-Bonnefil, 2008).

Clinical features of neurological disorders and results from epidemiological studies suggest an epigenetic contribution to etiology of these diseases. Epigenetic modulation has been well documented in brain development, plastic changes, and in brain diseases including AD and PD. The most compelling evidence on the role of epigenetics on AD comes from the results of treatment of AD patients with inhibitors of histone deacetylases (HDAC). HDAC is a key enzyme involved in histone acetylation (Graff et al., 2012). Also, in animal models of PD, HDAC inhibitor inhibits α-synuclein toxicity in the dopamine neuron, a common neuropathological feature of PD (Outeiro et al., 2007). Dysregulation of DNA methylation in AD and PD patients is also well documented. Recent evidence shows that AD patients have an elevated DNA methylation state of repetitive elements (Bollati et al., 2011). Hypomethylation of the tumor necrosis factor (*TNF)* gene in cortex and higher levels of TNF-α cytokine in the cerebrospinal fluid has been reported in patients with PD (Mogi et al., 1996). TNF-α is one of the main proinflammatory cytokines that play a central role in the inflammatory response. TNF-α is

also upregulated in AD patients and is involved in the pathogenesis of AD (Perry, Collins, Wiener, Acton, & Go, 2001). In dopaminergic regions of post-mortem brains, decreased methylation of the α-synuclein gene (*SNCA)* has been observed. The decreased methylation might be responsible for the accumulation of the protein α-synuclein, and thus the progression of PD (Jowaed, Schmitt, Kaut, & Wullner, 2010; Matsumoto et al., 2010b). Moreover, DNA methylation and histone acetylation have recently been identified as playing a role in depression (Sun, Kennedy, & Nestler, 2013), an important feature of neurodegenerative diseases (Rickards, 2005). Emerging evidence shows that epigenetic mechanisms contribute to the process of learning and memory formation (Jarome, Thomas, & Lubin, 2014; Levenson & Sweatt, 2005). Despite this evidence, to date, a comprehensive assessment of the role of epigenetic mechanisms in the development of AD and PD has not yet been done.

Therefore, we aimed to systematically review all available evidence in humans to assess the association of DNA methylation and histone modifications with the neurodegenerative disorders AD and PD.

MATERIAL AND METHODS

Literature Search

This review was conducted using a predefined protocol in accordance with the PRISMA (Moher, Liberati, Tetzlaff, Altman, & Group, 2009b) and MOOSE (Stroup et al., 2000) guidelines **(eAppendix 1 and 2)**. Eleven bibliographic databases (Embase.com, Medline (Ovid), Web-of-Science, Scopus, PubMed, Cinahl (EBSCOhost), Cochrane Central, ProQuest, Lilacs, Scielo and Google Scholar) were searched until July 11th 2016 (date last searched) without any language restrictions, with the help of an experienced medical information specialist (WMB). The search strategy combined terms related to exposure (e.g., epigenetics, DNA methylation, histone, CpG) and outcomes (e.g., neurological disorders, dementia, Alzheimer, Parkinson). In databases where a thesaurus was available (Embase, Medline and Cinahl) articles were searched by thesaurus terms, title and/or abstract; other databases were searched only by title and/or abstract. We restricted the search to studies on human adults. The full search strategies of all databases are provided in **eAppendix 3**. After eliminating duplications, we identified a total of 6927 potentially relevant citations. We retrieved reference lists of the studies and sought contact with experts to find further relevant publications.

Study Selection and Inclusion Criteria

Included studies either described an association between epigenetic marks (global, site specific or genome-wide methylation of DNA) or histone modifications (methylation, phosphorylation, acetylation, ubiquitylation, and sumoylation) and neurodegenerative outcomes defined as AD and PD. There was no restriction based on the tissue type examined for epigenetic marks, and therefore, epigenetic marks assessed in any tissue (e.g. brain, blood) were included. We included cross-sectional, prospective, case-cohort and nested case control studies. Studies were excluded if they (i) examined epigenetic marks other than DNA methylation and histone modifications, such as noncoding RNAs;

(ii) examined neurodegerative diseases other than AD and PD, such as Huntington's disease, Prion disease, Motor neurone diseases, Spinocerebellar ataxia, Spinal muscular atrophy; (iii) were case studies or letters to the editor. Two independent reviewers (KW/JM and KD/JN/TP/BK/AZ) screened the retrieved titles and abstracts and selected eligible studies. In cases of disagreement, decision was made through consensus or consultation with a third independent reviewer (TM). Full texts were retrieved for studies that satisfied all selection criteria.

Data Extraction

A predesigned data collection form was prepared to extract the relevant information from the selected studies, including study design, study population, location, age range, duration of follow up (for longitudinal studies), confounders, tissue sample, method used to assess epigenetic marks, type and numbers of neurodegenerative outcomes and reported measures of associations (e.g., correlation analysis, odds ratio, relative risks, confidence intervals). Two independent authors (KW and JM/TM) extracted the data.

Assessing the risk of bias

Bias within each individual study was evaluated by two independent reviewers (KW and JM) using the validated Newcastle-Ottawa Scale, a semi-quantitative scale designed to evaluate the quality of nonrandomized studies (Stang, 2010). The scores are provided in **eAppendix 4**. Study quality was judged based on the selection criteria of participants, comparability of cases and controls, and exposure and outcome assessment. Studies that received a score of 9 stars were judged to be at low risk of bias; studies that scored 7 or 8 stars were considered to be at medium risk; those that scored 6 or less were considered to be at high risk of bias.

Outcome Assessment and Statistical Methods

For each study, we defined whether an association was reported and whether direction and effect sizes were reported, when applicable. Heterogeneity permitting, we sought to pool the results using a random effects meta-analysis model. If pooled, results were expressed as the pooled estimate and the corresponding 95% confidence intervals.

RESULTS

We identified 6927 potentially relevant publications (**Figure 1**) after removal of duplicate citations. Based on the title and abstracts, 107 articles were selected for detailed evaluation of their full texts. Of those, 32 articles were excluded for either having the wrong exposure or outcome (n=28), reporting results from animal models (n=3), or unavailable full texts (n= 1) (**Figure 1 and eAppendix 4**). Seventy-five articles, based on 73 unique case-control studies, met our eligibility criteria and were included in this review.

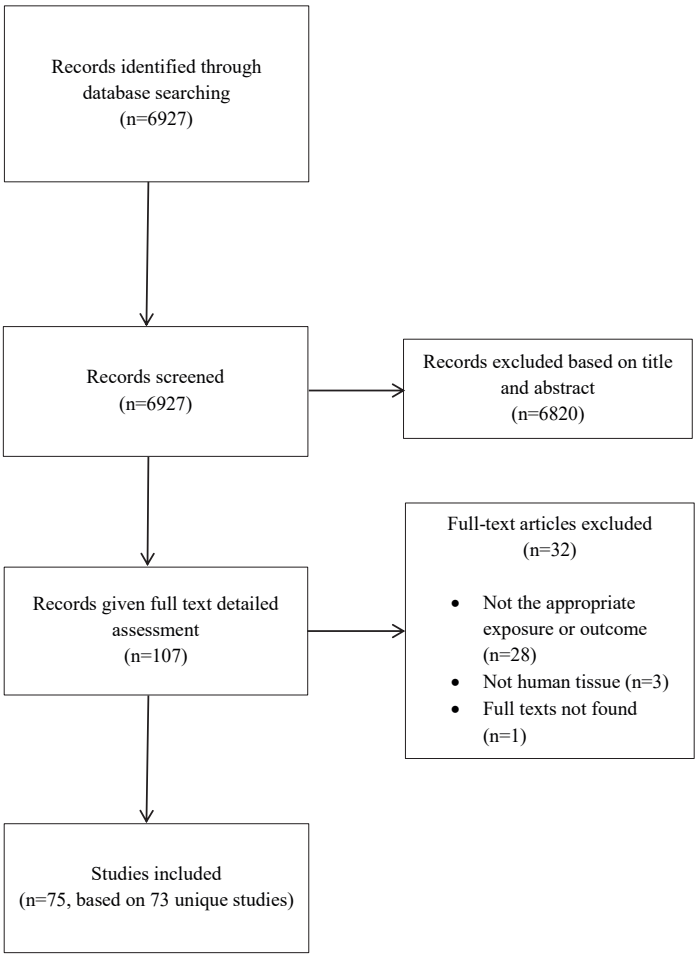

Figure 1. Flowchart of studies investigating epigenetic marks in relation to Alzheimer's disease and Parkinson's disease

Summary of Included Studies

Overall, 11453 individuals were included within the systematic review, with a total of 2640 for AD and 2368 for PD outcomes. Of the 73 unique studies included, 13 studies assessed global DNA-methylation, 45 studies assessed DNA methylation in specific candidate genes, 8 studies used genome-wide approaches, 1 study assessed both global DNA methylation, histone modifications and DNA methylation in specific candidate genes, and 6 studies examined histone modifications in relation to ND (**Tables 1-3**). Twenty-nine studies assessed DNA methylation and/or histone modifications only in blood, 35 in the brain tissue, 8 studies in both blood and brain tissue and 1 study assessed methylation in skin fibroblasts. Fifty-seven studies examined AD as an outcome while 18 studies examined PD. Twenty-four studies included participants from USA, 11 studies from China, 4 studies included participants from more than 1 country and the rest included participants solely from

Canada, Germany, United Kingdom, Italy, Spain, Japan, Sweden, Columbia, Australia, New Zealand, Serbia or Brazil (**Table 1-3).** Three studies were judged at low risk of bias whereas the rest were at medium and high risk of bias (**eAppendix 5**).

Global DNA Methylation

Global methylation refers to the overall level of methylcytosine in the genome, expressed as percentage of total cytosine. Many of the methylation sites within the genome are found in repeat sequences and transposable elements, such as Alu and long-interspersed nuclear element (LINE-1). They correlate with the total genomic methylation content. Measurements of methylation of the repetitive elements in the genome are used as a surrogate measurement for the overall methylation of the genome. Some studies quantified global DNA methylation by calculating the amount of methylated cytosines in the sample (5 mc) relative to global cytidine (5mC + dC) in a positive control. Other methods to assess global genomic DNA methylation (e.g., Luminometric Methylation Assay (LUMA) and the [^{3}H]-methyl acceptance based method) are primarily based on the digestion of genomic DNA by restriction enzymes HpaII, MspI and Dpn I.

(i) Alzheimer's Disease

Thirteen studies examined the association between global DNA methylation and AD (**Table 1**). Eight studies assessed DNA methylation in brain tissue and the rest of the studies assessed it in blood cells. Seven studies assessed global DNA methylation as a percentage of 5-methylcytosine in samples from brain. Of these seven studies, three studies (Chouliaras et al., 2013; Condliffe et al., 2014; Mastroeni et al., 2010) found lower levels of methylation in AD cases compared to controls, two studies (Bednarska-Makaruk et al., 2016; Lashley et al., 2014) found no difference, and two other studies (Coppieters et al., 2014; Rao, Keleshian, Klein, & Rapoport, 2012) reported higher levels of methylation in AD subjects. One study (Mastroeni et al., 2016) reported an increase in DNA 5-hydroxymethylation levels in AD compared to age-matched controls. One study (Hernandez, Mahecha, Mejia, Arboleda, & Forero, 2014) assessed global DNA methylation in LINE-1 elements in blood and showed no difference between AD patients and healthy controls. One study (Bollati et al., 2011) examined global DNA methylation in both LINE-1 and Alu elements. It reported no difference in global DNA methylation levels in Alu elements, and reported higher levels of methylation in LINE-1 elements in blood cells of AD compared to healthy controls. Three studies used other methods to assess global DNA methylation: two studies (Basile, Colacicco, Venezia, Kanduc, & Capurso, 1997; Di Francesco et al., 2015) reported DNA hypermethylation in AD individuals whereas one study (Schwob, Nalbantoglu, Hastings, Mikkelsen, & Cashman, 1990) showed no difference in global DNA methylation between AD cases and controls.

(ii) Parkinson's disease

There was only one study that examined the association between global DNA methylation at LINE-1 elements in blood and PD. The study showed no association (Nielsen et al., 2012) (**Table 1**).

Table 1. Global DNA methylation in Alzheimer's disease and Parkinson's disease

Author	Population	No of cases	Tissue	Adjustment	Association	Comment
ALZHEIMER'S DISEASE						
5mdC						
Mastroeni D et al. 2010 (Mastroeni et al., 2010)	USA, n=40, 60-97 years, M and F	20	Human post-mortem brain tissue (neurons of entorhinal cortex layer II and other regions-cerrebellum)		Inverse association	Methylation levels were decreased in AD cases compared to controls (91.3% ± 1.3 in non-AD cases and 39.9% ± 3.4%, P<0.0001). No difference in methylation frequency in other regions of the brain such as the cerebellum.
Chouliaras L Et al, 2013(Chouliaras et al., 2013)	USA, n=20 and one pair of monozygotic twins discordant for AD), 76.64 ± 4.9 years, M and W	10	Hippocampal tissue	Age and gender	Inverse association	Decreased 5-mC and 5-hmC immunoreactivity in AD hippocampus (-19.6%, p=0.006 and -20.2%, p=0.012). Decreased level of 5-mC immunoreactivity in glial cells in the CA3 and CA1 region of the hippocampus (-26.9%, p=0.016 and -25.7%, p=0.003 respectively) as well as in the neurons of the CA1 region (-21.1%, p=0.01). No differences in DG or CA3 neurons. Decreased level of 5-hmC immunoreactivity in cells of the DG and glial cells of the CA3 (-16.1%, p=0.042 and -34.2%, p=0.011 respectively).
Condliffe D. Et al, 2014(Condliffe et al., 2014)	UK, n=21, 78.18 ± 2.02 years, M and W	13	Cortical and cerebellar tissue	Age and gender	Inverse association	Significant decrease in 5-hmC in AD compared to controls (EC p<0.001, CER p=0.0476). No differences found in 5-mC levels between AD and controls, nor between brain regions. No estimates given.
Lashley T et al 2014 (Lashley et al., 2014)	UK, n=26, 71.8 ± 4.2 years, M and W	12	Brain tissue (entorhinal cortex and cerebellum)		No association	No significant differences detected between AD and control cases in either 5mC or 5hmC staining (both in immuno-histochemical analysis and ELISA).
Coppieters N. et al, 2014(Coppieters et al., 2014)	New Zealand, n=58, 75.35 ± 9.2 years, M and W	29	Cortical tissue: In middle frontal gyrus (MFG) and middle temporal gyrus (MTG)	Age at death and post-mortem delay matched	Positive association	Significant increase in global levels (integrated intensity per cell) of 5mC (p=0.0304) and 5hmC (p=0.0016) in MFG of AD cases compared to controls. Significant increase of 5mC (p<0.0001) and 5hmC (p<0.0001) each in MTG of AD cases compared to controls.
Rao JS et al. 2012(Rao et al., 2012)	USA, n=20, 70.4 ± 2.4, Gender not specified	10	Post-mortem frontal cortext (Brodmann area 9)		Positive association	The AD brains showed significant increases in global DNA methylation compared to age-matched controls.

Table 1. Global DNA methylation in Alzheimer's disease and Parkinson's disease (*continued*)

Author	Population	No of cases	Tissue	Adjustment	Association	Comment
Bednarska-Makaruk M et al. 2016(Bednarska-Makaruk et al., 2016)	Poland, 194, 71.1 ± 7.56, M and W	53	PB	Age	No Association	No significant differences detected between AD and control cases.
5hmeC (5-hydroxymethylation)						
Mastroeni D et al. 2016(Mastroeni et al., 2016)	USA, n=12, 79-96, M and W	N=6	Sub ventricular zone	Age	Positive association	There was an increase in DNA hydroxymethylation levels in AD compared to age-matched controls
LINE-1 methylation						
Bollati V. et al. 2011(Bollati et al., 2011)	Italy, n=81, 71.2 ± 8.3 years, M and W	43	PB	Age and gender	Positive association	LINE-1 methylation was significantly increased in AD patients compare to controls (83,6% vs. 83,1 p=0.04).
Hernandez H et al 2014(Hernandez et al., 2014)	Columbia, n=58, 76,2 ± 11,7 years, M and W	28	PBMCs	Age and sex	No association	No significant difference in median LINE-1 methylation levels between AD group and control group. There was also no differences between the groups when men and women were compared separately. There was also no difference seen when stratified for APOE-ε4 carrier status.
ALU						
Bollati V. et al, 2011(Bollati et al., 2011)	Italy, n=81, 71.2 ± 8.3 years, M and W	43	PB	Age and gender	No difference	No difference.
HpaII/MspI ratio						
Shwob NG et al. 1990(Schwob et al., 1990)	Canada, n=64, 45-92 years, M and W	44	Human post-mortem brain tissue (frontal cortex)		No difference	No difference in DNA methylation level between cases and controls (54.1 ± 2.26% vs. 52.9 ± 1.79%)
Basile AM. et. al, 1997(Basile et al., 1997)	Italy		Lymphocytes		Positive association	DNA hypermethylation characterized the AD individuals.
LUMA						
DiFrancesco A. et al, 2015(Di Francesco et al., 2015)	Italy, n=81, 79.5 ± 6.33 years, M and W	37	PBMCc		Positive association	Global DNA methylation levels were significantly increased in patients with LOAD compared to controls (p=0.0122).

Table 1. Global DNA methylation in Alzheimer's disease and Parkinson's disease (*continued*)

Author	Population	No of cases	Tissue	Adjustment	Association	Comment
H2						
Anderson KW et al. 2015(Anderson & Turko, 2015)	USA, n=16/ 72-92.1 years old, M and F	6	Post-mortem frontal cortex		No difference	No difference in isoforms K/R99 or without K/R99
H3						
Zhang K et al 2012(Zhang et al., 2012)	USA, n=15, 54-101 years, M and W	11	Temporal lobe		Inverse association	Histone H3(H3K18/ K23) acetylation in AD cases was lower than in controls (six fold and $p<0.02$). This study also showed that SRM-based targeted proteomics, compared to western blot method and LC-MS/ MS-TMT, showed higher throughput and therefore promises to be more suitable for clinical applications.
Rao JS et al. 2012 (Rao et al., 2012)	USA, n=20, 70.4 ± 2.4, Gender not specified	10	Post-mortem frontal cortex (Brodmann area 9)		Positive and no association	H3 phosphorylation was increased in AD brains compared to age-matched controls. No difference was observed in H3 acetylation.
Anderson KW et al. 2015(Anderson & Turko, 2015)	USA, n=16/ 72-92.1 years old, M and F	6	Post-mortem frontal cortex		No difference	K4- and K9-acetylated H3 did not show statistically significant changes between AD and control
Naryan PJ et al. 2015(Narayan et al., 2015)	New Zeland, n=67, 75.4 ± 9.2, W and F	29	Post-mortem inferior temporal gyrus		Positive association	Acetyl histone H3 and acetyl histone H4 levels, as well as total histone H3 and total histone H4 protein levels, were significantly increased in post-mortem Alzheimer's disease brain tissue compared to age- and sex-matched neurologically normal control brain tissue. The increase in acetyl histone H3 and H4 was observed in Neuronal N immunopositive pyramidal neurons in Alzheimer's disease brain.
H4						
Anderson KW et al. 2015(Anderson & Turko, 2015)	USA, n=16/ 72-92.1 years old, M and F	6	Post-mortem frontal cortex		Positive and no difference	K8-, K12- and K16-acetylated H4 did not show statistically significant changes between AD and control. However, there was a 25% increase in K12- and K-16 acetylated H4.
Plagg B et al. 2015(Plagg et al., 2015)	Austria, n=80, age and sex not defined	34	Monocytes		No difference	No difference in H4K12 acetylation was observed between AD patients and controls.

Table 1. Global DNA methylation in Alzheimer's disease and Parkinson's disease (*continued*)

Author	Population	No of cases	Tissue	Adjustment	Association	Comment
PARKINSON'S DISEASE						
LINE-1 methylation						
Nielsen SS et al. 2012 (Nielsen et al., 2012)	USA (n=693)/ (66.7 ± 9.5) years, M and W	292	PBMCs	Age, sex and smoking	No association	No association was observed between LINE-1 methylation and the presence of PD (p>0.40).
Histone						
Gebremedhin KG, et al. 2016(Gebremedhin & Rademacher, 2016)	USA, n=17, 71-87 years, M and W	9	Primary motor cortex		Positive and no difference	There was net increase inhistone H3 acetylation due to increased H3K14 and H3K18 acetylation. There was a decrease in H3K9acetylation. No between-groups difference was detected in H3K23 acetylation
Park G et al. 2016(Park et al., 2016)	USA, n=10, 67.8 to 79.2 years, M and W	5	Postmodern midbrain tissues	Age and sex	Positive	Levels of histone acetylation (H2Ak5, H2Bk15, H3k9, and H4k5) are markedly higher in midbrain dopaminergic neurons of PD patients compared to those of their matched control individuals.

Gene Specific DNA Methylation

DNA methylation, the addition of a methyl group to the 5' position of cytosine in a dinucleotide CpG site, is an important mechanism in gene expression regulation. The direction of association between DNA methylation and gene expression depends on where within the gene sequence the methylation occurs. DNA methylation in the promoter region of the gene down-regulates its expression whereas higher methylation in the gene body may promote the expression of the gene (Jones, 2012). However, in most instances DNA methylation represses gene expression. It is thought that methylation of DNA either directly prevents binding of DNA transcription factors, or it recruits proteins that bind to methylated DNA. Recruiting proteins may prevent transcription by influencing chromatin structure by histone modification (Curradi, Izzo, Badaracco, & Landsberger, 2002; Jones, 2012). The effects of DNA methylation allow for the evaluation of gene function by comparing individuals who have the methylated or unmethylated versions of a gene. These methylation patterns can be studied both in a candidate gene approach or a genome-wide approach.

Candidate Gene Studies

(i) Alzheimer's disease

Thirty-four studies examined methylation sites in or near known candidate genes for AD susceptibility in relation to AD (**Table 2**). The 34 studies showed that AD cases, compared to controls, have a higher degree of methylation of *OPRK1, UQCRC1*, *AR*, *BDNF* and *HTERT* in blood cells, *BDNF*, synaptophysin gene, *CREB* promoters, *APOE, TREM 2*, *TBX2AR*, *SORBS3* and *SPTBN4* in the brain, and lower methylation levels of 2-5a-synthetase gene in skin fibroblasts, *PIN1*, *FAAH*, *ALOX5* and DR4 gene in blood cells, *TNFA*, *COX-2*, NF-kβ gene and *S100A2* and *CRTC1* in the brain tissue. The most consistently reported epigenetic associations with AD were that of methylation at *BDNF* (Chang et al., 2014; Nagata et al., 2015; Rao et al., 2012) in both blood and brain tissue, and at *SORBS3* (Sanchez-Mut et al., 2013; Siegmund et al., 2007) in the frontal cortex, which were reported in three and two studies respectively. However, one study (Siegmund et al., 2007) did not find a difference in DNA methylation of *BDNF* gene in AD brain compared to healthy controls. The most studied epigenetic mark in relation to AD was the methylation pattern of the *APP* gene. The *APP* gene was investigated in five studies: three studies (two studies using brain samples (Iwata et al., 2014; West, Lee, & Maroun, 1995) and one study using blood cells (Hou et al., 2013)) showed hypomethylation of *APP* in AD cases compared to controls. Alternatively, two studies (Barrachina & Ferrer, 2009; Brohede, Rinde, Winblad, & Graff, 2010) showed no difference in DNA methylation of *APP* in brain tissue between AD and healthy controls. Fourteen studies found no difference or clear pattern in methylation of the following genes: *12-LOX* (Rao et al., 2012), debrin-like protein gene (Rao et al., 2012), p450 epoxygenase gene (Rao et al., 2012), *MAPT*, *PSEN1*, *UCHL1*, *SST* (Grosser, Neumann, Horsthemke, Zeschnigk, & Van de Nes, 2014), *SSTR4* (Grosser et al., 2014), *F2RL2* (Sanchez-Mut et al., 2013), *SOD-1* (West et al., 1995) and *GRN* (Banzhaf-Strathmann et al., 2013) in brain tissue; *PS1* (Hou et al., 2013), *PS2* (Hou et al., 2013) and tau1 (Hou et al., 2013), *SMARCA* 5 (P. N. O. Silva et al., 2008), *CHD1* (P. N. O. Silva et al., 2008), *BDNF* (Carboni et al., 2015), *SIRT1* (Carboni et al., 2015), *PSEN1* (Carboni et al., 2015{Tannorella, 2015 #2823), genes involved in DNA repair (Coppedè et al., 2016), genes involved in homocysteine pathway (Tannorella et al., 2015), *CTSB* (Ma, Tang, & Lam, 2016), *CTSD* (Ma et al., 2016), *DDT* (Ma et al., 2016), *TSC1* (Ma et al., 2016), *NRD1* (Ma et al., 2016)

and *NDUFA6* (Ma et al., 2016) in blood cells; *HSPA8* (P. N. Silva et al., 2014), *HSPA9* (P. N. Silva et al., 2014), *ApoE4* (Hou et al., 2013; Iwata et al., 2014), *SNAP25* (Furuya et al., 2012), *SORL 1*, *SIRT1* and *SIRT3* (Furuya et al., 2012; Hou et al., 2013; P. N. O. Silva et al., 2008) in both blood cells and brain tissue (**Table 2**). However, 7 studies showed differences in methylation patterns of CpG sites (within same gene some CpG sites were hypomethylated and some others were hypermethylated, in AD cases) examined at the following genes: *SORL1* (Yu et al., 2015), *ABCA7* (Yu et al., 2015), *SLC2A4* (Yu et al., 2015), *BIN1* (Yu et al., 2015), *HSPA8* (P. N. Silva et al., 2014), *HSPA9* (P. N. Silva et al., 2014), *DR4* gene (Ai et al., 2014), *BDNF4* (Chang et al., 2014; Nagata et al., 2015), *SIRT1* (Hou et al., 2013), *APP* (Iwata et al., 2014), *MAPT* (Iwata et al., 2014) and *GSK3B* (Iwata et al., 2014).

(ii) Parkinson's disease

There were 13 studies that examined methylation sites in or near known candidate genes for PD susceptibility (**Table 3**). Overall the studies looking at PD found lower levels of methylation of *NAPS2* and *NOS2* in blood cells and of *ADORA2A* in the brain tissue of PD cases, and higher levels of methylation of PGC–1α gene in brain tissue of PD patients. Six studies examined the methylation pattern of α-synuclein gene (*SNCA*) in blood and brain tissue in relation to PD: 5 studies (Ai et al., 2014; Jowaed et al., 2010; Matsumoto et al., 2010a; I. Schmitt et al., 2015; Y. Y. Tan et al., 2014) showed significantly decreased levels of methylation in PD patients compared to controls whereas 1 study (Y. Song et al., 2014) found a non-significant decrease in PD subjects. Four studies (Banzhaf-Strathmann et al., 2013; Cai, Tian, Zhao, Luo, & Zhang, 2011; Lin et al., 2012; Y. Tan et al., 2016) did not show any difference in DNA methylation of the following genes: Parkin gene, *DJ-1, PER1*, *PER2*, *CRY1*, *CRY2*, *CLOCK* and *BMAL1* in blood cells and of *GRN* in brain tissue. One study (Coupland et al., 2014) examined DNA methylation of the *MAPT* gene in blood cells and different areas of the brain and showed that the association between DNA methylation of *MAPT* and presence of PD differ by the tissue examined.

Genome-wide analysis

(i) Alzheimer's disease

Six studies looked for differentially methylated sites associated with AD in brain tissue; 1 study also looked in both brain tissue and blood cells (**Table 2**). Up to 1106 CpG sites were reported to be differentially methylated in the brains of AD cases compared to individuals without AD. One study (Bakulski et al., 2012) found 948 CpGs representing 918 unique genes in the frontal cortex were associated with late onset-AD status. In AD cases, there was mainly hypermethylation of genes related to molecular and biological processes involved in transcription, and hypomethylation of genes related to membrane transport and protein metabolism (e.g. *TMEM59*). One study reported that out of 137 CpGs in cortical brain tissue found to relate with the burden of natriuretic amyloid plaques (NP), 22 were also associated with the presence of AD (De Jager et al., 2014). Another study (Humphries et al., 2015) reported 1106 CpGs to be differentially methylated in late onset-AD subjects compared to healthy controls and that 87,3% of the CpG sites were hypomethylated. Among the CpGs found to differ in methylation frequency between AD patients and healthy controls in the initial analysis, only the hypermethylation of *DUSP22* gene in AD cases could be confirmed in the replication set (Sanchez-Mut et al., 2014). Two other studies (Bernstein et al., 2016; Watson et al., 2016) reported that differentially methylated regions in the brain tissue of AD patients were related to genes involved

Table 2. Specific gene methylation in Alzheimer's disease: gene and genome-wide approaches.

Author	Study design	Population/Age range/ Follow-up	Cases
Candidate gene approach			
An S et al. 1994(An, Khanna, & Wu, 1994)	CCS/ Comparison of skin fibroblasts of AD and age/sex-matched controls	N=4* Age and sex unspecified	N=2
Arosio B et al. 2012(D'Addario et al., 2012)	CCS/Comparison of subjects with late onset AD (LOAD) and age-matched controls	Italy, n=60, 79.7 ± 6.3 years, M and W	N=32
Bajic V et. al 2014(Bajic et al., 2014)	CCS/ Comparison of female AD patients and healthy age-matched controls	Serbia, n=20, 68.1 ± 6.5 years, W	N=10
Banzhaf-Strathmann J et al. 2013(Banzhaf-Strathmann et al., 2013)	CCS/ Comparison between AD patients and age-matched neurologically healthy controls.	Multiple countries, n=51, 70.5 ± 7.7 years, M and W.	N=8
Barrachina et. al, 2009(Barrachina & Ferrer, 2009)	CCS/ Comparison of AD (different stages) and controls.	European Brain Bank network (BrainNet Europe II, N=70, 73.1 ± 10.1 years	N=44
Brohede J et al, 2010(Brohede et al., 2010)	CCS	Sweden, n=6, Five men, one woman.	N=6
Chang L Et al, 2014(Chang et al., 2014)	CCS/ comparison of AD patients and age- and gender matched controls	China, n=106, Men and women.	N=44
D'addario C et al, 2012(D'Addario et al., 2012)	CCS/ comparison of LOAD cases and age-matched controls	Italy, n=66, 79.73 ± 7.77 years, Men and women	N=33
DiFrancesco A et al., 2013(Di Francesco et al., 2013)	CCS/ comparison of LOAD subjects with age-matched controls	Italy, n=55, 79.7 ± 6.34 years	N=27
Furuya T. et. al, 2012(Furuya et al., 2012)	CCS/ AD cases compared to healthy elderly and healthy young controls	Canada, Brain (n=22), PB (n=84)/ 62.9 ± 3.4 years, M and W	Brain: N=12 Blood: N=36
Furuya T. et al, 2012 (Furuya et al., 2012)	CCS/ AD cases compared to healthy elderly and healthy young controls	Canada, Brain (n=20), PB (n=79), 63.5 ± 5.1 years, M and W	Brain: N=10 Blood: N=34
Grosser C, et al, 2014 (Grosser et al., 2014)	CCS/ AD cases compared to controls	Netherlands, n=10), 77.5 ± 13.3 years, M and W	N=5
Hou Y. et al, 2013(Hou et al., 2013)	CCS/ AD cases compared to controls	China, n=135, 78.4 ± 13.3 years, M and W	N=63

Tissue type	Methylation sites/methods	Adjustments	Main finding
Skin fibroblasts	2-5A synthetase gene/ Methylation-sensitive restriction enzymes (HpaII).		Hypo-methylation
PBMCs	*PIN1* gene promoter region / bisulphite labelled RT-PCR		Hypo-methylation
PBMCs	Androgen receptor promoter (as a measure of X-inactivation pattern)/ MethySYBR Assay		Hyper-methylation
Human post-mortem brain tissue (frontal cortex)	*GRN* promoter/ Sequenom MassARRAY platform		No difference
Human post-mortem brain tissue	CpG methylation in *MAPT, PSEN1, APP, UCHL1*/ SEQUENOM (Hamburg, Germany) MassArray System. Other: evaluation of effect of post-mortem delay on methylation analysis; comparison to other pathologies (FTD, PD etc.)		No difference
Brain tissue (cortical and cerebellar).	12 CpG sites in the amyloid precursor protein gene (*APP*)/ bisulphite-PCR sequencing by 3100 Genetic analyzer		No difference
PB	*BDNF* promoter (4 CpG islands) / Pyromark Gold Q24 Reagents (Qiagen)	Age and gender matched	Hyper-methylation
PBMCs	Methylation at fatty acid amide hydrolase (*FAAH*) gene promotor (18 CpG sites)/ methylation-specific primer real-time PCR.	Age matched	Hypo-methylation
PBMCs	DNA methylation of *ALOX5* promoter	Age-matched controls	Hypo-methylation
Brain (entorhinal cortex, auditory cortex, hippocampus) and PBMCs	*SORL1* and *SIRT1* gene methylation/ Sequenom EpiTYPER		No difference
Brain (entorhinal cortex, auditory cortex, hippocampus) and PBMCs	*SNAP25* gene methylation/ Sequenom EpiTYPER	*ApoE4* status	No differences
Brain tissue (middle temporal and superior frontal gyrus)	Methylation of *SST* and *SSTR4* promoter CpG islands (27 and 44 CpGs)/ Bisulphite RT-PCR sequencing	Age matched	No difference
PBMCs	CpG islands of *SIRT1* (SI and SII1/SII2) and amplifiable regions of *APP, ApoE4, PS1, PS2* and *Tau* / Bisulphite pyrosequencing (EZ DNA methylation Gold Kit)	Age, sex, scholarity and vascular disease matched	*SIRT1*: Hyper-methylation *APP*: Hypo-methylation *Apoe4, PS1, PS2* and *Tau*: No difference

Table 2. Specific gene methylation in Alzheimer's disease: gene and genome-wide approaches. (*continued*)

Author	Study design	Population/Age range/ Follow-up	Cases
Iwata A et al, 2014(Iwata et al., 2014)	CCS/ AD cases compared to controls	Japan, n=158, 77.4 ± 6.1 years	N=62
Kaut O et al, 2014(Kaut et al., 2014)	CCS/ AD cases compared to controls	Germany, PB, n=105, 69.7 ± 7.6 years Cortical tissue, n=8, 77.15 ± 10.0 years M and W	N=55 and n=4
Nagata T et al, 2015(Nagata et al., 2015)	CCS/ Comparison of AD patients with age-matched controls.	Japan, n=40, 66.5 ± 5.09, M and W	N=20
Sanchez-Mut JV et al. 2013(Sanchez-Mut et al., 2013)	CCS/ Comparison of AD patients with age and gender matched non-AD subjects.	eBrainNet Europe Bank / n=40, 76,5 ± 2,5	N=20
Siegmund KD et al. 2007(Siegmund et al., 2007)	CCS/ Comparison of AD patients with controls (including schizophrenic subjects).	USA, N=58,60-104.3, M and W	N=18
Silva PN et al. 2014(P. N. Silva et al., 2014)	CCS/ Comparison of AD patients with non-AD controls.	Canada, n=79, 75,7 ± 8.2 years, M and W	N=46
Silva PNO et al 2008(P. N. O. Silva et al., 2008)	CCS/ Comparison of AD patients with age matched non-AD controls and young controls.	Brazil, n=145,, 55.32 ± 49 years, M and W	N=45
Wang SC et al. 2008(S. C. Wang, Oeize, & Schumacher, 2008)	CCS/ Comparison of late onset-AD patients with geographical location, ethnicity, age and sex matched non-AD controls	Germany, n=34, , 80,6 ± 9,4 years, M and W	N=24
Wang Y et al. 2014(Ai et al., 2014)	CCS/ Comparison of AD patients with age and sex matched non-AD controls.	China, n=50, 75.44 ± 9.1 (60-90) years. M and W	N=25
West RL et al. 1995(West et al., 1995)	CCS/ Comparison of female AD patients with age-matched controls.	USA, n=3, 83, 74 and 81 years, w	N=12

Tissue type	Methylation sites/methods	Adjustments	Main finding
Brain tissue (cerebellum, anterior parietal lobe and inferior temporal lobe)	203 CpGs for *ACE, APOE, APP, BACE1, GSK3B, MAPT, PSEN1*/ Bisulphite pyrosequencing by Pyromark Q24 analyzer (Qiagen)	Age-matched samples	
PBMCs and cortical tissue	TNF-α promoter. 10 CpGs analyzed by bisulphite sequencing PCR		Cortex: Hypo-methylation PBMC: No difference
PBMCs	*BDNF* promoter 20 CpGs/ bisulfite sequencing		Hyper-methylation
Human post-mortem brain tissue (grey matter of frontal cortex)	*F2RL2, SORB3, SPNB4* and *TBX2AR*/ bisulfite pyrosequencing		*TBX2AR, SORBS3* and *SPTBN4*: Hyper-methylation *F2RL2*: No difference
Human post-mortem brain tissue (temporal and frontal cortex)	50 loci related to central nervous system growth and development (*SORBS3, S100A2, LDLR, MYOD1, MGMT, LZTS1, GDNF, PYCARD, STK11, UIR, CRABP1, PLAGL1, DIRAS3, PGR, SERPINB5, NEUROD2, GAD1, RNR1, ALU, TFAP2A, MINT1, CDKN2A, NTF3, SASH1, PAX8, SYK, NEUROD1, PSEN1, ALU, GABRA2, DRD2, LTBR4, ALU, HOXA1, CALCA, DNAJC15, SMAD3, CDX1, SCGB3A1, MT1A, TNFRSF25, MTHFR, MGMT, FAM127A, AR, LPHN2, ALU, RASSF1, BDNF*)/ bisulfite pyrosequencing.		*SORB3*: Hyper-methylation *S100A2*: Hypo-methylation Other genes: No difference
PB and human post-mortem brain tissue	*HSPA8* and *HSPA9*, 22 and 34 CpGs respectively/ Sequenom EpiTyper MassARRAY		No difference overall, but differentially methylated CpG sites
PB	*SIRT3, SMARCA5, HTERT* and *CHD1* gene/ bisulfite pyrosequencing		*HTERT:* Hyper-methylation *SIRT3, SMARCA5* and *CHD1*: No difference
Human post-mortem brain tissue (prefrontal gyrus frontalis superior) and Blood lymphocytes	12 AD's susceptibility loci (*HTATIP, MTHRF, DNMT1, TFAM, SIN3A, NCSTN, BACE1, APP, PSEN1 APH1B* and *APOE*/ bisulfite pyrosequencing (MALDI-TOF mass spectrometry analysis)		
Blood lymphocytes.	DR4 gene promoter, 2 CpG islands (9 and 13 CpG sites each)/ Bisulfite sequencing		Hypo-methylation
Human post-mortem brain tissue (Brodmann's area 38)	Amyolid precursor protein (*APP*) and superoxide dismutase (*SOD-1*) genes/ Methylation-sensitive restriction enzymes (HpaII).		*APP:* Hypo-methylation *SOD-1*: No difference

Table 2. Specific gene methylation in Alzheimer's disease: gene and genome-wide approaches. (*continued*)

Author	Study design	Population/Age range/ Follow-up	Cases
Rao JS et al. 2012(Rao et al., 2012)	CCS/ Comparison of AD patients with age-matched controls.	USA, n=20, 70.4 ± 2.4 years, Gender not specified	N=10
Yu L et al. 2015(Yu et al., 2015)	CCS/ Comparison of AD patients with non-AD controls.	USA, n=740, 88 ± 6.7 years, M and W	N=447
Carboni L et al. 2015(Carboni et al., 2015)	CCS/ Comparison of AD patients with non-AD controls.	Italy, n=39, 75 ± 7, M	N=20
Celarain N et al. 2016(Celarain et al., 2016)	CCS/ Comparison of AD patients with non-AD controls.	Spain, n=42, 19 to 98 years, M and W	N=30
Coppedè F et al. 2016(Coppedè et al., 2016)	CCS/ Comparison of late onset-AD (LOAD) patients with non-AD controls.	Italy, n=111, 77.1 ± 8.8, M and W	N=56
Ferri E et al. 2016(Ferri et al., 2016)	CCS/ Comparison of AD patients with non-AD controls.	Italy, n=283, 79.4 ± 0.5, M and W	N=176
Foraker J et al. 2015(Foraker et al., 2015)	CCS/ Comparison of AD patients with non-AD controls.	USA, n=25, 83.6 ± 9, M and W	N=15
Ji H et al. 2015(Ji et al., 2015)	CCS/ Comparison of sporadic AD patients with non-AD controls.	China, n=106, 80.4 ± 8.4, M and W	N=48
Ma SL, et al. 2016(Ma et al., 2016)	CCS/ Comparison of AD patients with non-AD controls.	China, n=260, 81.3 ± 7.0, W	N=80
Tannorella P et al. 2015(Tannorella et al., 2015)	CCS/ Comparison of sporadic AD patients with non-AD controls.	Italy, n=223, 76.6 ± 8.2, M and W	N=120

Tissue type	Methylation sites/methods	Adjustments	Main finding
Human post-mortem brain tissue (Brodmann's area 9)	Promoter of *COX-2, BDNF, NF-k*β, *CREB, 12-LOX,* p450 epoxygenase, synaptophysin and debrin-like genes/ Methylation-sensitive restriction enzymes		*COX-2* and *NF-k*β: Hypo-methylation *BDNF*, synaptophysin and *CREB*: Hyper-methylation *12-LOX*, debrin-like protein or p450 epoxygenase: No difference
Human post-mortem brain tissue (gray matter)	28 reported AD loci/ Infinum HumanMethylation 450: Illumina)	Age, sex, batch, bisulfite conversion efficacy, macroscopic and microscopic infarcts and cortical Lewy bodies	Results vary per CpG sites
Peripheral blood	Promoter of Bdnf, Sirtuin 1 (Sirt1) and and Presenilin 1 (Psen1)/ Bisulfite sequencing		No difference
Frozen postmortem hippocampus samples	TREM2 transcription start site (TSS)-associated region / Bisulfite sequencing		Hypermethylation
PB	Genes involved in major DNA repair pathways: OGG1, PARP1, MRE11A, BRCA1, MLH1,and MGMT/ effectivePCR based methylation-sensitive high-resolution melting (MS-HRM) technique	Age, gender and multiple comparison	No difference
Peripheral blood mononuclear cells	Pin1 gene promoter, 5 CpG sites / Bisulfite sequencing	Age and gender	No difference
Postmortem brain, cerebellum, hippocampus, frontal lobe	APOE, 76 CpG sites/ Bisulfite sequencing	Age, sex, disease status, APOE genotype, CpG site, and tissue type, as well as all second-order interactions involving tissue	Hypermethylated
PB	Promoter OPRK1, 3 CpG sites/ Bisulphite pyrosequencing	History of smoking, diabetes and hypertension	Hypermethylated
PB	CTSB, CTSD, DDT, TSC1, NRD1, UQCRC1 and NDUFA6 / Bisulphite pyrosequencing		Hypermethylated and no difference
PB	The promoter/5-UTR regions of PSEN1, BACE1, MTHFR, DNMT1, DNMT3A, and DNMT3B / Bisulphite pyrosequencing	Age at sampling, gender, homocysteine, folate, vitamin B12 and batch	No difference

Table 2. Specific gene methylation in Alzheimer's disease: gene and genome-wide approaches. (*continued*)

Author	Study design	Population/Age range/ Follow-up	Cases
Mendioroz M et al. 2016(Mendioroz et al., 2016)	CCS/ Comparison of AD patients with non-AD controls.	Spain, n=42, age and sex not defined	N=30
Genome-wide approach			
Bakulski K et al. 2012(Bakulski et al., 2012)	CCS/Comparison of subjects with LOAD and age- and gender-matched controls	USA, n=24, 79.8 years (range 69-95) (13 additional matched pairs for the population validation phase, 78.2 years (range 61-95) M and W	N=12/ N=13
De Jager et al, 2014(De Jager et al., 2014)	CCS/ comparison of participants in a prospective cohort study, with post-mortem diagnosis of AD.	USA, n=708, M and W	60.8% (N=430) of subjects met a pathological diagnosis of AD.
Fernandez AF. et al, 2012(Fernandez, Assenov, & Martin-Subero, 2012)	CS/ whole genome methylation "fingerprint" including normal tissues, oncogenic tissues, and non-cancerous disease tissues (such as AD and DLB)	Europe, Asia and North America, n=1628 Men and women	N=11

	Tissue type	Methylation sites/methods	Adjustments	Main finding
	Hippocampus	CRTC1 gene / Bisulphite pyrosequencing		Hypomethylation
	Human post-mortem frontal cortex tissue	Genome-wide DNA methylation profile. 27,578 CpG sites spanning 14,475 genes/ Infinium HumanMethylation27 BeadArray (Illumina) Gene-specific DNA methylation/bisulfite-pyrosequencing on the Qiagen Pyromark MD (Valencia, CA). Other: gene expression, protein quantification	Age and gender	948 CpG sites representing 918 unique genes potentially associated with LOAD disease status (p<0.05). Across these sites the mean methylation difference between cases and controls is 2.9%. Hypermethylation in AD cases of molecular function and biological processes associated with transcription (e.g. RNA polymerase II transcription factor activity). Hypomethylation in AD cases of functions relating to membrane transport and protein metabolism. The CpG site in the promoter of the Transmembrane Protein 59 (*TMEM59*) gene is 7.3% hypomethylated in AD cases.
	Cortical brain tissue	Methylation at 425,848 discrete CpG dinucleotides in 708 subjects (Illumina HumanMethylation beadset) Other: Identification of genes near the associated CpGs.		137 CpGs were found to be associated with the burden of neuritic amyloid plaques (NP) (p<1.20x 10^-7). When corrected for the proportion of neurons and possible measurement artifacts, 71 CpG associations remained. 22 of the NP-associated CpG s were also associated with AD at a genome-wide level of significance, and all displayed at least (p<0.001) some evidence of association with AD. Associated methylated regions included *ABCA7* and *BIN1* genes, which are known AD susceptibility regions.
	Brain tissue and PBMCs	1322 CpG sites/ Golden Gate DNA methylation BeadArray (Illumina), Pyromark Q24 (Qiagen)		No significant difference was found between brain samples from AD patients and normal tissues.

Table 2. Specific gene methylation in Alzheimer's disease: gene and genome-wide approaches. (*continued*)

Author	Study design	Population/Age range/ Follow-up	Cases
Humphries C et. al 2015(Humphries et al., 2015)	CCS/ AD cases compared to healthy controls and diseased controls (DLB)	USA, n=30, 77.0 ± 4.5 years	N=8
Sanchez-Mut JV et al. 2014(Sanchez-Mut et al., 2014)	CCS/ Comparison of AD patients with non-AD subjects.	Spain Discovery set: n=20, 79.7 ± 1.9 Replication set: n=50, 71,7 ± 2,1. M and W	Discover set, n=15 Replication set, n=25
Bernstein AI et al. 2016(Bernstein et al., 2016)	Comparison of AD with control cases	USA, n=11, 78-91, M and W (both discovery and replication set)	N=6
Watson CT et al. 2016(Watson et al., 2016)	CCS/ Comparison of AD patients with non-AD subjects.	USA, n=68, 66-95, M and W	N=34

Tissue type	Methylation sites/methods	Adjustments	Main finding
Brain tissue	DNA methylation analysis including 5,147 CpG sites on 465 genes/ Illumina Infinium HumanMethylation 450 beadchip		1,106 CpG sites differed in LOAD-associated methylation network genes between LOAD and control subjects ($p<0.05$). Hypomethylation was observed in LOAD subjects in 87.3% of these CpG sites.
Human post-mortem brain tissue (grey matter, Brodmann area 9)	Illumina 27K array assay and bisulfite pyrosequencing		In the discovery set, four CpG methylation probes corresponding to 3 individual genes showed a significant difference between AD-cases and controls ($P<0.05$); two hypermethylated CpGs in dual specificity phosphatase 22 (*DUSP22*), 1 CpG in claudin 15 (*CLDN15*) and and 1 CpG in quiescin Q66 sulfhydryl oxidase 1 (*QSCN6*). In the replication set, the hypermethylation of *DUSP22* was confirmed.
Human post-mortem brain tissue (frontal cortex)	5-methylcytosine and 5-hydroxymethylcytosine (5hmC)		There were 325 genes containing differentially hydroxymethylated loci (DhMLs) in both discovery and replication datasets. These are enriched for pathways involved in neuron projection development and neurogenesis.
Bulk tissue samples from the superior temporal gyrus	461,272 autosomal CpGs / HumanMethylation450 platform	AOD, gender, race, array/batch, and neuronal/ glial cell composition.	There were 479 differentially methylated regions (DMR) ((increased in AD; hyper-DMRs = 321, hypo-DMRs = 158), with relevant roles in neuron function and development, as well as cellular metabolism. Top DMRs were close to following genes: MOV10L1, B3GALT4, DUSP6, TBX15, HLA-J, ZNRD1-AS1, PRDM16, ELOVL1, RIBC2, SMC1B, KLK7, TRIM6, FBRSL1, VAX2, CDH23, KIF25, NRG2, RNF39, CMYA5, TNXB, NAV2, TAP2, ZNF177, FLOT1

Table 3. Specific gene methylation in Parkinson's disease: gene and genome-wide approaches.

Author	Study design	Population/Age range/ Follow-up	Cases	Tissue type
Candidate gene approach				
Ai SX. et al, 2014(Ai et al., 2014)	CCS/ Comparison between PD patients and neurologically healthy controls	China, n=195, 61.8 ± 9.7 years, M and W	N=100	PBMCs
Banzhaf-Strathmann J et al. 2013(Banzhaf-Strathmann et al., 2013)	CCS/ Comparison between PD patients and age-matched neurologically healthy controls.	Multiple countries, n=51, 70.5 ± 7.7 years, M and W.	N=8	Human post-mortem brain tissue (frontal cortex)
Cai M. et al, 2011(Cai et al., 2011)	CCS/ Comparison between PD patients (with and without heterozygous Parkin gene mutations) and neurologically healthy controls	China, n=44 Men and women	N=34 (17 with heterozygous Parkin gene mutations and 17 without)	PBMCs
Coupland K.G. et al, 2014(Coupland et al., 2014)	CS	Australia (n = 1442 leukocyte samples + 109 PD brain tissue DNA samples)	N=386	Leukocyte DNA and brain tissue DNA
Jowaed A et. al, 2010(Jowaed et al., 2010)	CCS/ Comparison between PD patients and neurologically healthy controls	Germany, n=26, 77,5 ± 3,8 years, M and W	N=12	Brain tissue (substantia nigra pars compacta (SNpc) and cortex and putamen)
Song Y et al. 2014(Y. Song et al., 2014)	CCS/ Comparison of PD patients with age, gender, ethnicity and area of residence matched controls.	China, n=100, 72.26 ± 7.6 years, M and W	N=50	Blood leucocytes
Lin Q et al. 2012(Lin et al., 2012)	CCS/ Comparison of PD patients with age and gender non-PD controls.	China, n=386, 66.2 ± 3.4 years, M and W	N=206	Blood leucocytes

	Methylation sites/ methods	Adjustments	Main finding
	23 CpG sites in the *SNCA* gene/ Bisulphite pyrosequencing (Epitect Bisulfite Kite, Qiagen) Other: genotyping of Rep1 (polymorphic dinucleotide repeat upstream of SNCA), rt-PCR of *SNCA*	Age, gender and origin matched	Hypo-methylation
	GRN promoter/ Sequenom MassARRAY platform		No difference
	33 CpG sites in the Parkin gene promoter region/ Bisulphite sequencing (EZ DNA Methylation Kit, Zymo Research).	Age, gender and ethnicity matched	No difference
	Six CpGs in the *MAPT* gene. Methylation assessed by bisulphite pyrosequencing (PyroMark Q24, Qiagen) Other: in vitro *MAPT* promoter methylation assay and Vitamin E assay	In leukocytes, adjustment for (amongst others) smoking, L-dopa medication, gender, age, *MAPT* diplotype In brain tissue (cerebellum), adjustment for age, sex and *MAPT* diplotype	Hyper-methylation in the cerebellum. Hypo-methylation in the putamen.
	Bisulphite sequencing of 23 CpG sites in the *SNCA* gene		Hypo-methylation
	α-synuclein gene (*SNCA*), 13 CpGs/ bisulfite pyrosequencing		No difference
	Clock genes (*PER1, PER2, CRY1, CRY2, CLOCK, NPAS2* and *BMAL1*)/ bisulfite pyrosequencing		*NAPS2*: Hypo-methylation Other genes: No difference

Table 3. Specific gene methylation in Parkinson's disease: gene and genome-wide approaches. (*continued*)

Author	Study design	Population/Age range/ Follow-up	Cases	Tissue type
Tan Y et al. 2014(Y. Y. Tan et al., 2014)	CCS/ Comparison of PD patients with age and gender matched non-PD controls.	China, n=200, 65.23 ± 0.12 years, M and W	N=100	Blood leucocytes
Villar-Menendez I et al. 2014(Villar-Menendez et al., 2014)	CCS/ Comparison of PD patients with age matched non-PD controls.	Spain, n=19, 24-85 years, M and W	N=7	Human post-mortem brain tissue (putamen)
Nielsen SS et al. 2015(Searles Nielsen et al., 2015)	CCS/ Comparison of PD cases with non-PD controls.	USA, n=201, 25-65 years, M	N=49	WB
Matsumoto L et al. 2010(Matsumoto et al., 2010a)	CCS/ Comparison of PD cases with non-PD controls.	Japan, n=20, 57-87 years, M and W	N=11	Human post-mortem brain tissue (anterior cingulate, putamen and substantia nigra)
Tan Y et al. 2016(Y. Tan et al., 2016)	CCS/ Comparison of PD cases with non-PD controls.	China, n=80, 62.5 ± 7.8, M and W	N=40	Peripheral blood leukocytes
Su X et al. 2015(Su et al., 2015)	CCS/ Comparison of PD cases with non-PD controls.	USA, n=20, 78.3 ± 8.1, M and W	N=10	Substantia nigra
Schmitt I et al. 2015(I. Schmitt et al., 2015)	CCS/ Comparison of PD cases with non-PD controls.	Germany, n=975, 64.6 ± 9.6, M and W	N=490	PB
Genome-wide approach				
Kaut O. et al, 2012(Kaut et al., 2012)	Case control study/ Comparison between PD patients and neurologically healthy controls	Germany, n=18, 78,6 ± 10,1 years, M and W	N=6	Brain tissue (cortex and putamen)

	Methylation sites/ methods	Adjustments	Main finding
	α-synuclein gene (*SNCA*) (2CpGs islands, 30 CpGs) and *LRRK2* (1 CpG island, 34 CpGs) promoter/ bisulfite Specific PCR-based and bisulfite Specific Cloning-based		Hypo-methylation
	ADORA2A, 3 CpG island, 108 CpG sites/ Sequenom EpiTyper MassARRAY		Hypo-methylation
	NOS2, 3CpGs/ bisulfite pyrosequencing	Age, examiner and experimental plate	Hypo-methylation
	α-synuclein gene (*SNCA*), CpG-2 / bisulfite sequencing		Hypo-methylation
	DJ-1, 2 CpGs / bisulfite sequencing	Age	No difference
	Peroxisome proliferator-activated receptor gamma coactivator–1 α (PGC–1α)/ bisulfite sequencing	Age	Hypermethylated
	α-synuclein	Not clear	Hypomethylated
	Genome-wide methylation. 17,500 individual CpG sites from 14,495 genes (EZ DNA Methylation Gold Kit (Zymo Research) and Illumina Human-Methylation27 BeadChip).		In both cortex and putamen of PD patients, *CYP2E1* was hypomethylated (Mean β-value: 0.37 ±0.27 (control) vs. 0.07±0.06 (PD), p=0.04 and 0.48±0.17 (control) vs 0.07±0.01 (PD), p=0.0005 respectively). This difference remained when the analysis was stratified by gender. In the cortex of PD patients, the gene *PPP4R2* was hypomethylated (0.50±0.30 (control) vs. 0.32±0.05 (PD), p=0.02) in comparison to controls. In the putamen of PD patients, the gene *MGC 3207* was hypomethylated when compare to controls(0.47 ±0.22 (control) vs. 0.16±0.13 (PD), p=0.02).. In the putamen of PD patients, *DEFA1* and *CHFR* were hypermethylated.

Table 3. Specific gene methylation in Parkinson's disease: gene and genome-wide approaches. (*continued*)

Author	Study design	Population/Age range/ Follow-up	Cases	Tissue type
Masliah E et al. 2013(Masliah et al., 2013)	Genome-wide DNA methylation Case control study/ Comparison of PD cases with age matched non-PD controls.	USA (n=11) ND Men and women	N=5	Human post-mortem brain tissue (frontal cortex) and PBL

	Methylation sites/ methods	Adjustments	Main finding
	485386 CpG/ HumanMethylation 450k BeadChip (Illumina # WG-314-1003)		2908 CpG - 174 genes (317 hypermethylated-84 genes and 2591 – 233 genes hypomethylated-90 genes) in the brain and 3897 CpG (476 hypermethylated-127 genes and 3421 hypomethylated-106 genes) in the blood of PD cases were differentially methylated compared to controls. 30% (124/407) of the total autosomal annotated genes differentially methylated presented concordant changes in methylation between blood and brain (63 loci with increased methylation and 61 with decreased methylation), suggesting that a number of methylation changes in PD is shared between brain and blood, positioning these 124 genes that co-varied among tissues as candidates for biomarker discovery. Top 30 loci: hypermethylated in PD: *KCTD5, VAV2, MOG, TRIM10, HLA-DQA1, ARHGEF10, GFPT2, HLA-DRB5, TMEM9, MRI1, MAPT, HLA-DRB6, MAPT, HLA-DRB6, LASS3, GSTTP2* and *GSTTP1*; Hypomethylated in PD: *DNAJA3, JAKMIP3, FRK, LRRC27, DMBX1, LGALS7, FOXK1, APBA1, MAG12, APBA1, SLC25A24, GSTT1, MYOM2, MIR886, TUBA3E* and *TMCO3*. Gene ontology analysis showed that same functional groups were affected in brain and blood, with cell communication and cellular and metabolic processes being the more populated clusters, and including genes related to apoptosis, a molecular pathway largely implicated in PD. Overall methylation patterns of the brain and blood were similar, with more than 80% of the sites reported as differentially methylated being hypomethylated. While there were no differences between brain and blood in CpGs clustering in low-methylated fraction, there were more CpGs in the high-methylated fraction in PD blood in comparison to control subject's blood and also to PD brains ($P<0.001$). CpG neighbourhood context analysis and genomic location distribution was comparable between brain and blood samples and showed that loci with decreased methylation were more likely to locate at CG islands and associated with promoter regions including TSS1500, TSS200 and 1st exon sites; while CpG sites located further away from islands (open sea) and at the gene bodies were more likely to present increased methylation.

in neurogenesis, neuronal projection development and regulation of neuron differentiation, as well as β-amyloid and tau metabolism.

(ii) Parkinson's disease

Two studies conducted an epigenome-wide association study approach for PD. One study reported hypomethylation of *CYP2E1*, *PPP4R2* and *MGC3207* and hypermethylation of *DEFA1* and *CHFR* in the putamen and cortex of PD cases compared to controls (Kaut, Schmitt, & Wullner, 2012). Another study (Masliah, Dumaop, Galasko, & Desplats, 2013) found 2908 CpGs (317 hypermethylated and 2591 hypomethylated) in the brain tissue and 2897 CpGs (476 hypermethylated and 3421 hypomethylated) in the blood cells of PD patients to be differentially methylated compared to controls. The study found that 30% of the differentially methylated sites presented concordant changes in methylation between blood and brain. The identified genes were enriched for genes (known from genome-wide association studies) with epigenetic changes in biological pathways relevant to PD-development, such as cell communication and apoptosis (**Table 3**).

Histone Modifications and Neurodegenerative disorders

(i) Alzheimer's disease

Five studies (Anderson & Turko, 2015; Narayan, Lill, Faull, Curtis, & Dragunow, 2015; Plagg, Ehrlich, Kniewallner, Marksteiner, & Humpel, 2015; Rao et al., 2012; Zhang et al., 2012) examined histone modification in relation to AD. There were no consistent findings on the role of H3 or H4 acetylation in AD (**Table 1**). However, one of the studies (Rao et al., 2012) showed increased H3 phosphorylation in AD brains compared to age-matched controls (**Table 1**).

(ii) Parkinson's disease

There were two studies (Gebremedhin & Rademacher, 2016; Park et al., 2016) examining the role of histone modifications in PD. They mainly showed an increase in levels of histone acetylation in PD patients.

DISCUSSION

We have systematically reviewed the current knowledge about epigenetic associations with Alzheimer's disease (AD) and Parkinson's disease (PD). There is some evidence that DNA methylation may be related to the risk of neurological disease. Among gene-specific studies, DNA methylation at 24 genes was found to be associated with AD, while 7 genes were differentially methylated in PD.

The present review finds inconsistent associations between global DNA methylation and AD. These results are in line with previous studies showing contradictory results when studying the relationship between global DNA methylation and other health outcomes, including cardiovascular disease and diabetes (M. Kim et al., 2010; Luttmer et al., 2013; Muka, Koromani, et al., 2016; Muka, Nano, et al., 2016; Ribel-Madsen et al., 2012; P. Sharma et al., 2008; Wei et al., 2014). The use of different methods for assessing global DNA methylation, including the 5-methylcytosine ratio and the methylation of LINE-1 and Alu repeat elements, may account for some of these differences. LINE-1

and Alu repeat elements are used as a measure of global DNA methylation due to their ubiquitous presence in the genome. However, as they may have different functions, the resulting differences in methylation may explain some of the conflicting results (Nelson, Marsit, & Kelsey, 2011). DNA methylation at Alu is about one-third to one-fourth of methylation at LINE-1. The difference may suggest that epigenetic changes at LINE-1 and Alu measure different traits (Nelson et al., 2011). Global DNA methylation assessed by LUMA modestly correlates with LINE-1 methylation, suggesting that the differences in the reported results may come from the assay used to assess global DNA methylation (Terry, Delgado-Cruzata, Vin-Raviv, Wu, & Santella, 2011). Furthermore, as different tissue types (brain tissue or peripheral blood samples) are assessed between studies, tissue-specific DNA methylation patterns may partially explain the heterogeneous findings. Even within studies performed on brain tissue, samples are obtained from different areas of the brain, including cortical, cerebellar, and hippocampal tissue. This difference may limit comparability of the results as specific brain regions comprise different cell populations (astrocytes, neurons, microglia, oligodendrocytes). Furthermore, the same methylation pattern, depending on its position toward coding gene, can have different effects (Aran, Toperoff, Rosenberg, & Hellman, 2011; Jones, 2012). Therefore, global DNA methylation provides an oversimplified assessment of epigenetic dysregulation, as it neither quantitatively nor qualitatively acknowledges the co-existence of hypo- and hypermethylation within a gene or distinct genes within the same cell.

In our review, several genes were found to be differentially methylated in brain tissue or peripheral blood of AD patients when compared to controls. In particular, brain derived neurotrophic factor (*BDNF*) and *SORBS3* were each found in two different studies to be significantly more methylated in AD patients than in controls. These results parallel previous studies showing an association between *BDNF* hypermethylation in blood and depression, depressive symptoms and antidepressants response (Januar, Saffery, & Ryan, 2015). Similarly, previous studies have reported hypermethylation of *BDNF* and of its receptor (Tropomyosin-Related Kinase B) in brains of individuals who have committed suicide (Ernst et al., 2009; Keller et al., 2010). BDNF is a secretory protein with neuroprotective effects (Nagahara et al., 2009) which has been shown to be associated with neurodegenerative diseases, including AD, PD and Huntington's disease (Zuccato & Cattaneo, 2009). *BDNF* was shown to be hypermethylated in the peripheral blood of AD patients compared to controls, indicative of decreased expression of *BDNF.* This is consistent with findings in brain tissue of patients diagnosed postmortem with AD (Rao et al., 2012) and with other studies showing that *BDNF* promoter methylation is related to *BDNF* mRNA expression (Keller et al., 2010). As BDNF is able to cross the blood-brain barrier (Pan, Banks, Fasold, Bluth, & Kastin, 1998), DNA methylation in the peripheral tissue may exert effects on neuronal tissue and vice versa, highlighting the potential utility of peripheral *BDNF* methylation as a biomarker for AD. This is supported by the overlap of epigenetic changes in both AD-brain tissue and peripheral blood reported in this review. *SORBS3* is involved in neuronal signaling (Ito et al., 2007) and regulation of gene expression (Matsuyama et al., 2005), and was found in two studies to be hypermethylated in the frontal cortex of AD patients. However, its role in the pathogenesis of AD and whether methylation of *SORBS3* is consistent across tissue types remains to be investigated.

Also, genes of proteins implicated in AD pathogenesis, such as CREB, were differentially methylated in PD, but the evidence is too limited to draw a firm conclusion. AD is associated with a reduction of CREB activation. CREB is a histone acetyltransferase that functions as a co-activator

that catalyzes histone acetylation, causing a decrease in the transcription of memory-associated genes, and therefore, leading to memory impairment (Teich et al., 2015). Treatment targeting the transcription machinery interacting with CREB during memory formation has been suggested to be a useful strategy for treating AD (Teich et al., 2015). Furthermore, genes of proteins such as death receptor 4 (DR4) and NF-κB are involved in processes that may play a role in the pathogenesis of AD such as apopotosis and/or inflammation. DR4 and NF-κB genes were reported to be differentially methylated in AD cases (Edgunlu, Ozge, Yalin, Kul, & Erdal, 2013; Granic, Dolga, Nijholt, van Dijk, & Eisel, 2009). DR4 might impair the apoptotic signal transduction and may cause apoptosis of brain cells(Edgunlu et al., 2013). Polymorphisms of the DR4 gene have been shown to influence susceptibility to AD (Edgunlu et al., 2013). NF-κB activation is a common feature of many neurodegenerative diseases, particularly of AD (Granic et al., 2009). Activation of NF-κB leads to the expression of a large variety of pro-inflammatory molecules such as cytokines and chemokines, which could be in part responsible for the neurotoxicity seen in AD (Granic et al., 2009). The interaction of methylation of these genes with molecular pathways and how this affects risk of AD remains to be elucidated.

In PD patients, *SNCA* was consistently found to be hypomethylated in both peripheral blood cells and brain tissue. Known to be a causative gene of familial PD (Shulman, De Jager, & Feany, 2011), the overexpression of *SNCA* in sporadic PD cases (Chiba-Falek, Lopez, & Nussbaum, 2006; Grundemann, Schlaudraff, Haeckel, & Liss, 2008; Grundemann, Schlaudraff, & Liss, 2011) suggests a role in the pathogenesis of sporadic PD as well. The finding that *SNCA* is similarly hypomethylated in both peripheral blood and in brain tissues is in line with previous studies and indicates it may be useful as a biomarker in sporadic PD.

Also, several other genes involved in the pathogenesis of PD were reported to be differentially methylated in PD cases, including *NOS2* (hypomethylated), *ADORA2A* (hypomethylated), and *CYP2E1* (hypomethylated). *NOS2*, the gene coding for inducible nitric oxide synthase (iNOS) is primarily regulated at the transcriptional level, at least partially via DNA methylation (Chan et al., 2005). Hypomethylation of CpG sites in the 5′ promoter region of the gene might increase iNOS expression (Chan et al., 2005). Increased iNOS expression in turn promotes inflammation and may lead to PD (Aquilano, Baldelli, Rotilio, & Ciriolo, 2008). In line with this evidence, a selective iNOS inhibitor, GW274150 ([2-[(1-iminoethyl) amino] ethyl]-L-homocysteine) has been reported to have a neuroprotective effect in a model of PD (Broom et al., 2011). *ADORA2A* is the gene coding for adenosine A2A receptor (A2AR), which is highly expressed in the striatum. *ADORA2A* polymorphisms have been inversely associated with PD risk (Popat et al., 2011). Also, A2AR antagonists are effective in relieving parkinsonian motor symptoms and have been suggested as potential new drugs for PD treatment (Armentero et al., 2011). *CYP2E1* codes for Cytochrome P450 2E1, a member of the Cytochrome P450 enzyme family, which represent a major part of the cellular defense against xenobiotic exposure and have been implicated in PD pathophysiology since the mid-1980s (Riedl, Watts, Jenner, & Marsden, 1998). Decreased methylation of *CYP2E1* is related to increased expression of CYP2E1 messenger RNA in PD patients (Kaut et al., 2012). Enhanced CYP2E1 activity has been suggested to contribute to dopaminergic neurodegeneration in PD (Riedl et al., 1998; Viaggi, Vaglini, Pardini, Caramelli, & Corsini, 2009).

This review demonstrated that while epigenetic changes in AD and PD patients have been investigated via global methylation and gene-specific methylation studies, findings are lacking regarding

histone modification. Histone modifications are another epigenetic mark that play a pivotal role in the epigenetic regulation of transcription and other functions in cells, including neurons (Graff, Kim, Dobbin, & Tsai, 2011). Posttranslational histone modifications interfere with the transcriptional program inducing long-lasting phenotypic changes in neural plasticity including learning and memory (Peleg et al., 2010; M. Schmitt & Matthies, 1979). Many enzymes are involved in the regulation of histones including processes such as acetylation, methylation, phosphorylation, sumoylation and ubiquitination, which may play important roles in the pathogenesis of ND (C. Song, Kanthasamy, Anantharam, Sun, & Kanthasamy, 2010). Histone deacetylases (HDACs) has been reported to be active in these processes. Valproic acid, an inhibitor of HDACs, demonstrates neuroprotection against rotenone in a rat model of PD (Monti et al., 2010). Also in AD and PD animal models, histone acetylation has been linked to neurodegeneration (Francis et al., 2009; C. Song et al., 2010). One study in Huntington's disease patients found that most of the identified histone modifications in the brain are associated with genes that have known roles in neuronal signaling (Bai et al., 2015). Those findings suggest that histone modifications may be a relevant form of epigenetic change in patients of neurological diseases. Therefore, much information may still be gained from histone modification studies in AD or PD patients.

The strengths and limitations of the findings from this review merit careful consideration. The present report involves data from nearly 11,453 individuals. It is the first systematic review on the subject that has critically appraised the literature following an *a priori* designed protocol with clearly defined inclusion and exclusion criteria. Using a systemic search in medical databases, few reviews evaluating the role of epigenetics marks in AD and PD were found (Coppede, 2014; Lardenoije et al., 2015; Wullner, Kaut, deBoni, Piston, & Schmitt, 2016). Existing reviews were all narrative reviews (not performed systematically). Narrative reviews do not involve a systemic search and they are often focused on a subset of studies in the chosen area based on availability of the author selection. Therefore, they are more likely to experience selection bias. A number of limitations, however, need to be considered. The majority of studies included in our systematic review are cross-sectional assessments, making it difficult to draw conclusions on causality. Also, studies investigating epigenetic dysregulation in neurological diseases suffer from small sample size, the consequences of which include reduced statistical power and increased false discovery rates. In addition, although most of the epigenetic studies included in this review adjusted for age and sex and sampled from an ethnically homogenous population, a number of analyses are lacking adjustment for lifestyle and environmental factors. Factors including smoking and alcohol consumption are important risk factors for neurological disorders and can alter epigenetic mechanisms. Furthermore, when assessing epigenetic modifications, studies used different techniques, which may produce heterogeneous results. Also, genetically, AD and PD are usually divided into familial cases with Mendelian inheritance and sporadic cases with no familial aggregation (Piaceri, Nacmias, & Sorbi, 2013). The sporadic form is more complex and likely results from a combination of genetic and environmental influences. Therefore, examining whether epigenetic marks may have different role in the etiology of AD and PD types would be interesting (Piaceri et al., 2013). Most of the studies included in this review used post-mortem brain tissue, which can help to provide several insights about the nature of epigenetic medications in relation to neurodegenerative diseases, but can also present several limitations. Using post-mortem brain is problematic with respect to temporality between exposure and outcome(Harrison, 2011).

Second, untangling real effects from confounders (such as medications) can be challenging. Lastly, death often involves acidosis, which can alter genetic material, increasing the likelihood of misclassifying epigenetic modification and increasing the chances of spurious findings (Tomita et al., 2004; Vawter et al., 2006).

CONCLUSION

Overall, the findings from this review indicate that there are significant epigenetic differences between patients with neurodegenerative diseases and healthy individuals. Furthermore, candidate gene studies have shown that some genes known to play a role in maintenance and function of neurological tissues are differentially methylated in diseased individuals. In addition, a number of these genes, such as *BDNF* in AD patients and *SNCA* in PD patients, are similarly methylated in blood and brain tissue. Along the same lines, Epigenetic Wide Association Studies show that differentially methylated sites in neurological disorders present concordant changes in methylation between blood and brain. These data suggest that studies in peripheral blood can provide valuable information on the neuronal epigenetic changes and their consequences on cell function. Therefore, methylation profiling in peripheral blood to identify neurological disorders-related methylated regions has a high potential clinical utility. It may allow clinicians to identify high-risk individuals who may benefit from preventive and therapeutic interventions. However, due to the mostly cross-sectional design of included studies and lack of replication in the case of new findings, there remain many questions about the temporal relation between epigenetic modifications and neurological diseases, as well as the significance of the findings in disease pathology. Also, given the reversible nature of epigenetic aberrations, targeting the epigenome can be a novel preventive strategy and treatment for AD and PD. There is evidence showing that methyl donors such as folate and vitamin B12 may affect DNA methylation and the risk for several neurodegenerative conditions, including AD and PD (Clarke et al., 1998; Luchsinger, Tang, Miller, Green, & Mayeux, 2007). Studies from animal studies show that histone deacetylase inhibitors lowers Aβ levels and improves learning and memory in a mouse model of Alzheimer's disease. Those findings provide support that histone deacetylase inhibitors may serve as a novel therapeutic strategy for AD (Sung et al., 2013). Epigenetic therapy has been shown to successfully reverse several epigenetics marks and disease symptoms and have been approved by the FDA for use in cancer (S. Sharma, Kelly, & Jones, 2010). Therefore, studies in larger cohorts with longitudinal design may help to close the gap on identifying epigenetic changes that have clinical significance and could lead to strategies for intervention in neurological diseases.

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Supplementary Material

Supplementary Material can be found online: http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0167201#sec022

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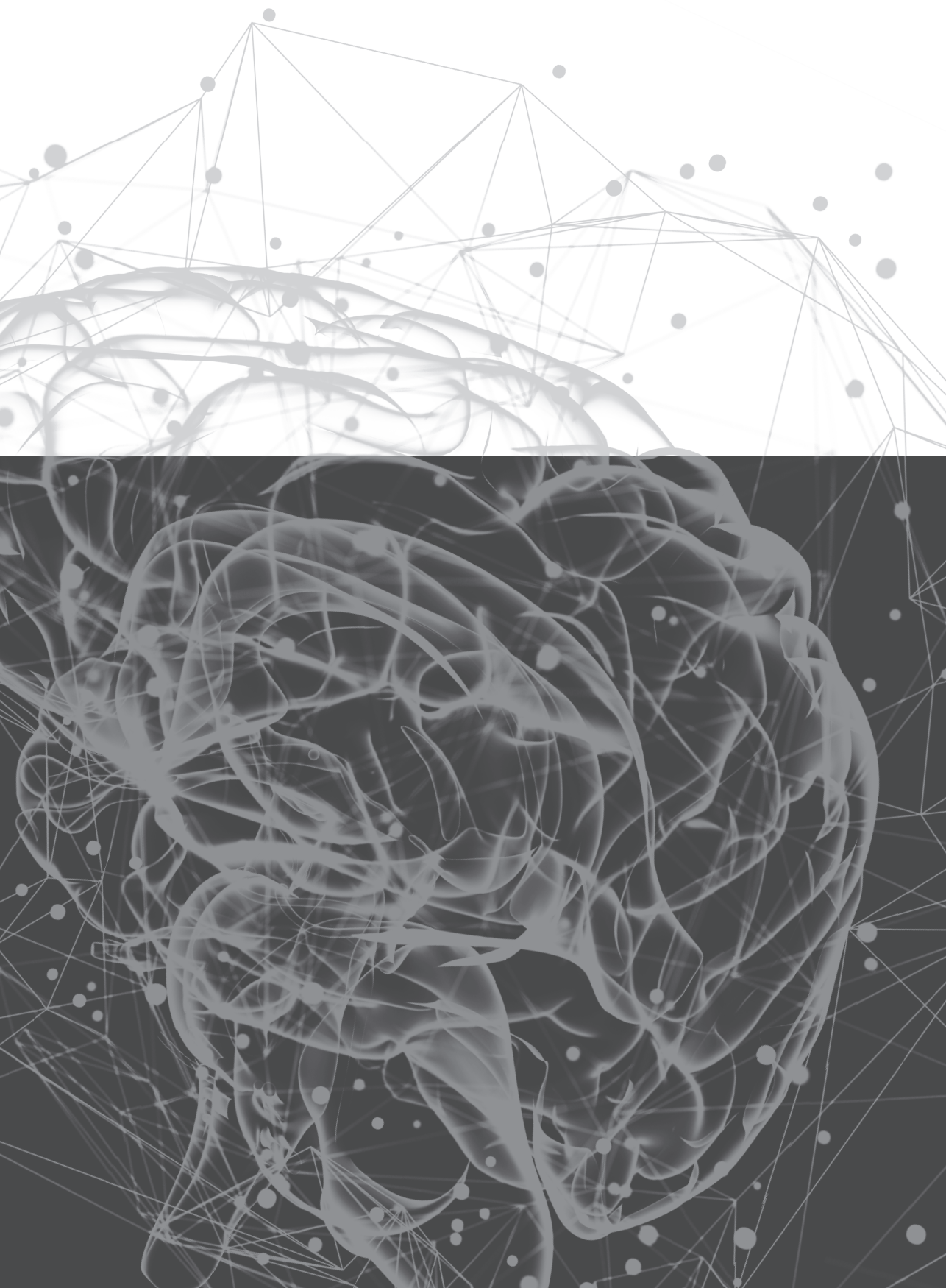
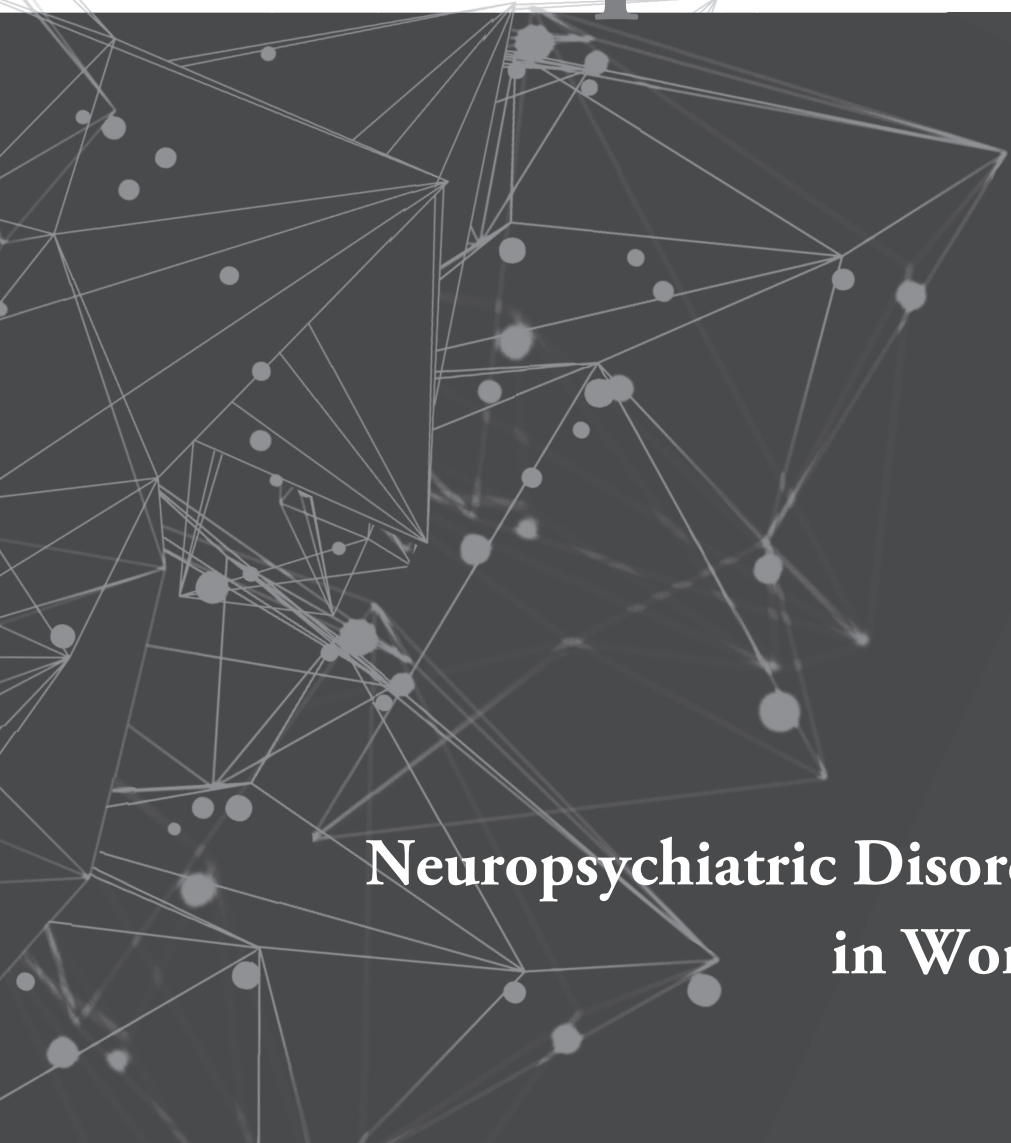


Chapter 3



Neuropsychiatric Disorders in Women

3.1

The Functions of Estrogen Receptor Beta in the Female Brain: A Systematic Review of Current Progress and Future Directions

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ABSTRACT

Females have unique and additional risk factors for neurological disorders. Among classical estrogen receptors, estrogen receptor beta (ERβ) has been suggested as a therapeutic target. However, little is known about the role of ERβ in the female brain. Six electronic databases were searched for articles evaluating the role of ERβ in the female brain and the influence of age and menopause on ERβ function. After screening 3186 titles and abstracts, 49 articles were included in the review, all of which were animal studies. Of these, 19 focused on cellular signalling, 7 on neuroendocrine pathways, 8 on neurological disorders, 4 on neuroprotection and 19 on psychological and psychiatric outcomes (6 studies evaluated two or more outcomes). Our findings showed that ERβ phosphorylated and activated intracellular second messenger proteins and regulated protein expression of genes involved in neurological functions. It also promoted neurogenesis, modulated the neuroendocrine regulation of stress response, conferred neuroprotection against ischemia and inflammation, and reduced anxiety- and depression-like behaviours. Targeting ERβ may constitute a novel treatment for menopausal symptoms, including anxiety, depression, and neurological diseases. However, to establish potential therapeutic and preventive strategies targeting ERβ, future studies should be conducted in humans to further our understanding of the importance of ERβ in women's mental and cognitive health.

INTRODUCTION

Non-communicable diseases including neurological disorders constitute a significant and increasing public health problem (Epstein, Fischer-Elber, & Al-Otaiba, 2007). The global burden of disease due to neurological disorders, measured in disability-adjusted life years, is expected to increase to 103 million DALYs in the next fifteen years; an approximate 10% increase from today's values (Epstein et al., 2007). The highest prevalence of many of the most common neurological disorders, including stroke, dementia, Parkinson's disease, and depression are found in the female population (Hofman, de Jong, van Duijn, & Breteler, 2006). Women are more prone to neurological disorders due to additional and unique risk factors: hypercoagulable states in relation to pregnancy and hormonal contraceptives, as well as longer lifespan predispose women to Alzheimer's disease (AD) and stroke (O'Neal, 2013). In addition, postmenopausal women lose the protective anti-inflammatory effects previously conferred by estrogen, and replenishment of the hypoestrogenic state through hormonal replacement therapy seems to be insufficient.(O'Neal, 2013) Estrogen, including estradiol, has many physiological roles in the body and brain, all of which are mediated by its receptors. A newly-discovered estrogen receptor beta (ERβ) has been found to be widely distributed throughout the brain (Sugiyama, Barros, Warner, & Gustafsson, 2010). Consequently, high expression of ERβ in certain regions such as the hippocampus, amygdala, and dorsal raphe nucleus has raised the question of whether these estrogenic receptors could be used as novel therapeutic agents against common neurological and behavioral disorders (Sugiyama et al., 2010). However, the definitive role of ERβ and its mechanism of action in estradiol-regulated brain areas remain to be further elucidated.

We conducted a systematic review of all the available evidence evaluating the function of ERβ in the female brain and the role of age and menopause on ERβ actions.

METHODS

2.1 Literature Search

This review was conducted using a predefined protocol and was conducted in accordance with the PRISMA and MOOSE guidelines (**eAppendix 1 and 2**). Six electronic databases (Medline, Embase, Web of Science, PubMed, Cochrane and Google Scholar) were searched until September 17th 2015 (date last searched) without any language or study design restriction, with the help of an experienced medical information specialist (WMB). The search strategy combined terms related to exposure (e.g., estrogen receptor beta) and outcome (e.g., nervous system, mental function, depression, AD, Parkinson's disease, cognition). In databases where a thesaurus was available (Embase and Medline), articles were searched by thesaurus terms and in title and / or abstract. In other databases, they were searched only by title and / or abstract. The full search strategies of all databases are provided in **eAppendix 3**. In total, we identified 3186 potentially relevant citations.

2.2 Study Selection and Inclusion Criteria

We included studies that evaluated the function of ERβ in the female brain on at least one of the following outcomes: 1) cellular signaling, including metabolic regulation, regulation of gene expres-

sion in the brain, posttranslational modification, and neurogenesis; 2) neuroendocrine pathways, i.e., hypothalamic-pituitary-adrenal axis; 3) neurological outcomes, such as hippocampal-dependent learning tasks or memory; 4) neuroprotection, including protection against neuroinflammation, and finally; 5) psychological and psychiatric disorders including cognitive function, anxiety-related behavior, aggression, pain, emotions and sexual dysfunction, depression, AD and Parkinson's disease. We considered studies that assessed the function of ERβ via its gene deletion, use of ERβ ligand, for example, diarylpropionitrile (DPN), antibodies/vectors or its expression levels. Studies performed either in female animals or adult humans were also included. We excluded conference abstracts, narrative reviews, studies evaluating the localization of ERβ only, as well as studies that assessed ERβ function in tissues other than the brain. Furthermore, we excluded studies that did not report the sex of participants or that did not show sex-specific results when both males and females were included.

Two independent reviewers screened the retrieved titles and abstracts and selected eligible studies. Any disagreements were discussed and resolved by consensus with the participation of a third investigator. Full texts were retrieved for studies that satisfied all selection criteria.

2.3 Data Extraction

Two reviewers (KGV and AZ) independently performed the extraction of the data. A predesigned extraction form was used to collect relevant information from the selected full-text articles, including lead author, year of publication, sample type, sex, age, and number of participants, menopausal status, brain region involved, method used to assess ERβ function, outcome measures, and results of each study. In case of disagreement, a decision was made through consensus or consultation with a third independent reviewer (TM).

2.4 Outcome Assessment

For each animal study, we defined whether a positive (enhanced), negative (decreased), or null effect was reported. For population-based studies, we reported the effect magnitude, direction and significance.

RESULTS

A total of 3186 potentially relevant citations were identified (Figure 1). After removing duplicates and excluding studies by titles and abstracts, 141 articles were retrieved for detailed assessment. Of these, 92 did not meet our selection criteria. Thus, 49 were included in our analysis.

3.1 General Characteristics of the Included Studies

General characteristics of the included studies are reported in **Table 1**. All studies were performed in animal models (24 used tissues from mice, 24 from rats and 1 used combined tissue from both mice and rats). Of the included studies, 19 focused on the function of ERß on cellular signaling, 7 focused on the neuroendocrine pathway, 4 studied whether neuroprotection was conferred, 8 dealt with neurological outcomes and 19 with psychological and psychiatric outcomes. Six studies evaluated and reported results on two or more major outcomes (Benmansour, Adeniji, Privratsky, & Frazer,

2015; Clark et al., 2012; Kudwa, McGivern, & Handa, 2014; Liu et al., 2008; Raval, Borges-Garcia, Javier Moreno, Perez-Pinzon, & Bramlett, 2013; Rossi et al., 2010).

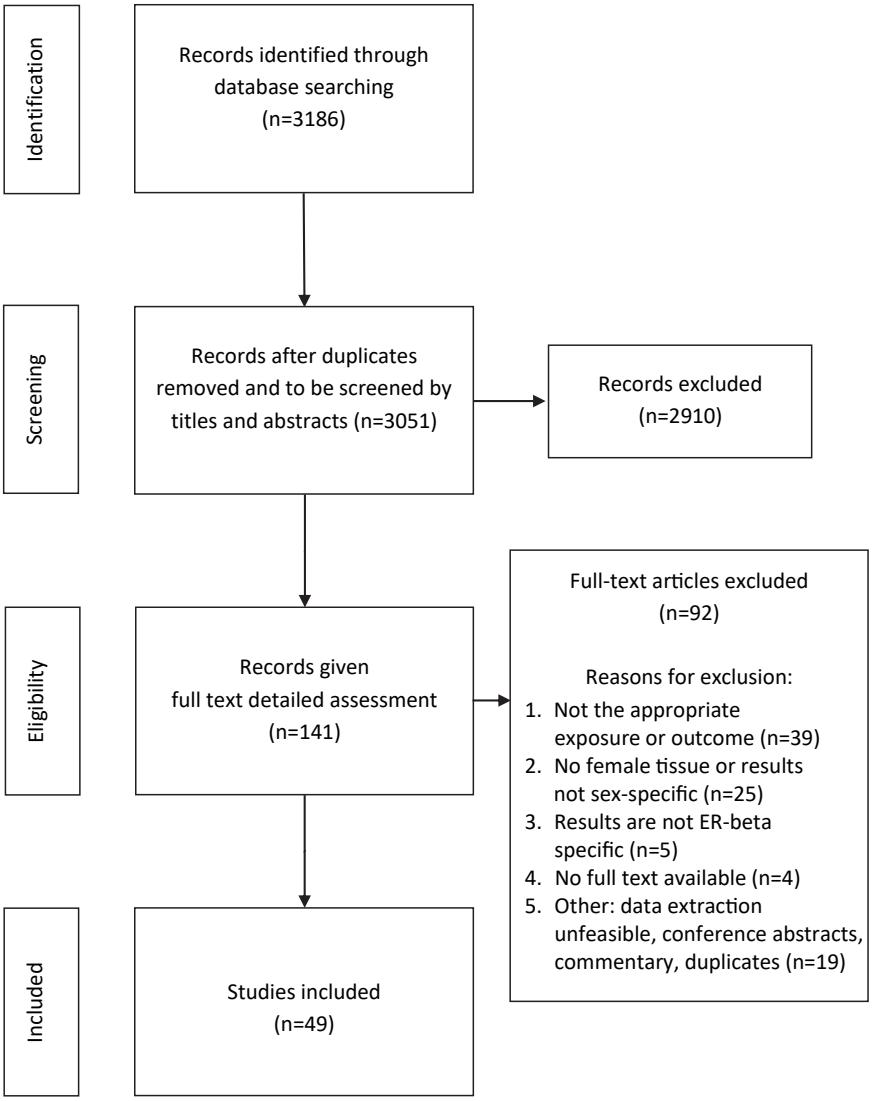

Figure 1 Flowchart of studies investigating the function of estrogen receptor beta in the female brain

Table 1. General Characteristics of the Animal Studies Included in this Review

No.	Author	Animal Type	N	Age	Assessment of ERβ function
1	(Abraham et al., 2003)	Mice	ND	40–54 days	Gene deletion, estradiol (E2)
2	(Abraham et al., 2004)	Mice	ND	ND	Gene deletion; 17-ß-estradiol
3	(Bansal & Chopra, 2015)	Rats (Sprague-Dawley)	8-12	ND	DPN
4	(Bastos et al., 2015)	C57BL/6 mice	ND	8-12 weeks	DPN, E2
5	(Benmansour et al., 2014)	Sprague–Dawley rats	ND	ND	DPN, E2
6	(Benmansour et al., 2015)	Sprague–Dawley rats	12-28	4 months	DPN
7	(Bosch et al., 2009)	Mice	ND	ND	Gene deletion, E2
8	(Boulware et al., 2005)	Rat pups	ND	1- to 2-days-old rat pups	DPN, 17 E- 1
9	(Boulware et al., 2013)	C57BL/6 mice	ND	8–12 weeks of age	DPN, E2, novel object recognition and object placement behavioral tests
10	(C. M. Brown et al., 2010)	Mice	ND	10–15 weeks of age	Gene deletion, E2, cytokine/chemokine quantification.
11	(Carswell et al., 2004)	C57BL/6J mice	44	3 months of age	DPN
12	(Cheong et al., 2012)	Mice	ND	2–3 months	Gene deletion, 17-ß-estradiol
13	(Choleris et al., 2003)	Mice	89	3–5 months old	Gene deletion, social recognition paradigm (tests) and behavioral analysis.
14	(Choleris et al., 2006)	Mice	88	3–4 months	Gene deletion, social recognition paradigm (tests) and behavioral analysis.
15	(Chu et al., 2009)	Mice	ND	2–3 months of age	DPN Estradiol
16	(Clark et al., 2012)	Mice	ND	ND	SERM-beta1, SERM-beta2, E2, gene deletion, forced swim test (FST)
17	(Donner & Handa, 2009)	Sprague–Dawley rats	ND	ND	DPN and behavioral testing
18	(Galvin & Ninan, 2014)	C57/BL6 mice	ND	3-5 months old	DPN

Tissue	Outcome
GnRH neurons in medial septum , rostral preoptic area, and anterior hypothalamus	Cellular signaling
Medial preoptic nucleus; medial septum;; caudate-putamen; the retrosplenial granulate cortex	Cellular signaling: phosphorylation; rapid intracellular signaling
NA	Psychological and psychiatric (depression-like and memory impairment)
Hippocampus	Psychological and psychiatric (depression-like and non-spatial memory)
CA3 region of the hippocampus	Psychological outcomes (mood)
Hippocampus	Cellular signaling: Psychological and psychiatric outcomes (depression-like)
Hypothalamic nuclei and pituitary calcium channel subunit: Cav3.1	Neuroendocrine pathway: excitability of hypothalamic neurons and modulation of pituitary secretion
CA3–CA1 Hippocampal pyramidal neurons	Cellular signaling: CREB phosphorylation
Dorsal hippocampus	Neurological outcomes: (hippocampal memory consolidation)
Cortex and striatum	Neuroprotection: anti-inflammatory effects (mediation of neuroinflammatory response)
Caudate nucleus and dorsal hippocampus	Neuroprotection
GnRH neurons from medial septum (MS) to the anterior hypothalamic area	Cellular signaling
NA	Psychological outcomes: social recognition and anxiety
NA	Psychological outcomes: binary social discrimination
Preoptic area (POA) and hypothalamus	Neuroendocrine pathway: GnRH release
Dorsal raphe nuclei, dentate gyrus in hippocampus	· Psychological outcomes: mood, antidepressive effect. · Neurogenesis
Brainstem (dorsal raphe nuclei)	Psychological outcomes: stress and anxiety-related behaviors
Infralimbic medial prefrontal cortex	Psychological outcomes: fear extinction

Table 1. General Characteristics of the Animal Studies Included in this Review (*continued*)

No.	Author	Animal Type	N	Age	Assessment of ERβ function
19	(Grove-Strawser et al., 2010)	Rat pups	ND	1–2 day old rat pups	DPN
20	(Gundlah et al., 2005)	Mice	ND	14 weeks	Gene deletion, estradiol
21	(Han et al., 2013)	Mice	163	4 months and 13–14 months	Gene deletion; Estradiol (17 ß-estradiol benzoate); Behavioral studies
22	(Imwalle et al., 2005)	Mice	80	10 week	Gene deletion;E2; behavioral testing (the elevated plus-maze test), monoamine HPLC
23	(Irwin et al., 2012)	Sprague–Dawley rats	ND	4–6 months old	DPN, E2
24	(Jacome et al., 2010)	Sprague–Dawley rats	32–34	2 months old	DPN; estradiol benzoate; behavioral tests (object recognition / placement memory tasks)
25	(Krezel et al., 2001)	Mice	ND	8-month-old	Gene deletion; behavioral testing; electrophysiological evaluation of amygdala functions
26	(Kudwa et al., 2014)	Sprague–Dawley rats	118	ND	DPN, behavioral and restraint testing
27	(Le & Belcher, 2010)	Neonatal Rats (Sprague Dawley)	ND	ND	Xenoestrogens, DPN
28	(Liu et al., 2008)	Mice	ND	ND	Gene deletion; WAY-200070 or WAY-202779; behavioral tests
29	(Lynch et al., 2014)	Long-Evans rats	146	90 days old	DPN; behavioral test
30	(Mazzucco et al., 2006)	Sprague-Dawley rats	69	Adults	DPN
31	(W. J. Miller et al., 2004)	Sprague-Dawley rats	5	Adults	Transfected ER beta 1
32	(N. R. Miller et al., 2005)	Sprague-Dawley rats	8	21 days	WAY 200070-2
33	(Morissette et al., 2008)	Sprague-Dawley rats	10	Adults	DPN
34	(Oyola et al., 2012)	Mice	ND	3-4 months	Gene deletion, S-DPN

Tissue	Outcome
Striatal neurons culture	Cellular signaling: CREB phosphorylation
Dorsal raphe nucleus (DRN) in midbrain.	Psychological outcomes: serotonin
Hippocampus	Neurological outcomes: maintenance of hippocampal-dependent memory.
Cingulate cortex, caudate putamen, nucleus accumbens, medial septum, stria terminalis, hippocampus; posterodorsal amygdale; substantia nigra; dorsal raphe, locus coeruleus	Psychological outcomes: modulation of estrogen effects on anxiety and catecholamine concentrations
Isolated brain mitochondria (of forebrain)	Cellular signaling: modulation of mitochondrial function in the brain
NA	Neurological outcomes: mediation of sub-chronic and acute effects of estrogens on recognition memory
Amygdala	Psychological outcomes: emotional behavior
NA	· Neuroendocrine pathway: HPA reactivity. · Psychological outcomes (anxiety-like behaviors).
Immature cerebellar granule cell cultures	Cellular signaling: rapid intracellular signaling
Hippocampus	Neurological outcomes: hippocampal-dependent memory + hippocampal synaptic plasticity
NA	Psychological outcomes: fear generalization
Hippocampus (Dentate gyrus)	Cellular signaling (cellular proliferation)
Hypothalamus	Neuroendocrine pathway
Hippocampus	Neuroprotection
Prefrontal cortex and hippocampal regions	Cellular signaling(NMDA receptor modulation)
NA	Psychological and psychiatric (anxiety-like behaviors)

Table 1. General Characteristics of the Animal Studies Included in this Review (*continued*)

No.	Author	Animal Type	N	Age	Assessment of ERβ function
35	(Raval et al., 2013)	Sprague-Dawley rats	ND	9-11 days	DPN, 17β-Estradiol (E2)
36	(Rocha et al., 2005)	Mice	24	ND	Gene deletion; 17β-Estradiol (E2)
37	(Rossi et al., 2010)	Sprague-Dawley rats	ND	Adults	DPN
38	(Sa et al., 2013)	Wistar rats	34	10 weeks	DPN, EB
39	(Sarvari et al., 2011)	Wistar rats	ND	Middle-aged	DPN, E2
40	(Sarvari et al., 2014)	Harlan-Wistar rats	13	13months	DPN, E2
41	(Spencer-Segal et al., 2012)	Mice	63	9-20 weeks	Gene deletion, estradiol benzoate (EB)
42	(S. Suzuki et al., 2007)	Mice	120	11 weeks	Gene deletion, E2
43	(H. Suzuki et al., 2013)	Mice	10	6 months	Gene deletion
44	(Walf & Frye, 2007)	Long-Evans rats	84	55+ days	DPN; E2
45	(J. M. Wang et al., 2006)	Sprague-Dawley rats	9	4-6 months	DPN
46	(Waters et al., 2009)	Sprague-Dawley rats	ND	Adults	DPN, estradiol benzoate
47	(Zeidan et al., 2011)	Sprague-Dawley rats	35	Adults	DPN
48	(Zhao et al., 2011)	Sprague-Dawley rats 3xTg-AD mice	ND	14-16 weeks 12 months	DPN; 17β-Estradiol
49	(Zhao et al., 2013)	OVX Triple transgenic AD (3xTg-AD) mice (3 months old)	ND	3 months old	Phytoestrogenic ER-ß selective modulator (SERM) Formulation, Y-Maze cognition-behavioral test

DPN, diarylpropionitrile; EB, estradiol benzoate; E2, 17β-Estradiol; NA, not available; ND, not defined.

Tissue	Outcome
Hippocampus	· Neuroprotection (against cerebral ischemia) · Neurological (learning and memory)
NA	Psychological and psychiatric (antidepressant-like effect)
Hypothalamic paraventricular nucleus	· Cellular signaling · Psychological and psychiatric
Hypothalamic ventromedial nucleus	Neuroendocrine pathway
Frontal cortex	Cellular signaling (neuroinflammatory genes regulation)
Hippocampus	Cellular signaling
Hippocampus (dorsal)	Cellular signaling (synaptic plasticity)
Subventricular zone	Cellular signaling (neurogenesis)
Dorsal raphe nucleus	Psychological and psychiatric
Hippocampus	Psychological and psychiatric
Hippocampus	Neurological (ApoE protein expression)
Hippocampus (dorsal region)	Cellular signaling (postsynaptic protein expression)
Ventromedial prefrontal cortex and amygdala	Psychological and psychiatric
Hippocampus	Neurological
Hippocampus, cortex, cerebellum	Neurological

3.2 Cellular signaling:

Nineteen studies evaluated the function of ERβ on cellular signaling (**Table 2A**). Of these, nine studies assessed the effect of phosphorylation in different subunits (Abraham, Han, Todman, Korach, & Herbison, 2003; Abraham, Todman, Korach, & Herbison, 2004; Benmansour et al., 2015; Boulware et al., 2005; Cheong et al., 2012; Grove-Strawser, Boulware, & Mermelstein, 2010; Le & Belcher, 2010; Raval et al., 2013; Spencer-Segal et al., 2012). Within these nine, four studies (Abraham et al., 2004; Benmansour et al., 2015; Le & Belcher, 2010; Spencer-Segal et al., 2012) reported that activation of ERβ was associated with increased immunoreactivity and expression of phosphorylated mitogen-activated protein kinase (MAPK/ERK), protein kinase B (Akt), and tropomyosin receptor kinase B (TrKB) receptor. Also, one study (Cheong et al., 2012) found that ERβ was involved in the phosphorylation of ERK1/2 in gonadotropin-releasing hormone (GnRH) neurons. Akt and TrkB receptors participate in synaptic plasticity involved in spatial memory, cognition, and other hippocampal-dependant behaviors (Spencer-Segal et al., 2012). MAPK/ERK signaling regulates a variety of cellular activities including proliferation, differentiation, survival, and death and has been implicated in the development of AD, Parkinson's Disease, and amyotrophic lateral sclerosis (E. K. Kim & Choi, 2010). Two other studies (Boulware et al., 2005; Grove-Strawser et al., 2010) showed that ERβ, via activation of metabotropic glutamate receptor (mGlur) 2 but not mGlur3, dephosphorylates L-type calcium channels and, therefore causes a reduction in L-type calcium channel-mediated cAMP response element-binding protein (CREB). Furthermore, two studies (Abraham et al., 2003; Cheong et al., 2012) reported an involvement of ERβ in CREB phosphorylation in GnRH neurons whereas another study (Abraham et al., 2004) found regional actions of ERβ on CREB phosphorylation. In the medial preoptic septum, phosphorylation of CREB was found to be dependent solely on ERß, whereas no role of this receptor on CREB phosphorylation was found in hypothalamic ventromedial nucleus, caudate-putamen, or retrosplenial granulate cortex. Moreover, one study tested periodic ERβ-activation on CREB phosphorylation in the hippocampus and showed increased protein expression of phosphorylated CREB (Raval et al., 2013). CREB has been found to be involved in the formation of long-term memories, long-term potentiation, and in the development of drug addiction, physiological dependence and Huntington's Disease (Bourtchuladze et al., 1994; DiRocco, Scheiner, Sindreu, Chan, & Storm, 2009; Nazarian et al., 2009; Rouaux, Loeffler, & Boutillier, 2004; Y. Wang, Ghezzi, Yin, & Atkinson, 2009).

Three studies assessed the function of ERβ on neurogenesis (Clark et al., 2012; Mazzucco et al., 2006; S. Suzuki et al., 2007). The first two studies were performed in the hippocampus under normal conditions, and the third in the subventricular zone after an induced stroke injury. Their conclusions were consistent in that the number of newborn cells was significantly increased with the activation of ERβ, and therefore support a role of ERβ in neurogenesis.

Five studies evaluated gene and protein expression (Irwin et al., 2012; Liu et al., 2008; Sarvari et al., 2011; Sarvari, Kallo, Hrabovszky, Solymosi, & Liposits, 2014; Waters et al., 2009). One study found that ERβ activation was required in order to potentiate mitochondrial function in the brain (Irwin et al., 2012). The second study found that ERβ agonist affected the expression of a specified set of genes (Iba1, Cd68, Cd11b and Cd18) that, in turn, affects neuronal control of microglia and complement expression (Sarvari et al., 2014). Two studies concluded that ERβ increased the regulation of post-synaptic PSD-95 and AMPA-type glutamate receptor subunit GluR1 (Liu et al., 2008; Waters et al.,

2009). One study evaluated the effect of DPN on the modulation of N-methyl-D-aspartate (NMDA) receptor specific binding and found no modulating effect (Morissette, Le Saux, & Di Paolo, 2008). One other study concluded that DPN upregulated the expression of more than 16 neuroinflammatory genes, thus leading to suspicion that ERβ may be a target to supress regulatory functions of glial cells in the E2-deprived female brain and inflammatory diseases. (Sarvari et al., 2011)

Finally, one study assessed whether DPN had an effect on the desensitization of serotonin 1A ($5\text{-}HT_{1A}$) receptor signaling and on the plasma levels of oxytocin and adrenocorticotropic hormone (ACTH) and found no effect.(Rossi et al., 2010)

3.3 Neuroendocrine Pathway Outcomes:

Seven studies evaluated the functions of ERβ on neuroendocrine pathway outcomes (**Table 2B**). Of these, one study found that ERß agonist activates GnRH firing and increases gamma-aminobutyric acid (GABA) transmission and postsynaptic response in GnRH neurons.(Chu, Andrade, Shupnik, & Moenter, 2009)

Two studies evaluated the involvement of ERβ in modulation of hypothalamus-pituitary-adrenal (HPA) axis reactivity.(Kudwa et al., 2014; W. J. Miller, Suzuki, Miller, Handa, & Uht, 2004) The first study found that administration of DPN reduced adrenal corticosterone (CORT) and ACTH responses to restraint stress. Based on the observed findings, the authors concluded that ERβ may modulate HPA/neuroendocrine stress reactivity.(Kudwa et al., 2014) The second study found that ERβ isoforms had a positive effect on regulation of corticotropin-releasing hormone (CRH) promoter activity; indicating possible involvement of ERβ in the mechanisms of the stress response and HPA axis disorders pathogenesis.(W. J. Miller et al., 2004) Furthermore, one study investigated the role of ERβ in oxytocin cells in desensitization of $5\text{-}HT_{1A}$ receptor signalling and showed no effect. (30) Thus, it was inferred that ERβ might not play a role in central regulation of $5\text{-}HT_{1A}$-mediated ACTH release.

The role of ERβ in the induction of progesterone receptors (PRs), which are critical for female sexual behavior, was assessed in two studies.(Clark et al., 2012; Sa, Pereira, Malikov, & Madeira, 2013) One study showed that ERβ activation in hypothalamic ventromedial nucleus averts the action of ERα in the induction of PRs (Sa et al., 2013), whereas one study reported that the ERβ agonist selective estrogen receptor modulator beta 2 (SERM-beta 2) dose-dependently increased PRs in the murine dorsal raphe nucleus but not in the hippocampus.(Clark et al., 2012)

Finally, one study assessed the role of ERβ in the expression and function of T-type calcium channels (subtype Cav3.2) in the hypothalamus. The authors found that the effect of estradiol on Cav3.2 was dependent on ERβ as well as on ERα indicating involvement of ERβ in excitability of hypothalamic neurons.(Bosch, Hou, Fang, Kelly, & Ronnekleiv, 2009)

3.4 Neurological outcomes:

Eight studies evaluated the function of ERβ on neurological outcomes (**Table 2C**). Of these, four investigated memory as their main outcome.(Boulware, Heisler, & Frick, 2013; Jacome et al., 2010; Liu et al., 2008; Raval et al., 2013) One study demonstrated that ERβ in dorsal hippocampus, through activation of mGluR1 signaling, enhanced novel object recognition and object placement memory. (Boulware et al., 2013) Another study (Liu et al., 2008) concluded that along with increased protein expression of the AMPA receptor (AMPAR) subunit GluR1 and PSD-95, ERβ activation induced

morphological changes in hippocampal neurons, including increased dendritic branching and increased density of mushroom-type spines. Furthermore, the same study showed that ERβ improved performance in hippocampus-dependent memory tasks. This finding implies that activation of ERβ could regulate hippocampal synaptic plasticity and improve hippocampus-dependent cognition.(Liu et al., 2008) Jacome at al found that ERβ activation increased recognition memory and altered the levels of monoamines (3-methoxy-4-hydroxyphenylglycol (MHPG) or the MHPG/norepinephrine (NE) ratio), dopamine's metabolite (homovanillic acid (HVA)), and serotonin metabolite (5-hydroxyindole acetic acid (5-HIAA)) in several areas of the brain, including the prefrontal cortex, ventral hippocampus and dentate gyrus, but not in the striatum or medial septum. This finding implies that ERβ may enhance recognition memory through alterations in monoaminergic containing systems primarily in the prefrontal cortex and hippocampus.(Jacome et al., 2010) One study assessed spatial learning, memory, and post-ischemic neuronal survival. It showed improvement in all these outcomes when ERβ agonist (DPN) was given periodically.(Raval et al., 2013)

The four other studies evaluated a neurological outcome based on gene regulation and protein expression.(Han et al., 2013; J. M. Wang, Irwin, & Brinton, 2006; Zhao, Mao, Chen, Schneider, & Brinton, 2013; Zhao et al., 2011) The first investigated the role of ERβ in regulating gene transcription and learning in the hippocampus. It found a positive effect and concluded that ERβ might regulate transcription involved in maintaining hippocampal function during aging.(Han et al., 2013) The second study on gene and protein expression demonstrated a decrease in apolipoprotein E (ApoE) mRNA protein expression (detrimental in AD) with the use of DPN.(J. M. Wang et al., 2006) The third publication studied whether any changes were produced at the insulin-degrading enzyme (IDE) level. IDE is an enzyme involved in the catabolism of amyloid beta (Aβ) protein in the brain and has been associated with the etiology and development of AD. The third publication found that DPN significantly increased IDE in hippocampal neurons via activation of phosphatidylinositol 3-kinase (PI3-K).(Zhao et al., 2011) Finally, the fourth study sought to determine the efficacy of a SERM formulation in the regulation of early stages of AD. A nine-month dietary supplementation of β-SERM formulation was found to promote physical health, prolonged survival and improved spatial recognition memory. It also revealed attenuation of both Aβ deposition and plaque formation in AD mice.(Zhao et al., 2013)

3.5 Neuroprotection outcomes:

Four studies set out to determine the role of ERβ on neuroprotection (**Table 2D**). Three of these considered whether protection against ischemia was conferred either by way of DPN (Carswell, Macrae, Gallagher, Harrop, & Horsburgh, 2004; Raval et al., 2013) or WAY 200070-3.(N. R. Miller, Jover, Cohen, Zukin, & Etgen, 2005) Regardless of the ERβ agonist used, their results were all similar in that protection of neurons in caudate nucleus and hippocampus against global ischemia-induced death was conferred. The fourth study evaluated two outcomes: regulation of cerebral inflammatory cytokine and chemokine levels as well as regulation of the blood brain barrier permeability. The authors found a positive effect for both and concluded that ERβ may also confer protection against neuroinflammation.(C. M. Brown, Mulcahey, Filipek, & Wise, 2010)

3.6 Psychological and psychiatric outcomes:

Nineteen studies assessed the function of ERβ agonists or behavioral testing after gene deletion or a combination of both (**Table 2E**). Of these, three studies considered anxiety-like behavior as their main outcome. The first evaluated anxiety-like behavior and measurement of brain serotonin and dopamine levels. It found enhanced anxiety and decreased concentrations of these neurotransmitters in the absence of functional ERβ.(Imwalle, Gustafsson, & Rissman, 2005) The second studied the contribution of estrogen receptors in modulation of anxiety and analyzed the effect of deleting ERβ gene in mice. Absence of this receptor was associated with expression of anxiety disorders and with a reduced threshold for synaptic plasticity in the amygdala, suggesting a role of ERβ in the processing of emotional behavior.(Krezel, Dupont, Krust, Chambon, & Chapman, 2001) The third study showed that S-enantiomer of diarylpropionitrile (S-DPN) reduced anxiety-like behavior and attenuated stress-induced corticosterone (CORT) and ACTH in wild-type mice, but not in mice lacking the ERβ gene, suggesting anxiolytic effects mediated by ERβ.(Oyola et al., 2012)

Seven studies focused on depression-like behavior.(Bansal & Chopra, 2015; Bastos et al., 2015; Benmansour et al., 2015; Benmansour, Privratsky, Adeniji, & Frazer, 2014; Clark et al., 2012; Donner & Handa, 2009; Rocha, Fleischer, Schaeffer, Rohrer, & Hickey, 2005) The first (Benmansour et al., 2014) described that activation of ERβ in the CA3 region of hippocampus induced by DPN slowed serotonin clearance via MAPK/ERK1/2 signaling and interactions with both TrKB and IGF-1 receptors whereas no role of PI3K/Akt signaling in mediating this effect was shown. These results show an antidepressant-like effect of ERβ.(Benmansour et al., 2014) Moreover, the other six studies used the widely used behavioural assays/tests, Porsolt test or forced swim test and found an anti-depressant effect of ERβ activation.(Bansal & Chopra, 2015; Bastos et al., 2015; Benmansour et al., 2015; Clark et al., 2012; Donner & Handa, 2009; Rocha et al., 2005)

Two other studies evaluated both anxiety- and depression-like behaviors simultaneously. They reached the same conclusion in that both outcomes were decreased after the use of ERβ agonists (Kudwa et al., 2014; Walf & Frye, 2007).

Four studies focused on the role of ERβ in tryptophan hydroxylase (TPH) 1 mRNA expression, which is involved in the synthesis of serotonin. Three of these studies found that ERβ was associated with increased expression of TPH1 (Clark et al., 2012; Donner & Handa, 2009; Gundlah et al., 2005) A fourth study concluded that high TPH levels could be maintained by using the selective ERβ agonist, LY3201) even after ovariectomy.(H. Suzuki et al., 2013)

Two publications dealt with social recognition.(Choleris et al., 2003; Choleris et al., 2006) Both used gene deletion, social recognition paradigm tests, and behavioral analysis. Their results were similar: ERβ is necessary for social discrimination and modulation of social behavior.(Choleris et al., 2003; Choleris et al., 2006)

Three publications looked to study the role of ERβ on extinction recall, which is the process of learning not to fear. Two of these authors concluded that DPN administration facilitated extinction memory consolidation.(Galvin & Ninan, 2014; Zeidan et al., 2011) However, one study found that fear generalization was increased via an effect on fear memory retrieval mechanisms through activation of ERβ.(Lynch et al., 2014)

Table 2A. Estrogen Receptor Beta and Cellular Signaling Outcomes

No.	Author	Outcome	Study Sample	Brain Tissue
1	(Abraham et al., 2003)	Phospho-cAMP response element binding protein (pCREB) CREB phosphorylation MAPK phosphorylation	OVX KO/WT Mice	GnRH neurons in medial septum (MS), rPOA, and anterior hypothalamus
2	(Abraham et al., 2004)	CREB phosphorylation MAPK/ ERK1/2 phosphorylation	OVX KO/WT Mice	Medial preoptic nucleus; medial septum; ventrolateral VNM; caudate-putamen; the retrosplenial granulate cortex
3	(Benmansour et al., 2015)	DPN Phosphorylation of both ERK and TrkB	OVX Sprague-Dawley rats	Hippocampus
4	(Boulware et al., 2005)	CREB phosphorylation L-type calcium channel-dependent CREB phosphorylation L-type calcium channel currents	Intact rat pups of both sexes	CA3–CA1 Hippocampal pyramidal neurons culture
5	(Cheong et al., 2012)	ERK1/2 and CREB phosphorylation in GnRH neurons	OVX GnRH neuron-specific KO/WT Mice and OVX C57BL/6 Mice	GnRH neurons from medial septum (MS) to the anterior hypothalamic area
6	(Clark et al., 2012)	Neurogenesis in the dentate gyrus	KO/WT mice and OVX C57BL/6 mice	Dorsal raphe nuclei, dentate gyrus in hippocampus

Assessment of ERß function	Conclusion	Comment
Gene deletion, estradiol (E2)	No clear pattern, it depends on brain region	The actions of ERß on CREB phosphorylation depends on the brain region: in the medial septum, phosphorylation of CREB was found to be dependent solely on ERß, whereas no role of this receptor on CREB phosphorylation was found in hypothalamic ventromedial nucleus, caudate-putamen and retrosplenial granulate cortex. ERß plays a role in MAPK phosphorylation in the medial preoptic septum but not in the retrosplenial granulate cortex.
Gene deletion; 17-ß-estradiol	No clear pattern, it depends on brain region	The actions of ERß on CREB phosphorylation depends on the brain region: in the medial preoptic septum, phosphorylation of CREB was found to be dependent solely on ERß, whereas no role of this receptor on CREB phosphorylation was found in hypothalamic ventromedial nucleus, caudate-putamen and retrosplenial granulate cortex. ERß plays a role in MAPK phosphorylation in the medial preoptic septum but not in the retrosplenial granulate cortex.
DPN	Increased	DPN did not alter phosphorylation of Akt. All of these signaling pathways may mediate neuroprotection and cognitive function.
DPN, 17 E-1	CRCREB phosphorylation: No effect B) L-type calcium channel-dependent CREB phosphorylation: reduced (attenuated or decreased) L-type calcium channel currents: reduced (attenuated or decreased)	ERβ activates mGlur2 and/or mGlurR3 which leads to diminished cAMP concentrations and a reduction in PKA activity, ultimately resulting in dephosphorylation of L-type calcium channels and a reduction in L-type calcium channel-mediated CREB phosphorylation.
Gene deletion, 17-ß-estradiol	Direct and indirect positive effect in GnRH neurons	E2 acts through calcium/calmodulin-dependent protein kinase type II and protein kinase A to rapidly phosphorylate ERK1/2, which then acts to phosphorylate CREB in adult female GnRH neurons. These effects of E2 are dependent upon both direct ERß mechanisms as well as indirect actions mediated by afferent inputs to GnRH neurons.
SERM-beta1,	Positive effect	ERß stimulates neurogenesis in the dentate gyrus.
SERM-beta2, E2, gene deletion, forced swim test (FST)	Positive effect	

Table 2A. Estrogen Receptor Beta and Cellular Signaling Outcomes *(continued)*

No.	Author	Outcome	Study Sample	Brain Tissue
7	(Grove-Strawser et al., 2010)	L-type calcium channel-mediated CREB phosphorylation Mglur2 and Mglu3	Intact female rat pups	Striatal neurons culture
8	(Irwin et al., 2012)	Mitochondrial DNA-encoded COX I expression;	OVX Sprague–Dawley Rats	Isolated brain mitochondria from forebrain
9	(Le & Belcher, 2010)	ERK phosphorylation	Intact neonatal female Sprague Dawley rats	Immature cerebellar granule cell cultures
10	(Liu et al., 2008)	Protein expression of the AMPAR subunit GluR1 and PSD-95; LTP; Dendritic branching and spine number; Spatial memory	OVX KO/WT Mice	Hippocampus
11	(Mazzucco et al., 2006)	hippocampal neurogenesis (hippocampal cell proliferation)	OVX Sprague–Dawley rats n=95	Hippocampus
12	(Morissette et al., 2008)	Modulation of NMDA receptor specific binding	OVX Sprague–Dawley rats n=50	Prefrontal cortex and hippocampus

	Assessment of ERß function	Conclusion	Comment
	DPN	CRCREB phosphorylation: No effect B) L-type calcium channel-dependent CREB phosphorylation: reduced (attenuated or decreased) C) ERβ activates mGlur3 but not mGlurR2	ERβ activates mGlur2 and therefore leads to a reduction in L-type calcium channel-mediated CREB phosphorylation.
	DPN E2	Positive effect	Activation of ERß is differentially required to potentiate mitochondrial function in brain.
	Xenoestrogens; DPN	Positive effect	Rapid intracellular signaling of estrogen is mediated by ER beta.
	Gene deletion; WAY-200070 or WAY-202779; Behavioral tests	 Positive effect Positive effect Positive effect Positive effect	Activation of ERß can regulate hippocampal synaptic plasticity and improve hippocampus-dependent cognition.
	DPN, E2	Increased	Neurogenesis: (hippocampal cell proliferation) ERß is involved in estradiol-enhanced cell proliferation.
	DPN, E2	No effect	Cellular signaling outcomes: (modulation of NMDA receptors) ERß agonist had no effect on hippocampal NMDA receptor-specific binding and NMDAR2B levels. More potent ERß agonist and/or higher concentrations of DPN may be effective to modulate hippocampal NMDA receptors.

Table 2A. Estrogen Receptor Beta and Cellular Signaling Outcomes *(continued)*

No.	Author	Outcome	Study Sample	Brain Tissue
13	(Raval et al., 2013)	Protection of Hippocampal CA1 Neurons in Oxygen-Glucose Deprivation Model	*In vitro*: brain hippocampal slices from Sprague-Dawley female rats (9–11 days old) *In vivo*: OVX Sprague–Dawley rats	Hippocampus
14	(Rossi et al., 2010)	Desensitization of HT_{1A} receptor signaling.	OVX, rats	Hypothalamic paraventricular nucleus
15	(Sarvari et al., 2011)	Neuroinflammatory genes regulation	OVX Wistar rats	Frontal cortex
16	(Sarvari et al., 2014)	mRNA expression in the hippocampus after gonadal hormone withdrawal	OVX Harlan-Wistar rats n=34	Hippocampus
17	(Spencer-Segal et al., 2012)	Gene transcription and protein expression of pAkt-ir, PSD-95-ir and pTrkBir	OVX KO/WT Mice n=63 9-20 weeks	Hippocampus
18	(S. Suzuki et al., 2007)	Neurogenesis in animal model of ischemic stroke	OVX C57BL/6J mice n=90 11 weeks old. Age-matched OVX KO/WT Mice n=30	Subventricular zone (SVZ) of the brain
19	(Waters et al., 2009)	Expression of synaptic proteins PSD-95 and AMPA-type glutamate receptor subunits GluR1, GluR2 and GluR3.	OVX, rats	Hippocampus

DPN, diarylpropionitrile; EB, estradiol benzoate; E2, 17β-Estradiol; NA, not available; ND, not defined.

	Assessment of ERß function	Conclusion	Comment
	DPN E2 Behavioral tests	Positive effect	Neuroprotection (against cerebral ischemia).
	DPN	No effect	DPN did not alter plasma baseline levels of ACTH or oxytocin.
	DPN, E2	Positive effect	DPN upregulated the expression of more than sixteen neuroinflammatory genes. ERß may be a target to supress regulatory functions of glial cells in the E2-deprived female brain and inflammatory diseases.
	DPN, E2	ERß agonist was capable of attenuating the expression of several macrophage-associated and complement genes.	After ovariectomy, E2 and DPN restore the mRNA expression of genes Iba1, Cd68, Cd11b and Cd18. This shows that estrogen replacement partly restores neuronal control of microglia and modulates complement expression.
	Gene deletion, E2	ERß mediates increase in pAkt-ir expression after E2 treatment lasting 6 hrs. Estradiol tended to decrease PSD-95 in BERKO mice: However, this trend was not statistically significant. ERß mediates increase in pTrkB-ir expression after E2 treatment lasting 48 hrs.	Cellular signaling outcomes (synaptic plasticity). ERß mediates effects of estradiol on pathways important for synaptic plasticity in the mouse hippocampal formation.
	Gene deletion, E2	Enhanced	Neurogenesis: (in an animal model of ischemic stroke). ERß mediates effects of estradiol treatment on increase in number of newborn neurons in the dorsal SVZ of the adult mouse and on enhancement of neurogenesis in an animal model of ischemic stroke.
	DPN	DPN administration increased expression of PSD-95 and AMPA-type glutamate receptor subunit GluR1. DPN administration increased expression of AMPA receptor subunits GluR2 and decreased expression of AMPA receptor subunits GluR3.	Cellular signaling outcomes (expression of synaptic proteins in rat hippocampus) ERβ specific agonists regulate the expression of synaptic proteins in rat hippocampus which is important for hippocampal-dependent cognitive performance.

Table 2B. Estrogen Receptor Beta and Neuroendocrine Pathway Outcomes

No.	Author	Outcome	Study Sample	Brain Tissue
1	(Bosch et al., 2009)	T-type calcium channel (subtype Cav3.2) expression and function	KO/WT Mice and OVX C57BL/6 mice	Hypothalamic nuclei and pituitary calcium channel subunit: Cav3.1
2	(Clark et al., 2012)	Tryptophan hydroxylase-1 (TPH-1) mRNA expression; progesterone receptor expression	KO/WT Mice and OVX C57BL/6 Mice	Dorsal raphe nuclei; dentate gyrus in hippocampus
3	(Chu et al., 2009)	Firing of GnRH neurons GABA transmission	OVX Mice expressing enhanced green fluorescent protein under the control of the GnRH promoter	Preoptic area (POA) / hypothalamus
4	(Kudwa et al., 2014)	CORT and ACTH response to restraint stress	Sprague–Dawley rats of both sexes (OVX females) n=118	NA
5	(W. J. Miller et al., 2004)	regulation of the corticotropin-releasing hormone (CRH) promoter	OVX Sprague–Dawley rats n=5	Hypothalamus
6	(Rossi et al., 2010)	Desensitization of 5-HT_{1A} receptor signaling	OVX Sprague–Dawley rats	Hypothalamic paraventricular nucleus
7	(Sa et al., 2013)	estrogen induction of progesterone receptors (PRs)	OVX Wistar rats (10 weeks of age)	the ventrolateral division of the hypothalamic ventromedial nucleus (VMNvl)

DPN, diarylpropionitrile; EB, estradiol benzoate; E2, 17β-Estradiol; NA, not available; ND, not defined.

Assessment of ERß function	Conclusion	Comment
Gene deletion, Estradiol (E2)	Positive effect	Involvement in excitability of hypothalamic neurons.
SERM-beta1, SERM-beta2, E2, gene deletion, forced swim test (FST)	Positive effect	SERM-beta1 and 2 exhibited antidepressant-like effects / ERß may play an important role in modulating mood
Estradiol, DPN	Supporting effect	ERß agonist activates GnRH firing, reduces after hyperpolarizing potential (AHP) and increased slow afterdepolarization amplitudes (ADP), and reduced I_{AHP} and enhanced I_{ADP}. Also, ERß agonist increased GABA transmission and postsynaptic response in GnRH neuron
DPN, behavioral and restraint testing	DPN reduced CORT and ACTH responses in both males and females.	ERβ may modulate HPA (neuroendocrine stress) reactivity.
Expression vectors: ERß isoforms	Enhanced (Increased) (positive effect)	Neuroendocrine pathway: (regulation of the corticotropin-releasing hormone promoter). ERß regulates CRH in the PVH (paraventricular nucleus) of the hypothalamus.
DPN, recombinant adenovirus-containing ERß siRNAs	No effect	Desensitization of 5-HT_{1A} receptor signaling does not appear to be mediated by ERß in oxytocin cells. ERß together with GPR30, may play a complex role in central regulation of 5-HT_{1A} -mediated ACTH release.
DPN E2	No effect	Neuroendocrine pathway: (induction of progesterone receptors in hypothalamus). Since PR induction by estradiol is required for the display of female sexual behavior obtained data point to the conclusion that activation of the ERß in OVX rats does not facilitate the expression of female sexual behavior.

Table 2C. Estrogen Receptor Beta and Neurological Outcomes

No.	Author	Outcome	Study Sample	Brain Tissue
1	(Boulware et al., 2013)	Hippocampal memory consolidation p42 ERK phosphorylation	OVX C57BL/6 mice (8–12 weeks of age)	dorsal hippocampus
2	(Liu et al., 2008)	Protein expression of the AMPAR subunit GluR1 and PSD-95; LTP; Dendritic branching and spine number; Spatial memory	OVX KO/WT Mice	Hippocampus
3	(Han et al., 2013)	Regulation of gene transcription and learning in the hippocampus	OVX KO/WT Mice n=163	Hippocampus
4	(Jacome et al., 2010)	Recognition memory Brain monoamines levels	OVX Sprague Dawley rats n=32-34	NA
5	(Raval et al., 2013)	Post-ischemic Learning and Memory	*In vitro*: brain hippocampal slices from Sprague-Dawley female rats (9–11 days old) *In vivo*: OVX Sprague–Dawley rats	Hippocampus

	Assessment of ERß function	Conclusion	Comment
	DPN, E2, novel object recognition and object placement behavioral tests	Increased	Neurological outcomes: (hippocampal memory consolidation) ERß/mGluR signaling can mediate the beneficial effects of E2 on hippocampal memory consolidation.
		Increased	
	Gene deletion; WAY-200070 or WAY-202779; Behavioral tests	Positive effect	Activation of ERß can regulate hippocampal synaptic plasticity and improve hippocampus-dependent cognition.
		Positive effect	
		Positive effect	
		Positive effect	
	Gene deletion, estradiol (17 ß-estradiol benzoate); behavioral studies	Positive effect	ERß interacts with estradiol levels to regulate transcription involved in maintaining hippocampal function (hippocampal dependent memory) during aging
	DPN, estradiol benzoate; behavioral tests (object recognition / placement memory tasks)	Positive effect	ERß mediates sub chronic and acute effects of estrogens on recognition memory. Memory enhancements may occur, in part, through alterations in monoaminergic-containing systems primarily in PFC and hippocampus.
		Increased	
	DPN, E2, behavioral tests	Improved by periodic activation of ERß	Neurological outcome: (learning and memory). ERß intracellular signaling mediates improvement of post-ischemic outcome and cognition induced by long-term periodic E2 treatment in the hippocampus of OvX female rats.

Table 2C. Estrogen Receptor Beta and Neurological Outcomes (*continued*)

No.	Author	Outcome	Study Sample	Brain Tissue
6	(J. M. Wang et al., 2006)	Apolipoprotein E expression in hippocampus *in vitro* and *in vivo*	Rat Hippocampal Neurons in Primary Culture / OVX Sprague–Dawley rats n=9 (4-6 months old)	Hippocampus
7	(Zhao et al., 2011)	Insulin-degrading enzyme(IDE) mRNA and protein expression	OVX Sprague–Dawley rats(14 to 16-week-old) A triple transgenic AD(3xTg-AD) mice	Hippocampus, cortex and cerebellum
8	(Zhao et al., 2013)	Regulation of early stages of physical and neurological changes associated with AD	OVX Triple transgenic AD (3xTg-AD) mice (3 months old)	Hippocampus, cortex, cerebellum

DPN, diarylpropionitrile; EB, estradiol benzoate; E2, 17β-Estradiol; NA, not available; ND, not defined.

	Assessment of ERß function	Conclusion	Comment
	DPN	Decreased / ERβ selective agonist, DPN, down-regulated ApoE mRNA and protein expression.	Neurological outcome: AD Use of ER-selective ligands could provide therapeutic benefit to reduce the risk of AD by increasing ApoE expression in ApoE2/3 allele carriers and decreasing ApoE expression in ApoE4 allele carriers.
	DPN, E2	Treatment with DPN increased IDE protein expression in rat hippocampal neurons	Neurological outcome: AD prevention; ERß/PI3-K pathway modulates effects of 17ß-E2 on regulation of insulin-degrading enzyme (IDE) expression in a brain region-specific manner. Such regulatory role in the hippocampus could serve as a direct mechanism underlying estrogen-mediated preventative effect against AD when initiated at the onset of menopause.
	Phytoestrogenic ER-ß selective modulator (SERM) formulation containing equal parts of genistein, daidzein, and equol. Y-Maze cognition-behavioral test	When initiated prior to the appearance of AD pathology, a 9-month dietary supplementation with the phyto- ß -SERM formulation promoted physical health, prolonged survival, improved spatial recognition memory, and attenuated amyloid-ß deposition and plaque formation in the brains of treated AD mice.	Neurological outcome: AD Obtained results support the therapeutic potential of the phyto- ß –SERM formulation for prevention and/or early intervention of AD, and warrant further investigations in human studies.

Table 2D. Estrogen Receptor Beta and Neuroprotection Outcomes

No.	Author	Outcome	Study Sample	Brain Tissue
1	(C. M. Brown et al., 2010)	Regulation of brain cytokine and chemokine levels; Regulation of BBB permeability	OVX KO/WT Mice	Cortex and striatum
2	(Carswell et al., 2004)	Induction of transient global ischemia by bilateral carotid artery occlusion	OVX C57BL/6J mice n=44	Caudate nucleus and dorsal hippocampus
3	(N. R. Miller et al., 2005)	Protection of hippocampal neurons in a global ischemia model	OVX Sprague–Dawley rats (21 days old)	Hippocampus
4	(Raval et al., 2013)	Protection of Hippocampal CA1 Neurons in Oxygen-Glucose Deprivation Model	*In vitro*: brain hippocampal slices from Sprague-Dawley female rats (9–11 days old) *In vivo*: OVX Sprague–Dawley rats	Hippocampus

DPN, diarylpropionitrile; EB, estradiol benzoate; E2, 17β-Estradiol; NA, not available; ND, not defined.

	Assessment of ERß function	Conclusion	Comment
	Gene deletion; E2; Cytokine/ chemokine quantification.	Positive effect	ERß regulates proinflammatory cytokine and chemokine production through E2-dependent and E2-independent mechanisms. ERß is essential for E2-mediated regulation of BBB permeability.
		Positive effect	
	DPN	DPN significantly reduced ischemic damage by 70% in the caudate nucleus and 55% in the CA1 region compared with vehicle controls.	ERß activation is neuroprotective in global cerebral ischemia mouse model.
	WAY 200070-3	ERß-selective agonist WAY 200070–3 produced nearly complete protection of CA1 neurons in approximately 50% of the animals.	Neuroprotection: (protection of hippocampal neurons in global ischemia model) ERß mediates estradiol protection of CA1 hippocampal neurons from global ischemia-induced cell death.
	DPN E2 Behavioral tests	Positive effect	Neuroprotection (against cerebral ischemia).

Table 2E. Estrogen Receptor Beta and Psychological/Psychiatric Outcomes

No.	Author	Outcome	Study Sample	Brain Tissue
1	(Bansal & Chopra, 2015)	Depression-like behavior Memory impairment	OVX Sprague–Dawley rats	NA
2	(Bastos et al., 2015)	Depression-like behavior Non-spatial memory	OVX C57BL/6 mice	Hippocampus
3	(Benmansour et al., 2014)	Assessment of serotonin clearance	OVX Sprague–Dawley rats	CA3 region of the hippocampus
4	(Benmansour et al., 2015)	Depression-like behavior	OVX Sprague-Dawley rats	NA
5	(Choleris et al., 2003)	Social recognition Social anxiety	KO/WT Mice and OVX Swiss Webster mice n=89	NA
6	(Choleris et al., 2006)	Social recognition (binary social discrimination assay)	KO/WT Mice and OVX Swiss Webster mice n=88	NA
7	(Clark et al., 2012)	Tryptophan hydroxylase-1 (TPH-1) mRNA expression; progesterone receptor expression	KO/WT Mice and OVX C57BL/6 Mice	Dorsal raphe nuclei; dentate gyrus in hippocampus
8	(Donner & Handa, 2009)	Tryptophan-Hydroxylase 2 mRNA expression Anxiety-like behaviors Despair-like behavior	OVX Sprague–Dawley rats	Brainstem (dorsal raphe nuclei)

	Assessment of ERß function	Conclusion	Comment
	DPN	DPN markedly prevented memory impairment and decreased immobility time in OVX or diabetic rats	DPN decreased immobility time in the forced swim test when compared to controls. Memory improvement was suggested by DPN decreasing the total distance traveled in a water maze.
	DPN, E2	DPN decreased the immobility time; the depressive-like behavior of mice in the forced swim test. DPN rescued object recognition memory by acting on early stages of memory consolidation.	These effects were observed up to 12 weeks after ovariectomy; demonstrating that 12 weeks of OVX is not a sufficient time to abolish the beneficial effects of E2.
	DPN, E2	The E2-induced slowing of serotonin clearance via activation of ERβ required MAPK/ERK1/2 signaling pathways and involved interactions both with TrkB and IGF-1R.	Activation of ERβ induced by E2 slows serotonin clearance / Antidepressant-like effect of estradiol is mediated by ERβ.
	DPN	DPN at 5 or 10 micrograms induced antidepressant-like effects during the forced swim test.	Higher doses of DPN were not found to produce antidepressant-like effects.
	Gene deletion, social recognition paradigm (tests) and behavioral analysis.	ß-ERKO mice were selectively impaired in social recognition but not in overall activity. The reduced social anxiety was found in the ß-ERKO mice.	Genes for ERß play a crucial role in oxytocin-dependent social recognition. Estrogen modulation of female psychosocial anxiety depends on ER-ß.
	Gene deletion, social recognition paradigm (tests) and behavioral analysis.	ß-ERKO mice showed partially impaired social discrimination.	ERß genes are necessary for social discrimination and, thus, for the modulation of social behavior (e.g., aggression, affiliation).
	SERM-beta1, SERM-beta2, E2, gene deletion, forced swim test (FST)	Positive effect	SERM-beta1 and 2 exhibited antidepressant-like effects / ERß may play an important role in modulating mood
	Systemic and local application of DPN, Behavioral testing	Positive effect Systemic delivery of DPN decreased anxiety-like behavior, while local administration of DPN failed to have the same effect. Local DPN administration in animals showed decreased despair-like behavior. does not alter anxiety-like behaviors, but enhances active stress-coping behavior.	ERß acts at the level of the rat DRN to modulate tph2 mRNA expression and thereby influence 5-HT synthesis in DRN subregions. Local activation of ERß neurons in the DRN may be sufficient to decrease despair-like behavior, but not anxiolytic behaviors.

Table 2E. Estrogen Receptor Beta and Psychological/Psychiatric Outcomes (*continued*)

No.	Author	Outcome	Study Sample	Brain Tissue
9	(Galvin & Ninan, 2014)	Fear extinction	C57/BL6 mice	Infralimbic medial prefrontal cortex
10	(Gundlah et al., 2005)	TPH1 mRNA expression	KO/WT mice and OVX C57BL/6 mice	Dorsal raphe nucleus (DRN) in midbrain
11	(Imwalle et al., 2005)	Anxiety-like behavior Measurement of brain serotonin and dopamine levels	OVX KO/WT mice n=80	Cingulate cortex; caudate putamen; nucleus accumbens; medial septum; stria terminalis; hippocampus; posterodorsal amygdale; substantia nigra; dorsal raphe, locus coeruleus
12	(Krezel et al., 2001)	Anxiety-like behavior 5-HT1a receptor expression in amygdala Synaptic plasticity in the amygdala	KO/WT mice both sexes	Amygdala
13	(Kudwa et al., 2014)	Anxiety and depressive-like behavior	Sprague–Dawley rats of both sexes (OVX females) n=118	NA
14	(Lynch et al., 2014)	Fear generalization	Long-Evans rats	NA
15	(Oyola et al., 2012)	Anxiety-like behaviors	OVX KO/WT Mice 3-4 months of age	Anterodorsal medial amygdala and bed nucleus of the stria terminalis

Assessment of ERß function	Conclusion	Comment
DPN	Increased	ERß activation facilitates the infralimbic medial prefrontal cortex to undergo potentiation; necessary for the regulation of fear extinction.
Gene deletion, estradiol	Positive effect	ERß might be responsible for mediating estrogen regulated TPH1 expression in the murine DRN. / ERß might be a key mediator of estrogen action in serotonergic neurons.
Gene deletion, E2, behavioral testing (the elevated plus-maze test), monoamine HPLC	In the absence of functional ERß female mice exhibited enhanced anxiety and decreased concentrations of 5-HT or dopamine in several brain regions.	ERß may modulate estrogen effects on anxiety and catecholamine concentrations.
Gene deletion, behavioral testing; electrophysiological evaluation of amygdala functions.	ERß mutant females showed increased anxiety. Positive effect Enhanced anxiety is associated with a reduced threshold for synaptic plasticity in the amygdala, principally in female mice.	ERß mediates estrogen signaling in the processing of emotional behavior particularly in females. / ERß may be critically involved in the expression of anxiety disorders to which females may be particularly susceptible.
DPN, behavioral and restraint testing	DPN effectively increased anxiolytic behaviors more effectively in females. Females responded to DPN with an anti-depressive like response whereas males did not respond to treatment.	ERβ may reduce anxiety-like behavior.
DPN, behavioral test.	Increased	DPN at a 2.5 mg dose increased fear generalization through an effect on fear memory retrieval mechanisms by activation of ERß.
Gene deletion, S-DPN, E2, behavioral tests	Reduced by DPN administration	Psychological outcomes: (mediation of anxiety-like behaviors). ERß mediates anxiolytic actions of estradiol.

Table 2E. Estrogen Receptor Beta and Psychological/Psychiatric Outcomes (*continued*)

No.	Author	Outcome	Study Sample	Brain Tissue
16	(Rocha et al., 2005)	Depression-like behavior through total duration of immobility in the Forced Swim Test (FST)	OVX: CD1 Mice (n=171) 129S6 Mice (n=35) KO/WT Mice (n=24)	NA
17	(H. Suzuki et al., 2013)	Expression of tryptophan hydroxylase (TPH) in the Dorsal raphe (DR), Alterations in glucose homeostasis.	OVX KO/WT Mice n=45 6 months old	Dorsal raphe nucleus
18	(Walf & Frye, 2007)	Anxiety and depressive behavior	OVX Long–Evans rats (n=84) (55+ d old)	Hippocampus
19	(Zeidan et al., 2011)	Extinction memory expression during extinction recall (learning not to fear).	Sprague–Dawley naturally cycling rats n=81	Ventromedial prefrontal cortex (vmPFC) and amygdala

DPN, diarylpropionitrile; EB, estradiol benzoate; E2, 17β-Estradiol; NA, not available; ND, not defined

Assessment of ERß function	Conclusion	Comment
Gene deletion, E2, behavioral tests	E2 did not reduce immobility in the FST in BERKO mice	Psychological outcomes: (antidepressant-like effects). ER-β receptors are implicated in the antidepressant-like effects of E2 in mice.
Gene deletion, LY3201 (selective ERß agonist), E2	3-day treatment with LY3201 L has a rapid onset effect on serotonergic activity with increased expression of TPH and ERß. LY3201 only restored TPH expression in the DR when the duration of estrogen deprivation was less than 10 weeks. 3-day treatment with LY3201 causes only mild alterations in glucose homeostasis but effectively restored TPH expression in the DR.	Psychological outcomes: (serotonergic activity). ERß agonists could be useful pharmaceuticals in maintaining functional DR neurons to treat postmenopausal depression.
Estrogen receptor beta-specific selective estrogen Receptor modulators (DPN), behavioral testing	ERβ-selective SERMs to the hippocampus, but not the ventral tegmental area, decreased anxiety and depressive behavior.	Psychological outcomes: (Anxiety and depressive behavior) Administration of estrogen receptor beta-specific selective estrogen receptor modulators to the hippocampus decrease anxiety and depressive behavior of ovariectomized rats.
DPN and behavioral tests (extinction recall learning trials) in animals	Administration of the estrogen-receptor beta agonist facilitated extinction recall.	Psychological outcomes: (fear extinction recall) ERß modulates effects of estradiol on facilitation of extinction memory consolidation in a time-dependent manner in rodents.

DISCUSSION

This review summarizes 49 studies published worldwide that investigate the functions of ERβ in the female brain. The studies suggest multiple functions of ERβ in the female brain that might contribute in a diversity of normal neurophysiologic functions **(Figure 2)**. Our results support non-genomic actions of ERβ on the phosphorylation status and activity of multiple different signaling pathways in the brain. ERβ may act in the female brain through MAPK/ERK1/2 and PI3K/Akt signaling pathways and interactions of ERβ with TrkB and IGF-1R may facilitate activation of these kinases. Akt and TrkB are markers of hippocampal synaptic plasticity.(Nakai et al., 2014) Spenser-Segal et al have demonstrated that ERβ mediates the increase in pAkt-ir expression after treatment with estradiol for 6 hours. The kinase pathways in the brain play a role in cell differentiation, survival, apoptosis, and cell proliferation among other functions, all of which might determine the initiation and progression of neurodegenerative disorders.(Spencer-Segal et al., 2012) Also, ERβ interacts with metabotropic glutamate receptors (mGluRs) signaling, which may depend upon caveolin proteins that are essential for the trafficking and clustering of signaling molecules.(Boulware et al., 2005) Among mGluRs, ERβ may activate cell membrane-localized mGluRs type 2 and 3. Their activation would lead to decreased concentrations of cAMP and a reduction in PKA activity, which would, in turn, result in

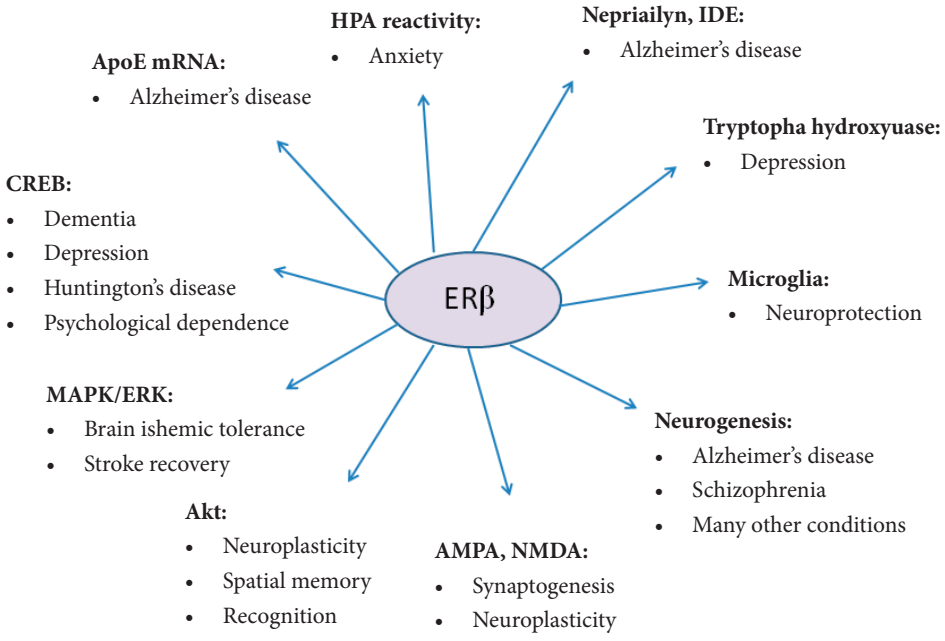

Figure 2 Potential pathways for ERβ function in the female brain.
Legend: *mRNA, Apolipoprotein E messenger RNA; HPA, hypothalamus-pituitary-adrenal; IDE, insulin-degrading enzyme; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NMDA, N-Methyl-D-aspartic acid; Akt, Protein kinase B; MAPK, Mitogen-activated protein kinase; ERK, extracellular signal-regulated kinases; CREB, c-AMP response-element binding protein.*

dephosphorylation of L-type calcium ion channels and a reduction of L-type calcium ion channel-mediated CREB phosphorylation. Phosphorylation of CREB in the medial septum, for example, is undoubtedly due to action of ERβ since it is the only estrogen receptor type expressed in that part of the brain. Alternatively, other regions devoid of both ERα and ERβ, such as the caudate putamen or granulate cortex, do not show signs of CREB phosphorylation. CREB-mediated transcription has been described as promoting synapse formation and transmission in long-term memory and conferring protection against striatal neuronal death. Both of these attributes may be used as rationale for potential therapeutic interventions against Huntington's disease.(Choi et al., 2009; Liu et al., 2008) Accordingly, an alteration of CREB functioning has also been associated with major depressive disorder, drug addiction and psychological dependence; conditions which may be better treated with a more thorough understanding of ERβ's biological function.(Belmaker & Agam, 2008; McPherson & Lawrence, 2007) Similarly, mGluRs act as a second messenger in the signaling pathway that modulates synaptic transmission and neuronal excitability, suggesting a potential therapeutic utility in AD, Parkinson disease, schizophrenia, anxiety and depression.(Niswender & Conn, 2010; Pomierny-Chamiolo et al., 2014) Consequently, further research into these components may prove to be potential opportunities for treatment for Huntington's disease, AD, epilepsy, and trauma.(Boulle et al., 2012; Chong, Shang, Wang, & Maiese, 2012)

The selected studies in this review consistently showed that ERβ agonists increased hypothalamic proteins PSD-95, synaptophysin and AMPA-receptor subunit GluR1, and dendritic branching and mushroom-type spines. Altogether, these effects of ERβ may suggest a role of this receptor in enhancing synaptic plasticity by persistent strengthening of synapses (long-term potentiation) and improving hippocampus-dependent memory and cognition (13,21). Further evidence in this systematic review demonstrates that mGluR1 from the dorsal hippocampus interacts with other membrane components such as p42 ERK and Gq proteins to enhance novel object recognition and object placement memory consolidation (27,29). These may constitute potential targets for treatment of memory impairment conditions. The effect of ERβ on object recognition and object placement memory tasks, however, may only be exerted in certain regions of the brain and in response to a dose-dependent administration of estradiol. In this regard, ERβ and its agonist DPN have been associated with alteration of the monoamines 3-methoxy-4-hydroxyphenylglycol (MHPG) and metabolites of dopamine and serotonin: homovanillic acid (HVA) and 5-hydroxyindole acetic acid, respectively. These findings were noted only in the prefrontal cortex and dentate gyrus but not in the striatum or medial septum. (Jacome et al., 2010) Although ERβ is present in many areas of the neocortex, it is highly expressed in the hippocampus and frontal cortex, areas that are important for memory.(Mitra et al., 2003)

ERβ also plays a key role in neurogenesis. Three of the studies included in this systematic review show a consistent effect of increased cell formation in the hippocampal dentate gyrus and subventricular zone (Clark et al., 2012; Mazzucco et al., 2006; S. Suzuki et al., 2007), a finding supported by results from previous studies in adult animal models.(Ormerod, Lee, & Galea, 2003; Tanapat, Hastings, & Gould, 2005) These particular areas of the brain are known to produce a high expression of ERβ, which may explain their unique function of producing newborn cells. Neurogenesis in the hippocampus promotes the formation of new episodic memories and may even contribute to the therapeutic actions of antidepressant treatment.(Malberg, Eisch, Nestler, & Duman, 2000) Similarly, newly formed astrocytes from the subventricular zone could stimulate brain repair after ischemic

injury and may limit the extension of neurodegenerative changes and traumatic brain injuries.(S. Suzuki et al., 2007) However, the functional outcome of cell proliferation may become more important if we consider the exclusive presence and potential action of ERβ in other cell types of the brain, such as neurons of the paraventricular, suprachiasmatic, and tuberal hypothalamic nuclei, as well as the cerebellum and pineal gland.(Weiser, Foradori, & Handa, 2008)

Other mechanisms of ERβ action have been described in the neuroendocrine system. The activation of the HPA axis normally responds to a stress stimulus and is controlled by neurons in the paraventricular nucleus of the hypothalamus, an area of the brain rich in ERβ mRNA. Part of this response involves the action of CRH whose promoter activity may be regulated by ERβ isoforms. Furthermore, ERβ may also decrease stress-induced HPA activation through oxytocin neurons in the hypothalamus. ERβ and oxytocin are highly co-expressed in that part of the brain and both their actions may be mediated by CRH. Control of ERβ signaling may be better appreciated in the female population given that females are known to secrete higher levels of glucocorticoid in response to stress than are their male counterparts. Thus, targeting ERβ may be of benefit in the treatment of anorexia nervosa and depression, conditions characterized by dysregulation of the HPA axis.(Kudwa et al., 2014; W. J. Miller et al., 2004) ERβ was also found to increase postsynaptic response of GnRH neurons and could therefore prove to be important in regulating ovulation, fertility, and maternal behavior.(Brooks, Le, Chung, & Tsai, 2012; Chu et al., 2009) However, the display of lordosis, another female sexual behavior in mice, could not be attributed to ERβ. Its lack of relationship to ERβ was implied from the null effect on the expression of progesterone receptors in the hypothalamic ventromedial nucleus.(Sa et al., 2013) The raphe nucleus, on the other hand, may respond to the ERβ agonist SERM Beta2 in a dose-dependent manner in order to increase progesterone receptor expression. (Clark et al., 2012) Another significant contribution of ERβ may be observed in the hypothalamus by way of regulation of low voltage-activated (T-type) calcium channels that participate in burst firing and neurotransmission. These phenomena, however, may also be dependent on the co-expression of other estrogen receptors, including ERα.(Bosch et al., 2009)

ERβ has also gained particular interest in the prevention of AD. Several studies included in this systematic review have shown that ERβ agonist DPN may induce a significant reduction of hippocampal ApoE mRNA and protein expression, an established risk factor for late-onset AD. In an aging study sample with low estradiol level, ERβ may compensate to maintain hippocampal function, provided that estradiol level is increased.(Han et al., 2013; J. M. Wang et al., 2006; Zhao et al., 2013) Another mechanism may involve catabolism of Aβ by insulin-degrading enzyme (IDE). ERβ, in conjunction with the activation of PI3-K, may regulate the expression of this protease in normal and early-stage AD brains. IDE induction, which is reported also to respond rapidly to estradiol administration, may prove to be a target for prevention of AD.(Zhao et al., 2011) Functional significance of ERβ is also supported by findings from clinical studies in which genetic variations of ERβ were found to increase the risk of AD in women.(Pirskanen et al., 2005)

Neuroprotection has also been consistently reported in this systematic review. ERβ may reduce global ischemia in the caudate nucleus and CA1 pyramidal layer by enhancing expression of estrogen-regulated genes such as ApoE or *bcl-2*. Other proposed vehicles for neuroprotection are activation of CREB or preservation of mitochondrial function.(Carswell et al., 2004; Raval et al., 2013) As ischemic neuronal death is mainly caused by mitochondrial dysfunction disrupting calcium homeostasis and

increasing oxidative stress, interest has increased in studying the pathway by which ERβ may potentiate mitochondrial function.(Irwin et al., 2012; Raval et al., 2013) Although the exact mechanism of action for this protective effect remains to be further explained, its potential benefits may help prevent loss of synaptic transmission after cerebral ischemia as well as age-related decline in cognition. Reducing neuroinflammation may also confer brain protection. A number of cytokines and chemokines (interleukins) including IL-1 β, IL-6 and IL-12p40 in the brain may be regulated by ERβ through alteration of the blood brain barrier. These findings may contribute to decrease proinflammatory cytokines commonly described in postmenopausal females and thought to induce subclinical states of neurodegeneration and cognitive decline.(Abu-Taha et al., 2009)

This systematic review also shows consistency in the role of ERβ in modulating estrogen signaling in the process of emotional behavior (Imwalle et al., 2005; Krezel et al., 2001; Kudwa et al., 2014; W. J. Miller et al., 2004; Oyola et al., 2012) and other types of behavior such as social recognition and extinction recall, which is learning not to fear.(Choleris et al., 2003; Choleris et al., 2006; Zeidan et al., 2011) The use of ERβ knockout animal models and adapted behavioral tests were particularly useful in determining its functional role. Three studies in this review demonstrated that anxiety-like behavior was enhanced, while serotonin levels decreased, with the absence of functional ERβ. These effects may be region-specific to the stria terminalis, preoptic area, hippocampus, and possibly the dorsal raphe nucleus. The reduction of other monoamines such dopamine and dihydroxyphenylacetate may also be associated with increased anxiety in the absence of ERβ. Similarly, the addition of ERβ specific agonist DPN was found to attenuate the levels of stress-induced CORT and ACTH; which may contribute to decrease anxiety-like behaviors in different test settings. Furthermore, ERβ may reduce not only anxiety but also depression-like behavior, which may be mediated by genomic effects of ERβ in 5-hydroxytryptamine (5-HT) neurons of the dorsal raphe nucleus.(Benmansour et al., 2014) This effect was described after undergoing tests such as the open field, elevated plus maze, and the forced swim test. ERβ activation may increase expression of TPH and thereby induce slowing of 5-HT clearance.(Benmansour et al., 2014) Consequently, 5-HT in the inter-synaptic spaces would be present in higher concentrations and would have more time to exert its antidepressant effect; further suggesting that ERβ likely promotes an antidepressant-like effect.

To our knowledge, this is the first systematic review on the subject that critically appraised the literature following an *a priori* designed protocol with clearly defined inclusion and exclusion criteria. Using a systemic search in medical databases, few reviews evaluating the role of ERβ in the brain were found. They were all narrative reviews (not performed systematically) mainly focused on estrogen signaling and not specifically on ERβ.(H. J. Kim & Casadesus, 2010; Lokuge, Frey, Foster, Soares, & Steiner, 2010; Sugiyama et al., 2010; Weiser et al., 2008) In contrast to systematic reviews, narrative reviews do not involve a systemic search, and they are often focused on a subset of studies in the chosen area based on availability of the author selection. Therefore, they are more likely to suffer from selection bias(Garg, Hackam, & Tonelli, 2008; Uman, 2011). Additionally, the identified narrative reviews, contrary to our study, covered only specific-limited areas of the function of ERβ in the brain. A number of limitations, however, need to be considered. We included studies that were highly heterogeneous in the input parameters, assumptions, and study design, and therefore, performing a quantitative pooling of the existing data was unfeasible. Also, all the included studies used animal tissue and so caution should be taken when extrapolating the results of this review to human subjects.

Finally, publication bias may be a concern, as with all systematic reviews. However, we tried to minimize the impact of publication bias by employing a thorough search strategy in six databases in which no restrictions were applied on language or time of publication, checking reference lists of identified studies and contacting experts in the field

Overall, the results of the current systematic review, including only animal studies, show abundant functions of ERβ in the female brain and support the notion that future therapies targeting ERβ could constitute a novel preventive strategy and treatment for neurological diseases in females. ERβ agonists can mimic the actions of 17β-estradiol in the brain without causing other physiological responses mediated by other estrogen receptors. In view of the high burden of depressive disorders and neurodegenerative disease and despite advances in their prevention and treatment, transfer of these novel therapeutic venues on the field of neurological diseases could constitute a suitable alternative in the future. However, to establish potential therapeutic and preventive strategies targeting ERβ, future studies should be conducted in humans to further our understanding of the importance of ERβ in women's mental health.

Competing interest

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Role of the Funder/Sponsor

Metagenics Inc. with the steering committee were involved in study design; collection, analysis and interpretation of data; writing of the report; and decision to submit for publication. The funder/sponsor did not have the ability to veto publication of study results.

Supplementary Material

Supplementary Material can be found online: http://www.sciencedirect.com/science/article/pii/S0378512216301220?via%3Dihub#sec0100

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3.2

Menopause, ageing and alcohol use disorders in women

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ABSTRACT

Alcohol affects the female body differently than it affects males, caused by females' lower levels of dehydrogenase enzymes, the enzyme that breaks down alcohol, coupled with the higher ratio of fat to water. Both of these factors cause alcohol levels to rise more quickly in women than in men. Sex differences in the metabolism of alcohol make women are more vulnerable to alcohol's harmful effects, and women tend to develop alcohol-related diseases and other consequences of drinking earlier in life than do men. As women age and go through menopause, there is an age-related decline in the dehydrogenase enzyme, and women experience changes in body composition and life changes like retirement or loss of a loved one. In this period, alcohol problems and dependence also become more prevalent among women (Epstein, Fischer-Elber, & Al-Otaiba, 2007). Furthermore, aging women may experience additional stress and depression caused by the perception that their youth has ended (by virtue of losing the ability to bear children) and 'empty nest' syndrome. Under these conditions, alcohol may seem like an acceptable remedy. There are many consequences of alcohol abuse on the addicts' quality of life and the positive effects of moderate alcohol intake are miniscule in comparison to the adverse effects caused by its abuse. Further, due to social stigmas, women tend to have more difficulty gaining access to treatment and recovering from alcohol dependence than do men. Current research on interventions and treatments that aim to reduce alcohol consumption and prevent/treat alcohol dependence in middle-aged and elderly women is limited. In this study, we provide an overview of prevalences of drinking patterns and alcohol dependence, risk factors, health impacts and treatment challenges for women as they progress through middle and older age.

INTRODUCTION

Neurophysiology of alcohol use disorder (AUD)

The human brain graditution (reward) pathways make alcohol a powerful drug. The involuntary association between the positive effects of alcohol like its relaxing, and sociability-enhancing properties "rewires" the brain chemistry such that individuals who consume it wish to have more of it. Alcohol affects several neurological pathways, including the dopaminergic pathway, serotoninergic, gamma-amino butyric acid (Rubin et al., 1996; Solfrizzi et al., 2011) and glutamate pathways, which cause significant changes in the brain. Due to its anxiolytic effect as a facilitator of sociability, alcohol is culturally accepted worldwide, and is the most widely used recreational drug.

Aging process

Alcohol consumption declines with age for most adults, but some begin to experience alcohol dependence-related problems at or after ages 55 or 60 (Hajema, Knibbe, & Drop, 1997). Older age introduces multiple changes in health, lifestyle , family obligations, work roles and sources of support (Singh & Misra, 2009) . Also it is highly likely that older age is accompanied with physical pain (Patel, Guralnik, Dansie, & Turk, 2013), stress (Jeon & Dunkle, 2009) , loneliness (Singh & Misra, 2009) and loss of mobility (Manini, 2013) . All these reasons can trigger or worsen previously existing Alcohol use disorders (AUD) in elderly. In this review we focus on AUD in menopausal and postmenopausal women. With age, the impact of AUD related injuries becomes more severe, falls are more frequent which is especially harmful for menopausal and postmenopausal women due to onset and consequences of osteoporosis that is more severe than in men in the same age groups (Cawthon, 2011).The risk of harmful medication interactions is very high, as self-medication is one of the most important health concerns, especially in women in menopause and the postmenopausal period (Afshary et al., 2014) . The general physical effects of AUD are more debilitating (Alomar, 2014).

Epidemiological data

In the past 30 years, studies have shown that the prevalence of alcohol abuse and drinking problems among elderly adults ranges from 1% to 16% (Ahlström S, 2008; Moore et al., 1999; Substance Abuse and Mental Health Services Administration, 2004).

Due to increased life expectancy and the gender gap in longevity, women make up a significantly larger share of the older population. Although prevalence of drinking, binge drinking and volumes of alcohol consumption are higher in men compared to women (Grenard, Dent, & Stacy, 2013; Makela, Tigerstedt, & Mustonen, 2012), the prevalence, health and social impact of women's AUD is expected to increase in the near future. Aging of the baby boom and younger generations, and reduced disparity in levels of alcohol consumption between men and women at younger ages are likely to occur (Sanjuan & Langenbucher, 1999). This gap is closing in younger cohorts (Delker, Brown, & Hasin, 2016; Kraus, Piontek, Atzendorf, & Matos, 2016) During the past decades, alcoholic drinks have become more readily available, more advertised and worldwide cultural acceptance of women's consumption of alcohol has significantly increased (Grenard et al., 2013; Österberg, 2002; Slade et al., 2016; Sudhinaraset, Wigglesworth, & Takeuchi, 2016). In a study of Finnish women aged 59 to 79 from the mid-1980s to the early 2000s, the prevalence of consuming at least 5 units of alcohol

per week increased in all age groups (Sulander, Martelin, Rahkonen, Nissinen, & Uutela, 2005). A previous study indicates that the frequency of alcohol consumption among women is higher after menopause (Petri et al., 2004). This study included 13,074 Danish women combined from 6 population cohorts, aged 20 to 91 years. Women were followed for present or prospectively diagnosed breast cancer. Participants reported current drinking of alcohol in the following manner: light drinking (less than one drink per week) was present in 9% of premenopausal women were, 25% of postmenopausal women below 70 years of age and 20% women above 70 years of age. 20%. Low-moderate drinking (<7 drinks per week) was present in 22% of the premenopausal women, 33% of postmenopausal women below 70 years of age and 14% of the women above 70%years of age. Sensible drinking limit (from 7 - 13 drinks per week) was present in 7% of premenopausal women, 11 %of postmenopausal women under 70 years of age and in 14% of the women above 70 years of age. Moderate to severe drinking (categories of 14 to 27 drinks per week and over 27 drinks per week combined) was present in 4% of premenopausal women , 7% of postmenopausal women below 70 years of age and in 3% of women above 70 years of age (Petri et al., 2004). Generally, previous studies show a considerably higher prevalence of alcohol consumption within postmenopausal women, having in mind that this topic is still understudied. A Dutch population cohort study (age >55) (Vliegenthart et al., 2002) explored effects of moderate alcohol consumption on cardiovascular disease risk reduction. Data showed that among 2,486 females with a mean age of 67 years of age, 75 percentage reported current drinking of alcohol. Out of 75 % , 51,6% were light drinkers (≤10g per day), 12, 7% were moderate drinkers (>10 - ≤ 20 g per day) and 10.4% were severe drinkers (>20g per day), 25.3% were nondrinkers and considered abstainers (Vliegenthart et al., 2002).

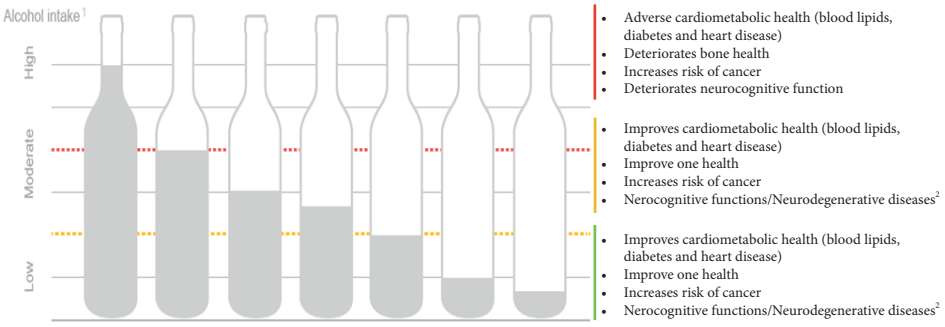

Figure 1. Dosage dependent risk factors and benefits of moderate intake

1. *For women, low-risk drinking is defined as no more than 3 drinks on any single day and no more than 7 drinks per week. Moderate drinking: up to up to 1 drink per; Severe drinking: 4 alcoholic drinks per day for women on 5 or more days in the past month.*
   One unit of alcohol is defined as 10ml (8g) of pure alcohol.
2. *Current Evidence is not persuasive for a beneficial effect of low/moderate alcohol consumption on cognitive functions and/or risk of developing neurodegenerative diseases*

Source of the classification: The National Institute on Alcohol Abuse & Alcoholism (NIAAA). (2015). Drinking Levels Defined. Retrieved from US: https://www.niaaa.nih.gov/alcohol-health/overview-alcohol-consumption/moderate-binge-drinking

Psychological aspects

The menopausal transition can also be associated with social impairment, depression, anxiety, stress, fatigue, embarrassment due to end of reproductive period, diminished libido, disturbed sleep, and changes in cognitive function and processing speed, all factors that can trigger or worsen existing AUD (Lewis Alexander, LaRosa, Bder, & Garfield, 2009; Maki et al., 2010; H. D. Nelson, 2008; Reed, Newton, LaCroix, Grothaus, & Ehrlich, 2007) . Among elderly females, death of a loved one, particularly a spouse (Rozenzweig, Prigerson, Miller, & Reynolds, 1997), is generally accepted to be a common and traumatic life event (Rozenzweig et al., 1997) that can cause late onset of AUD (Rigler, 2000). Depression (along with suicide), anxiety, substance abuse, and symptoms of "complicated" grief are among the more important psychiatric sequelae of spousal bereavement and the challenges of adapting widowhood (Rozenzweig et al., 1997) .

Socioecomic determinants and risks of developing AUD

Also, socioecomic determinants and risks of developing AUD in women exist. Previous studies show that effect is larger effect on when lower socioeconomic status is present (Lewer, Meier, Beard, Boniface, & Kaner, 2016). As already mentioned, menopausal women seem to be especially at risk of experience, divorce and departure of children from the home.(Allan & Cooke, 1985). Furthermore, in postmenopausal age, women may be more prone to AUD due to retirement (Hurt, Finlayson, Morse, & Davis, 1988). Published evidence show sociocultural factors influencing the initiation and continued use of alcohol and risky drinking behaviors being more common among women who are past or current smokers (Powers, Anderson, Byles, Mishra, & Loxton, 2015) We present Biopsychosocial model of female AUD - Risk factors in Figure 2.

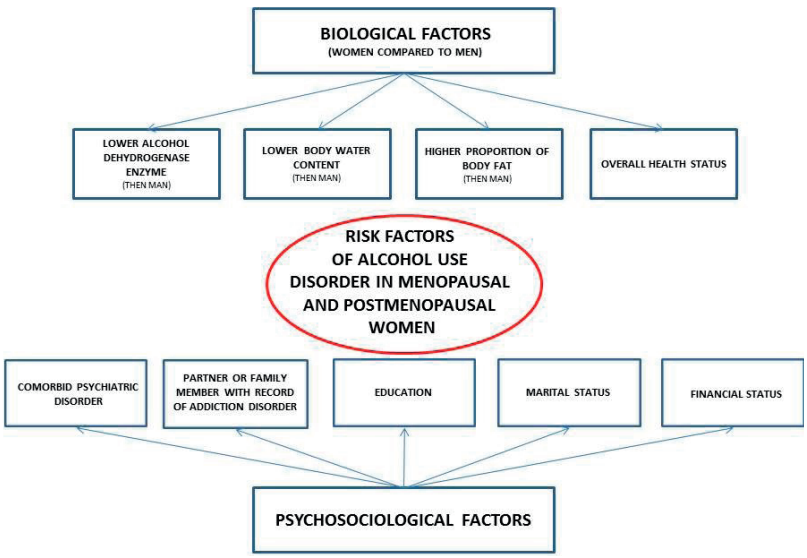

Figure 2. Biopsychosocial model of female AUD - Risk factors

Why is AUD in women understudied?

There is a noticeable lack of studies about gender differences in AUD. This lack may be due to lack of resources and awareness and stigma or taboo about women's substance use (Lal, Deb, & Kedia, 2015; Stone, 2015). AUD among men has been the predominant focus of the research (W. J. Lynch, Roth, & Carroll, 2002). The United Nations Global Illicit Drug Trends report from 2002 discusses gender differences with respect to substance abuse (United Nations publication SNEX, 2002) . It indicates that women represent an estimated 10% of substance abusers in some traditional Asian societies, 20% in countries of the former USSR and Latin America and about 40% in North America and some European countries, a finding that calls for more studies that focus on alcohol and substance abuse in women.

Vulnerability of females to AUD – a major public health concern

The vulnerability of females to alcohol-related harm is a major public health concern because as social norms have shifted away from traditional gender roles, young women start to exhibit higher frequency, higher volume, and more binge drinking, leading to a closing of the gender gap not only in consumption but also in terms of health consequences, with more pronounced health outcomes at the same level of consumption for all-cause mortality, cancers, gastrointestinal diseases, and cardiovascular disease (WHO, 2014). Alcohol-attributable cardiovascular disease and diabetes are more common in females than men (33.6% vs. 10.6% of the global disease burden, respectively 3). In line with these observations, alcohol-attributable morbidity has increased more steeply among women than among men during the past two decades (Erol & Karpyak, 2015). Therefore in the current overview we try to address all aforementioned issues.

METHODS

In this review we performed a narrative synthesis using 2 electronic database relevant to our topic (PubMed and Cochrane). In order to identify published work within the medical and psychological literature on AUD in in women in menopause and the postmenopausal period we selected key words, defined inclusion criteria and justified publication restrictions (we excluded economic analysis or clinical practice guideline development). The scoping review considered all study designs including qualitative and quantitative methodologies. We decided to select the studies published within the approximate 40-years period: 1977–2018. We choose our Keywords following PICO criteria and filtered our search accordingly. We used the following keywords in order to define population : Female, Women, Menopause, Menopause transition, Postmenopausa, Psychological aspects, Neurobiological aspects, Elderly; in order to define intervention: Psychiatric treatment, Psychotherapy treatment, Pharmaco-treatment ; in order to have a comparator group : Male AUD, Gender differences in AUD, Elderly men, Geriatric male drinking; in order to define outcome: AUD, Alcohol addiction, Alcohol consumption, Heavy drinking. Identifying the themes that emerge from the set of studies, we synthetized published evidence and we draw our conclusion with all authors agreeing on which studies to include in the review. We used the experience of each of the lead authors in the area of alcohol consumption, cardiometabolic diseases, menopause and women's health, addictology and

psychotherapy, to synthetize all the main insights on drinking patterns and alcohol dependence, risk factors, health impacts and treatment challenges for women as they progress through middle and older ages. Moreover, each author was encouraged to discuss theory and context to provoke thought and possible controversy by presenting philosophical perspectives in a balanced manner regarding clash between subjective perspective and published material. Identifying the themes that emerged from the set of studies, we synthetized published evidence and we draw our conclusion by identifying potential interventions to support the female patients with AUD, as well as their family members or surrounding environment in this process.

RESULTS AND DISCUSSION

Current definitions of alcohol consumption

This review touches on a wide spectrum of different patterns of alcohol consumption . ICD-10 distinguishes "harmful use" from "dependence", whereas DSM-5 combines the two into "alcohol use disorder-AUD" Under DSM–5, anyone meeting any two of the 11 criteria during the same 12-month period would receive a diagnosis of AUD. The severity of AUD—mild, moderate, or severe—is based on the number of criteria met (National Institute on alcohol Abuse and Alcoholism, 2016) (Supplementary table 1). Also, there is an extensive literature on safe drinking levels/patterns, international comparison on guidelines, government panels documents (Alcohol Guidelines development group, 2016; Kalinowski & Humphreys, 2016; UK Chief Medical Officers, 2016) that take sex differences into account.

There are several ways to define drinking at low risk of developing Alcohol Use Disorder (AUD). Firstly, we can refer to new DSM V definition (supplementary table 1). Next, we can refer to the US National Institute on Alcohol Abuse & Alcoholism (NIAAA): low drinking is considered to be one or less than one alcoholic drink for men and women in any single day; and a maximum of 7 drinks for men or 3 drinks for women per week. Moderate drinking is up to four alcoholic drinks for men and three for women in any single day, and a maximum of 14 drinks for men and 7 drinks for women per week. Severe drinking is four alcoholic drinks for men and three for women in any single day, and a maximum of over 14 drinks for men and over 7 drinks for women per week (The National Institute on Alcohol Abuse & Alcoholism (NIAAA, 2015). Since definition on units is not consistent and differs from country to country, to have more clear understanding, The National Health Service (The National Health Service (NHS)), UK, states that: one unit of alcohol is defined as 10 mL (8 g) of pure alcohol (NIAAA, 1999). However, variation in the number of grams of alcohol in a standard drinks between countries are wide. The lowest number of grams of alcohol is in the UK unit at 8 grams and the highest in Austria at 20 grams. Eleven European countries have 10 grams of alcohol in a standard drink and five have 12 grams in a standard drink (Mongan D & Long J, 2015). Further, due to the metabolic differences between men and women, as explained below in details, a woman will absorb about 30% more alcohol into her bloodstream than a man of the same weight who has consumed an equal amount. Thus, alcohol recommendations are sex specific (Lewis Alexander et al., 2009).

Though some health benefits of alcohol consumption have been demonstrated, alcohol consumption can result in physical and mental health problems if consumed irresponsibly and excessively.

We can divide this health problems in 2 groups: a) Chronic diseases and conditions that are alcohol attributed by definition (Table 1) and b) Chronic diseases and conditions for which alcohol is a component cause (Table 2) .

Table 1. Chronic diseases and conditions: alcohol attributed by definition (Listing from ICD-10)

ICD-10 Code	Disease
F10	Mental and behavioral disorders attributed to the use of alcohol
F10.0	Acute intoxication
F10.1	Harmful use
F10.2	Dependence syndrome
F10.3	Withdrawal state
F10.4	Withdrawal state with delirium
F10.5	Psychotic disorder
F10.6	Amnesic syndrome
F10.7	Residual and late-onset psychotic disorder
F10.8	Other mental and behavioral disorders
F10.9	Unspecified mental and behavioral disorders
G31.2	Degeneration of nervous system attributed to alcohol
G62.1	Alcoholic polyneuropathy
G72.1	Alcoholic myopathy
I42.6	Alcoholic cardiomyopathy
K29.2	Alcoholic gastritis
K70	Alcoholic liver disease
K70.0	Alcoholic fatty liver
K70.1	Alcoholic hepatitis
K70.2	Alcoholic fibrosis and sclerosis of liver
K70.3	Alcoholic cirrhosis of liver
K70.4	Alcoholic hepatic failure
K70.9	Alcohol liver disease, unspecified
K85.2	Alcohol-induced acute pancreatitis
K86.0	Alcohol-induced chronic pancreatitis
P04.3	Fetus and newborn affected by maternal use of alcohol
Q86.0	Fetal alcohol syndrome (dysmorphic)

** Table 1 does not contain: "Chronic Diseases and Conditions for Which Alcohol Is a Component Cause" (Alcohol is a component cause for more than 200 other diseases and conditions and they are all listed in: "2005 Global Burden of Disease (GBD)(Rehm et al., 2009) ")*
Source: Shield, K. D., Parry, C., & Rehm, J. (2014). Chronic Diseases and Conditions Related to Alcohol Use. Alcohol Research : Current Reviews, 35(2), 155-171. (Kevin D. Shield, Parry, & Rehm, 2014)

Table 2. Chronic diseases and conditions for which alcohol is a component cause

No. of 2005	Disease	ICD–10	Effect	Level of Evidence	Used if Included in the GBD Study
GBD Code				**Meta-Analysis**	
IIA	Malignant neoplasms				
IIA1	Mouth cancer	C00–C08	Detrimental	Causally related	International Agency for Research on Cancer 2008[+]
IIA2	Nasopharynx cancer and other pharynx cancers	C09–C13	Detrimental	Causally related	International Agency for Research on Cancer 2008[+]
IIA3	Esophagus cancer	C15	Detrimental	Causally related	International Agency for Research on Cancer 2008[+]
IIA4	Stomach cancer	C16	Detrimental	Insufficient causal evidence	
IIA5	Colon and rectum cancers	C18–C21	Detrimental	Causally related	International Agency for Research on Cancer 2008[+]
IIA6	Liver cancer	C22	Detrimental	Causally related	International Agency for Research on Cancer 2008[+]
IIA9	Larynx cancer	C32	Detrimental	Causally related	International Agency for Research on Cancer 2008[+]
IIA10	Trachea, bronchus, and lung cancers	C33–C34	Detrimental	Insufficient causal evidence	
IIA13	Breast cancer*	C50	Detrimental	Causally related	International Agency for Research on Cancer 2008[+]
IIA16	Ovarian cancer	C56	Detrimental	Insufficient causal evidence	
IIA17	Prostate cancer	C61	Detrimental	Insufficient causal evidence	
IIA19	Kidney and other urinary organ cancers	C64–C66, $C68_1$	Beneficial[i]	Insufficient causal evidence	
IIA23	Hodgkins	C81	Beneficial	Insufficient	

Table 2. Chronic diseases and conditions for which alcohol is a component cause (*continued*)

No. of 2005	Disease	ICD–10	Effect	Level of Evidence	Used if Included in the GBD Study
	lymphoma			causal evidence	
IIA24	Non-Hodgkins	C82–C85,	Beneficial	Insufficient	
	lymphoma	C96		causal evidence	
IIB	Other neoplasms	D00–D48$_2$	Detrimental		
IIC	Diabetes	E10–E13	Beneficial[ii]	Causally related	(Baliunas et al. 2009)
No. of 2005 GBD Code	Disease	ICD–10	Effect	Level of Evidence Meta-Analysis	Used if Included in the GBD Study
IIE	Mental and behavioural disorders				
IIE1	Unipolar depressive disorders	F32–F33, F34.1	Detrimental	Causally related	
IIF	Neurological conditions				
IIF1	Alzheimer's disease and other dementias	F01–F03, G30–G31	Conflicting evidence[iii]	Insufficient causal evidence	
IIF3	Epilepsy	G40–G41	Detrimental	Causally related	(Samokhvalov et al. 2010a)
IIH	Cardiovascular and circulatory diseases				
IIH2	Hypertensive heart disease	I11–I13	Beneficial[ii]	Causally related	(Taylor et al. 2010)
IIH3	Ischemic heart disease	I20–I25	Beneficial[ii]	Causally related	(Roerecke and Rehm 2010)
IIH4	Cerebrovascular				

Table 2. Chronic diseases and conditions for which alcohol is a component cause (*continued*)

No. of 2005	Disease	ICD–10	Effect	Level of Evidence	Used if Included in the GBD Study
	diseases				
IIH4a	Ischemic stroke	I63–I67, I69.3	Beneficial[ii]	Causally related	(Patra et al. 2010)

* Women only
1 Except C68.9
2 Except D09.9, D37.9, D38.6, D39.9, D40.9, D41.9, D48.9
[i] Renal cell carcinoma only
[ii] However, this depends on drinking patterns and volume of consumption
[iii] Mainly beneficial
+Based on relative risks from Corrao et al. 2004
Source: Listed studies are identified by Various Meta-Analyses and Reviews and Listed in the 2005 Global Burden of Disease (GBD) Study, 2005, classified and presented in study of Shield, K. D., Parry, C., & Rehm, J. (2014)*
*Shield, K. D., Parry, C., & Rehm, J. (2014). Chronic Diseases and Conditions Related to Alcohol Use. Alcohol Research : Current Reviews, 35(2), 155-171.

Physiological processes that make women more vulnerable to the effects of alcohol

Between the genders, alcohol has a different effect based on physiological processes and functioning (Lieber, 1997; Mumenthaler, Taylor, O'Hara, & Yesavage, 1999; Nolen-Hoeksema, 2004; Plant, 2002; Wilsnack RW, 1997). Women are more susceptible to long-term negative effects of alcohol on health, develop alcohol-related medical problems after shorter duration and lower levels of consumption compared to men (Erol & Karpyak, 2015) Subsequent to same dose of alcohol intake , women have higher blood ethanol levels than do men (Cederbaum, 2012; Nolen-Hoeksema, 2004) . Firstly, there are differences between men and women in the enzymatic processes that break down ethanol and eliminate it from the body. Women generally have lower levels of alcohol dehydrogenase enzyme, which makes them more susceptible to the effects of alcohol (Lieber, 1997; Nolen-Hoeksema, 2004). Secondly, the difference in the effect of alcohol between the sexes is due in part to the fact that women generally weigh less than men and that their bodies contain a lower proportion of water and a higher proportion of fat, causing the concentration of ethanol to rise more quickly in the blood (Cederbaum, 2012). Because alcohol is water-soluble, this sex difference in body composition means that, for a given dose of alcohol, the concentration of alcohol in the blood system is greater in women than in men (Cederbaum, 2012). A previous study states that the risk of liver cirrhosis, a disease predominantly attributable to alcohol, has been shown to be greater for women than men in relation to alcohol consumption (Parrish, Higuchi, & Dufour, 1991). Possible mechanisms that underlie the gender difference in blood-alcohol level comprise gender differences in the metabolism of alcohol (Baraona et al., 2001), the interaction of alcohol dehydrogenase (ADH) with female sex hormones and a decreased first pass effect (also known as first-pass metabolism or pre-systemic metabolism) (Baraona et al., 2001; Parlesak, Billinger, Bode, & Bode, 2002). First-pass metabolism is a phenomenon of drug metabolism whereby the concentration of a drug is greatly reduced before it reaches the systemic circulation . In women this phenomenon occurs because of lower levels of gastric ADH, and more rapid metabolism of alcohol in the liver (Baraona et al., 2001; Cederbaum, 2012; Parlesak et al., 2002; Thomasson, 1995). However, the only biological difference consistently related to increased blood-alcohol level that is easy to measure is body water (Graham, Wilsnack, Dawson, & Vogeltanz, 1998). The lower volume of body water in women gives less volume for distribution of alcohol than men, which may explain their higher blood-alcohol level at comparable quantities of alcohol consumed. In general this factor has been used to explain gender differences in AUD (Cederbaum, 2012; Ely, Hardy, Longford, & Wadsworth, 1999). Thirdly, the human body further differentiates with aging. Age-related changes in body composition are important factors in the study of ethanol metabolism and its effects. Lean body mass and total volume of distribution of the ethanol is negatively correlated with age (Vestal et al., 1977). The smaller volume of distribution, in association with the decreased lean body mass, could possibly be the reason that the higher peak ethanol concentration is found in the blood after intake of same alcohol dose in the old as compared with the younger (Vestal et al., 1977). As Lean body mass decreases with age, in women - body fat increases after menopause, and total body water content also decreases Thus, sex differences in the effect of alcohol become even more prominent. Therefore, especially in women the amount of alcohol that may previously have had little effect can cause intoxication in women after menopause (Vestal et al., 1977).

Having in mind all the aforementioned physiological factors that are positively associated with age, postmenopausal women are more vulnerable to AUD-related medical problems at lower levels of consumption than men or premenopausal women (Bradley, Badrinath, Bush, Boyd-Wickizer, & Anawalt, 1998; Erol & Karpyak, 2015). These findings strongly suggest that male/female differences in alcohol-related harm is likely to be heavily influenced by physiological (biological) aspects and further enhanced or controlled by psycho-social factors (Erol & Karpyak, 2015).The need for additional research on women is heightened by the fact that female physiology is more complex than male physiology. Research has shown that, although there are no gender differences in the development of addiction behaviors following first administration, women typically become dependent on substances more quickly than do men. (W. J. Lynch et al., 2002; Reynolds, 2003; The National Institute on Alcohol Abuse and Alcoholism, 1999; The National Institute on Drug Abuse (NIDA), 2000; Westermeyer & Boedicker, 2000).

We can clearly comprehend that physiological processes that make women more vulnerable to the adverse effects of alcohol intake represent a main focus of potential predisposition of developing AUD.

Finally, factors modifying the alcohol elimination rate which significantly influences metabolism of alcohol absorption, vary in humans because of various genetic and environmental factors (Cederbaum, 2012). Some of those are: race, food, exercise, AUD (especially severe AUD), circadian rhythm, and usage of other medications or drugs (Cederbaum, 2012).

Health consequences

The fact that the sex differences in the adverse effects of alcohol largely result from hormone physiology adds to the overall discussion of cardio-protective effects of moderate consumption (Roerecke & Rehm, 2014) and the effect on hormone-related cancers (Rehm, 2015) . Namely, moderate alcohol consumption has been inversely associated with the risk of cardiovascular disease , serum cholesterol levels (Koppes, Twisk, Snel, Van Mechelen, & Kemper, 2000; Sillanaukee, Koivula, Jokela, Pitkajarvi, & Seppa, 2000), diabetes (de Vegt et al., 2002; Kao, Brancati, Boland, Watson, & Puddey, 1998; Knott, Bell, & Britton, 2015; Wannamethee, Camargo, Manson, Willett, & Rimm, 2003), and mortality (Kendler, Ohlsson, Sundquist, & Sundquist, 2016; Knott et al., 2015; Roerecke & Rehm, 2014) while reversely associated with hormone-related cancers (Rehm, 2015). The potential benefits of moderate alcohol need to be weighed against the health risks, and cancer is of particular concern, because alcohol causes a large number of neoplasms, even at low levels of consumption (Bagnardi et al., 2015). In some drinking patterns it creates the opposite effect: binge drinking substantially raises the risk of cardiovascular disease and liver disease (Roerecke & Rehm, 2014). While some evidence suggests that women experience stronger cardio-protective associations but also quicker upturn to a detrimental effect at lower levels of average alcohol consumption compared to men, other studies (C. Wang et al., 2014; Zheng et al., 2015) indicated that women with moderate to heavy alcohol intake had a greater risk of total mortality and coronary heart disease than men. Among women, greater risk of AUD and total mortality is predominantly seen in postmenopausal women due to drop of estrogen. Specially, because of biological differences, women tend to become severely intoxicated more quickly than men (even when consuming less) and are at greater risk for developing a number of health problems including liver cancer, breast cancer, and accelerated brain atrophy (Wilsnack, Wilsnack, & Kantor,

2014). A systematic review and meta-analysis with studies enrolling both men and women showed that moderate alcohol consumption was associated immediately upon consumption with a higher risk of cardiovascular event, though the risk was attenuated after 24 hours, even showing protective effects against myocardial infarction and hemorrhagic stroke (≈2–4 drinks: relative risk=30% lower risk) and ischemic stroke within 1 week (≈6 drinks: 19% lower risk) (Mostofsky, Chahal, Mukamal, Rimm, & Mittleman, 2016).

For postmenopausal women, although not conclusive and debatable, moderate consumption of alcohol may be beneficial regarding the progression of osteoporosis and the severity of bone fracture (Charles P, 1999; Felson, Zhang, Hannan, Kannel, & Kiel, 1995; Naves Diaz, O'Neill, & Silman, 1997; Rapuri, Gallagher, Balhorn, & Ryschon, 2000; Smeets-Goevaers et al., 1998; Williams, Cherkas, Spector, & MacGregor, 2005). Osteoporosis is very common among postmenopausal and older women, and compared with abstainers, women who consume between 0.5 and 1.0 alcoholic drink per day were found to have a 20% lower risk of hip fracture (Berg et al., 2008). Furthermore, although the evidence is not consistent, light-to-moderate alcohol use may be associated with a reduced risk of incident dementia and Alzheimer's disease (Letenneur, 2004). Current evidence also suggests that moderate alcohol consumption may be protective from cognitive decline and pre-dementia syndromes (Solfrizzi et al., 2011).

Available evidence suggests a favorable effect of low and moderate alcohol consumption on cardio-metabolic health, bone density, and neuropsychiatric health, but a precise range of beneficial alcohol consumption cannot be determined. Several observational studies report a J-shaped relationship between alcohol consumption and cardio-metabolic and bone health outcomes (Berg et al., 2008). Emerging evidence indicates that the amount of alcohol associated with cardiovascular benefits is lower among women than it is among men, meaning that the threshold where drinking becomes harmful is lower in females than males (Mostofsky et al., 2016). For example, a Danish study showed that 14 drinks per week was associated with the lowest risk of diabetes among men, for women, the lowest risk of diabetes was observed at 9 drinks/week (Holst, Becker, Jorgensen, Gronbaek, & Tolstrup, 2017). Given that different preference for alcoholic beverages exist between men and women, differential associations between alcohol consumption and disease risk might exist for different type of alcohol beverage in female drinking. For example, women are likelier to have a preference for wine while men may prefer to drink beer (Sluik et al., 2016). Wine contains polyphenols such as resveratrol and various studies have suggested that resveratrol may foster beneficial effects on cardiovascular, inflammatory, neurodegenerative, and metabolic diseases as well as various forms of cancer (Weiskirchen & Weiskirchen, 2016; Zhou et al., 2016). Although wine has been suggested to have particularly protective effects in relation to cardio-metabolic outcomes in either sex, epidemiological studies are inconclusive when it comes to the specific effects of beer and spirits and the role of sex (Artero, Artero, Tarín, & Cano; Koloverou et al., 2015). More recent studies show that using data on both amount and frequency of alcohol consumption, alcohol intake at least 3 to 4 days per week is associated with a lower risk of myocardial infarction among menopausal and postmenopausal women, and HDL cholesterol, fibrinogen, and HbA1c accounted for 75% of the association (Djousse, Lee, Buring, & Gaziano, 2009). A range of potential health benefits for the general population associated with low to moderate alcohol consumption has been described, from a link between low and moderate alcohol intake with reduced coronary heart disease (Baer et al., 2002; Camargo, 1999; Fuchs et al.,

1995; Grobbee, Rimm, Keil, & Renaud, 1999) to benefits related to serum cholesterol levels (Koppes et al., 2000; Sillanaukee et al., 2000), and to a protective effect against type 2 diabetes (de Vegt et al., 2002; Kao et al., 1998; Wannamethee et al., 2003).

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While moderate alcohol consumption has been shown to have some health benefits, severe intake leading to severe AUD have adverse effects on health. Severe AUD can increase risk for coronary heart disease, certain types of stroke, liver cirrhosis, and diabetes (National Institute on Alcohol abuse and Alcoholism 2015). For instance, results from several meta-analyses have shown that up to 30 g of alcohol consumed per day may be protective for health, including cardiovascular diseases and type 2 diabetes, but that alcohol consumption of more than 60 g per day may have a negative impact (Koppes, Dekker, Hendriks, Bouter, & Heine, 2005; Ronksley, Brien, Turner, Mukamal, & Ghali, 2011). Combined analysis of data from 53 studies worldwide suggests a dose-response relationship between alcohol consumption and increased risk of breast cancer (Schutze et al., 2011). Despite these findings, there is little discussion among health agencies that women specifically should limit their alcohol consumption to a lower reference intake level than men (Nolen-Hoeksema, 2004) since acetaldehyde, the first and most toxic ethanol metabolite, is considered to be carcinogenic (Seitz & Becker, 2007). It has been estimated that for each 10g/day increase in alcohol, there is an increased risk of 5-10% for incident breast cancer (Hamajima et al., 2002; Schutze et al., 2011). Alcohol is causing breast cancer, both in pre-menopausal and post-menopausal women , and the risk increases in a linear dose-response manner, with risk appearing even at low consumption levels (K. D. Shield, Soerjomataram, & Rehm, 2016). Therefore, we can infer that dosage-dependent risk factors and benefits of moderate intake exist and should be considered in parallel (Figure 1).

These results suggest that women also seem to be more sensitive than men to the alcohol-related causes of death (C. Wang et al., 2014; Zheng et al., 2015). A recent meta-analysis found a J-shaped relationship between alcohol consumption and all cause cancer; as for sex-specific dose-response relation, a J-shape was also found in males but not in females (Jin et al., 2013). It was suggested that women are at higher risk for all-cause mortality at moderate-to-light levels of consumption due to

increased risk of alcohol-related cancers (Jin et al., 2013). Further, among men, drinking more frequently seems to have greater impact than the actual amount consumed; effects are less clear among women (Breslow & Mukamal, 2014).

These biological factors have not been fully understood and include, but are not limited to the variability in alcohol pharmacokinetics, alcohol effects on the brain, sex hormone levels (Agabio, Campesi, Pisanu, Gessa, & Franconi, 2016; Erol & Karpyak, 2015) . These findings strongly suggest that male/female differences in alcohol-related harm is likely to be heavily influenced by biological rather than psycho-social factors (Erol & Karpyak, 2015). Differences in sex-specific drinking patterns and the resulting medical consequences have recently been outlined for future research (Breslow & Mukamal, 2014). The higher female vulnerability seems to be multifactorial and only partly related to the higher alcohol blood levels achieved by women after drinking equivalent amounts (Agabio et al., 2016; Erol & Karpyak, 2015).

AUD challenges: identifying optimal therapeutic choice for women

Most people with an AUD benefit from using both medications and psychotherapy. This approach appears to give the optimum results. Also, a support group such as Alcoholics Anonymous (AA) is very helpful . People with severe AUD may need intensive treatment. They may go to a residential treatment center for rehabilitation (rehab) with highly structured. It usually includes several different kinds of behavioral therapies and medicines for detox (medical treatment for alcohol withdrawal) and/or for treating the AUD. Currently, naltrexone and disulfiram are dominantly used for treating AUD in the general adult population (Rogers & Wiese, 2011). Naltrexone is a competitive μ -opioid receptor , antagonist that blocks the effects of opiates. It also belongs to a group of "anti-craving" medications that reduce the craving effect of opiates and it is approved by US Federal Drag Association. Accumulating data have suggested that men and women with alcohol or drug dependence respond differently to the same pharmacological treatment, typically with men having better treatment outcomes than women (Garbutt et al., 2005; Hernandez-Avila et al., 2006; Nich et al., 2004; Pettinati, Volpicelli, Pierce, & O'Brien, 2000; Thom, 1987). Conversely, a previous study on Naltrexone has suggested that alcohol-dependent women (mean age 47.9 years) treated with 50 mg/day of naltrexone abstained from alcohol for a longer duration than men (mean age 45.6 years) treated with the same dosage (Kiefer, Jahn, & Wiedemann, 2005). Additional information is necessary to defect optimal age and gender targeted pharmacotherapy, leading to better outcomes for postmenopausal women suffering from AUD .

It is also important to point out which psychological/psycho-therapies can treat alcohol use disorder.

Published evidence reveals that Behavioral therapy might be a good solution for elderly suffering AUD (D. & H., 2011) . Cognitive-behavioral therapy (CBT) helps to identify the feelings and situations that can lead to heavy drinking. It empowers coping skills, with focus on how to how to manage stress and how to change the "drinking-thoughts". Clinical intervention studies indicate that these treatments are effective in addressing many common problems in late life, including alcohol abuse and dependence (D. & H., 2011). Other helpful type psychotherapies are: Motivational and Family therapy (US National Library of Medicine - MedlinePlus, 2018). Motivational enhancement therapy helps to build the motivation to change drinking behavior. It includes couple (4 to 8) ses-

sions in a short period of time. The therapy identifies the pros and cons of entering into treatment. Subsequently the therapist forms a plan for restructuring drinking patterns. Further sessions focus on building confidence and developing the skills necessary to stick to the plan (US National Library of Medicine - MedlinePlus, 2018).Also, marital and family counseling involves spouses and other family members as co-patients. It focuses on repairing and improving family relationships, that may help the person to stay away from drinking (U.S National Library of Medicine - MedklinePlus, 2018).

CONCLUSION

While in recent decades our understanding of gender differences in developing AUD has increased, several gaps remain regarding factors that can improve treatment options to address the needs of women. Targeted screening and preventive programs for women should be developed, trending towards damage control and formation of cultures around smart drinking behaviors. In order to achieve optimal prevention and treatment for menopausal and postmenopausal women , we must increase our understanding of the drinking patterns that are beneficial and harmful to women of these age groups; including more information on types of alcohol, and levels and frequency of consumption. Moreover, future studies should be conducted to investigate factors that might contribute to increased consumption of alcohol in menopausal and postmenopausal women, as well as sex-specific factors that predict better treatment outcomes in women suffering from AUD.

Limitations of the review

Our review has several limitations. Firstly, the nature of the method that we selected for this review is highly subjective (in the determination of which studies to include, the way the studies are analyzed, and the conclusions drawn). Secondly, we might be misled in drawing conclusions due to limited availability of evidence as complex interactions in interpretation of the context and dynamics of AUD in females, especially postmenopausal women are understudied.

Clinical implications

We underline gender and age - relevance, as older women are more vulnerable to effects and consequences of AUD. Importantly, women are highly responsive to psychotherapy and recruiting the female patients with any form of AUD in such treatment improves chances of preventing grief and correlated depressive and anxiety symptomatology.

Overall comprehension of this problem can add knowledge and expertise to a multidisciplinary team of healthcare professionals to prevent development of severe AUD and its consequences.

Contributors

JM, TM and OHF were responsible for the design and development of the review and critical revision of the manuscript for important intellectual content. Literature review and drafting of the manuscript: JM, MG, TV, LPB, EA, LZRS, JT, JCKJ, EB. Critical revision of the manuscript for important intellectual content: JM, TV, MG, TV, EB, JCKJ. Language editing: JT Figure design: JM and MG. All authors saw and approved the final version.

Conflict of interest

Declarations of interest: none

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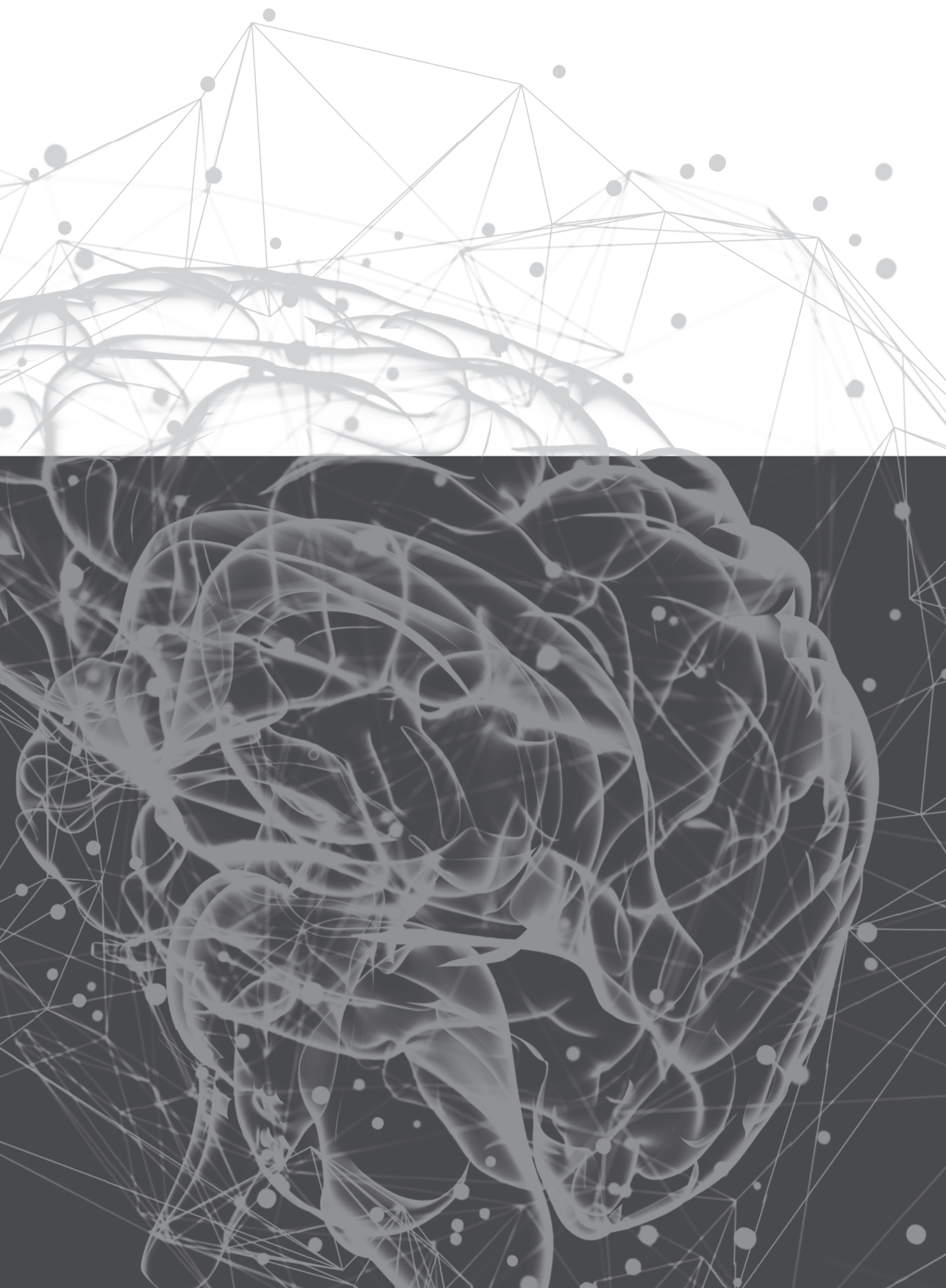
SUPPLEMENTARY MATERIAL

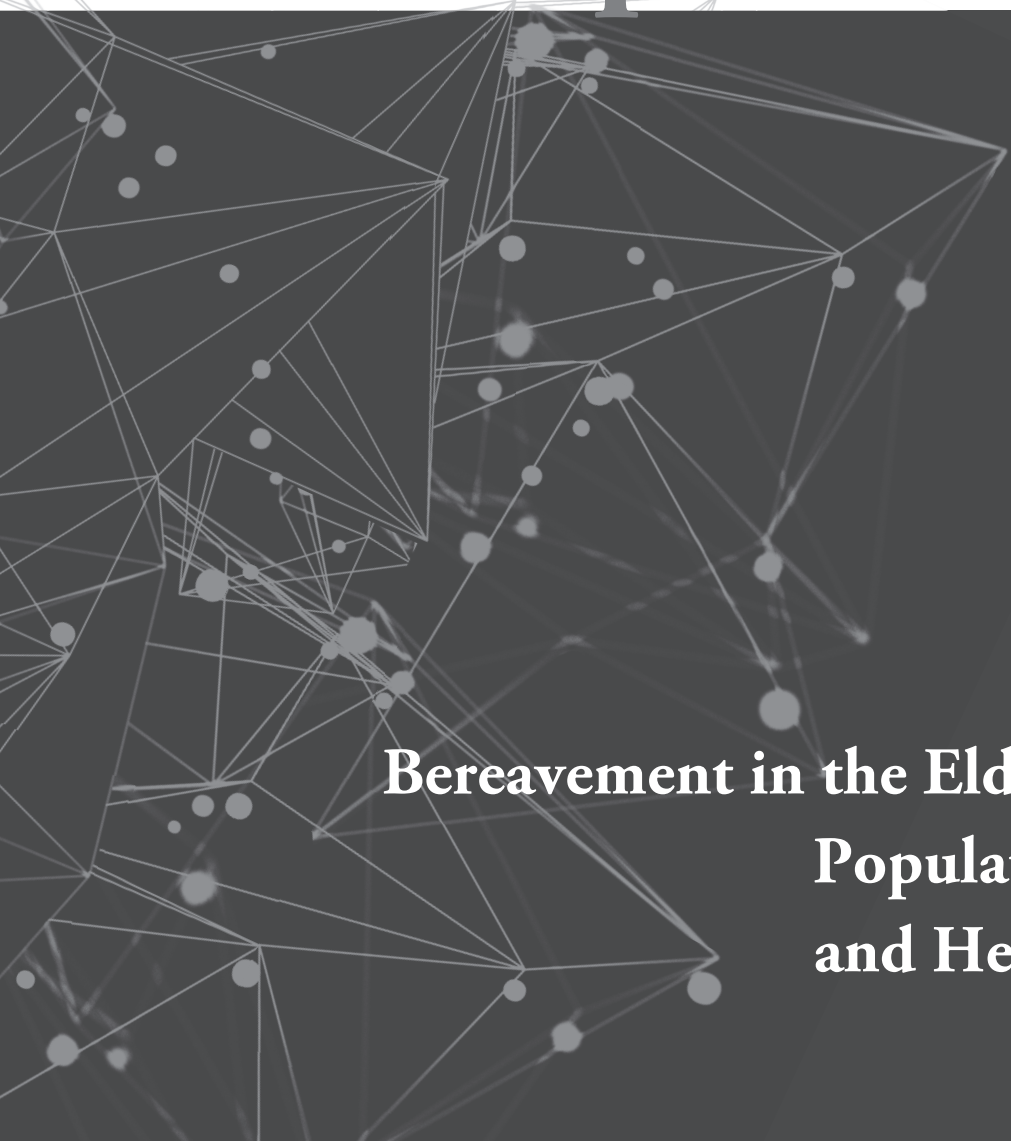
Supplementary table 1. DSM-5 criteria for establishing and classifying AUD

	DSM-5	
	In the past year, have you:	
1	Had times when you ended up drinking more, or longer, than you intended?	
2	More than once wanted to cut down or stop drinking, or tried to, but couldn't?	
3	Spent a lot of time drinking? Or being sick or getting over other aftereffects?	
4	Wanted a drink so badly you couldn't think of anything else? ****This is new to DSM-5****	
5	Found that drinking - or being sick from drinking - often interfered with taking care of your home or family? Or caused job troubles? Or school problems?	The presence of at least 2 of these symptoms indicates an
6	Continued to drink even though it was causing trouble with your family or friends?	**Alcohol Use Disorder (AUD)**.
7	Given up or cut back on activities that were important or interesting to you, or gave you pleasure, in order to drink?	The severity of the AUD is defined as:
8	More than once gotten into situations while or after drinking that increased your chances of getting hurt (such as driving, swimming, using machinery, walking in a dangerous area, or having unsafe sex?	**Mild:** The presence of 2 to 3 symptoms
9	Continued to drink even though it was making you feel depressed or anxious or adding to another health problem? Or after having had a memory blackout?	**Moderate:** The presence of 4 to 5 symptoms
10	Had to drink much more than you once did to get the effect you want? Or found that your usual number of drinks had much less effect than before?	**Severe:** The presence of 6 or more symptoms
11	Found that when the effects of alcohol were wearing off, you had withdrawal symptoms, such as trouble sleeping, shakiness, restlessness, nausea, sweating, a racing heart, or a seizure? Or sensed things that were not there?	

Source: National Institute of Alcohol Abuse and Alcoholism. Alcohol Use Disorder: A Comparison Between DSM–IV and DSM–5.20. National Institute of health (NIH) Publication No. 13–7999,Reviewed July 2016. Available online: https://pubs.niaaa.nih.gov/publications/dsmfactsheet/dsmfact.pdf



Chapter 4



Bereavement in the Elderly Population and Health

4.1

The impact of complicated grief on diurnal cortisol levels two years after loss. A population-based study

Heidi C. Saavedra Pérez, Nese Direk, Jelena Milic, M. Arfan Ikram, Albert Hofman, Henning Tiemeier

ABSTRACT

Objective: Few studies have focused on the effect of complicated grief - unresolved and prolonged grief - on the neuroendocrine systems. The present study examined the association of complicated grief and normal grief with the diurnal cortisol patterns in a large population-based study.

Methods: This study was set in the Rotterdam Study and comprised 2084 persons aged older than 55 years (mean [SD] age 64.9 [5.5] years). Participants were assessed with the Complicated Grief Inventory and classified into no grief (*n* = 1922), normal grief (*n* = 131), or complicated grief (*n* = 31) if they experienced the loss in the last 2 years. Saliva samples were collected to measure cortisol levels. Morning cortisol and summary measures (area under the curve and the slope) were studied to account for the diurnal pattern of cortisol. Persons with depressive disorders were excluded and analyses were additionally adjusted for depressive symptoms.

Results: Compared to normal grievers, participants with complicated grief showed lower levels of morning cortisol (11.26 vs 15.51 nmol/L; difference, −4.24; 95% confidence interval [CI] = −7.87 to −0.62; p = .022), and lower levels of overall diurnal cortisol (6.89 vs 8.98 nmol/L; difference, −2.09; 95% CI = −3.81 to −0.37; p = .017). No difference was observed in slope between both groups. Participants with complicated grief also showed lower levels of morning cortisol than the nongrievers (11.26 vs 14.71; difference, −3.46; 95% CI = −6.78 to −0.13; p = .042). In contrast, cortisol secretion patterns did not differ between persons with normal grief and nongrieving controls. cortisol levels characteristic for a chronic stress reaction.

Acronyms: RS Rotterdam Study, CG complicated grief, ICG Inventory of complicated grief, AUC area under the curve, CES-D Centre for epidemiologic studies of depression, BMI body mass index, MMSE mini mental score, SD standard deviation, HPA hypothalamic–pituitary–adrenocortical axis , ACTH adrenocorticotropic hormone.

INTRODUCTION

Bereavement is defined as having experienced a significant loss (Shah & Meeks, 2012). Its most common reaction is grief (Rozenzweig, Prigerson, Miller, & Reynolds, 1997). Grief knows many different manifestations and courses, but grief itself is not a mental disorder. However, approximately 9% to 20 % of the population experiencing bereavement show symptoms of an unresolved and prolonged grief, termed "complicated grief" (Newson, Boelen, Hek, Hofman, & Tiemeier, 2011). Complicated grief has been referred to as "traumatic grief", "complicated grief disorder" and "prolonged grief" and is distinctly different from "depression accounted for by bereavement", as mentioned in the DSM-IV (Shear et al., 2011). Recently, complicated grief has been included within the section "conditions for further study" in DSM-5 Section as Persistent Complex Bereavement Disorder (American Psychiatric Association 2013). Complicated grief includes a set of symptoms such as persistent intense yearning for the deceased, intense feelings of loneliness, feelings of disbelief or that life is empty, being emotionally numb or troubling accepting the death, bitterness, intrusion and rumination of thoughts or images of the dead person, and hearing or seeing the deceased person, lasting for at least 6 months (Pasternak et al., 1991; Prigerson et al., 1999; Shear et al., 2011).The etiology of complicated grief is not well-established, although psychosocial risk factors have been described, such as an unexpected death or suicide of the deceased (Ginzburg , Geron , & Solomon, 2002; Mitchell, Kim, Prigerson, & Mortimer-Stephens, 2004), lack of social support, or fewer pre-loss coping resources (lower self-perceived coping efficacy; lower religiosity) (Hansson, 2014; Ott, 2003), excessive dependence on the deceased (Bonanno et al., 2002), and pessimistic temperament (Robinson & Marwit, 2006).

The death of a loved one can be one of the most stressful events a person must endure (Stroebe, Schut, & Stroebe, 2007). Under conditions of stress, the hypothalamic-pituitary-adrenocortical (HPA) axis is stimulated and activates the secretion of cortisol into the bloodstream. Acute psychosocial stress is typically accompanied by increased secretion of cortisol, as an adaptation to the stressor and then decreases to normal levels; but it is the chronic dysregulation of cortisol that is implicated in a host of psychological and physical health conditions (Dickerson & Kemeny, 2004). A recent meta-analysis found that, compared to non-stressed controls, chronically stressed persons more often had a dysregulated pattern of cortisol secretion. This pattern was characterized by lower morning secretion and higher secretion across the rest of the day, yielding a flattened diurnal pattern (Miller, Chen, & Zhou, 2007). In depressed persons, higher levels of cortisol secretion have been reported widely, although in chronic depression and in community dwelling depressed persons low levels of morning cortisol have also been observed (Stetler & Miller, 2011). Likewise, low cortisol levels are reported in studies of chronic fatigue syndrome (Cleare, 2003, 2004; Roberts, Wessely, Chalder, Papadopoulos, & Cleare, 2004; Strickland et al., 2002; Tak et al., 2011; Ter Wolbeek, van Doornen, Coffeng, Kavelaars, & Heijnen, 2007) and in studies of post-traumatic stress disorder and after traumatic events (Yehuda, 2002b, 2006; Yehuda et al., 1995). Previous studies of grief and cortisol showed that the loss of a loved one is associated with more dysregulated cortisol patterns and an increased mortality risk of the bereaved person (Miller et al., 2007; Stroebe et al., 2007). Richardson et al. studied bereaved spouses and the effect of a prolonged forewarning of the death (i.e., knowing at least one month before the death that the person is going to die in the coming months). The group of bereaved persons who reported a forewarning of death showed higher cortisol levels at six month than those bereaved who

did not experience prolonged forewarning (Richardson et al., 2015). Few studies have investigated the relationship between cortisol and complicated grief. One study compared 12 women with complicated grief to 12 women with normal grief, showing a flatter slope across the day in those with complicated grief (O'Connor, Wellisch, Stanton, Olmstead, & Irwin, 2012). Another study compared 56 depressed adults, divided in three groups of non-bereaved, bereaved without signs of complicated grief, and bereaved with complicated grief symptoms. Interestingly, the depressed bereaved persons had lower levels of log-cortisol at wake and flatter diurnal slopes compared with the depressed non bereaved independent of, complicated grief symptoms (Holland et al., 2014).

Against this background and given the phenotypical correlation of complicated grief and chronic depression and PTSD, both of which have been related to lower cortisol values, we examined the association between complicated grief and cortisol measures.

We focused on persons with duration of complicated grief up to two years and five years after the loss (Maciejewski, Zhang, Block, & Prigerson, 2007). The aim of our study was to examine the association of the morning cortisol and summary cortisol measures with grief and complicated grief. We tested two hypotheses. First, persons with complicated grief symptoms have a lower diurnal cortisol secretion than those without grief or with normal grief. Second, persons with normal grief have a similar cortisol secretion pattern as those without grief.

METHODS

Study participants

This study was set in the Rotterdam Study (RS), a large prospective population-based cohort designed to examine the occurrence and risk factors of chronic diseases. The study has been described in detail elsewhere (Hofman et al., 2011). In 1990, all residents in a district of Rotterdam who were aged 55 years and over were invited to participate. Every four years, participants undergo an extensive home interview and physical examination at a research center. The Medical Ethics Committee of the Erasmus University of Rotterdam approved the study, and informed consent was obtained from all participants. The current study is based on the fourth examination of the original cohort members (n=7983). The examination was performed in 2002-2004 with 3550 participants (74% response rate), and assessed complicated grief and salivary cortisol levels.

For the present study we excluded participants with incomplete complicated grief inventory (n = 187), those using corticosteroids (n = 78), without salivary cortisol measures (n = 840), and persons with complicated grief less than six months after loss (n = 18) or grieving more than two years (n = 313). This resulted in 2084 elderly persons aged older than 55 years (M [SD] age 64 [5.5] years, 55% women) for the analyses of two years after loss. Another 130 grieving persons were additionally included in the analyses of all persons that experienced grief in the last five years.

Assessment of complicated grief

All participants were asked if they were currently grieving (Newson et al., 2011). If the answer was positive we asked whom they were grieving over (spouse, partner, child, parent, sibling, other family member, close friend, other, several people — including or excluding spouse) and the time elapsed

since the death. The participants, who answered the first question affirmatively, were assessed for complicated grief with the Dutch version of the Inventory of Complicated Grief (ICG).

In the present study, we used two different cut-offs to limit the time since loss. The primary analyses used a 2 year cut-off since bereavement and included all participants with grief (≤ 2 years) and complicated grief (≥ 6 months to 2 years). The second and contrasting analyses used a 5 years cut off since bereavement, and included all participants with grief and complicated grief up to 5 years after bereavement. However, at least six month duration of symptoms was required in accordance with recommendations for diagnostic criteria and to exclude acute stress reaction (Prigerson et al., 2009). The choice of the two year post loss cut-off was made to study the association with more recent complicated grief in line with a previous study (Newson et al., 2011) and the cut-off has also been used in previous analyses of the present cohort (Saavedra Perez et al., 2015). However, we also present analyses including all persons (N=292) with grief or complicated grief up to five years post loss. Complicated grief was diagnosed with the Dutch version of the Inventory of Complicated Grief (ICG). Questions represent symptoms of complicated grief on the basis of the most recent proposed criteria. The ICG is considered the gold standard for measurement of complicated grief in older adults because it has high internal consistency, good convergent and criterion validity (Prigerson et al., 1995). The inventory represents a single underlying construct of complicated grief. As described before (Newson et al., 2011), seventeen questions were asked and responses were provided on a 5-point scale to reflect an increase in severity (0-never, 1-seldom, 2-sometimes, 3-often, and 4-always). In the current setting, one item, "I feel bitter over this person's death", was removed from the original inventory as a pilot study revealed that this sentiment had a very similar meaning within the Dutch language as the included item: "I feel anger over this person's death". Two further items (relating to seeing and hearing the deceased) were combined into one due to their similarity and a pilot study indicating these symptoms were low in frequency and too often overlapped ("I hear the voice of, or see, the person who died").

A summary score for the ICG was calculated by totaling each individual item score (responses from 0-never to 4-always) across the 17-items providing a potential score range of 0 to 68. Participants with a score of less than 22 were considered as participants with grief symptoms in line with previous studies (Newson et al., 2011).

We defined complicated grief based on the severity of symptoms and did not define severity by duration of symptoms. Participants with a score of less than 22 were considered as participants with grief symptoms in line with previous studies (Newson et al., 2011). Participants with a score of 22 or greater and with symptoms lasting after for at least six months were considered to have complicated grief. This cut-off was based on the cut-off in the original version of the ICG (original cut-off of 25 from 19-items). We classified participants into three groups; no grief (control group), persons with normal grief (experiencing non-complicated grief as shown by an ICG score < 22) and those with complicated grief (ICG score ≥22). The non-grieving control group included persons who had experienced bereavement in the past but were not grieving at the time of interview. Likewise persons mourning over someone with severe disease, but who were still alive; or a pet, were included in the control group.

Salivary cortisol protocol

Saliva samples were collected on awakening (T1), 30 min after awakening (T2), at 1700 h (T3), and at bedtime (T4). Saliva samples were collected on awakening (T1), 30 min after awakening (T2), at 1700 h (T3), and at bedtime (T4). Salivary cortisol concentrations were measured using a commercial immunoassay with chemiluminescence detection (CLIA; IBL Hamburg, Hamburg, Germany). Intraassay and interassay coefficients of variation were less than 6 and 9%, respectively. The lower limit of detection was 0.4 nmol/liter. Data were screened for quality of cortisol measurements. For each time point, cortisol values that were above the 98th percentile in the original cortisol data set were excluded from the final data set to normalize the distribution of cortisol measurements and to exclude misclassification due to possible measurement errors. After this exclusion, cortisol levels followed a normal distribution (Dekker et al., 2008). The individual measures of cortisol were combined in summary measures to provide valid information about the diurnal pattern of cortisol. We calculated the area under the curve with respect to the ground (AUCg) and the slope. The AUCg summarizes overall diurnal cortisol exposure, and was calculated as the local area under the curve from the individual cortisol measures on the Y-axis and the time between cortisol measures on the X-axis. It takes into account both sensitivity (the difference between the single measurements from each other) and intensity (the distance of these measures from ground) (Fekedulegn et al., 2007; Pruessner, Kirschbaum, Meinlschmid, & Hellhammer, 2003). In order not to include the effect of the morning rise as part of the AUC, we did not include T2 in the AUC calculation. Diurnal decline was assessed by a slope, which was calculated by fitting a linear regression line for each participant, which predicted the cortisol values from time since awakening. A greater decline in the daytime cortisol means that the slope of the regression line is steeper, whereas a lesser decline means that the slope is flatter.

Assessment of other variables

Age, sex, education level, smoking status, body mass index, cognitive status, major depression, depressive symptoms and anxiety symptoms were evaluated as potential confounders on the basis of previous publication (Corruble, Falissard, & Gorwood, 2011; Direk, Newson, Hofman, Kirschbaum, & Tiemeier, 2011; Lee et al., 2007; Rosnick, Small, & Burton, 2010). The time between subsequent cortisol measurements within the same day was use as a covariate in the analyses of the AUC and the slope. Information was collected in home interviews and physical examination at baseline (Vermeer et al., 2003).

Level of education was assessed during home interview and was classified into those with primary education only, intermediate education, and high education (university studies). Participants were asked about their smoking status (never, current, and former). Current smokers were asked how many cigarettes they smoked daily and how long they had been smoking. Former smokers were asked about their smoking history (Direk et al., 2011). Height and weight were measured in participants without shoes and heavy clothes and body mass index was calculated as kg/m2, and used as a continuous variable (Visscher et al., 2001). Cognitive status was evaluated with the Mini Mental Status Examination (Folstein, Folstein, & McHugh, 1975) and used as a continuous variable. Depressive symptoms were assessed with a validated Dutch version of the Centre for Epidemiologic Studies Depression (CES-D) scale (range 0-60) to obtain a continuous measure of depressive symptoms (Beekman et al., 1997; Beekman, van Limbeek, Deeg, Wouters, & van Tilburg, 1994; Folstein et al., 1975). All participants

with clinically relevant depressive symptoms (i.e., those with a scores of 16 or greater on the CES-D, see above), were interviewed using the Present State Examination, a semi-structured psychiatric interview included in the Schedules for Clinical Assessment in Neuropsychiatry. All interviews were conducted by two experienced clinicians. Major depression was classified according to the Diagnostic and Statistical Manual of Mental Disorders-IV (World Health Organization - Division of Mental Health 1994).

Anxiety disorders were diagnosed with an adapted version of the Munich version of the Composite International Diagnostic Interview (M-CIDI) to assess the following anxiety disorders: GAD, panic disorder with or without history of agoraphobia, agoraphobia, social phobia and specific phobia. Obsessive compulsive disorder and post-traumatic stress disorder, which are part of the anxiety disorders in the DSM-IV, were not assessed. The M-CIDI was specifically designed to obtain DSM-IV diagnoses of mental disorders (Hek et al., 2011).

Assessment of Exclusion Criteria

Participants using corticosteroid were excluded. Pharmacy records were used to collect data on systemic corticosteroid use (Direk et al., 2011).

Statistical Analysis

Information on demographic characteristics was compared among the groups using a $\chi 2$ test for categorical data and an analysis of variance for continuous variables. We investigated the association of complicated grief with cortisol, the single (T1 morning cortisol) and the summary measures (AUC and slope). The primary analyses included as cases only persons who grieved after a loss up to two years ago; as this cut-off was used previously (Maciejewski et al., 2007). The second analyses included persons who were grieving after bereavement five or less years ago. We compared cortisol measures of the reference population to participants with normal grief and to those with complicated grief using analysis of covariance.

The analyses were adjusted for age and sex, level of education, smoking status, body mass index, Mini Mental State Examination, current depressive symptoms, and time between cortisol measures (for TI, T2, T3, and T4). Covariates were imputed using the Expectation-Maximization Algorithm. All covariates had less than 3% missing values. Missing values were imputed on the basis of the entire baseline population (n = 3550).

Although the ICG is designed as a screening measure and not to assess severity of grief, we performed continuous analyses in persons grieving using the scores on the ICG to test whether there is a dose–response relationship between the grief symptoms score and cortisol levels independently of the predefined cutoff.

RESULTS

The study population comprised 1922 persons with no grief, 131 with normal grief and 31 with complicated grief of maximal two years duration. In the sensitivity of persons with up to five years after loss, 210 persons with normal grief, and 82 persons with complicated grief were included.

Table 1 presents the characteristics of the study population. The sample was composed of 55 % females and the mean age of the participants was 64.9 years (SD=5.5). Participants with complicated grief were more often women and they were older than persons without grief. The main cause of grieving in those with complicated grief was death of the partner (61%), and in those with normal grief it was death of a partner (29%) or a sibling (22%). Participants with complicated grief had more clinical relevant depressive symptoms (18.4%), major depression (13.2%) and anxiety symptoms (18.4%) than persons without grief or those with normal grief.

Table 1. Baseline characteristics of the study population, N=2084

Characteristic	No Grief N=1922			Grief N=131			Complicated Grief N=31		
	N	%	Mean±(SD)	N	%	Mean±(SD)	N	%	Mean±(SD)
Sex, (women)	1056	(55)		89	(68)		21	(68)	
Age			63.5(5.6)			65.3(6.3)			65.9(4.7)
CESD score			5.1(6.1)			7.6(6.6)			13.8(9.8)
BMI (kg/m²)			27.4(3.9)			27.2(4.2)			26.1(2.4)
MMSE score			27.5(2.2)			27.4(2.5)			27.6(1.9)
Education Primary	515	(27)		37	(28)		10	(32)	
Education Intermediate	1380	(72)		90	(69)		21	(68)	
Education High	27	(1.4)		4	(3.1)		0	(0)	
Smoking status									
Never smoked	603	(31)		49	(37)		14	(45)	
Current smoker	234	(12)		12	(9)		3	(10)	
Former smoker	1085	(57)		70	(53)		14	(45)	
Anxiety Symtoms	144	(7.5)		12	(9)		6	(19)	
Who died?									
Partner	-	-		38	(29)		19	(61)	
Child	-	-		7	(5)		1	(3)	
Parent	-	-		4	(3)		0	(0)	
Brother/sister	-	-		29	(22)		4	(13)	
Others(another family member, good friend, several)				52	(40)		7	(23)	

Participants with grief (ICG <22) from 0 months to 2 years post loss and participants with complicated grief (ICG≥22) from 6 months to 2 years post loss were included. Participants using corticoids were excluded. Chi-square test and analysis of variance were performed.
SD: standard deviation. BMI: body mass index. MMSE: Mini Mental State Examination

Table 2 shows the cortisol saliva measures (morning cortisol, area under the curve, and slope) of the participants with no grief, normal grievers and complicated grievers who experienced the loss in the last two years. Participants with complicated grief had lower levels of morning cortisol than normal grievers [11.26 versus 15.51 nmol/l; difference -4.24, 95% CI (-7.87, -0.62), p=0.02]. Persons with complicated grief also had lower overall diurnal cortisol levels (AUCg) [6.89 versus 8.98 nmol/l;

Table2. Cortisol saliva summary measures in persons up to 2 years after loss.

Outcome salivary Cortisol measure after 2 year post loss	No Grief Reference category N=1922	Grief N=131	Complicated Grief N=31	Comparison Complicated Grief - No Grief		Comparison Complicated Grief – Grief	
	Estimated mean	Estimated mean	Estimated mean	Difference with reference (95%CI)	p-value	Difference with reference (95%CI)	p-value
Morning Cortisol (nmol/l)	14.72	15.51	11.26	-3.46 (-6.78, -0.13)	0.042	-4.25 (-7.87, -0.62)	0.022
Area Under the Curve (nmol/l)	8.31	8.98	6.89	-1.42 (-2.99, 0.16)	0.078	-2.01 (-3.81, -0.37)	0.017
Slope (nmol/l/h)	-0.83	-0.83	-0.64	0.19 (-0.04, 0.06)	0.113	0.19 (-0.06, 0.44)	0.134

The table presents estimated means, which are adjusted values. The analyses included persons who grieved after a loss up to two years ago. ANCOVA was performed and adjusted values are presented. Participants using corticosteroid were excluded. All analyses were adjusted for age, sex, level of education, smoking, body mass index, CES-D score, MDD, anxiety symptoms and mini mental state examination score. P-values are Bonferroni corrected accounting for the three groups.
CES-D: Center of Epidemiological Studies-Depression scale, MDD: Major Depressive Disor

Table3. Cortisol saliva summary measures in persons up to 5 years after loss.

Outcome salivary Cortisol measure after 2 year post loss	No Grief Reference category N=1922	Grief N=210	Complicated Grief N=82	Comparison Complicated Grief - No Grief		Comparison Complicated Grief – Grief	
	Estimated mean	Estimated mean	Estimated mean	Difference with reference (95%CI)	p-value	Difference with reference (95%CI)	p-value
Morning Cortisol (nmol/l)	14.72	15.18	14.44	-0.26 (-2.34, 1.81)	0.802	-0.74 (-3.09, 1.60)	0.535
Area Under the Curve (nmol/l)	8.31	8.57	8.39	0.07 (-0.91, 1.05)	0.884	-0.18 (-1.29, 0.95)	0.743
Slope (nmol/l/h)	-0.83	-0.83	0.04	0.04 (-0.10, 0.18)	0.596	0.19 (-0.12, 0.20)	0.636

The table presents estimated means, which are adjusted values. The analyses included persons who grieved after a loss up to two years ago. ANCOVA was performed and adjusted values are presented. Participants using corticosteroid were excluded. All analyses were adjusted for age, sex, level of education, smoking, body mass index, CES-D score, MDD, anxiety symptoms and mini mental state examination score. P-values are Bonferroni corrected accounting for the three groups.
CES-D: Center of Epidemiological Studies-Depression scale, MDD: Major Depressive Disorder.

difference -2.09, 95% CI (-3.81, -0.37), p=0.017]. The difference in the slope of cortisol observed between both groups did not reach significance.

Consistently, participants with complicated grief were found to have lower levels of morning cortisol than non-grievers [11.26 versus 14.71; difference -3.46, 95% CI (-6.78, -0.13), p=0.042]. Neither the difference in overall cortisol levels nor the difference in cortisol slope reached statistical significance; although the effect estimates suggest a meaningful differrence.

The table does not show the formal contrast between those with grief and without grief in the table; none of the group means between those with normal grief and those without grief reached significance (data not shown). However, the table clearly shows that the means of all cortisol measures are very similar in these two groups.

Table 3 presents the results of our sensitivity analyses. The groups now include all persons that experienced grief or complicated grief up to five years ago (those experiencing grief two to five years were added in these analysis). No differences between the participants with grief, complicated grief and those without any grief, in the cortisol morning levels or the two sum measures were observed anymore - whatever the reference and comparison group. Consequently, we tested if the cortisol measures of persons with complicated grief differed depending on time since bereavement. To this aim we defined exclusive groups of complicated grievers less than two years and between two and five years since bereavement. Both the AUCg (CG 2 years: 6.84 nmol/l, CG>2-5 years: 9.23 nmol/l, difference: -2.393, CI (-4.21, -0.57); F-statistic: 3.33, p-value 0.01) and the morning cortisol levels (CG 2 years: 11.85 nmol/l, CG>2-5 years: 16.44 nmol/l, difference: -4.58, CI (-8.46, -0.70); F-statistic: 2.69, p-value 0.021) differed in persons with complicated grief depending on the time since bereavement.

The continuous analyses in persons with grief experienced in the last five years showed that higher scores on the ICG were associated with lower morning cortisol (B=-0.270, 95% CI-11.94 to -0.72, p=0.027).

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DISCUSSION

In this population-based study, we studied whether diurnal cortisol secretion of persons with complicated grief or persons with normal grief differed from those not experiencing grief in the last two years. As hypothesized, the present study demonstrated that participants with complicated grief two years post loss showed lower level of morning cortisol and lower overall diurnal cortisol exposure than the participants with grief or without grief. In contrast, persons with grief showed similar cortisol secretion patterns as those without grief.

Cortisol is frequently referred to as the "stress hormone". Stressful life circumstances stimulate hypothalamic–pituitary–adrenocortical axis HPA, activating a hormonal response system that results in increased blood levels of cortisol (Beekman et al., 1997). However, hypocortisolism has been observed in patients, who developed post-traumatic stress disorder (PTSD) (Beekman et al., 1994; Roberts et al., 2004; World Health Organization - Division of Mental Health 1994), and has also been reported in patients suffering from bodily disorders, such as burnout with physical complaints, chronic fatigue syndrome, fibromyalgia, and chronic pelvic pain (Cleare, 2003; Hek et al., 2011; Lovallo 2005; Miller et al., 2007; Stetler & Miller, 2011; Ter Wolbeek et al., 2007). Similar findings have been reported for healthy individuals living under conditions of chronic stress, chronic depression, as well as for patients with rheumatoid arthritis, and asthma (Lovallo 2005; Robinson & Marwit, 2006; Stroebe et al., 2007). Our observations in persons with complicated grief extend these latter findings. Whereas a previous study of complicated grief and diurnal cortisol showed that those with complicated grief have a flatter slope across the day (Yehuda, 2006), morning cortisol or area under the curve have not been examined in persons with complicated grief.

Several mechanisms may be involved in the development of hypocortisolism. First, reduced biosynthesis of cortisol as part of an adaptive down-regulation has been postulated in persons experiencing severe trauma (Hek et al., 2011; Yehuda, Teicher, Trestman, Levengood, & Siever, 1996). Second, increased sensitivity of the HPA axis for negative feedback (Yehuda, 2002a; Yehuda & Seckl, 2011) or corticotropin releasing factor hypersecretion from the hypothalamus, are discussed, as these can result in reduced ACTH and lower cortisol levels (Lovallo 2005). Third, a very different but not necessarily exclusive mechanism is suggested by prior work from our group. We have previously shown that persons with complicated grief are characterized by more brain atrophy than persons with grief. Neuronal loss may disrupt the microstructural integrity of the fascicles connecting prefrontal cortex with the subcortical areas (amygdala and hippocampus) (Maciejewski et al., 2007; Nugent et al., 2015), which are involved in the inhibitory regulation of the HPA axis (Heim, Ehlert, & Hellhammer, 2000; Meewisse, Reitsma, de Vries, Gersons, & Olff, 2007; Yehuda & Seckl, 2011). Morphological brain changes: have also been described in patients with PTSD, such as a reduced volume of the

hippocampus, which is predominantly involved in the inhibitory regulation of the HPA axis (Heim et al., 2000; Meewisse et al., 2007; Yehuda & Seckl, 2011). Our data, however, suggest also that any such mechanisms behind hypocortisolism may sometimes be reversible. In the present study, no effect of grief or complicated grief on cortisol secretion patterns was observed, if persons with longer periods since the loss were included. This suggests that with longer follow-up time the association between grief and cortisol may atenuatte. We can only carefully speculate about coping in this interval. Participants with more than two years post loss may have adapted to the loss and the resulting changes, instead of avoiding the loss. Several studies proposed that CG reactions only persist when people engage in avoidance behaviors trying to impede habituation to painful memories and interfering with the integration of the loss (Bremner et al., 1997; Stein, Koverola, Hanna, Torchia, & McClarty, 1997; Yehuda, Lowy, Southwick, Shaffer, & Giller, 1991).

In our study, we found no differences in diurnal secretion cortisol and persons with normal grief and the controls. These findings were not unexpected if one views grief as a normal life event, unrelated to pre-existing vulnerabilities. Effects on the HPA axis may be visible after several months or even years only if a person develops complicated grief or has risk factors for complicated grief such as a dependent relationship with the dead person, unexpected dead, lack of social support, and the loss of someone who was ambivalently loved (Maciejewski et al., 2007). Or results suggest that persons with normal grief may have a more healthy coping style. Alternatively, they experience less of those grief-related feelings and behaviors, which tend to dominate severe bereavement and may affect the HPA axis (Boelen, 2006).

Strengths of our study include the sample size, the population-based setting, and the multiple daily cortisol measurements. A large control group of non-grieving persons was provided the reference for participants with normal grief or complicated grief. Also, we controlled for clinically relevant depressive symptoms and major depression.

Some limitations of the current study should also be mentioned. First, it is not possible to evaluate if these associations were causal due to the cross-sectional design of the study. In particular, we do not have cortisol assessments prior to grief, nor in the next examination round. Thus, we cannot rule out that existing alterations in HPA axis activity made some persons more susceptible to develop complicated grief. Second, not all the participants with Inventory of complicated grief participated in the saliva sampling. Third, selection effects may have lead to the inclusion of more grievers with healthy coping style in those persons whose loss events occurred long ago. This would have diluted the effects. Fourth, is not possible to account for complicated grief with PTSD because in the Rotterdam Study this was not assessed. Fifth, we do not have longitudinal data to study the factors that might be involved on the reversibility of the cortisol secretion. Also, we can not rule out that existing alterations in HPA axis activity made some persons more susceptible to develop complicated grief.

The importance of our study is that cortisol, which is involved in cognitive functions such as memory performance and executive function and regulates the the inflammatory responses is altered in persons experience complicated grief (Bonanno et al., 2002; Hek et al., 2011; Lovallo 2005; Parkes, 1998). This implies that persons with complicated grief may be more vulnerable to develop cognitive problems (Maciejewski et al., 2007), depression and medical conditions than persons with normal grief. Follow-up studies are needed, however, to demonstrate the clinical consequences of our observations.

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4.2

The Longitudinal and Cross-Sectional Associations of Grief and Complicated Grief With Sleep Quality in Older Adults

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ABSTRACT

Objective/Background: About 15% of grievers experience complicated grief. We determined cross-sectional and longitudinal relations of grief and complicated grief with sleep duration and quality in the general population of elderly adults.

Participants: We included 5,421 men and women from the prospective population-based Rotterdam Study.

Methods: The Inventory of Complicated Grief was used to define grief and complicated grief. We assessed sleep with the Pittsburgh Sleep Quality Index.

Results: After 6 years, 3,511 (80% of survivors) underwent the follow-up interview. Complicated grief was cross-sectionally associated with shorter sleep duration and lower sleep quality. These associations were explained by the presence of depressive symptoms. The prospective analyses showed that sleep duration and sleep quality did not decline further during follow-up of persons who experienced grief or complicated grief.

Conclusion: In community-dwelling, middle-aged and older adults, persons with normal and complicated grief had both a shorter sleep duration and a lower sleep quality, mainly explained by depressive symptoms. However, prospective analyses showed that sleep quality and sleep duration do not decline further in persons with normal grief and complicated grief.

INTRODUCTION

The death of a loved one is a common life event in older adults (Boelen & Hoijtink, 2009; Boelen & van den Bout, 2008; Shear, 2015. Very few persons make it through old age without having to cope with this kind of loss, once or several different times. The loss of a partner, child, parent, or close family member can be very distressing (Monk, Germain, & Reynolds, 2008). However, even if it is experienced as a traumatic event, after a delimited period of grief, the majority of people recover. An estimated 15% of bereaved people continue to grieve for an extended period; they experience disbelief and are preoccupied by the deceased (Prigerson et al., 1995). This state is known as complicated grief (Prigerson et al., 2009). Complicated grief is an important mental health issue for the aging population, affecting social functioning and well-being (Newson, Boelen, Hek, Hofman, & Tiemeier, 2011). However, our knowledge about complicated grief is limited. Previous studies suggest that symptoms of complicated grief are distinct from those of depression and anxiety and have incremental validity predicting impairments in social and interpersonal daily functioning (Boelen, van de Schoot, van den Hout, de Keijser, & van den Bout, 2010; Newson et al., 2011; Prigerson & Jacobs, 2001). In addition, the severe emotional strain of the loss of a loved one can trigger profound changes in lifestyle. These changes often induce reductions in financial security, perceived personal safety, and freedom of action. All of these facets of grief could lead to changes in sleep patterns. Several studies (Hall et al., 1997; Kowalski & Bondmass, 2008; Monk, Begley, et al., 2008) suggest that grief is associated with significant sleep impairment. However, our knowledge regarding the associations of complicated grief with sleep is limited, as only a few studies with small sample size and a cross-sectional design have been conducted (Boelen & Lancee, 2013; Germain, Caroff, Buysse, & Shear, 2005; Maytal et al., 2007; Monk, Begley, et al., 2008; Purebl, Pilling, Konkoly, Bodizs, & Kopp, 2012; Spira, Stone, Beaudreau, Ancoli-Israel, & Yaffe, 2009). An exploratory study of the effects of complicated grief on sleep by McDermott et al. (1997) conducted analyses on 65 bereaved persons. The results showed mild subjective sleep impairment is associated with complicated grief, but no effect was detected using the electroencephalographic sleep measures. Germain et al. (2005) evaluated the severity of sleep disturbances in a group of 105 adults meeting criteria for complicated grief. They showed an association of complicated grief with an overall poor sleep quality. Comorbid depression (Adrien, 2002; Germain et al, 2005; Hall et al., 1997; Maytal et al., 2007; Monk, Begley, et al., 2008; Nutt, Wilson, & Paterson, 2008; Purebl et al., 2012; Spira et al., 2009), but not posttraumatic stress disorder, further worsened sleep quality.

Taking into account the lack of high-powered longitudinal studies in normal populations of elderly adults, we aim to determine whether in adults aged 55 years and above, grief or complicated grief was related to sleep duration and sleep quality, cross-sectionally and longitudinally.

We hypothesized that if studied cross-sectionally, persons with grief and complicated grief have shorter sleep duration and a lower sleep quality than persons who did not experience grief due to the stress that death of the loved one brings to person's life. Second, we hypothesized that complicated grief remains a risk factor for further decline of sleep duration and poor sleep quality over time due to coping mechanisms that may not always be successful.

METHODS

Settings and Study Population

This study was embedded in The Rotterdam Study, an ongoing prospective cohort of older adults designed to examine the occurrence and risk factors of chronic diseases. The study design and objectives are described in Hofman et al. (2013). The Rotterdam Study comprises two cohorts, which were combined in the current analysis. Between 2002 and 2005, complicated grief and sleep quality were assessed during a home interview, referred to as baseline. The baseline interview was conducted in 5,481 participants. Of these participants, 60 persons did not complete the grief or sleep questionnaire. This left 5,421 participants with assessment of grief and sleep characteristics for cross-sectional analysis. In part of the follow-up examination (2009– 2011), both components of sleep (duration and quality) were assessed at the research center. After an average of 6.33 years (*SD* = 0.42), 3,511 (80%) of the 4,601 surviving participants underwent the follow-up interview for sleep duration, and 3,003 (71%) for sleep quality. The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus Medical Center (Erasmus MC) and by the Ministry of Health of the Netherlands, implementing the Wet Bevolkingsonderzoek: ERGO (Erasmus Rotterdam Gezondheid Onderzoek; Population Studies Act: Rotterdam Study). All participants provided written informed consent to participate in the study and to obtain information from their treating physicians.

Assessment of Complicated Grief

Complicated grief was diagnosed at the baseline examination (2002 and 2005), with a Dutch version of the 17-item Inventory of Complicated Grief (ICG) originally constructed by Prigerson et al. (which contains 19 items). First, participants were asked if they were currently grieving. If a positive answer was received, the ICG was administered, but if not, they were categorized as nongrievers (the reference group). The ICG is the most widely used instrument to measure complicated grief. Questions represent symptoms of complicated grief such as those in the most recent proposed criteria for the condition suggested by (Prigerson et al., 2009). Some of the symptoms include intense yearning for the lost person, anger over the death, distrust and detachment from others as a consequence of the death, survivor guilt, and loneliness. The measure has high internal consistency and convergent and criterion validity and it is considered the gold standard for measurement of complicated grief in older adults. The inventory is shown to represent a single underlying construct of complicated grief (Boelen & Hoijtink, 2009). The Dutch version of the Inventory of Complicated Grief contains 17 items and has been previously validated (Boelen et al., 2003). These 17 questions were asked and responses were provided on a 5-point scale to reflect an increase in severity (0-never, 1-seldom, 2-sometimes, 3-often, 4-always). In the current study one item from the original inventory, "I feel bitter over this person's death," was removed from the original ICG because a pilot study revealed that this sentiment had the same meaning within the Dutch language as the included item, "I feel anger over this person's death." Two further items (relating to seeing and hearing the deceased) were collapsed into one due to their similarity and to a pilot study indicating these symptoms were low in frequency and often overlapped ("I hear the voice of, or see, the person who died"). Several studies give further details on the interpretation of ICG (Boelen et al., 2010; Newson et al., 2011; Prigerson & Jacobs, 2001). We divided all interviewed participants into nongrievers (reference group), normal grievers, and

complicated grievers. Complicated grief symptoms were assessed as present among participants who scored equal or greater than 22 on the ICG score and grieved longer than 6 months (Newson et al., 2011; Saavedra Perez et al., 2015).

Assessment of Sleep

Sleep duration and sleep quality were measured with the Pittsburgh Sleep Quality Index (PSQI), a self-reported questionnaire (Buysse, Reynolds, Monk, Berman, & Kupfer, 1989). The PSQI consists of 19 self-rated questions. Questions are grouped into seven component scores, each weighted equally on a 0–3 scale. The seven component scores are then summed to yield a global PSQI score, which is used in all further analyses. This score has a range of 0–21; higher scores indicate worse sleep quality. The seven components are subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbances, use of sleeping medications, and daytime dysfunction. In the current study, we used total sleep time in hours to indicate sleep duration, and a total score of PSQI to indicate sleep quality. Finally, we presented a sample of PSQI (Supplement A).

Assessment of potential confounders

Age, sex, education, cognitive functioning, activities of daily living, body mass index (BMI), and depressive symptoms were considered as potential confounders. Education was assessed routinely in the home interview and subdivided into low, intermediate, and high education. Cognitive functioning was measured using the Mini-Mental State Examination (Folstein, Folstein, & McHugh, 1975) during one of the visits to our center. The ability to perform activities of daily living was measured with the Stanford Health Assessment Questionnaire (Bruce & Fries, 2003; Fries, Spitz, & Young, 1982). Height and weight were measured without shoes and heavy clothing to calculate the BMI (kg/m2). Depressive symptoms were measured with the Center for Epidemiological Studies Depression scale (CES-D). In our baseline table, we also showed the presence of depressive symptoms among participants who scored 16 or above, suggesting clinically relevant depressive symptoms on the Center for Epidemiological Studies Depression scale.

Statistical Analyses

To explore the association between grief and sleep parameters we used linear regression. Model 1 was adjusted for age and sex. Model 2 was additionally adjusted for education, cognitive functioning, activities of daily living, and BMI. Model 3 was further adjusted for depressive symptoms. In the longitudinal analyses, to examine whether grief status was prospectively associated with sleep duration and sleep quality, we used sleep duration and sleep quality assessed during the follow-up as outcomes. We selected the same covariates as in the cross-sectional analyses and adjusted for the respective baseline values of sleep duration or sleep quality.

We conducted a series of sensitivity analyses. First, we reran the analysis, not only for depressive symptoms, but to exclude all patients with major depression disorder at the baseline. We evaluated the presence of Major Depressive Disorder in those with a CES-D score, or above the established screening cutoff of 16, using the Schedules for Clinical Assessment in Neuropsychiatry (SCAN) interview (Wing et al., 1990). This semistructured clinical assessment was performed by trained clinicians to determine which participants fulfilled the DSM-IV major depressive disorder. We performed

this sensitivity analysis in order to minimize the depressive disorders on sleep quality. Also, in our study, Major Depressive Disorder (MDD) was assessed at baseline only (prevalence of 2%). Second, to test the effect of the more recent sleep, we performed longitudinal analysis restricted to those who experienced the bereavement in the years prior to baseline assessment. Third, we repeated the cross-sectional linear regression only in those participants who had attended the follow-up assessment, to test whether any between–cross-sectional and longitudinal analysis reflected a selection effect. Adjustments for these analyses were conducted as in the main linear regressions. Fourth, since sleep duration component of PSQI, we performed a sensitivity analyses calculating the PSQI total without the component of sleep duration. Fifth, in order to explore the possibility of reverse causality, in the longitudinal analysis, we excluded people who had poor sleep at the baseline (defined as the total score of all components of PSQI greater than 5 points). Sixth, we explore whether, cross-sectionally and longitudinally, sleep duration and sleep quality differ between grievers (reference) and complicated grievers. We also explored the association between grief status and depressive symptoms (CES-D). In multivariable linear regression models, we examined whether baseline grief status was prospectively associated with CES-D at follow-up, further adjusted for the baseline value of depressive symptoms.

With respect to the remaining data, missing values were imputed using multiple imputations (Rubin, 2004). In the present study, for each missing value five draws were performed providing five substituted items of data, which in turn created five completed data sets. Analyses were performed separately on each completed data set and thereafter combined into one pooled estimate. The percentage of missing values within the population for the analyses was lower than 20% (ranging from 0 to 18%). Age and sex had no missing values, education had 18%, cognitive score had 8%, activities of daily living score had 1%, BMI had 14%, and CES-D had 0.2%. Analyses were performed using SPSS Statistics (version 20; SPSS, Chicago, IL, USA).

RESULTS

Of the 5,421 eligible participants, 4,378 (80%) were classified as experiencing "no grief," 795 (15%) as experiencing "normal grief," and 248 (5%) as experiencing "complicated grief" at baseline. Table 1 presents the characteristics of the study population. Participants classified as experiencing complicated grief were older, were more likely to be female, had a lower level of education, and were more likely to have clinically relevant depressive symptoms. The main cause for grief was death of a partner (36% of those with normal grief and 56% of those with complicated grief), or a child (10% of those with normal grief and 22% of those with complicated grief).

Table 2 shows the cross-sectional associations of grief with sleep duration and sleep quality. In the age-and-gender adjusted analysis, we found a consistent association pattern of grief and complicated grief with sleep duration as well as with sleep quality. Further, adjustment for education level, activities of daily living, cognitive functioning, and body mass index did not change these associations. However, the association between grief and sleep indicators was explained by depressive symptoms (model 3).

Table 1. Baseline characteristics of study participants 2002-2005 (n=5421)

Characteristics	Non-grievers N=4378 (80%)		Normal grievers N=795 (15%)		Complicated grievers N=248 (5%)	
Age, years (S.D.)	72.4	(7.7)	73.4	(8.1)[a]	74.7	(7.5)[b]
Women, (%)	55		73[a]		72 [b]	
Education [a,b]						
Primary (%)	19		22		30	
Intermediate (%)	67		66		63	
High (%)	14		12		7	
Cognitive functioning, score (S.D.)	27.41	(2.58)	27.39	(2.52)	26.99	(3.04)[b,c]
Depressive symptoms, score (S.D.)	5.42	(6.67)	8.62	(8.49)[a]	14.12	(11.35)[b,c]
Clinically relevant depressive symptoms,(%)	4		8[a,]		25 [b,c]	
Who died?,						
Partner, (%)	-	-	36		56	
Child, (%)	-	-	10		22	
Parent, (%)	-	-	12		6	
Brother/sister, (%)	-	-	17		7	
Others, (%)	-	-	25		9	
Activities of daily living, score (SD)	0.51	(0.57)	0.59	(0.57)[a]	0.66	(0.60)[b]
Body mass index (kg/m^2), (SD)	27.56	(4.03)	27.67	(4.34)	27.75	(4.38)
Sleep quality, score (SD)	3.37	(2.91)	4.28	(3.20) [a]	5.08	(3.67)[b,c]
Sleep duration (hours), (*SD*)	6.91	(1.30)	6.69	(1.36)[a]	6.44	(1.50)[b,c]

Group comparisons were performed with χ^2 (categorical variables) or T-test (continuous variables) for independent-samples.
[a]*Comparison of non-grieving participants with grieving participants (p<0.05).*
[b]*Comparison of non-grieving participants with complicated-grief participants (p<0.05).*
[c]*Comparison of grief participants with complicated-grief participants* (p<0.05).

Table 2. Cross-sectional associations of grief and complicated grief with sleep

		Sleep Duration (hours)											
		Model I				Model II				Model III			
N		B	95% CI		*p*	B	95% CI		*p*	B	95% CI		*p*
Grieving status													
Non-Grievers (ref.)	4378	-	-	-	-	-	-	-	-	-	-	-	-
Grievers	795	-0.15	-0.25	0.05	0.003	-0.15	-0.25	-0.05	0.004	-0.05	-0.15	0.05	0.30
Complicated Grievers	248	-0.41	-0.57	-0.24	≤0.0001	-0.40	-0.57	-0.24	≤0.0001	-0.12	-0.30	0.05	0.16

		PSQI (hours)											
		Model I				Model II				Model III			
N		B	95% CI		*p*	B	95% CI		*p*	B	95% CI		*p*
Grieving status													
Non-Grievers (ref.)	4378	-	-	-	-	-	-	-	-	-	-	-	-
Grievers	795	0.64	0.39	0.90	≤0.0001	0.63	0.38	0.88	≤0.0001	0.15	-0.08	0.39	0.20
Complicated Grievers	248	1.52	1.09	1.95	≤0.0001	1.48	1.06	1.90	≤0.0001	0.08	-0.33	0.48	0.71

Model I was adjusted for age and sex; Model II was adjusted for age, sex, education, activities of daily living, cognitive functioning, BMI and the respective baseline values of sleep duration and Pittsburgh Sleep Quality Index to model change; Model III was adjusted for age, sex, education, depressive symptoms, activities of daily living, cognitive functioning, and BMI

In Table 3 we present the prospective association of grief with sleep duration and sleep quality (both assessed at follow up exam after 6.33 years on average (*SD* = 0.42)). We did not find an association of grief or complicated grief with changes in sleep duration or sleep quality, either in the age-and-gender adjusted or in the fully adjusted analyses. Next, we performed a series of sensitivity analyses. First, we excluded persons with major depression from our study population and reran the analysis; our result remained essentially unchanged. Then we limited the cases to those who experienced the bereavement leading to complicated grief in the last 2 years prior to baseline assessment (Supplement B). Our result showed no association between more recent lost event and sleep parameters in the longitudinal analysis. Also, to test whether the differences between cross-sectional and longitudinal analysis reflect a selection effect, we reran the cross-sectional analysis in participants who attended the follow-up assessment. Results remained essentially unchanged; the cross-sectional associations of complicated grief with sleep duration and sleep quality were similar to our original cross-sectional findings (data shown in Supplement C). Also, the results did not change when we reran the analysis calculating the PSQI score without including the sleep duration component (Supplement D). Next, in the longitudinal analysis, exclusion of subjects who had poor sleep quality at baseline did not change the results (Supplement E). Furthermore, we did not find any difference in sleep duration or sleep quality between grievers and complicated grievers in both cross-sectional and longitudinal analysis (data not shown). Last, we did not find an association between baseline grief status and depressive symptoms at follow-up (grievers: β = –0.22, 95% CI: –1.51—1.07, $p = 0.74$; complicated grievers: β = 1.00, 95% CI: –1.30—3.31, $p = 0.39$).

Table 3. The longitudinal associations of grief and complicated grief with sleep.

		Sleep Duration (hours)											
		Model I				Model II				Model III			
N		B	95% CI		*p*	B	95% CI		*p*	B	95% CI		*p*
Grieving status													
Non-Grievers (ref.)	2876	-	-	-	-	-	-	-	-	-	-	-	-
Grievers	495	0.02	-0.10	0.14	0.71	0.02	-0.10	0.14	0.72	0.01	-0.11	0.13	0.91
Complicated Grievers	140	0.06	-0.15	0.27	0.57	0.06	-0.15	0.27	0.57	0.01	-0.20	0.23	0.91

		PSQI (hours)											
		Model I				Model II				Model III			
N		B	95% CI		*p*	B	95% CI		*p*	B	95% CI		*p*
Grieving status													
Non-Grievers (ref.)	2482	-	-	-	-	-	-	-	-	-	-	-	-
Grievers	413	-0.09	-0.39	0.21	0.55	-0.09	-0.39	0.20	0.53	-0.07	-0.37	0.23	0.66
Complicated Grievers	108	0.06	-0.49	0.61	0.83	0.07	-0.49	0.62	0.82	0.15	-0.42	0.71	0.61

Model I was adjusted for age and sex; Model II was adjusted for age, sex, education, activities of daily living, cognitive functioning, BMI and the respective baseline values of sleep duration and Pittsburgh Sleep Quality Index to model change; Model III was adjusted for age, sex, education, depressive symptoms, activities of daily living, cognitive functioning, BMI and the respective baseline values of sleep duration and Pittsburgh Sleep Quality Index to model change.

DISCUSSION

In this large population-based study of middle-aged and elderly persons, we investigated whether persons with grief or complicated grief had a different sleep duration and sleep quality than participants without grief. Our cross-sectional findings showed that normal and complicated grief were associated with shorter sleep and lower sleep quality. These associations were mainly explained by the presence of depressive symptoms. No further changes in sleep duration and sleep quality between the groups were observed after an average follow-up of more than 6 years.

Complicated grief can be regarded as a bereavement situation for which sleep duration is likely to be affected (Monk, Germain, et al., 2008). A cross-sectional study of duration of sleep among unselected grievers, that is, most probably including persons with complicated grief, has been reported previously (Monk, Germain, et al., 2008). The authors conducted a laboratory study of sleep and circadian rhythm in 38 spousal bereaved seniors (≥ 60 years) observed 4 or more months after their loss event. On average, the bereaved seniors achieved only about 6 hr of sleep. In a large Japanese population-based prevalence study of 1,871 participants conducted by Doi, Minowa, Okawa, and Uchiyama (2000), the authors showed that being widowed or without a partner was associated with lower sleep quality.

There is evidence suggesting that behavioral changes associated with grief such as decreased activity levels or overall changes in social rhythm stability could lead bereavement to sleep disturbances. After the loss of a loved one, there are profound changes in lifestyle, often accompanied by reductions in financial security, perceived personal safety, and freedom of action (Monk, Germain, et al., 2008), all of which are likely to lead to sleep disruption. Also, the loss of a loved one is associated with psychological problems such as rumination or anxiety, which are shown to impair sleep (Carney, Edinger, Meyer, Lindman, & Istre, 2006; K. Shear et al., 2007). Sleep disturbances are particularly prevalent in depressed bereaved persons; even bereaved persons who fail to meet a formal diagnosis of depression have measurable sleep impairment (Reynolds et al., 1992). Indeed, our cross-sectional analysis showed that the association between grief and complicated grief with sleep indicators was largely explained by depressive symptoms. However, we ran multivariable linear regression models to see if baseline grief status was prospectively associated with CES-D at follow-up and found no association, providing support that depressive symptoms are not a mediator in the association between grief and sleep parameters. Further, reversed causality should be taken into account. Since relatively few studies have yet examined sleep difficulties as a risk factor for post-loss psychopathology, we cannot rule out that existing sleep problems make individuals vulnerable to more severe or prolonged grief or complicated grief. Indeed, as Boelen and Lancee (2013) pointed out, poor sleep quality is a known risk factor for many different forms of a psychopathology, including depression and PTSD.

We did not find a prospective association between grief and sleep parameters. Different explanations for these null findings are possible: First, the lack of findings can reflect the insufficient power to detect an association. Although fewer participants could be included in the longitudinal analyses, sufficient power to detect any effect similar to that observed in the cross-sectional analyses remained. Thus, these findings suggest that there was no further change in sleep duration and sleep quality once a person had reported bereavement at our baseline assessment. We carefully infer that the results could be explained with mechanisms of adaptive coping (S. S. Rubin, 1999; Stroebe & Schut, 1999)

developed by the grieving participants during prolonged exposure to grief. Possibly, persons grieving reached a "stable state," that is, with no further change of sleep quality, when participating in the follow-up assessment on average 6 years after the event. Sleep quality might have been affected before the occurrence of complicated grief. Due to the lack of prebereavement sleep assessment, it is not possible to evaluate the directional effects in the cross-sectional analysis, that is, whether bereavement triggered the decline of sleep duration and quality or whether sleep impairment preceded the grief reaction. However, our sensitivity analysis in which we excluded participants with poor sleep quality provides no evidence for reverse causality. Also, grief was assessed only at baseline. Consequently, we cannot account for the change in grief status, whether the feelings of grief remitted, persisted, or worsened.

However, the majority of clinical diseases and conditions are characterized by a progression of symptoms and their consequences; against this background, we had hypothesized a continuous decline of sleep problems, having in mind that sleep duration and quality among persons with complicated grief is of potential value for prognosis of grieving persons and potentially even of relevance for therapeutic interventions that rely on the cognitive behavioral interventions focused on sleep difficulties as discussed by Boelen and Lancee (2013). It suggests that the impact of grief on this important aspect of well-being is not accumulating over time and can potentially be overcome.

To the best of our knowledge, other longitudinal studies have not been performed previously in the general population. Also, our study is characterized by a long follow-up period and a large sample size. Furthermore, a middle-aged and elderly sample was used, which is the main vulnerable population for complicated grievers, as late-life loss of a loved one is among the most common life events. However, some limitations of the current study should be mentioned. First, in a population-based study, it is not feasible to ascertain grief and sleep directly after a loss event. Most important, complicated grief cannot be diagnosed if the event occurred less than 6 months before. Therefore, we performed sensitivity analyses restricting the study population to those who experienced the bereavement more recently (in the last 2 years before the baseline assessment). Second, we miss information on whether these persons are still suffering from complicated grief or MDD at follow-up. Further studies should be performed including this kind of prospective reassessment.

CONCLUSION

In community-dwelling middle-aged and older adults, persons with normal and complicated grief had both a shorter sleep duration and a lower sleep quality, mainly explained by depressive symptoms. However, prospective analyses showed that sleep quality and sleep duration do not decline further in persons with normal grief and complicated grief.

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Supplemental material

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4.3

Quality of Life and Bereavement: A Systematic Review

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ABSTRACT

Background Bereavement is a common experience in the life course of every person. The death of a loved one leads to grief characterized by yearning and longing, decreased interest in ongoing activities, and frequent thoughts of the deceased which might hamper the quality of life (QoL). However, little is known whether bereavement is associated with QoL.

Methods Seven bibliographic databases were systematically searched for studies assessing the association between any form of grief and QoL. Reference lists and contacts with experts were also used to identify relevant studies.

Results Fourteen studies (5 cross-sectional, 5 case control studies, 1 retrospective study and 3 prospective studies) were included with data on 9,108 middle-aged and elderly participants. Eight studies investigated grief induced by spousal loss, two focused on parental bereavement, three reported on grief in caregivers, one study compared bereavement in subjects with anxiety disorder to those with no anxiety disorder. Overall, bereavement, more precise the grief severity were associated with lower QoL, especially in women. Nevertheless, the evidence on grief and QoL is limited and hampered by the suboptimal of quality of the studies on this topic.

Conclusion Different types of bereavement and grief severity show associations with lower QoL. However, this review highlights the scarcity and low quality of the available information with important gaps including lack of large-scale prospective investigations, needed to reliably determine the association of grief with QoL and its domains.

INTRODUCTION

Experiencing a death of a loved one is a common event in the life course of every person (Boelen & Hoijtink, 2009; Monk, Germain, & Reynolds, 2008; Shear, 2015). Although it is a traumatic event, after a certain period of grief most people recover (Kacel, Gao, & Prigerson, 2011). However, an estimated 15% of bereaved people continue to grieve for a prolonged period, imprisoned by memories, regrets and a sense of guilt. This complex condition is called a Prolonged Grief Disorder (PGD), also referred to as 'complicated grief'(Prigerson et al., 2009). Grief affects many aspects of life from social functioning to well-being (Newson, Boelen, Hek, Hofman, & Tiemeier, 2011). Symptoms of complicated grief are distinct from those of depression and anxiety and can lead to impairment in social and interpersonal daily functioning (Boelen, van de Schoot, van den Hout, de Keijser, & van den Bout, 2010). Schwartz et al. explored the response shift phenomenon- how changes in general health status affect quality of life (QoL) (Schwartz & Sprangers, 1999). It is based on the hypothesis that internal standards, values and the conceptualization of QoL can change during a life course (Schwartz & Sprangers, 1999). This response shift is a subjective perception where the person is assigning a new meaning to the QoL construct. Thus, expectations, personality traits, and cognitive, affective or behavioral processes determine this new meaning (Schwartz & Sprangers, 1999). Presumable, the aforementioned aspects are affecting coping mechanisms in grieving persons. Also, the changes in individual's physical, psychological and social responses of a grieving person affect the individual's level of satisfaction and sense of self-worth (Cousson-Gelie, de Chalvron, Zozaya, & Lafaye, 2013; Ozer, Firat, & Bektas, 2009). Consequently, the impact of grief on a person's QoL might be considerable. However, the extent to which grief is associated with QoL and its domains remains unclear and the available studies examining this hypothesis are yet to be rigorously reviewed. As bereavement is a common event in life, understanding QoL related with bereavement and its types would allow health and social care providers and researchers to better understand the bereaved individuals views as having the greatest impact on their QoL.

In the present study, we aimed to synthesize all available evidence on various forms of grief in relation to QoL.

METHODS

Data Sources, Search Strategy and Eligibility Criteria

This review was conducted in accordance with the PRISMA (Moher, Liberati, Tetzlaff, Altman, & Group, 2009) and MOOSE (Stroup et al., 2000) guidelines (S1 and S2 Appendices). Seven electronic databases (Medline via Ovid, EMBASE, Cochrane Central, Web of Science, PUBMED, Psycinfo via Ovid and Google Scholar) were searched with the help of a medical librarian from inception until 29th of June, 2017 (date last searched). The searches combined terms related to the exposure (eg. bereavement, grief, PGD or complicated grief) and outcome (eg, quality of life), without language restriction. Details on the search strategy are provided in S3 Appendix. Reference lists of selected studies and reviews identified on the topic were searched to identify additional publications. Experts in the field were also contacted to identify missing studies.

Study Selection

Studies were eligible if they (i) were observational studies (cross-sectional, case-control and cohort studies), or randomized clinical trials; (ii) assessed any form of grief (bereavement, grief, PGD and complicated grief), (iii) collected endpoints for QoL; and (iv) examined the association between any form of grief and QoL). Anticipatory or preparatory (pre loss) grief was not the focus of this review, and therefore studies that investigated these forms of grief were excluded.

Two independent reviewers JM and BK, working in pairs, screened the titles and abstracts of all initially identified studies according to the selection criteria. In case of disagreement, a decision was reached through consensus or consultation with a third independent author TM. Full texts were retrieved from studies that satisfied all selection criteria.

Data Extraction

Data were extracted by two independent authors JM and BK. A predesigned data extraction form was used to collect relevant information. This included questions on study size; study design; baseline population; location; age at baseline; duration of follow-up (for prospective studies); form of grief (defined as bereavement, grief, PGD or complicated grief) and methods used to define grief and QoL. In the case of multiple publications, the most up-to-date or comprehensive information was included.

Assessing the Risk of Bias

Bias within each individual study was evaluated by two independent reviewers JM and OHF using the validated Newcastle-Ottawa Scale (NOS), a semi-quantitative scale designed to evaluate the quality of observational studies (Stang, 2010). Study quality was judged on the selection criteria of participants, and exposure and outcome assessment. The NOS assigns a maximum of four or five points for selection, one or two points for comparability and three points for outcome, depending on study design. Studies that received a score of eight and nine stars were judged to be of at low risk of bias; studies that scored six or seven stars were considered at medium risk; those that scored five or less were considered at high risk of bias.

Outcome Assessment and Statistical Methods

For each study, we defined whether an association was reported, and when applicable, the direction of effect sizes was reported. For the current study, due to the limited number of included studies and high heterogeneity in exposure and outcome assessment of the individual studies, meta-analysis was not feasible.

RESULTS

In total, we identified 1,604 potentially relevant citations. Based on the title and abstracts, 45 articles were selected for detailed evaluation of their full texts. Of those, 1559 were excluded for reasons shown in Figure 1. Therefore, 14 articles, based on 14 unique studies, met our eligibility criteria and were included in the analysis.

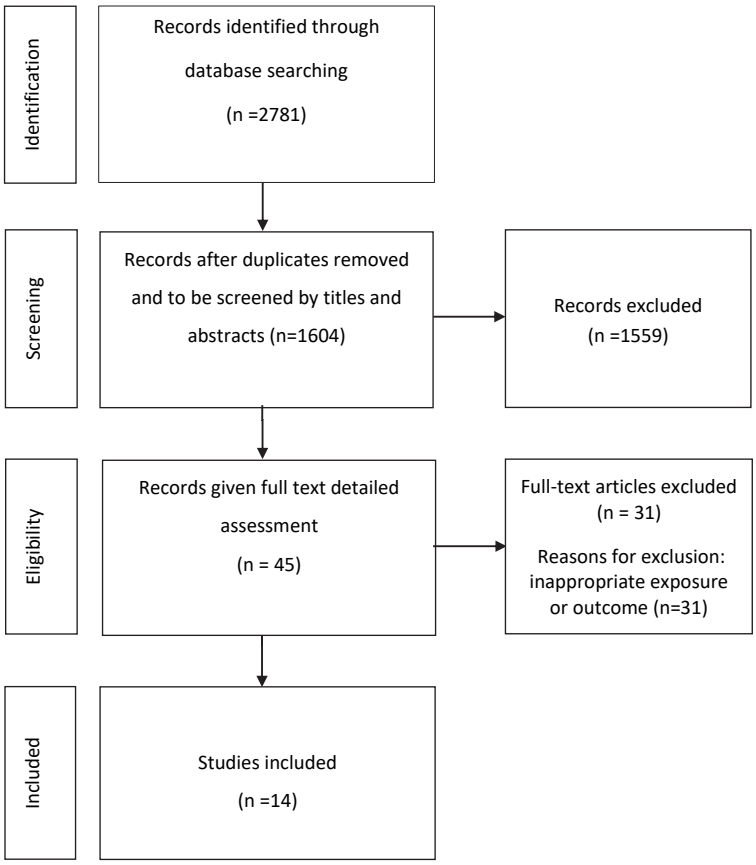

Figure 1. Flowchart of studies investigating the associated between bereavement and quality of life

Summary of Included Studies

Characteristics of the included studies are summarized in Table 1. Overall, data were available on 9,631 unique participants. The mean age of the participants at baseline across the included studies ranged from 42 to 72 years. Seven studies included participants from Europe, three studies participants form Asia, three studies from USA and one study included participants from Canada. All studies had an observational design: 5 studies were cross-sectional 4 were longitudinal studies (3 prospective and 1 retrospective) and 5 case–control studies. The year of baseline survey in these studies ranged from 1985 to 2016. Of the 14 included studies, 10 studies were judged to be at medium risk of bias and the 4 studies were judged to be at high risk of bias, whereas no study was judged to be at low risk of bias.

Bereavement Assessment

Eight studies investigated grief induced by spousal loss in relation to QoL, two focused on parental bereavement, three reported on grief in caregivers and one study compared bereavement in subjects with anxiety disorder to those with no anxiety disorder (Table 1). Bereavement assessment varied

Table 1 General characteristics of the included studies in the current systematic review

Lead author, Publication Date	Location/ Name of the study	Study Design	Baseline age	Average follow up
Boelen et al. 2007	The Nether-lands	Longitudinal	Sample 1: (recruited through mental health care workers): 48.0 ±14.8 years Sample 2: (recruited through advertisement on internet site) 45.9±14.5 years	6 and 15 months after the baseline assessment (follow up n=96))
Bourassa et al. 2016	EU/ Data used from: Multinational, representative study of Health, Ageing and Retirement in Europe (SHARE)	Case-control	Widowed 70.0 ±9.5 years Non-Widowed 61.9±8.4 years	*Time 1* (data collected 2 and 4 years prior to partner's death) *Time 2* (data collected 2 and 4 years post partner's death)
Cheng et al. 2000	Hong Kong/ China	Cross-sectional	80% (n=56) of respondents were ≤ 50 years.	n/a
Fry at al. 2001	Alberta/ Canada	Longitudinal	Range 65 to 85 years	18 Months after the baseline assessment
Grimby A. 1993	Goberborg/ Sweden IVEG Study (Intervention Study of Elderly in *Goteborg*)	Case-control	71-74 (mean 72.7)	1 year after the baseline assessment ; grief severity was measured 1st, 3rd and the 12th month .

Total participants	Outcome Assessed **QoL Instrument described in Table 3*	Exposure Assessed	Estimates reported
Total n=346 (80% women)	QoL assessed by RAND 36-Item Health Survey (RAND-36)	Grief severity in spouses 6 and 15 months post loss	Quality of life was lower and predictable in participants with prolonged grief disorder distinct from depression and anxiety.
Total n=3112 Widowed n=546 (69% women) Non-widowed n=2566 (50% women)	QoL and psycho -logical functioning was asessed by the Control, Autonomy, Self-Realization and Pleasure.Scale (CASP-19)	Bereaved spouses (6 to 24 months post loss)	Quality of life measured on a partner prior to his/her death predicted spouses' after loss quality of life(β= 0.16, 95% 0.05, 0.26, p = .003). The association between partners' earlier quality of life and their spouse's later quality of life was not significantly different for widowed vs. non-widowed people.
Total n=70 (67% women)	QoL was assessed by scale evaluating bereavement adjustments (Ho's Quality of Life Scale)	Bereaved spouses (6 to 24 months after the loss) No control group (non-bereaved)	Quality of life of the bereaved spouses was moderately lower; impact of age and sex was ruled out (mean score 6.20± 1.21),
Total n=211 (56% women)	Qol was assessed by abbreviated Health-Related Quality of Life Perceptions Scale based on three subscales of SF-36: general health, vitality and mental health.	Bereaved spouses (4 to 6 months after the loss) No control group (non-bereaved) Global and Domain-Specific Efficacy (DSEI) (widows vs widowers)	Quality of life related to health was significantly lower (p<0.01) in widows (43.8±4.4) vs. widowers (37.4 ±6.1). Self-reported quality of life: multidimensional efficacy showed significantly higher quality of life in widows vs. widowers.
Total n=42 (72% women)	QoL was assessed by scale assessing the dimensions of self-esteem, anxiety, psychosomatic health and satisfaction with life (based on methodology Rubenowitz and Berg)	Recently bereaved spouses (<2 weeks after loss): (n=47) Non recently bereaved: -Married (n=237) -Single (n=35) -Divorced (n=30) -Previously (≥2 weeks after loss)bereaved (n=101)	Quality of life of the recently bereaved (<2 weeks after loss) was lower than in married, single (never married) and not recently bereaved persons of the same age (p<0.05).

Table 1 General characteristics of the included studies in the current systematic review (continued)

Lead author, Publication Date	Location/ Name of the study	Study Design	Baseline age	Average follow up
Lannen et al. 2008	Sweden Swedish Population Register	Retrospective population based cohor	Age (%) <30 (15%) 30-39 (52%) ≥ 40 (32%) Not stated (1%)	4 to 9 years prior to study start (Parents who lost a child to cancer)
Marques at al. 2013	USA	Cross-sectional	bereaved with no anxiety disorder 43.0±13.6 bereaved with anxiety disorder 41.5±13.1	n/a
Onrust et al. 2007	The Netherlands	Cross-sectional	>55 (mean 68.8)	n/a
Ott et al. 2007	USA/ Yale Bereavement Study (YBS)	Longitudinal	60 to 91 years (70.35±6.42)	Measurement of grief scores are taken at 3 points during bereavement 1.Wave 1 at 4.36 months after loss (SD = 1.97) 2.Wave 2 at 9.22 months after loss (SD=1.92) 3.Wave 3 at 18.22 months after the loss (SD = 1.97)
Rebollo et al. 2005	Spain/ Dying elderly in Catalonia	Cross-sectional	56.40 ± 13.20	n/a

Total participants	Outcome Assessed **QoL Instrument described in Table 3*	Exposure Assessed	Estimates reported
Total n=449 (56% women)	QoL was assessed by the Tibblin score evaluating physical and psychological well-being	Parental grief Resolved(n=333) vs. un-resolved grief (n=116)	Quality of life of parents with unresolved grief showed to be significantly worse than parents with resolved grief .
Total n=397 (46% women) Bereaved with anxiety disorder N=242 (44%women) Bereaved without anxiety disorder N=155 (51% women)	QoL assessed by Quality of life Satisfaction and Enjoyment Questionnaire - Short Form (Q-LES-Q)	Bereaved people with or without anxiety disorder Bereaved with anxiety disorder N=242 (44%women) Bereaved without anxiety disorder N=155 (51% women)	Quality of life of bereaved with anxiety disorder associated with severe grief was lower (β = -0.140, P = 0.023) and with greater impairment (β = 0.141, P = 0.035) compared to bereaved without anxiety disorder
Total n=216 (13% women)	Qol assessed by EuroQoL (EQ-5D)	Spousal bereavement (loss event 6 to 9 months prior to assessment)	Quality of life of the spousal-bereaved was reduced. The average quality of life of the sample had a value of 0.79 ±0.22, whereas full health has a value of 1.
Total n=141 (69% women)	QoL was assessed by SF-12 Health Survey	Spousal bereavement: clusters based on grief scores: 1.common grief 2. chronic grief (dominantly complicated grievers) 3. resilient grief	Quality of life was lower in the common and chronic cluster compared to the resilient cluster.
Total n=130 (80% women)	QoL assessed by the SF-36	Recent bereavement of caregivers	Quality of life scores of the caregivers' were lower compared to standardized population scores.

Table 1 General characteristics of the included studies in the current systematic review (continued)

Lead author, Publication Date	Location/ Name of the study	Study Design	Baseline age	Average follow up
Song et al, 2010	USA/WLS: The Wisconsin longitudinal study	Nested case-control	Bereaved parents: Males n=64.1±3.8 Females n=66.6±4.5 Non-bereaved parents : Males n=63.4±4.3 Females n=66.3±4.8	n/a
Song et al, 2012	South Korea, Seoul/ Fourth Korean National Health and Nutrition Examination Survey	Case-control	53.2±12.5	n/a
Wiese et al, 2010	Germany	Cross-sectional	Caregivers: 58	n/a
Zhou et al. 2016	China	Cross-sectional	Married elders 67.5 ±6.0 Widowed elders 73.5 ±8.0	n/a

among the included studies (Table 2). Five studies evaluated grief using evaluation criteria that by Prigerson, Horowitz and Jacobs developed over time, or some of variation (Boelen & Prigerson, 2007; Marques et al., 2013; Onrust, Cuijpers, Smit, & Bohlmeijer, 2007; Ott, Lueger, Kelber, & Prigerson, 2007; Wiese, 2003). Parental bereavement assessed by Lannen et al, categorized patients in two groups, the ones who had and the ones who had not worked through their grief, asking "Do you think that you have worked through your grief?" (Lannen, Wolfe, Prigerson, Onelov, & Kreicbergs, 2008). Song

Total participants	Outcome Assessed *QoL Instrument described in Table 3*	Exposure Assessed	Estimates reported
Total n=462 couples (50% women) Bereaved couples (n=233) Non-bereaved couples (n=229)	QoL assessed by Health Utilities Index Mark 3 (HUI-3)	Parental bereavement examined by Cause of child death and Marital closeness	Quality of life of the bereaved parents was significantly lower than of the comparison group of parents.
Total n=501 (58% female) Bereaved family members: (n= 353) Non-bereaved controls: (n=353)	QoL assessed by EuroQoL (EQ-5D)	Bereavement in family members of patients with terminal cancer vs. non bereaved controls from general population	The overall quality of life as measured by the EQ-5D score , was significantly lower in bereaved family members than in controls (0.88±0.20 vs 0.93±0.13, p=0.002).
Total n= 46 (70% female)	QoL was assessed by SF-12's Mentale Component Score (MCS)	Post-mortal bereavement of family caregivers of patients with terminal cancer.	Quality of life of the caregivers was not reduced by complicated grief compared to those who showed normal grieving reactions (median MCS-12: 40 vs. 43; P > 0.05).
Total n=2985 Married elders n=1925 (43% Women) Widowed elders n=1060 (70% Women)	QoL assessed by SF-12 (V2 in Chinese) to measure the health-related QOL of elders.	Widowed elders vs. married counterparts; data from a large sample were analyzed instead of first search for widowed and then matched with controls (non-widowed)	Quality of life of widowers ws significantly lower than of their married counterparts (P<0.01). In widows Quality of life declined significantly with age: ; differences between widowed and married women did not reach statistical significance

et al. coded parental bereavement status as a dichotomous variable (1 = bereaved; 0 = comparison) (Song, Floyd, Seltzer, Greenberg, & Hong, 2010). Song et al used the Questionnaire from Korean National Health And Nutrition Examination Survey (Song et al., 2012). The other studies developed novel specific questionnaires tested in pilot studies. (Bourassa, Knowles, Sbarra, & O'Connor, 2015; Cheng & Ma, 2000; Fry, 2001; Grimby, 1993; Rebollo et al., 2005; Zhou & Hearst, 2016)

Table 2. Assessment of study quality through the Newcastle-Ottawa Scale

Author, Publication year	Selection	Comparability	Outcome/Exposure	Overall Score
Boelen at al.,2007	***	**	**	**7**
Bourassa et al.,2016	***	**	**	**7**
Cheng et al., 2000	**	**	*	**5**
Fry at al., 2001	**	**	**	**6**
Grimby at al.,1993	***	**	**	**7**
Lannen et al. 2008	***	**	**	**7**
Marques at al., 2013	***	**	**	**7**
Onrust et al., 2007	**	**	*	**5**
Ott et al., 2007	***	**	**	**7**
Rebollo et al., 2005	***	*	*	**5**
Song at al., 2010	***	**	**	**7**
Song at al., 2012	**	**	**	**6**
Wiese et al, 2010	*	**	**	**5**
Zhou et al. 2016	***	**	**	**7**

Assessment of Quality of Life

Assessment of QoL was heterogeneous among included studies (Table 2). Rebollo et al. used a Spanish version of the SF-36 Health Survey (Rebollo et al., 2005). Fry et al. used The Health-Related Quality of Life Perceptions Scale- HRQOL developed by using three subscales of the SF-36 (Fry, 2001). Ott et al utilized the SF-12 Health Survey (Ott et al., 2007). Lannen et al. estimated QoL using The Tibblin Score (Lannen et al., 2008). Grimby et al. used a 23-question questionnaire revised from Rubenowitz and Berg. Song et al assessed health-related QoL with the EQ-5D , including an a summary score and a visual analog scale (EQ-VAS) (Song et al., 2012). Marques et al. assessed QoL using the short form of Q-LES-Q, while Boelen et al. used RAND 36 (Boelen & Prigerson, 2007; Marques et al., 2013). Bourassa at al. (Bourassa et al., 2015) used 12 items of the CASP-19 (Cheng & Ma, 2000), a scale developed specifically for assessing quality of life. Cheng at al,(Cheng & Ma, 2000) used Ho's (Condliffe et al., 2014). Zhou and Hearst used SF12 questionnaires and information about individual and household characteristics (Zhou & Hearst, 2016). Wiese at al. measured psychosocial and physical distress of the care-giving relatives in the inquiry measured through a sub-score of SF-12 to validate the quality of life of the relatives with the mental component score (MCS-12). (Wiese et al., 2010)

Spousal bereavement and quality of life

Three cross-sectional studies and four prospective studies examined the association between spousal bereavement and QoL (Table 1). After a follow-up of one year, Grimby et al. found that the QoL of the recently bereaved (<2 weeks) 42 spouses was lower than that of married single/never married and not recently bereaved individuals ($p<0.05$) of the same age (Grimby, 1993). However, QoL was not significantly different in comparison with divorced individuals. Among 211 bereaved spouses, Fry et al. found that, after 18-months follow-up, comparing to bereaved women, widowers scored significantly

Table 3. Methods used to define exposure and outcome in each individual study included in the current review

Lead Author, Publication Date	Exposure assessment Grief or other	Outcome assessment of QoL
Boelen PA et al. 2007	Two samples of bereaved individuals were recruited for the research program on cognitive variables in grief, Symptoms of prolonged grief disorder. Items to assess PGD were taken from the Inventory of Complicated Grief-revised (ICG-r). The ICG-r was developed by Prigerson and Jacobs (Ref) as an extended version of the Inventory of Complicated Grief (Prigerson, 2001) It taps most criteria for PGD and other problematic grief reactions. Respondents rate the presence of symptoms in the last month on 5-point scales ranging from 1 (never) to 5 (always). The 29-item Dutch ICG-r has good psychometric properties.	QoL was assessed with the Rand 36-item Health Survey (RAND 36) (Ware, 1992). This questionnaire assesses subjective health status and functioning in eight domains: physical functioning (10 items), social functioning (2 items), role limitations due to emotional problems (3 items), role limitations due to physical problems (4 items), mental health (5 items), pain (2 items), energy (4 items), and general health perception (5 items). In addition, one item is included to assess the direction of change in health over the preceding year. Domain total scores are calculated such that higher scores reflect better functioning. The items of the RAND 36 are identical to those of the well-validated Medical Outcomes Survey Short Form-36 (Ware at al. , 1996). The instrument has yielded adequate psychometric properties in original (Cunningham at al, 2006) and Dutch version (van der Zee KI & Sanderman, 1993). In the current study the subscale physical functioning was not administered. The subscale pain was only administered at T3.
Bourassa KJ et al. 2016	This study used data from the multinational, representative Study of Health, Ageing and Retirement in Europe (SHARE). Spousal bereavement was detected by database information of couple's, in which one of the spouses died. Survived spouse was included in the study as a bereaved participant. No severity of grief was assessed	Quality of life was assessed by the CASP-19 (Ref), scale developed specifically for use in assessing quality of life, life satisfaction, and wellbeing in aging populations, which shows concurrent validity with similar measures..

Table 3. Methods used to define exposure and outcome in each individual study included in the current review (continued)

Lead Author, Publication Date	Exposure assessment Grief or other	Outcome assessment of QoL
Cheng at al. 2000	Bereaved participants were included via clinical referral from 11 hospitals and 1 clinic in China. The Stress of Bereavement Scale was used , this self-constructed assessment scale has two parts: measuring the stressors and perceived stress of the bereaved spouses. Each sub-scale consisted of 30 items, which measured different dimensions of stress including the physical, the psychological and the social domain. The respondents were asked to respond to both the objective and the subjective measures. These measures were the same, but the questions were differently stated. The answers to the questions that measured the stressors ranged from frequently (5), quite often (4), sometimes (3), seldom (2) and never (1), while the answers of the second part ranged from very stressful (5), quite stressful (4), somewhat stressful (3), slightly stressful 92) and not stressful (1). Internal consistency of Stressor and the perceived Stress Sub-scales was satisfactory with Cronbach's alphas of 0.82 and 0.85 respectively.	Quality of life was assessed by Ho's (1991) Quality of Life Scale measuring the adjustment outcome of the bereaved spouses. Questions 3 was changed from "your living, ability, movement/ action, interest" to "your participation in activities, cultivating interest". Higher scores denoted better quality of life. The Cronbach's alpha of this scale was 0.89.

Table 3. Methods used to define exposure and outcome in each individual study included in the current review (continued)

Lead Author, Publication Date	Exposure assessment Grief or other	Outcome assessment of QoL
Fry at al. 2001 (Fry, 2001)(Fry, 2001)(Fry, 2001) (Fry, 2001)	Bereavement was identified in the regional community-support project offering social support to recently bereaved elderly adults (see Fry, 1998,2001a, 2001b). Eligibility criteria included spousal loss approximately 4 to 6 months ago. No Instrument for grief severity was used, but severity of grief was assessed through the reported change on self-efficacy of the bereaved person (pre and post loss efficacy). Namely, domain Specific Efficacy and Global Efficacy Index.	An abbreviated measure of the Health-Related Quality of Life Perceptions Scale (HRQOL) was developed using three subscales of the SF-36 to measure quality of life. These three subscale measures are (a) the Subscale for General Health Perceptions, which evaluates current general health as well as whether or not the person believes his or her health will deteriorate (five questions), (b) the Vitality Subscale, which determines the degree to which the person feels full of energy and "pep" as well as how often he or she is tired or worn out (four questions), and (c) the Mental Health Subscale, which examines nervousness and feelings of depression, sadness, peacefulness, and happiness (five questions). Responses to the 14 questions included in the composite measure of the three subscales of the HRQOL were arranged on a 5-point Likert type scale ranging from strongly disagree (1) to strongly agree (5). The higher the cumulative score, the more positive are the HRQOL perceptions. The range of scores on this measure is 14 to 70. The HRQOL score for each individual is based on the subscale scores of general health perceptions, vitality, and mental health. Cronbach's alphas for this survey were 0.75 and 0.82 for widows and widowers, respectively.

Table 3. Methods used to define exposure and outcome in each individual study included in the current review (continued)

Lead Author, Publication Date	Exposure assessment Grief or other	Outcome assessment of QoL
Grimby et al. 1993	A semi structured interview of 1.5 to 2h was used to assess the severity of the grief. This interview was based on 13 grief reactions found in research (tested in a pilot study of 15 bereaved people). The reactions were rated by the psychologist with respect to frequency (presence or absence) and to intensity using a 5-point scale from 1 (mild) to 5 (very severe). In the statistical analysis the grief reactions were grouped into 3 dimensions: low mood (dysphoria, loneliness, crying and pessimism), cognitive dysfunctioning (fatigue, concentration problems, lack of interest, indecisiveness and memory problems) and self-reliance (anxiety, self-reproach, anger and lowered self-esteem). After 12 months follow-up grief reactions intensity was categorized as: significant or non-significant decrease. The control group was classified as follows: married, single, divorced and previously bereaved.	Quality of life assessment was performed with an instrument developed by Rubenowitz and Berg, measuring the dimensions of self-esteem, anxiety, psychosomatic health and satisfaction with life. The questionnaire contained 23 questions with answers scored from bad to very good. The sum score for each of the 3 dimension was obtained by dividing (100 times the sum of the score of each item) by the sum of the maximum for all items.
Lannen et al. 2008	Bereaved parents were included upon identification of the death of a child via the national register of causes of death and the national register of cancers. Severity of the bereavement was assessed via parental resolution of grief assessed with the question: "Do you think that you have worked through your grief?" Possible answers: not at all, somewhat, a lot, or completely. Parents who stated not at all or somewhat were placed in the category of those parents who had not worked through their grief.	The Tibblin Score was used to assess quality of life. Quality of life according to physical and psychological well-being was self-assessed by the parents with a seven point Visual Digital Scale.
Marques at al. 2013	Bereaved participants were recruited through advertisement or clinical referrals. They reported the loss of a close relative or significant other. The 19-item, self-report Inventory of Complicated Grief (Prigerson et al.,1995)	The 16-item self-report Quality of Life Satisfaction and Enjoyment Questionnaire–Short Form [Q-LES-Q) (Ref) was used to assess quality of life.

Table 3. Methods used to define exposure and outcome in each individual study included in the current review (continued)

Lead Author, Publication Date	Exposure assessment Grief or other	Outcome assessment of QoL
Onrust et al. 2007	Older widowed individuals were identified via the registers of birth , death and marriages in 18 Dutch municipalities. Traumatic or complicated grief was measured using the Inventory of Complicated Grief-revised (ICG-r) (Prigerson et al., 1995), a self-report use questionnaire of Psychological adjustment after bereavement with good psychometric properties (Boelen et al., 2003). The ICG-r consists of 29 items referring to cognitions, emotions and behaviours that define traumatic or complicated grief, such as preoccupation with thoughts of the deceased, yearning and searching for the deceased and feeling stunned by the death. Respondents were asked to indicate how often they had experienced each symptom during the past month, using the response categories "never," "hardly ever," "sometimes," "regularly," or "always." The corresponding scores sum to a total score ranging from 29 to 145.	Quality of life was assessed with the EuroQol (EQ-5D) (Brooks and EuroQol Group, 1996) The EuroQol is made up of five dimensions: mobility, self-care, usual activities, pain/discomfort, and, anxiety/depression. Respondents were asked to indicate for each dimension whether they experienced "no problems," "some problems," or "extreme problems." The separate scores were combined into the EQ-5D Index, a health status index, which is used to evaluate health status (QALY).
Ott et al. 2007	The bereaved participants were derived from the Yale bereavement study. Severity of the grief was assessed by a structured interview using the Inventory of Complicated Grief-Revised (ICG-R; Prigerson et al., 1995b; Silverman et al., 2000) Severity of grief was measured using the sum of the responses from nine questions from the ICG-R proposed for the DSM diagnostic criteria.	Quality of life was assessed at all three waves of data collection with the SF-12 Health Survey (Ware et al., 1996). The SF-12 is a well validated short form (12 items) of the SF-36 Health Survey
Rebollo et al. 2005	Bereaved individuals in this study were identified via a dataset derived from a project, 'Dying elderly in Catalonia', whose objective was to describe the place and the circumstances of the death of elderly persons (Ramon et al., submitted for publication)). The study surveyed the caregivers or 'the person, family member or otherwise, mainly responsible for delivering care to the deceased in the months directly prior to death; that is, the person most familiar with the state of health and care needs of the deceased.	Quality of life was assessed via two components of the Spanish version of the SF-36 Health Survey: Physical Component Summary (PCS) and Mental Component Summary (MCS) scores of the SF-36

Table 3. Methods used to define exposure and outcome in each individual study included in the current review (continued)

Lead Author, Publication Date	Exposure assessment Grief or other	Outcome assessment of QoL
Song, et al. 2010	Bereaved parents were included from the Wisconsin longitudinal study. Parental bereavement status was coded as a dichotomous variable (1 = *bereaved*; 0 = *comparison*). Cause of child death was taken into account as indicator of more severe grief . Marital closeness was assessed as a mitigator of negative effects of bereavement (reducing grief severity).	Quality of Life was measured by the Health Utilities Index Mark 3 (HUI-3). This is a multidimensional self-report measure of overall health status (Ref). The measure evaluates eight attributes: vision, hearing, speech, ambulation, dexterity, emotion, cognition, and pain. Each attribute is assessed by five or six levels of health status or functionality.
Song JI et al. 2012	Bereaved participants in this study were identified through a data set gathered via a national initiative to evaluate the quality of palliative care services The severity of bereavement was not assessed . Bereavement in family members of patients with terminal cancer vs. non-bereaved controls from general population	Quality of life was assessed by EQ-5D to evaluate health-related quality of life , including a summary score and a visual analog scale (EQ-VAS) (Ref).
Wiese CH et al. 2010	Bereaved participants were recruited via mental health institutions. Evaluation of bereavement used the validated criteria of Horowitz and Prigerson, together with the definition of complicated grief according to ICD-10.	Quality of life was assessed by sub-scores of SF-12: 1.Psychosocial and physical distress of the care-giving relatives in the inquiry was measured through a sub-score of SF-12® to validate the quality of life of the relatives (MCS-12). 2.Retrospective evaluation of the subjective psychosocial distress of family caregivers during specialized PCT care. 3.Correspondence of the results with reference to indications of complicated bereavement
Zhou JF et al. 2016	Bereaved elderly spouses in a rural part of the china, were identified via a local household registration information system in each town. For the duration of the bereavement the question was asked to give the year in which the spouse had died; (exact date was not asked because a pre-test indicated that widows often could not remember this) Severity of the bereavement was not assessed.	This study used SF-12 (V2 in Chinese) to measure the health-related QOL of elderly persons (Ref).

lower in the self-report measures of multidimensional efficacy (Fry, 2001). Widows also scored higher in the overall health-related QoL perceptions (widows = 43.8±4.4; widowers = 37.4±6.1, P<0.01). A prospective cohort study by Ott et al. compared three clusters of 141 spousal mourners, based on grieving scores (common, chronic and resilient) (Ott et al., 2007). The resilient cluster experienced the lowest levels of grief and depression, and the highest quality of life. While, the majority of subjects in the chronic cluster met the criteria for a diagnosis of complicated grief, and experienced the highest levels of grief and depression, were more prone to sudden deaths, had the lowest self-esteem, and the highest marital dependency (Ott et al., 2007). In a case-control study among 546 widowed and 2 566 non-widowed men and women, Bourassa at al. tested whether deceased spouses' characteristics were associated with their widowed partners' later quality of life using couples drawn from a multinational sample of aging adults. Independent subsamples were assessed before and after a spouse's death. Quality of life before the death of deceased partner's predicted their spouses' later quality of life after their passing (β= 0.16, 95% 0.05, 0.26, p = 0.003). In additon, as compared to people who remained married, widowed subjects had lower absolute levels of quality of life—both before (β=-0.23, 95% CI -0.33,-0.13,p<0.001) and after their partners' death (β=-0.24;95% CI -0.34;-0.13,p<0.001). Finally, the strength of independence in couples' quality of life was not significantly different between widowed and non-widowed subjects (β = −0.01, 95% CI −0.19, 0.17, p > 0.250) (Bourassa et al., 2015). Zhou and Hearst performed a study that comprised 3053 elders aged 60 and above in rural China. The SF12 questionnaire was administered and individual and household characteristics were obtained. Health related QoL was compared between 1 925 married and 1 060 widowed persons. Physical component summary (PCS) and mental component summary (MCS) scores between married elders and widowed elders in a bivariate model stratifying by gender and age group and in a general factorial ANOVA multivariate analysis were performed. Overall, widowed elderly Chinese people from rural areas had lower physical and mental quality of life than their married counterparts. Also, widowed men had lower PCS and MCS scores than married men after controlling for age. Although in widowed and married women QoL declined significantly with age, and overall PCS was lower in widowed women, the differencee in MCS scores between widowed and married women was not statistically significant (Zhou & Hearst, 2016).

Finally, we found two studies that examined the quality of life of spousal bereavement but with no comparison group. Onrust et al.(Onrust et al., 2007) demonstrated a reduced QoL among widows (widows had quality of life score of 0.79 ±0.22 whereas full health has a value of 1) in a cross-sectional study among 216 bereaved spouses. Cheng at al. (Cheng & Ma, 2000) conducted research on spouses six months to two years into bereavement. This study comprised 70 respondents (provided by eleven hospitals and one clinic). The mean scores of the respondents on the quality of life ranged from 3 to 8.78 (mean 6.20, SD 1.21). Overall, quality of life of the respondents was moderately good and age, gender and the other demographic characteristics of included participants did no alter their quality of life.

Bereavement in caregivers and quality of life.

There were three observational studies that evaluated the association between bereavement in caregivers and health related QoL (Table 1). Song at al. compared QoL of 353 bereaved family members of patients with terminal cancer with 353 non-bereaved controls from the general popula-

tion (Song et al., 2012). Quality of life in bereaved family members was significantly lower than in controls (0.88±0.20 vs 0.93±0.13, p=0.002). Also, a negative correlation with health-related QOL was observed with female gender, spousal relationship, lower income, and longer duration of survival after palliative care referral. Similarly, Wiese at al. studied 46 care-giving relatives of deceased patients with terminal cancer 2 to 24 months after the patient had died. The MCS-12, a subscore of SF-12, showed that the psychological strain in the caregivers was higher than that in the general population (range 46–60; median 52). Based on Prigerson criteria 30% of care-giving relatives had criteria for the diagnosis of complicated grief, while using the definition of ICD-10 only 13% fulfilled the criteria for this diagnosis. Further, the caregivers who experienced complicated grief did not present significantly higher psychosocial distress and limitations of their quality of life than those who showed normal grieving reactions (median MCS-12: 40 vs. 43; P > 0.05) Subjective evaluation of psychosocial distress that caregivers suffered at the time of the interview revealed increased values of MCS-12. Caregivers with decreased MCS-12 values and, consequently, increased psychosocial distress considered themselves to be more stressed (Wiese et al., 2010)- Rebollo. et al. compared recent (median of 139 days) bereavement of 130 mainly female caregivers of a deceased elderly relative compared to standardized population scores (Rebollo et al., 2005). Caregivers' SF-36 scores were lower than expected in the general population for mental health, role emotional and social functioning. The recent death, first-degree family relationship and younger age of deceased were found to be important factors to influence the health of caregivers.

Parental bereavement and quality of life

One retrospective cohort and one cross-sectional study examined the association between parental bereavement and QoL (Table 1). Lannen et al. investigated the QoL among 449 parents who lost a child due to cancer before the age of 25 (Lannen et al., 2008). Parents with unresolved grief (26%) were compared with parents who had worked through their grief. They were more likely to report poorer QoL 4 to 9 years after child loss; relative risks of Tibblin score <10 % were 2.7 (95% CI 1.2-6.1) and 4.4 (95% CI 1.7-11.5) among mothers and fathers respectively. Fathers had higher risk of sleep difficulties (crude RR 2.6, 95%CI 1.3-5.1) while mothers reported increased visits to physicians due to anxiety/depression (adjusted RR 2.1, 95% CI 1.1-3.9) and greater likelihood of taking sick leave (crude RR 2.1, 95% CI 1.2-3.5) when they had not worked through their grief. Also, while the number of fathers with unresolved grief was significantly decreased 4 to 9 years after loss (p<0.02) this was not seen among mothers. A longitudinal study by Song et al, compared 233 bereaved couples with 229 non-bereaved couples to investigate the long-term effects of child loss (that occurred on average two decades earlier) on parent's health related QoL using the Health Utilities Index Mark 3 (HUI-3) (Song et al., 2010). After controlling for demographic factors, bereaved parents had significantly worse health related QoL than non-bereaved parents. The age of the child or time since child's loss did not significantly predict health outcomes. Also, bereaved parents whose child died in violent circumstances had particularly low levels of QoL. Furthermore, marital closeness was a positive predictor, while having experienced a depressive episode was a negative predictor of QoL.

Severity of bereavement and QOL

Two studies, one cross-sectional and one longitudinal, reported on severity of bereavement and QoL. In a cross-sectional study Marques et al. compared 155 bereaved subjects with primary anxiety disorder and 242 bereaved subjects without anxiety disorder (Table 1) (Marques et al., 2013). After adjustment for age, sex, education level, comorbidity and major depressive disorder, complicated grief was associated with lower quality of life (β = -0.140, P = 0.023) and greater impairment (β = 0.141, P = 0.035) among individuals with anxiety disorder. Boelen et al. conducted a longitudinal study to investigate the association of grief severity and QoL 6 and 15 months post loss among 346 subjects (Table 1) (Boelen & Prigerson, 2007). Results showed that prolonged grief disorder, independently of depression and anxiety, is predictive of reduced QoL and mental health.

DISCUSSION

This is the first study to systematically review the evidence on the association between grief status and QoL. Overall, we found that bereaved persons and grief severity show associations with lower QoL. Also, our findings suggest that women experience changes in grief related QoL in a different way than men. After the loss of a child, females suffer from lower QoL then men. Also, females have more difficulties recovering from grief; this prolongs the grieving process and consequentially might lower life quality. Nevertheless, the evidence on grief and QoL is limited and hampered by the scarcity and suboptimal quality of the studies on the topic, and therefore no firm conclusion can still be drawn.

Interpretation of findings

Despite increasing efforts in bereavement research, little research has focused on QoL related to bereavement. Nevertheless, the current evidence, as supported by our findings, suggests that different types of bereavement and bereavement severity hamper the QoL in people reporting the loss of a loved one. Bereavement after losing a partner or a child shows associations with lower QoL. Also, subjects with complicated grief, a disorder characterized by persistent high levels of bereavement, experience persistent deficits in QoL.

Even a century ago, grief was officially regarded as a cause of death (Parkes, 1964). Currently QoL remains an important predictor of mortality. QOL is a broad concept that comprises a range of life domains of the individual, such as social relationships, role functioning, physical abilities and engagement in daily activities, and mental health functioning (Brown , Thompson , Zack , Arnold , & Barile 2015). The severity one grieves with after the death of a loved one may differ. Likewise, expression of the grief also differs depending on the social relations or how grieving is culturally/socially justified or expected. Loss of a loved one results in long-term changes in one's social situations and relationships, since grieving individuals have lack of clarity of their role in society contributing to a sense of social isolation (Wiese, 2003). Coping strategies after a loss help resolve the isolation in case of less severity and shorter duration of grief, which further prevents lower QoL. Another important aspect of the human stress response is the tendency to seek support and protection when feeling threatened (Aple, 2011). The hormone oxytocin motivates individuals to connect with others, but it has also been found to dampen the fight-or-flight response, which could be one explanation of why social

support is beneficial in the face of stress (Saavedra Perez et al., 2016). Many people experiencing grief might also suffer from more severe or triggered disability (d'Epinay, Cavalli, & Guillet, 2009). This is somewhat expected and can be explained through isolation processes or lifestyle changes (Cornwell & Waite, 2009). Lack of willingness to interact and retrieving to loneliness also triggers higher disability. Previous studies have shown bereavement is associated with a decline in functional impairment (d'Epinay et al., 2009). The majority of people experiencing a loss of a dear one can also experience a different mental health comorbidity such as, anxiety, anger, depressive symptoms or suicidal thoughts (Kristensen, Weisaeth, & Heir, 2012; Parkes, 1964). Although health-related factors, both physical and mental, have been hypothesized to influence the duration and severity of grieving, there is no clear explanation of mechanisms that can lead to this scenario (Feinson, 1982; Kristensen et al., 2012). On the other hand, recent evidence suggests that grief affects the same brain area as general pain mechanisms, which could explain the physical pain experienced by those who lose a loved one (Bratt, 2016).

Our findings suggest that bereavement may be associated with QoL in a gender-specific manner, with female grievers having lower QoL. These gender differences might be explained by multiple adaptive mechanisms as well as relation or compliance, and differences in male/female social support/ network (Trofimova, 2013). Stroebe, reports gender differences in the needs and benefits of bereaved men compared with bereaved women., for example, widowers benefit more from emotion-focused interventions while widows from problem-focused interventions (Stroebe 2001). Also, in elderly populations, a certain life style is present with women leading the household and taking care about the stability of daily rhythm, while men providing financial and logistic means. By the death of the woman, man would be left alone with difficulties in learning to suddenly take over control of household, self-care and overall setting and lifestyle (Stroebe 1998; Umberson, Wortman, & Kessler, 1992). By the death of the man traditionally women will experience difficulties learning to manage financial issues. This difference will probably disappear over time, due to changing marital roles. In both male and female spousal bereavement, persons have lower physical and mental quality of life than their married counterparts. Consequent to loss event, support from children is associated with better QOL. Namely, these elders rely on their children for care, and a supportive family is associated with better QOL and, seems to mitigate negative effects of widowhood (Song et al., 2010). Further in the palliative care setting, the findings suggest that care from a specialized palliative-care team providing psychological and social support may reduce the risk of complicated grief (Wiese et al., 2010). Careful exploration of possible risk factors for complicated grief is important for optimal care. Healthcare providers play an important role in helping family caregivers to manage the multiple burdens and the grieving reaction. Family-focused grief therapy may prevent complicated grieving reactions (Wiese et al., 2010).

Methodological Concerns and Future Recommendations

The magnitude of the effect size on the association between bereavement and QoL is currently difficult to assess because of the scarcity of the evidence. The available studies suffer from large heterogeneity in bereavement assessment and QoL instruments, study design and quality.. Factors that might hamper the quality of recording grieving states need consideration. These include the potential effect of recall bias and the challenges of objective interpretation of emotional status as it is generally

under-reported or not realistic if not checked or re-assessed by healthcare professionals. Beyond the caution needed in interpretation of grief length and severity, future studies must also consider other aspects associated with grieving. For instance, most of the studies define the state of grief as present or absent after the loss event, not taking into account the severity of grief and its duration (complicated grief or prolonged grief disorder). Only few of the included studies acknowledged grief severity and in a different manner (Boelen & Prigerson, 2007; Marques et al., 2013). Therefore, the continuation/persistence of all degrees of severity in the bereavement process needs more research effort in the future, in order to harmonize the definitions and distinct different bereavement status. Most studies lack appropriate adjustment for confounding factors since they did not consider important determinants of grief and QoL such as socioeconomic factors, social support, depressive symptoms and treatment. The majority of studies used small samples (ranging from 42 to 501) and there were methodological concerns related to the selection of participants, compromising generalization of the findings. Moreover, studies used different methods to assess QoL and in some studies the instrument used was not validated. In the future, it is necessary to harmonize bereavement and QoL assessment, as well as design better studies that adjust for a broad range of confounders and select a representative sample. Of special importance, prospective studies will be needed before any level of causality can be inferred from the existing findings, and residual confounding could be considered as an explanation for the associations observed. Future research, by taking into account all these methodological considerations, would provide a more reliable assessment of the association between bereavement and QoL, which might have numerous benefits. Understanding QoL related with bereavement and its types would allow health and social care providers and researchers to better understand what aspects of the bereavement and treatment/intervention the bereaved individuals views as having the greatest impact on their QoL. It may also give insights on whether specific interventions to enhance adaptation to bereavement, or treat health consequences related to bereavement might outweigh its potential benefits. QoL effects of bereavement may impact individuals sustaining health and social care activities and care providers decision-making. Understanding QoL related to bereavement may also be useful in shaping better interventions in individuals experiencing bereavement and improve outcomes related to bereavement. Also, if our findings on an association between bereavement and QoL are confirmed with further studies we could carefully infer that QoL could be assessed as an additional intervention/treatment bereavement outcome.

Strengths and limitations of the review

Previous studies have evaluated the effect of bereavement on some of the aspects or overall quality of life, but our study is the first attempt to systematically review and critically appraise the literature on the subject, and put together the different existing studies that evaluated the associations of bereavement and it's severity with overall QoL. On the other side, a meaningful quantitative pooling of the existing data was unfeasible due to different types of bereavements examined by individual studies, different methods used to define QoL, heterogeneity in the study designs and because the estimates were not reported in some of the studies. Furthermore, the findings reported in the current study do not carry any implications on causality because all included studies are observational.

CONCLUSIONS

Different types of bereavement and bereavement severity show associations with lower QoL. Researchers and providers evaluating bereavement care interventions might consider including QoL outcome measures in their projects. However, future large prospective studies are warranted to confirm our findings and to examine specific domains of QoL affected by bereavement, as well as assessing sex-specific effects of bereavement on QoL.

Contributors

1. Conceptualization: JT TM OHF.
2. Formal analysis: JM LZR BK WMB EB TM.
3. Investigation: JM LZS BK WMB EB TM OHF.
4. Methodology: JM TM OHF.
5. Project administration: JM TM OHF.
6. Supervision: HV HG HT EB TM OHF.
7. Writing – original draft: JM LZR HT HG EB OHF TM.
8. Writing – review & editing: JM LZR BK HT HG HV WBM EB TM OHF.

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SUPPLEMENTARY MATERIAL

Included in the online Supplementary Material:

APPENDIX 1. PRISMA 2009 CHECK-LIST

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	3-4
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	3
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	4
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	4-5
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	4
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	Appendix 3
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	4-5
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	5
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	5
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	5
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	-
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	-

Section/topic	#	Checklist item	Reported on page #
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	5
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	-
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	5-6; Figure 1
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	6-12; Table 1
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	-
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	6-12; Table1 & 3
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	-
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	-
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	-
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	12-15
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	17
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	17
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	18

APPENDIX 2. MOOSE CHECKLIST

Criteria	Brief description of how the criteria were handled in the meta-analysis
Reporting of background should include	
✓ Problem definition	It remains unclear if bereavement effects quality of life of the person losing a loved one.
✓ Hypothesis statement	Bereavement effects on of the person losing a loved one
✓ Description of study outcomes	Quality of life
✓ Type of exposure or intervention used	Bereavement, bereavement counseling, grief, mourn, loss of loved one
✓ Type of study designs used	Studies were eligible if they (i) were observational studies (cross-sectional, case-control and cohort studies), or randomized clinical trials; (ii) assessed any form of grief (bereavement, grief, PGD and complicated grief), (iii) collected endpoints for QoL; and (iv) examined the association between any form of grief and QoL)
✓ Study population	Bereaved family members or caregivers
Reporting of search strategy should include	
✓ Qualifications of searchers	The credentials of the investigators are indicated in the authors list.
✓ Search strategy, including time period included in the synthesis and keywords	Search strategy and time periods are detailed in page 5 of the manuscript and in the Appendix 3.
✓ Databases and registries searched	Medline, EMBASE, Web of Science, PubMed, Cochrane and Google Scholar
✓ Search software used, name and version, including special features	We did not employ any search software.
✓ Use of hand searching	We did not hand searched studies given that we used a thorough search strategy.
✓ List of citations located and those excluded, including justifications	Details of the literature search process are outlined in the flow chart. The citation list for excluded studies is available upon request.
✓ Method of addressing articles published in languages other than English	We placed no restrictions on language. All identified studies were in English.
✓ Method of handling abstracts and unpublished studies	No unpublished studies were identified
✓ Description of any contact with authors	None
Reporting of methods should include	
✓ Description of relevance or appropriateness of studies assembled for assessing the hypothesis to be tested	Detailed inclusion and exclusion criteria are described in the Methods section.
✓ Rationale for the selection and coding of data	Data extracted from each of the included studies were relevant to the population characteristics, study design, exposure, and outcome.
✓ Assessment of confounding	Confounding was assessed for each of the included study and if found described in Table 1 in “covariates adjusted” column.
✓ Assessment of study quality, including blinding of quality assessors; stratification or regression on possible predictors of study results	Assessed for each included study by New-castle Ottawa scale

Criteria	Brief description of how the criteria were handled in the meta-analysis
✓ Assessment of heterogeneity	Due to limited number of included studies and high heterogeneity in exposure and outcome assessment by the individual studies, we concluded that meta-analysis was not feasible
✓ Description of statistical methods in sufficient detail to be replicated	Not applicable.
✓ Provision of appropriate tables and graphics	We included 1 main figure, 3main tables and 3 supplementary tables
Reporting of results should include	
✓ Graph summarizing individual study estimates and overall estimate	Figures 1
✓ Table giving descriptive information for each study included	Tables 1, 2 and 3
✓ Results of sensitivity testing	Not applicable
✓ Indication of statistical uncertainty of findings	Not applicable
Reporting of discussion should include	
✓ Quantitative assessment of bias	Not applicable
✓ Justification for exclusion	We excluded, conference abstracts and studies with inappropriate exposure or outcome
✓ Assessment of quality of included studies	Assessed for each included study by New-castle Ottawa scale
Reporting of conclusions should include	
✓ Consideration of alternative explanations for observed results	In discussion of the review we elaborate our observation.
✓ Generalization of the conclusions	Our findings apply to middle age and elderly people who lost their loved ones . However, most of the studies were performed among western and Caucasian populations and generalization of findings to other populations and ethnicities is somewhat limited.
✓ Guidelines for future research	Most studies lack appropriate adjustment for confounding factors since they did not consider important determinants of grief and QoL such as socioeconomic factors, social support, depressive symptoms and treatment. The majority of studies used small samples (ranging from 42 to 501) and there were methodological concerns related to the selection of participants, compromising generalization of the findings. Moreover, studies used different methods to assess QoL and in some studies the instrument used was not validated. In the future, it is necessary to harmonize bereavement and QoL assessment, as well as design better studies that adjust for a broad range of confounders and select a representative sample. Of special importance, prospective studies will be needed before any level of causality can be inferred from the existing findings, and residual confounding could be considered as an explanation for the associations observed.
✓ Disclosure of funding source	Funding was disclosed on page 20.

APPENDIX 3. LITERATURE SEARCH STRATEGY

Embase.com

(grief/exp OR bereavement/de OR 'bereavement counseling'/exp OR (grief OR grieve* OR mourn* OR sorrow* OR bereave* OR ((loss OR lose OR lost) NEAR/3 (spous* OR husband OR wife OR partner* OR sibling* OR partner* OR brother* OR sister*))):ab,ti) AND ('quality of life'/exp OR ((qualit* NEAR/3 life)):ab,ti) NOT ([Conference Abstract]/lim OR [Letter]/lim OR [Note]/lim OR [Editorial]/lim) AND [english]/lim

Medline ovid

(exp bereavement/ OR (grief OR grieve* OR mourn* OR sorrow* OR bereave* OR ((loss OR lose OR lost) ADJ3 (spous* OR husband OR wife OR partner* OR sibling* OR partner* OR brother* OR sister*))).ab,ti,kf.) AND ("quality of life"/ OR ((qualit* ADJ3 life)).ab,ti,kf.) NOT ((letter OR news OR comment OR editorial OR congresses OR abstracts).pt.) AND english.la.

Psycinfo ovid

(exp bereavement/ OR (grief OR grieve* OR mourn* OR sorrow* OR bereave* OR ((loss OR lose OR lost) ADJ3 (spous* OR husband OR wife OR partner* OR sibling* OR partner* OR brother* OR sister*))).ab,ti.) AND ("quality of life"/ OR ((qualit* ADJ3 life)).ab,ti.) NOT ((letter OR news OR comment OR editorial OR congresses OR abstracts OR books).pt.) AND english.la.

Cochrane

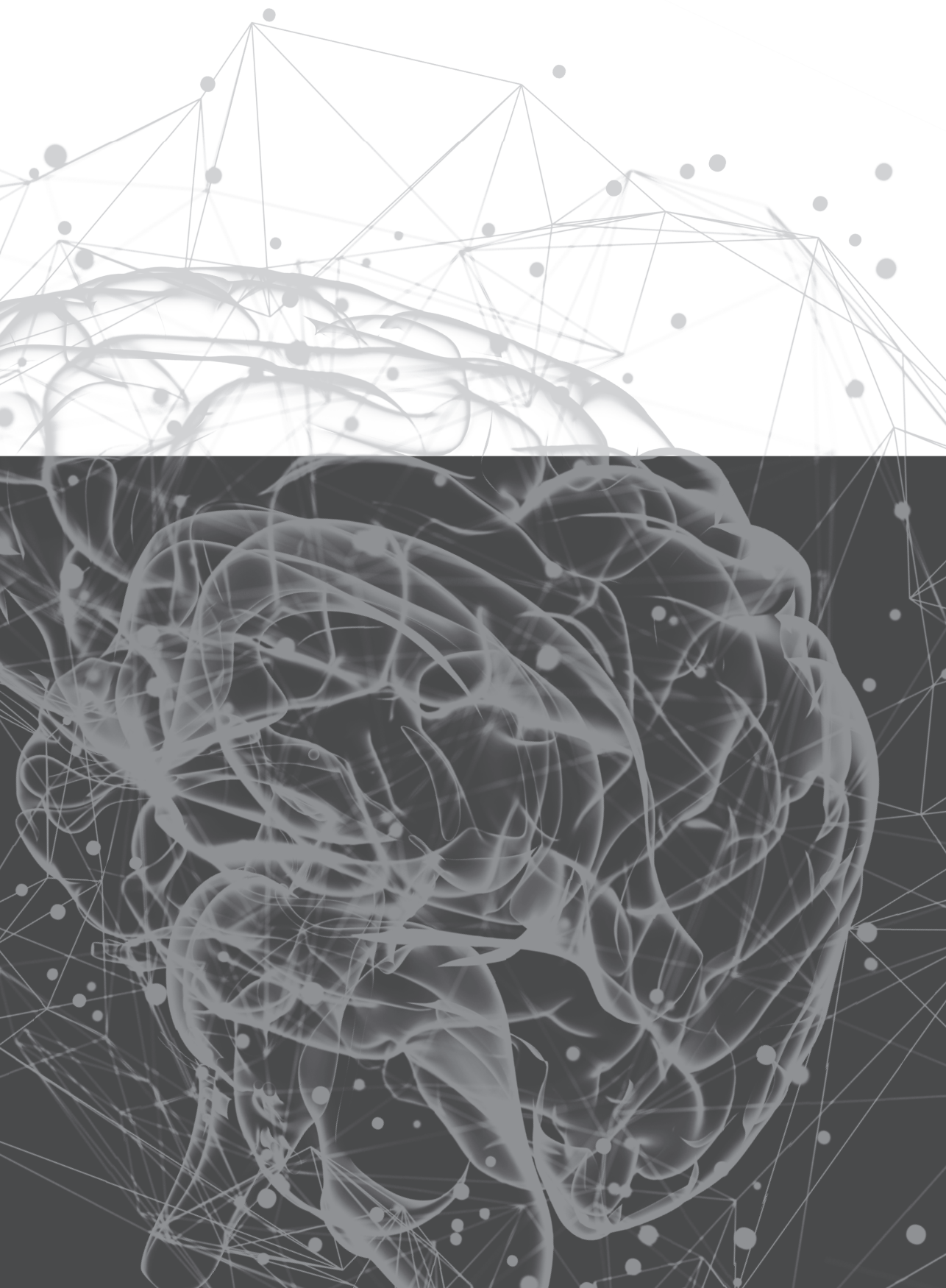
((grief OR grieve* OR mourn* OR sorrow* OR bereave* OR ((loss OR lose OR lost) NEAR/3 (spous* OR husband OR wife OR partner* OR sibling* OR partner* OR brother* OR sister*))):ab,ti) AND (((qualit* NEAR/3 life)):ab,ti)

Web-of-science

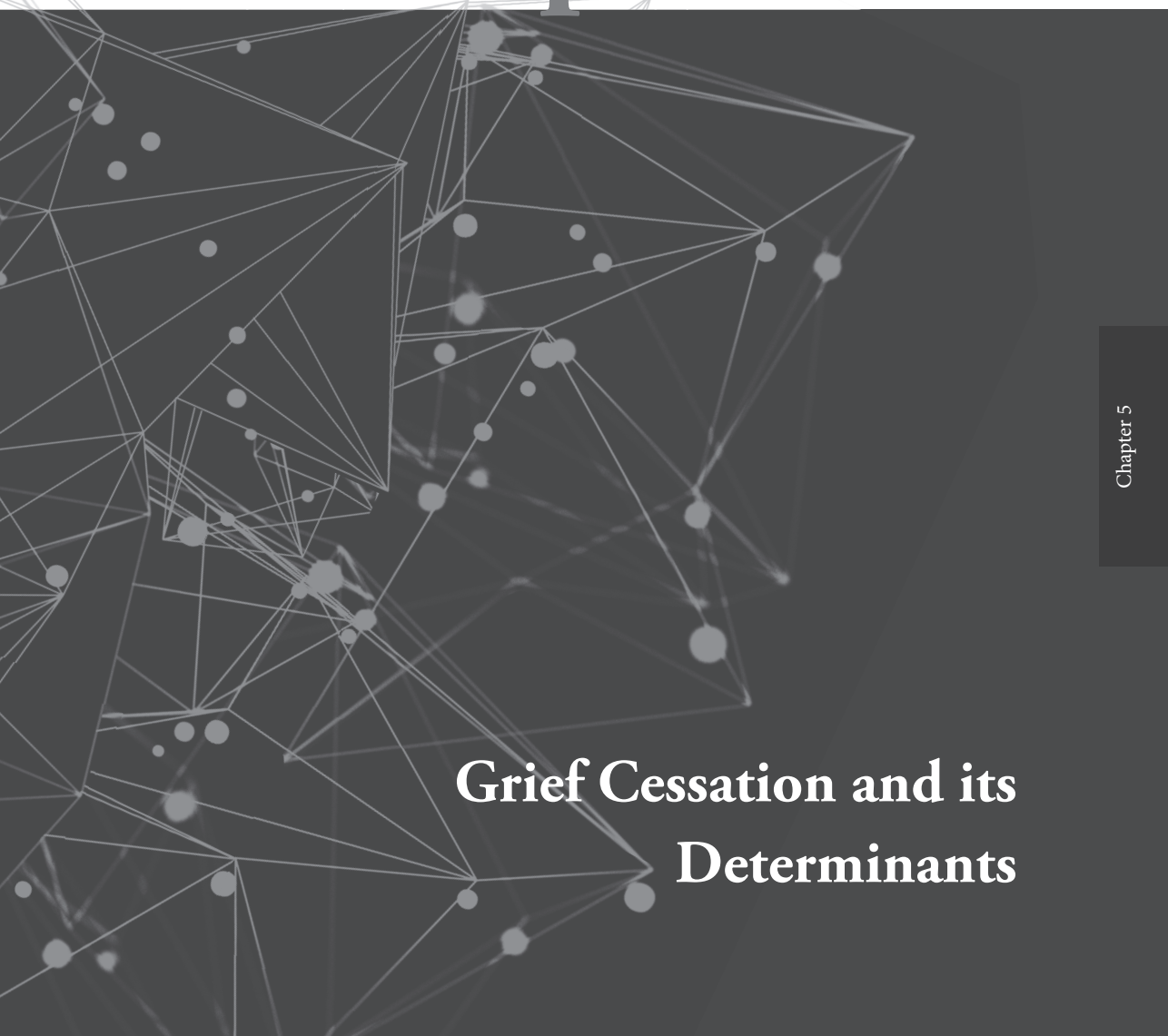
TS=(((grief OR grieve* OR mourn* OR sorrow* OR bereave* OR ((loss OR lose OR lost) NEAR/2 (spous* OR husband OR wife OR partner* OR sibling* OR partner* OR brother* OR sister*)))) AND (((qualit* NEAR/2 life)))) AND DT=(article) AND LA=(english)

Google scholar

grief|bereavement "quality * life"|wellbeing|"well-being"|hopelessness|loneliness



Chapter 5



Grief Cessation and its Determinants

5.1

Determinants and Predictors of Grief Severity and Persistence: The Rotterdam Study

Jelena Milic, Taulant Muka, M. Arfan Ikram, Oscar H. Franco and Henning Tiemeier

ABSTRACT

Objective: We aimed to explore correlates and predictors of bereavement severity and persistence (triggered by "loss of a loved one"; referent group partner loss) in the Rotterdam cohort. Method: We used linear regression to examine factors associated with grief severity using a cross-sectional analysis and logistic regression to determine prospective associations. Results: Cross-sectionaly, females, child-lost, higher depressive symptoms, lower education, and difficulties in daily activities were independently associated with a higher bereavement severity. Prospectively (6 years; response rate 71%), the baseline value of the grief severity was the single predictor significantly associated with grief persistence. Discussion: Our results suggest that only grief severity is independently associated with grief persistence. Further studies are needed to confirm our findings.

INTRODUCTION

Bereavement has been cited as one of the most traumatic life events (Clark & Georgellis, 2013). Death of a loved one is associated with severe stress and increased risk of occurrence of depressive symptoms, major depressive episodes, anxiety-related disorders (Alexopoulos, 2005; Kreicbergs, Valdimarsdottir, Onelov, Henter, & Steineck, 2004; Mendes de Leon, Kasl, & Jacobs, 1994; Prigerson et al., 1997), impaired immune function (Zisook et al., 1994), lower quality of life (Grimby, 1993), suicide, and increased risk of mortality (Kaprio, Koskenvuo, & Rita, 1987). After 6 to 12 months, most bereaved persons will adapt to the loss with a reduction in grief intensity and a return to a different but meaningful and satisfying life without the deceased (Bonanno et al., 2002; Prigerson et al., 2009). However, for some bereaved individuals, the adaptation might be complicated, slowed, or halted, leading to persistence of grief (Boelen & van den Bout, 2008; Shear et al., 2007). Persistent grief is associated with functional impairment, sleep disturbances, high-risk behaviors, and increased risk of cancer and cardiovascular disease (Simon et al., 2005; Simon et al., 2007). It is therefore important to identify the factors associated with persistent grief to identify the population at risk of developing prolonged or complicated grief in need of support and adequate treatment. Several factors have been suggested to influence the duration and severity of grief. First, the effects of grief on morbidity and mortality are greater for the widowers in the acute grieving period because women benefit more from social support (Stroebe, 2001). Likewise, with respect to gender differences in coping styles, women are more confronted and expressive of their emotions than men. That can be a predisposition for their better recovery (Stroebe & Stroebe, 1983). Second, due to life experience, people develop better coping strategies. Tempestuous emotions tend to be damped down, and it is less common for people to respond excitable to worries. The previous study showed that restlessness symptom was significantly higher in young widows and the irritability symptom showed an interaction effect—high for young (sudden death) and middle-aged (prolonged grief) widows (Ball, 1977). Thirdly, race influenced the grief severity. Previous study (Fitzpatrick & Tran, 2002) revealed that bereavement was a significant factor affecting the health of White Americans, particularly in the youngest and oldest age groups. However, no bereavement effects were observed among African Americans within any of the age groups. These findings raise questions as to the differential effects of bereavement between different races. Fourth, in the acute grieving phase, lower educated people had worse coping skills, while in prolonged grief, education level was no longer significant (Boelen, van den Bout, & de Keijser, 2003). This might be due to lack of intellectual capability to work on a coping system or lack of possibilities to seek comfort in spiritual and intellectual aspects of life and social networks. Fifth, employment might play a role in younger or middle-aged adults, but in elderly, it helps dominantly as an additional source for seeking peers for social networking after a loss event. Also, working people might experience difficulties going back to work while suffering severe grief due to fear of underperforming. Sixth, spousal bereavement increases the grief severity. The previous study shows that elderly participants who lost their spouse had a higher risk of developing complicated grief when compared with participants losing someone else (Fujisawa et al., 2010). Next, bereaved spouses with prolonged grief had difficulty accessing positive memories of the deceased, and higher recalled marital adjustment. (Mancini, Sinan, & Bonanno, 2015). Mood-incongruency effects are due to a mood-regulatory process in which people retrieve memories to repair moods. ::

The fact that one is not able to retrieve negative but only positive memories, increases the severity of grief. The same phenomenon is present in the more positive recalled marital adjustment when people tend to forget or repress the negative memories. Rumination keeps refocusing the griever on the thought of the deceased. Equally important for grief severity, experiential avoidance of situations that serve as reminders of the loss is also common. All of the above have been implicated as predictors of prolonged grief (Morina, 2011). Also, studies report severe depressive symptoms to be associated with prolonged grief, but findings have not been consistent (Tsai et al., 2016; Tsuboya et al., 2016). Most of the studies examine factors associated with grief severity and persistence solely on the basis of spousal bereavement, while other types of loss events have not been explored. Although health-related factors, both physical and mental (Onrust, Cuijpers, Smit, & Bohlmeijer, 2007), have been hypothesized to influence duration and severity of grieving, few longitudinal studies have examined their role in grief persistence among older adults. Grieving has been reported to differ by age, given the differential circumstances and mechanisms of adaptation that might occur within individuals at different stages of mental maturation. Mortality risk increases with age. Consequently, the probability of losing a spouse or partner also increases. Loss of a spouse or partner, might result in transferring dependency to others in the family or to the social surrounding .Elderly bereaved often lacks the necessary social support. This desolation further aggravates the grief process. Previous longitudinal studies on this topic had short follow-up (up to 24 months) and did not explore the determinants of grief related to long-term bereavement and different kind of loss (Bonanno et al., 2002; Prigerson et al., 2009; Tsai et al., 2016; Zisook, Paulus, Shuchter, & Judd, 1997; Zisook & Shuchter, 1991; Zisook et al., 1994). Therefore, our aim was to examine cross-sectionally factors associated with grief severity in adults aged 55 years and older taking into account all kind of kinship and also loved ones (not family related). Furthermore, we aimed to determine whether these factors were associated with grief persistence using a longitudinal data.

METHOD

Settings and Study Population

The study was performed within the framework of the Rotterdam Study, a population-based cohort, among persons 55 years and older living in the Ommoord district of Rotterdam, the Netherlands. The rationale and design of the Rotterdam Study is described elsewhere (Hofman et al., 2013). In brief, in 1990 all inhabitants of a well-defined district of Rotterdam were invited (RSI) and in 2000 an additional 3011 participants were enrolled (RSII), consisting of all persons living in the study district who had become 55 years of age. There were no eligibility criteria to enter the Rotterdam Study cohorts except the minimum age and residential area based on ZIP codes. The Rotterdam Study has been approved by the medical ethics committee according to the Population Screening Act: Rotterdam Study, executed by the Ministry of Health, Welfare and Sports of the Netherlands. All participants in the present analysis provided written informed consent to participate and to obtain information from their treating. Baseline measurements were performed in the fourth visit of the first cohort (RSI-4 year 2002-2004, n = 3,554) and the first visit of the second cohort (RSII-2 year 2004-2005; n = 2,468). Overall, there were 6,018 eligible participants (Figure 1). Of these participants, 597 persons did not

answer whether they had experienced a death that they were still grieving (consequently, they did not undergo an Inventory of Complicated Grief [ICG] interview). Additionally 1,146 participants were excluded because of cognitive impairment defined as a score ≤26 on the Mini-Mental State Examination (MMSE; Folstein, Folstein, & McHugh, 1975). Of the remaining 4,275 participants, 3,469 were classified as nongrievers: participants who replied negative to the question, "In the past years, have you lost someone who you still grieve over?" or they did not answer sufficiently the ICG interview (less than 75% of the answers was present), leaving 771 (468 from RSI-5 and 303 from RSII-3) persons who constituted the study population. Furthermore, 73 participants died during the follow-up period and out of 698 eligible, 223 persons did not complete the grief questionnaire (lost to follow-up), leaving 475 persons who constituted the follow-up study population. Follow-up measurements were performed in the fifth visit of the first cohort (RSI-5, year 2009-2011) and the second visit of the second cohort (RSII-3 year 2011-2012). The same grief questionnaire was administered at baseline and a follow-up exam with average time of 6.3 years between baseline and follow-up assessment.

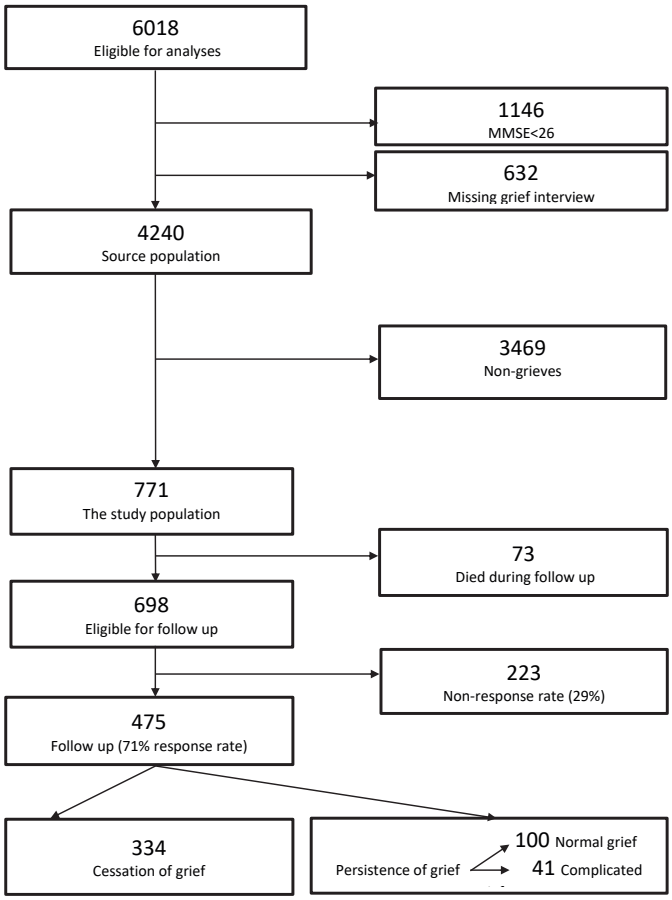


Figure 1. Characteristics of study participants; for cross-sectional and longitudinal analyses

Assessment of Grief–Interview of Complicated Grief (ICG)

Complicated grief was diagnosed with a Dutch version of the 19-item ICG (Prigerson et al., 1995). Participants underwent face-to-face interviews. They were first asked whether in the past years they had lost someone whom they were still grieving over. If yes, these participants were classified as grievers and were further assessed with the ICG to measure grief severity. Complicated grief symptoms were assessed as present among participants who scored equal or greater than 22 on the ICG score and grieved longer than 6 months (Newson, Boelen, Hek, Hofman, & Tiemeier, 2011; Saavedra Perez et al., 2015). ICG is composed to represent symptoms of complicated grief such as those in the most recent proposed criteria for the condition (Newson et al., 2011; Prigerson et al., 2009; Shear et al., 2011). Among the described symptoms are yearning for the lost person, anger over the death, distrust and detachment from others as a consequence of a death, survivor guilt, and loneliness. The measure has a high internal consistency and convergent and criterion validity, and it is the gold standard for measurement of complicated grief in older adults (Boelen, Van Den Bout, De Keijser, & Hoijtink, 2003). The inventory represents a single underlying construct of complicated grief (Boelen & Hoijtink, 2009). The original ICG consisted of 19 questions. Responses were provided on a 5-point scale to reflect an increase in severity (0 = never, 1 = seldom, 2 = sometimes, 3 = often, 4 = always; Prigerson et al., 1995). In this study, one item from the original inventory, "I feel bitter over this person's death," was removed from the original ICG as a pilot study revealed that this sentiment had the same meaning within the Dutch language as the included item, "I feel anger over this person's death" (Boelen et al., 2003) Two further items (relating to seeing and hearing the deceased) were collapsed into one due to their similarity after a pilot study indicated these symptoms were low in frequency and often overlapped ("I hear the voice of, or see, the person who died"; Boelen et al., 2003). Furthermore, at the baseline we collect the date of the loss event to identify complicated grievers (complicated grief defined as grieving longer than 6 months from the time of the death). No limit for the time since loss was set. : In our pilot study we found that adults may grieve over the loss of a dear one even more than 20 years. This provides the time frame for experienced loss. Information about the deceased person was obtained with question, "Who is the dear one you have lost?" Answers were subdivided into "Loss of the spouse (reference group)," "Loss of the child," "Loss of the others" (defined as one of the parents, a brother/a sister (Fujisawa et al., 2010), another family member, good friend, another dear person (not described relation-wise so far), multiple deaths (including a spouse and/or child or not including a spouse and/or child; Table 1).

Assessment of Potential Factors Associated With Grief

Age, sex, education, cognitive functioning, activities of daily living, body mass index (BMI), use of psycholeptics, and the presence of comorbidities and depressive symptoms were considered as potential confounders and assessed at the baseline examination. Education was defined as low (primary education, lower vocational education and/or lower/intermediate general education), intermediate (intermediate vocational education and/or higher general education), or high (higher vocational education or university). Information about the deceased person was obtained with the question: "Who is the dear one you have lost?" Answers were subdivided into loss of a partner, loss of a child, or other. Social support was assessed with social support interview, a self-reported questionnaire (Meltzer, Gill, Pettricrew, & Hinds, 1995). The social support interview consists of five self-rated

questions; answers scored equally for each question, on a 3-point scale (0 = not true, 1 = partly true, 2 = certainly true) to yield a global score of maximum 10 points. A higher score indicates the better level of social support. Depressive symptoms were assessed with a valid Dutch version of the Centre for Epidemiological Studies Depression (CES-D) scale (range = 0-60) where people score over 16 points (cutoff defined as score ≥ 16 points) identified persons with the clinically relevant depressive symptom (Beekman, 1997). The ability to perform activities of daily living was measured with the Stanford Health Assessment Questionnaire (Bruce & Fries, 2003; Fries, Spitz, & Young, 1982) that measures disability in eight fields (dressing and grooming, rising, reach, hygiene, eating, walking, grip, and activity). A Disability Index (DI) from 0.50 to 1.00 was considered as mild disability, while a DI of 1.00 or higher was regarded as a severe disability (Odding, Valkenburg, Stam, & Hofman, 2001). Psycholeptic drug use data were obtained from the Rotterdam Study pharmacies network at the baseline. This information was later on verified by a physician/cabinet check. Psycholeptics included benzodiazepines, antidepressants, neuroleptics, and anticonvulsants (Hofman et al., 2013; Stricker & Stijnen, 2010). Cognitive functioning was measured using the MMSE, during the baseline visits (Folstein et al., 1975). Participants with an MMSE score < 26 were excluded from the study. All comorbidities were assessed at the baseline by medical history, home inter- view, or by a clinician, and later on validated by relevant clinical test (if necessary). Comorbidities were categorized as present or absent and included one or more of the prevalence of diabetes mellitus, heart failure, stroke, myocardial infarction, chronic obstructive pulmonary disease (COPD), and joint problems that include osteoarthritis, rheumatoid arthritis gout, back pain, and ankylosing spondylitis. Diabetes mellitus was defined as a fasting glucose level of ≥7.0 mmol/L or use of blood glucose–lowering medication. Prevalent heart failure at baseline was measured using a validated score that was based on the heart failure definition of the European Society of Cardiology. A history of stroke was determined by interview or proxy informant interview. Confirmation of stroke diagnosis by a treating physician was required. A previous myocardial infarction was determined using an electrocardiogram.Diagnosis of COPD was confirmed by spirometry. Joint problems were obtained from home interview and a physician also measured the participants for joint complaints. Radiographic assessment for the diagnosis of osteoarthritis and rheumatoid arthritis were made at the research center. The presence of major depressive disorder (MDD) was assessed usingSchedules for Clinical Assessment in Neuropsychiatry (SCAN) interview. The SCAN is a set of tools created by World Health Organization aimeddiagnosing and measuring mental illness that occur in adult life (Wing et1990). This semistructured clinical interview was performed by trained clinician to determine which participants fulfilled the Diagnostic and Statistical Manual of Mental Disorders (4th ed.; DSM-IV; American Psychiatric Association, 1994), criteria for MDD.

Statistical Analyses

Continuous variables were reported as mean ± SD unless stated otherwise, and categorical variables were presented as percentages. Multivariable linear regression models were used to determine the cross-sectional association between potential determinants and ICG score. In our cross-sectional and longitudinal analyses, each potential factor was first analyzed in models adjusted for age and gender (basic adjusted model), followed by analyses that were additionally adjusted for all potential factors (fully adjusted model). For the longitudinal association on potential predictors of grief persistence,

logistic regression models were used (the outcome was dichotomized: complicated grief at follow-up and griever but not-complicated grief at follow-up, the last being the reference group) because standard transformations did not achieve a near normal distribution (more than70% had an ICG score of 0 at follow-up). No multicollinearity was observed in any of our analysis as assessed by variance inflation factor (all values were <1 and cannot inflate Type I error). To examine whether the presence of a depressive disorder could influence the results, we re-ran all analyses excluding individuals with MDD at baseline. We also examined potential interaction terms before running fully adjusted models. To examine the potential selection bias, we perform sensitivity analysis testing whether there was any significant difference in characteristics between responders and nonresponders at follow-up examination. Furthermore, we explored for potential outliers and whether there was any violation of assumptions, but we did not find any issue related to these two aspects of the analysis. Also, to test the association between more severe bereavement and potential factors, we repeated the prospective logistic regression analyses including only those participants who had complicated grief at baseline (persistence vs. cessation as a reference group). To adjust for the potential bias associated with missing data, we used multiple imputation procedures (n = 5 imputations). Rubin's method was used for the pooled regression coefficients (β) and 95% Confidence Intervals. The percentage of missing values within the population for the analyses was lower than 3% (ranging from 0% to 2%). The percentage of missing values within our study population was lower than 3% (ranging from 0% to 2%). Age, sex, cognitive score, MDD, CES-D, and comorbidities had no missing values; activities of the daily living score had 0.4%; psycholeptics drugs had 0.5%; and education had 2%. Analyses were performed using SPSS Statistics (version 20; SPSS, Chicago, IL, USA).

RESULTS

Table 1 presents the characteristics of the study population for cross-sectional and longitudinal analyses. Mean age of study participants was 72.7 years. Half of study participants had lost their partner (50%), 11% lost a child, and 39% lost someone else (parent, sibling, cousin, friend, or someone else participants felt close to). MDD was present in 4% participants. The majority of the study participants had depressive symptoms with the value of below 16 points and mild problems in activities of daily living (Table 1). Table 2 shows the results of the cross-sectional association between potential correlates and ICG score In our fully adjusted models, female sex (β = 1.94, 95% CI: [0.35, 3.52]), loss of the child (β = 4.24, 95% CI: [1.91, 6.58]) or of the other dear person (β = −3.56, 95% CI: [−5.07, −2.04]), higher depressive symptoms score (β = 0.46, 95% CI: [0.37, 0.55]), middle (β = 3.14, 95% CI: [1.04, 5.23]) and primary education level (β = 4.81, 95% CI: [1.89, 7.73]), were associated with higher ICG score. More difficulties in activities of daily living were associated with lower levels of ICG score (β = −2.20, 95% CI: [−3.69, −0.71]). After 6 years of follow-up, 475 (71%) of the 698 surviving participants underwent the ICG interview and were, therefore, included in our longitudinal analysis. Out of those, 334 participants reported complete cessation of grief and 141 still grieved. Out of grieving participants, 100 experienced normal grief and 41 complicated grief (Figure 1). In Table 3, we present the results of the prospective association of the correlates of grief severity with grief persistence (cessation as reference). In the age and gender-adjusted analyses, CES-D (OR = 1.04,

Table 1. Characteristics of study participants; for cross-sectional and longitudinal analyses

	Cross-sectional analysis		Longitudinal analysis			
	Grievers (N = 771)		Cessation of grief (N = 334)		Continuous grief (N = 141)	
Age, years (S.D.)	72.6	(7.6)	70.0	(6.6)	70.0	(6.4)
Gender (female), (%)	73		73		81	
Education						
Primary (%)	11		6		11	
Intermediate (%)	76		79		73	
High (%)	13		15		16	
Depressive symptoms score, (S.D.)	8.8	(8.6)	7.1	(7.2)	10.5	(10.9)
Activities of daily living problems score, (S.D.)	0.5	(0.5)	0.4	(0.4)	0.5	(0.5)
Social support score,(S.D.)	9.2	(1.4)	9.3	(1.5)	9.3	(1.3)
Diagnosed MDD, (%)	4		2		8	
Who died?						
Partner (reference), %	50		56		45	
Child, %	11		9		14	
Other,%	39		36		41	
Psycholeptics* use, yes (%)	20		14		24	

Values are presented as means, standard deviation (S.D) or percentages (%)
Psycholeptics includes: benzodiazepines, antidepressants, neuroleptics and anticonvulsants

95% CI: [1.02, 1.07]) and the baseline value of ICG score were associated with grief persistence (OR = 1.06, 95% CI: [1.04, 1.08]); other correlates were not associated with grief persistence. In the fully adjusted analyses, baseline value of ICG score was associated with grief persistence (OR = 1.05, 95% CI: [1.03, 1.08]), while no association was observed for the other factors examined (Table 3).Next, we performed a series of sensitivity analyses. First, we excluded persons with major depression from our study population and reran the analysis (Supplement Table A).; our result remained essentially unchanged. Then we limited the cases to those who experienced continuous complicated grief (non grievers as the reference group) and reran the longitudinal analyses(data not shown). Again, our result remained essentially unchanged. Last, to test for selection bias, we tested whether there was any significant difference in characteristics of the population between responders and nonresponders to follow-up exam. Compared with responders, nonresponders were more likely to be men, older age, and lower education; have more depressive symptoms, higher comorbidities, lower activities of daily living score, lower cognitive score, higher ICG score; and reported more use of psycholeptic medications (Supplement Table B).

Table 2. Potential determinants of grief persistence; cross-sectional analysis Grief severity at the baseline – ICG score

	Model 1*				Model 2†			
Potential determinants	N=771				N=771			
	B	95% CI		*p*	B	95% CI		*p*
Gender (female)	0.35	-1.30	2.00	0.68	1.94	0.35	3.52	**0.02**
Age, years	0.07	-0.02	0.17	0.13	-0.06	-0.16	0.04	0.24
Social support, score	-0.70	-1.23	-0.18	**0.009**	-0.28	-0.72	0.26	0.36
Who died?								
Partner (reference), %	ref.	-	-	-	ref.	-	-	-
Child, %	2.44	-0.01	4.90	**0.05**	4.24	1.91	6.58	**≤0.0001**
Other, %	-5.32	-6.88	-3.76	**≤0.0001**	-3.56	-5.07	-2.04	**≤0.0001**
Depressive symptoms , per 1 point of score	0.48	0.40	0.56	**≤0.0001**	0.46	0.37	0.55	**≤0.0001**
Education								
High (reference) ,%	ref.	-	-	-	ref.	-	-	-
Middle,%	3.21	0.91	5.50	**0.006**	3.14	1.04	5.23	**0.003**
Primary,%	5.07	1.89	8.26	**0.002**	4.81	1.89	7.73	**0.001**
Activities of daily living problems, score	0.19	-1.34	1.73	0.80	- 2.20	-3.69	-0.71	**0.004**
Psycholeptics‡ use, yes (%)	-3.70	-5.58	-1.81	**≤0.0001**	0.83	-0.96	2.63	0.36
Comorbidities§, yes (%)	0.49	-1.03	2.00	0.53	0.02	-1.40	1.44	0.98
Cognitive functioning, MMSE‖, - score	-0.66	-1.27	-0.06	**0.03**	-0.30	-0.86	0.26	0.30

***Model 1 was adjusted for gender and age for each covariate**

†Model 2 was adjusted for all listed covariates

‡Psycholeptics includes: benzodiazepines, antidepressants, neuroleptics and anticonvulsants

§Comorbidities includes: the prevalence of MI, heart failure, stroke, joint problems that include osteoarthritis, rheumatoid arthritis gout, back pain and ankylosing spondylitis, COPD and Diabetes mellitus

‖MMSE - The Mini–mental state examination (MMSE) test for cognitive impermanent

Table 3. Determinants of grief persistence; a six year longitudinal analysis Persistence of grief

	Model 1*				Model 2†			
Covariates/determinants	N=141/475				N=141/475			
	OR	95% CI		*p*	OR	95% CI		*p*
Gender (female)	0.64	0.39	1.06	0.08	0.67	0.39	1.20	0.18
Age, years	0.99	0.96	1.02	0.62	0.98	0.95	1.02	0.39
Social support, score	1.12	0.94	1.34	0.20	1.19	0.98	1.44	0.08
Who died?								
Partner (reference), %	ref.	-	-	-	ref.	-	-	-
Child, %	1.22	0.61	2.42	0.57	1.40	0.67	2.92	0.65
Other, %	1.00	0.61	1.63	0.99	1.13	0.67	1.92	0.37
Depressive symptoms‡ , per 1 point of score	1.02	0.99	1.04	0.17	1.02	0.99	1.06	0.13
Education								
High (reference) ,%	ref.	-	-	-	ref.	-	-	-
Middle,%	0.67	0.36	1.23	0.20	0.72	0.38	1.36	0.31
Primary,%	1.23	0.49	3.08	0.66	1.47	0.56	3.88	0.44
Activities of daily living problems, score	1.11	0.69	1.81	0.66	0.90	0.52	1.56	0.70
Psycholeptics‡ use, yes (%)	0.73	0.42	1.24	0.24	1.25	0.69	2.28	0.46
Comorbidities§, yes (%)	1.41	0.92	2.16	0.11	1.45	0.91	2.29	0.12
Cognitive functioning, MMSE‖, - score	1.08	0.90	1.28	0.41	1.08	0.90	1.30	0.41
Baseline value of ICG, score	1.06	1.04	1.08	**≤0.0001**	1.05	1.03	1.08	**≤0.0001**

Note: Grief persisted In 141 of 475 participants, 334 persons reported no grief at follow up (reference category)

*Model1 was adjusted for gender and age per covariant

†Model 2 was adjusted for all listed covariates

‡Psycholeptics includes: benzodiazepines, antidepressants, neuroleptics and anticonvulsants

§Comorbidities includes: the prevalence of MI, heart failure, stroke, joint problems that include osteoarthritis, rheumatoid arthritis gout, back pain and ankylosing spondylitis, COPD and Diabetes mellitus

‖MMSE - The Mini–mental state examination (MMSE) test for cognitive impermanent

DISCUSSION

In this population-based study of middle-aged and elderly persons, we investigated the factors related to grief severity in cross-sectional analyses and the determinants of grief persistence in longitudinal analyses. Our cross-sectional findings showed that grief severity was associated with female sex, having low to intermediate education level, the presence of depressive symptoms, and loss of a partner or a child, while more difficulties in daily living activities were associated with less grief severity. In this study of elderly individuals, several factors are associated with grief severity in our cross sectional analyses. These gender differences are based on the fact that females have better compliance and response to the social support/network (Trofimova, 2013).Stroebe et al. (2001) reports gender differ-

ences in the efficacy of different types of counseling, showing systematically gender differences in the needs and benefits of bereaved men compared with bereaved women: Widowers benefit more from emotion-focused interventions, while widows benefit more from problem-focused interventions. However, the only determinant of grief persistence after an average follow-up of more than 6 years was a lower severity of baseline grief. Similar to our results, previous studies have reported that women grieve more frequently and in a more complicated way than men (Ofstedal, Reidy, & Knodel, 2004). Beneria and Permanyer (2010) conducted an in-depth analysis of 43 participants (21 males and 22 female) at a Monrovia college fair. Findings revealed that men would rather not talk about grief because talking about the deceased would not help the situation while women thought otherwise. Women considered interiorizing the loss was a bad thing and consider being extroverted about grief a positive way of dealing with it. Moreover, any emotional expressions about a loss were recognized as weakness in men but not in women (Beneria & Permanyer, 2010). Other studies reported a higher level of bereavement among elderly women (Ofstedal et al., 2004). Second, we observed an association between kinship (who died) and grief severity. The spousal bereavement is reported as one of the most stressful events that anyone might experience, although its impact might diminish later in life than when it occurs during young adulthood or middle age (Holmes & Rahe, 1967; Moss, Moss, & Hanson, 2001). Two previous studies reported that even older bereaved spouses have higher rates of mortality and morbidity, impaired immune system, more depressive symptoms, more chronic conditions, and functional disabilities (Buckley et al., 2012; Fiske, Wetherell, & Gatz, 2009; Shahar, Schultz, Shahar, & Wing, 2001). Bonanno et al. (2002) stated that almost everyone experiences an initial increase in grief symptoms, but for most people, these symptoms subside 18 months after the loss. Resilient widows and widowers generally are more accepting of death, more extraverted, more emotionally stable, and less dependent on spouses than their nonresilient peers (Spahni, Morselli, Perrig-Chiello, & Benn ett, 2015). As about 50% of our participants experienced loss of the partner, we defined spousal bereavement as the reference group when studying the association between kinship and severity of grief. In the current study, loss of the partner is negatively associated with severity of grief, but we did not find a longitudinal association between kinship and grief persistence. Third, we found that difficulties in activities of daily living were associated with less grief severity. Current literature in the field suggests that the more a person is active prior to spousal death, the easier the adjustment process may be (Utz, Carr, Nesse, & Wortman, 2002). Documenting the effects of widowhood on physical function and activity of older adults is important because significant declines may place individuals at risk for compromised health (Utz et al., 2002). It is important to note that persistent strains prior to spousal loss (caregiving responsibilities) affect the well-being of widowed elders (Schulz et al., 2001) and likely affect their willingness and ability to engage in the activity (Utz et al., 2002). Differences between our findings and those available in the literature might be due to the timing of measurement of disabilities and grief as the directionality of the presence of both cannot be disentangled adequately at baseline. Furthermore, our results were only observed once we adjusted for depressive symptoms. Thus, our findings must be interpreted as the direct effect of activities of daily living on grief severity once the indirect effect via depressive symptoms is controlled for. Also, there might be other factors explaining these associations that were not collected during baseline exami- nation (e.g., environmental, social capital, networks) and that could add residual confounding to our analyses and results. It is important to note that persistent strains prior to spousal

loss (caregiving responsibilities) affect the well-being of widowed elders (Schulz et al., 2001) and likely affect their willingness and ability to engage in activity (Utz et al., 2002). However, more longitudinal studies are needed to examine the levels of physical function and activity prior to spousal death as well as after spousal death in relation to grief severity and persistence. Furthermore, we would like to tackle with several points to raise awareness of the further possibility for research questions. Namely, we would like to point out several possibly relevant predictors of the grief that are not assessed in the current study: coping style, relationship with deceased (not kinship but closeness to the observed/interviewed person), and do on. Also, we noticed that predictors vary by group, that there is interaction triggered by whom you lost, as well as gender. Unfortunately, we have no power to detect mentioned interaction. Furthermore, grief severity captures many aspects and includes information on potential predictors such as depression which opens a "chicken or egg" dilemma—what comes first. Further studies are necessary to assess predictors before bereavement, in particular, complicated grief. In the current study, reverse causality obscures relation that could otherwise have been observed (given the data set availability). In the longitudinal analysis, we observed that the baseline value of ICG score was independently related to grief persistence, whereas no independent associations were observed between CES-D and other correlates with grief persistence. Contrary to the CES-D questionnaire that assesses overall feelings of sadness and melancholy, the ICG questionnaire assesses specific coping abilities to loss of the loved one. We hypothesize that the baseline severity of grief captures psychological features that predict grief cessation such as poor coping, anger, and detachment from others. Bereaved individuals with good coping abilities might change their lifestyle leading to empowerment of self and present relationships, creating new ones, while preserving health and well-being (Neimeyer, 2006). However, further studies are needed to investigate whether different coping mechanisms are related to grief severity and duration.Our prospective findings imply that complicated grief is a dynamic condition/state that changes over time. Though the previous study stated that up to 40% of grievers never recover and stay in complicated grief, for the rest of the grievers it is expected that the symptoms get less severe (ICG < 22) or that they even completely stop feeling the grief (Goldsmith, Morrison, Vanderwerker, & Prigerson, 2008; Ott, Lueger, Kelber, & Prigerson, 2007; Piper, Ogrodniczuk, Azim, & Weideman, 2001; Prigerson et al., 1995). Our findings indicate that 7 out of 10 participants did not report any symptoms of complicated grief at follow-up which is in line with previous studies (Goldsmith et al., 2008; Ott et al., 2007; Piper et al., 2001; Prigerson et al., 1995). The major strengths of this study are prospective design and the population-based setting. To the best of our knowledge, this is the study with the longest follow-up to explore the determinants of grief and complicated grief in a population-based study of older adults. Furthermore, this study did not restrict the relationship of the person who died to the participant. We took in an account all kinds of kinship and also loved ones (not family related). We took in an account all kinds of relationships wit dear persons (both familly and not-family). Period after the loss event triggering grief , varies greatly among our participants (oldest date of loss event: was July 15 th, 1947, while the date of the most recent lost event was December 15th, 2011). Within this interval, we have people who grieve over 60 years. This long postdeath period is also a strength of our study because it reflects the variation of the time frame of grief in the general population and there is no evidence for an optimal cutoff to define maximum duration. Also, restriction of our analysis among participants, who scored lower than 26 points on MMSE, limited the possibility of misclassification of grieving status

and/or recall bias. Furthermore, the interview was conducted face to face in the privacy of the participant's own home by trained health care professionals and researchers, thus enhancing the reliability and validity of complicated grief measurement. There are also some limitations to our study that need to be considered. First, the screening question which determined who undertook the ICG allowed participants the opportunity to identify themselves as grievers, which may inadvertently result in an over- or underestimation of the number of grievers. However, in a pilot study, we found that nongrievers cannot meaningfully reply to the questions. We found differences in the group of participants grieving at the follow-up and the one that did not grieve anymore. Our nonresponse analysis showed that older persons and less healthy participants were more likely to be lost to follow-up. We might carefully infer that unhealthy persons were less likely to participate in the follow-up study, compared with the more healthy agers. Therefore, as inherent to all cohort studies, the possibility of health selection bias cannot be ruled out. This selection effect may bias our results and can impact generalizability to older and more frailer populations. Moreover, nearly all participants were of Caucasian descent. Therefore, the generalizability of our findings may be hampered. Another limitation of the current study might be the imprecise definition of grieving (asked within a time frame covering previous years which varies between participants) that did not consider individuals who experience someone's death but did not feel that they were still grieving (emotional detachment from self as a modus of defend), and therefore selection bias might have been introduced. Also, while in the current study we have defined very precisely the state of complicated grief and persistent grief, there are challenges in operationalizing and distinguishing these conditions. Previous attempts have been taken to tackle this issue and different terms have been suggested, but a consensus has not been achieved yet (Boelen & Hoijtink, 2009). The continuation/persistence of all degrees of severity in grieving process needs more research effort in the future, in order to harmonize the definitions and distinctions between different grief status. In conclusion, among elderly, grieving is a life event often experienced. Female sex, lower education, depressive symptoms, and loss of a partner orchild were associated with grief severity. Among those grieving approximately 25% had persistent grief after 6 years, which was associated with the severity of bereavement. Further longitudinal studies are needed to confirm and further explore our findings and to identify whether more potential predictors of grief severity and duration exist.

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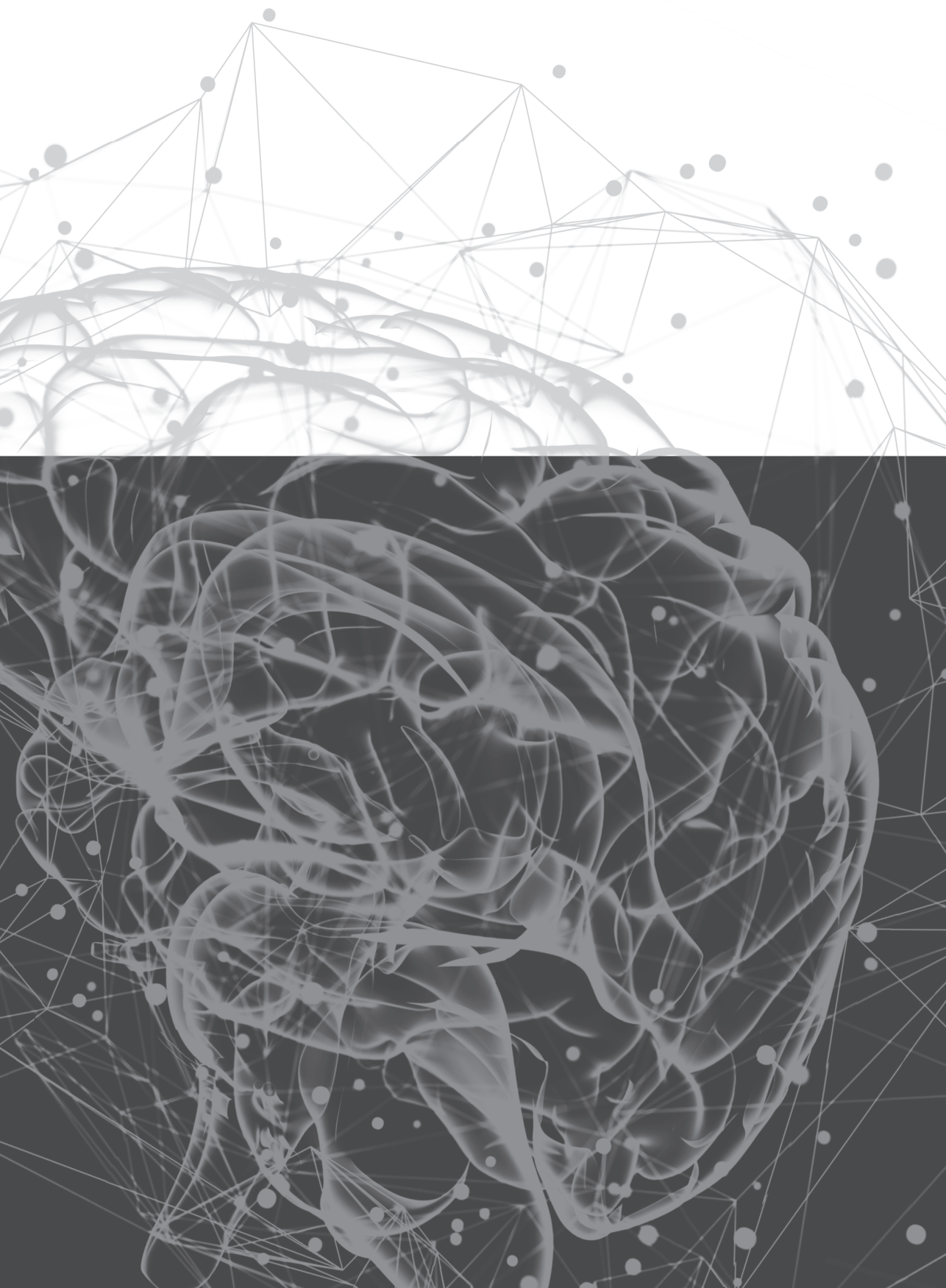

Chapter 6

General Discussion

The WHO reports show that 20% of adults aged 60 years and over suffer from a mental and/or neurological disorder that accounts for 6.6% of all disability (disability adjusted life years-DALYs) and account for 17.4% of Years Lived with Disability (YLDs) (WHO, 2016) among elderly adults. The most common neuropsychiatric disorders are dementia and depression (WHO 2016). As the population tends toward longer life, populations will get older and the negative impact of depression and the burden of age-related neurodegerative diseases such as AD and PD will increase (Dorsey, George, Leff, & Willis, 2013; McGovern Institute for Brain Research, 2014). Obtaining care for these type of disorders is becoming more important as the population ages (Dorsey et al., 2013). Therefore, identifying risk factors and pathways that contribute to the development of neuropsychiatric diseases could shed more light into the complex pathophysiology of these diseases and would facilitate the development of preventive strategies to reduce the burden of these diseases. Numerous genes, molecular pathways and environmental factors have already been identified that partly explain the complex pathophysiology underlying neurodegenerative diseases. The role of epigenetic determinants, such as DNA methylation and histone modifications, is increasingly being recognized as a potential link between environmental exposure and disease risk. Thus, epigenetic determinants may be a benchmark to capture the influences of environmental exposure and risk of developing neurodegenerative diseases. Knowledge of epigenetic influences on development of neurodegenerative diseases may help further our understanding of the mechanisms of AD and PD.

Sex is also increasingly being recognized as an important determinant of neuropsychiatric diseases. Mental health problems such as depression and AD are more common in women than in men, and other neurological disorders such as stroke present more severely in women. Estrogen signaling and hormonal changes during menopause may play a role in the increased risk that women face regarding neuropsychiatric disorders, including alcohol misuse and dependence (Wen et al., 2016).

Many elderly people experience the death of a loved one (Boelen & Hoijtink, 2009; Monk, Germain, et al., 2008; Shear, 2015). Grieving, defined as the emotional process of coping with a loss, can lead to health problems including depression, chronic stress, and impaired daily functioning (Newson et al., 2011). Grief can also affect the neuroendocrine system, immune function, and sleep patterns. Consequentially, grief can worsen quality of life and reduce life expectancy. An estimated 10-20% of elderly adults will suffer chronic and severe grief reactions, but the factors that contribute to serious symptoms of grief for prolonged periods of time are not understood. Understanding the determinants and predictors of grief cessation will help us to understand the most influential aspects of grief on everyday life, as well as identifying people at risk of severe or prolonged grief who may benefit from preventive strategies.

In this chapter I present an overview of the main findings of the studies in the thesis. I also provide suggestions for future research and the practical implications of the research. Furthermore, I discuss the methodological issues of the thesis.

MAIN FINDINGS

The Role of DNA Methylation and Histone Modifications in Neurodegenerative Diseases

Epigenetic mechanisms may account for the missing heritability determinants of complex diseases such AD and PD. Taking an epigenetic perspective in AD and PD research may provide new information in the etiology and treatment of these neurodegenerative diseases. In general, epigenetic studies focus on global methylation, gene-specific DNA methylation and histone modifications. Global methylation refers to the overall level of methylcytosine in the genome, expressed as percentage of total cytosine.

Global levels of DNA methylation increase in the first years of life and then decrease beginning in late adulthood. The fluctuation of levels of DNA methylation suggests that global methylation plays a role in healthy aging and age-related diseases (Jones, Goodman, & Kobor, 2015). In chapter 2.1, however, I found inconsistent associations between global DNA methylation pattern in blood and brain tissue and presence of AD and PD. Nevertheless, in brain tissue and/or peripheral blood, we found that epigenetic regulation of 31 genes involved in cell communication, apoptosis, and neurogenesis to be differentially methylated in individuals with AD and/or PD. Of those 31 genes, methylation at the genes brain derived neurotrophic factor (BDNF), Sorbin and SH3 Domain Containing 3 (SORBS3), and Amyloid precursor protein (APP) were the most consistently associated with AD. In both peripheral blood cells and brain tissue, methylation of α-synuclein gene (SNCA) was also found to be consistently associated with PD. In our review, seven studies reported histone protein alterations in AD and PD, underscoring a gap in the literature concerning the role of histone modifications in neurodegenerative diseases.

I identified several methodological concerns of the studies reviewed. The majority of the studies included in the review were cross-sectional assessments, making it difficult to draw conclusions regarding causality. Also, studies that investigate epigenetic dysregulation in AD and PD often suffer from small sample size, which may result in lack of statistical power and increased false discovery rates. In addition, the majority of studies were classified as low quality due to lack of proper adjustments. The studies included in the review lacked adjustment for basic covariates including lifestyle and environmental factors, such as smoking and alcohol consumption, which are important risk factors for neurodegenerative disorders and can alter epigenetic mechanisms. Furthermore, the studies used a variety of techniques to assess epigenetic modifications that may produce heterogeneous results.

The Functions of Estrogen Receptor Beta in the Female Brain

In chapter 3.1 estrogen loss associated with menopause may contribute to the development of neuropsychiatric diseases (Dalal & Agarwal, 2015; Wend, Wend, & Krum, 2012). Emerging evidence indicates that estrogen provides important benefits in the female brain, including its role in learning, memory, mood, and neurodevelopmental and neurodegenerative processes (Gillies & McArthur, 2010). All of the actions of estrogen in the human body are mediated by its two main receptors, alpha and beta (ERβ) (Deroo & Korach, 2006; Lee, Kim, & Choi, 2012; Paterni, Granchi, Katzenellenbogen, & Minutolo, 2014). The newly discovered ERβ is widely distributed in the female brain and may offer a new opportunity for pharmacological interventions to prevent and treat neuropsychiatric diseases

in women (Gogos et al., 2015; Nilsson et al., 2000; Peri & Serio, 2008; Warner & Gustafsson, 2015). In summarizing the evidence from 49 studies, we found that ERβ phosphorylated and activated intracellular second messenger proteins and regulated protein expression of the genes that are involved in neurological functions. It also promotes neurogenesis, modulates the neuroendocrine regulation of stress response, confers neuroprotection against ischemia and inflammation, and reduces anxiety- and depression-like behaviors. Also, ERβ may induce a significant reduction of hippocampal Apolipoprotein E, and may help to maintain hippocampal function, suggesting a protective effect of ERβ in AD. Further, ERβ may be a target for treatment of memory impairment conditions. (Figure 1.)

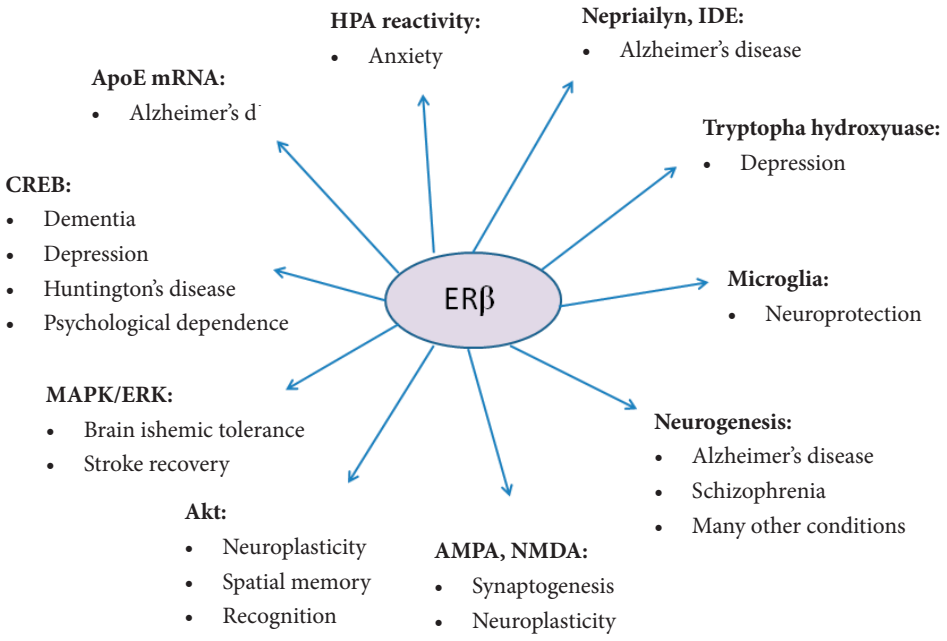

Figure 1. Potential pathways for ERβ function in the female brain.
Legend: *mRNA, Apolipoprotein E messenger RNA; HPA, hypothalamus-pituitary-adrenal; IDE, insulin-degrading enzyme; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NMDA, N-Methyl-D-aspartic acid; Akt, Protein kinase B; MAPK, Mitogen-activated protein kinase; ERK, extracellular signal-regulated kinases; CREB, c-AMP response-element binding protein.*

Overall, the data we reviewed showed that targeting ERβ may be a novel treatment for menopausal symptoms including anxiety, depression, and neurological diseases. However, since all studies have been based on animal models, these findings contribute to hypothesis generation. Caution should be taken when extrapolating the results of this review to human subjects. Studies in humans may confirm whether isoform-selective ERβ-ligands could be a therapeutic target for prevention and treatment of neuropsychiatric diseases. We did not find any studies that examined whether ERβ signaling may differ by age or menopausal status. Studies in tissues other than the brain, such as in the cardiovascular system have shown that age and menopause status may play a role in ERβ signaling (Muka et al., 2016).

Menopause, Ageing and Alcohol Use Disorders in Women

In chapter 3.2 I provide an overview of the prevalence of drinking patterns and dependence, risk factors, health impacts and treatment challenges for women as they progress through middle and older age. Moderate drinking frequency and alcohol intoxication is higher in younger women, compared to older women. As the disparity between men's and women's rates of alcohol consumption decreases and as women's life expectancy increases, we may see a higher number of female drinkers and a greater impact of alcohol problems and dependence in women (Geels et al., 2012). The prevalence and impact of older female drinkers is expected to increase as members of the Baby Boomer generation reach older age and younger generations move toward older adulthood. Younger generations have greater parity in alcohol consumption between the genders and women's alcohol consumption is also increasing for other reasons (Sanjuan & Langenbucher, 1999., Wilsnack, & Wilsnack, 1994). Moderate drinking frequency and alcohol intoxication has increased in both genders over time, but the increase has been greater among women. (Makela, Tigerstedt & Mustonen, 2012)

There are also differences between men and women in the metabolism of alcohol. As a result, it generally takes less alcohol to cause somatic and psychological harm in women than it does in men. Thus, women are more vulnerable to alcohol's harmful effects, and tend to develop alcohol-related diseases and other consequences of drinking sooner than men, and after drinking smaller cumulative amounts of alcohol. Additionally, as women age and go through menopause, they face new health and societal challenges such as menopause, retirement, and illness, all of which make them more susceptible to the effects of alcohol misuse. Lean body mass decreases with age and women experience an increase in body fat after menopause, so total body water also decreases. For this reason, after menopause the same amount of alcohol that previously had little effect on a woman can cause intoxication (Vestal et al., 1977).

Several observational studies have reported a J-shaped association between alcohol consumption and various health outcomes (e.g. cardiovascular disease), while other studies have shown a clear dose-response relationship between alcohol consumption and other health outcomes (e.g. for cancer) (Figure 2).

Probably due to the pro-estrogenic effect of alcohol, low levels of alcohol can delay menopause onset and reduce the risk of menopause-related diseases such as osteoporosis, diabetes and cardiovascular disease. Alternatively, consumption of high amounts of alcohol can reverse these benefits. The current research does not establish causality between alcohol consumption and health impacts, nor does it distinguish between the effects of different types of alcoholic beverages (e.g. beer, wine) on health. Despite the potential favorable effects of alcohol, even low-risk drinking (e.g. one standard drink per day) can lead toward alcohol dependence. Due to stigma and social inequalities women may have more difficulty gaining access to treatment for alcohol dependence. Current research on women-specific interventions and for prevention and treatment aimed at reducing alcohol consumption and alcohol dependence in middle-aged and elderly women is limited. Future research should aim to identify female-specific treatment options that take into account the challenges associated with woman's life stage and circumstances related to alcohol consumption. Clinical trials should be designed and implemented in which women of different ages and with different socioeconomic background are represented. Another avenue of future research could be the potential interactions of alcohol consumption and pharmacological medications or other drugs. .

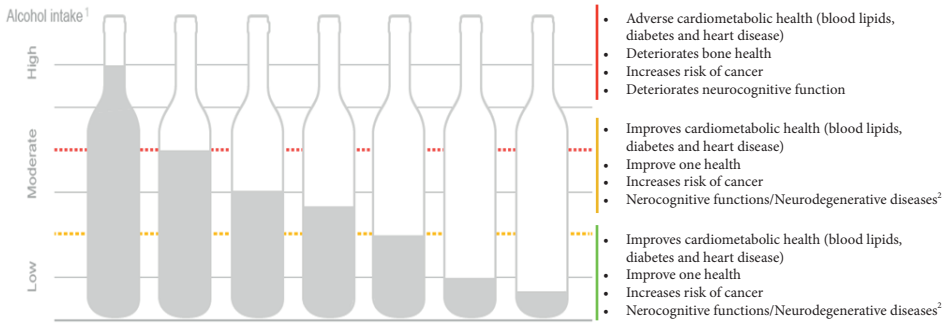

Figure 2. Dosage dependent risk factors and benefits of moderate intake

1. *Low drinking: less than 1 alcoholic drink for men and women in any single day, and a maximum of 7 drinks for men or 3 drinks for women per week; Moderate drinking: up to 4 alcoholic drinks for men and 3 for women in any single day, and a maximum of 14 drinks for men and 7 drinks for women per week; Severe drinking: 4 alcoholic drinks for men and 3 for women in any single day, and a maximum of over 14 drinks for men and over 7 drinks for women per week. One unit of alcohol is defined as 10ml (8g) of pure alcohol.*
2. *Current Evidence is not persuasive for a beneficial effect of low/moderate alcohol consumption on cognitive functions and/or risk of developing neurodegeneratie diseases*

The Impact of Complicated Grief on Diurnal Cortisol Levels

In chapter 4.1 by using data from a population-based study I investigated whether diurnal cortisol secretion differed in persons with normal grief, complicated grief, and those who have not experienced grief in the past 2 years. As hypothesized, participants who had complicated grief two years after their initial loss showed lower levels of morning cortisol and lower overall diurnal cortisol exposure than did the participants with normal grief or without grief. Persons with normal grief showed similar cortisol secretion patterns as those without grief. A previous study of complicated grief and diurnal cortisol showed that those with complicated grief have less oscillation in cortisol levels across the day (Yehuda, 2006). However, neither morning cortisol nor AUC had been examined in persons with complicated grief. Moreover, no effect of grief or complicated grief on cortisol secretion patterns was observed. Since higher levels of cortisol are known to appear in acute stress phase, it may be presumed that a person with more severe grief had a more acute/severe reaction at the moment of loss, while, over time, the cortisol secretion may normalize. This suggests that with a longer period of grieving, the association between grief and cortisol may attenuate. We might argue, then, that a coping mechanism helps to resolve the most severe grief consequences over time and even complicated grief is no longer an acute stressor. We can only carefully speculate about mechanisms of coping with grief, as has already been suggested in previous studies (Bremner et al., 1997; Stein et al., 1997; Yehuda et al., 1991; Boelen et al., 2006). Effects on the HPA axis may be visible after several months or even years only if a person develops complicated grief or has risk factors for complicated grief, such as a dependent relationship with the dead person, unexpected death, lack of social support, and the loss of someone who was ambivalently loved (Maciejewski et al., 2007). The significance of the study I performed is that it revealed that cortisol, which is involved in cognitive functions such as memory performance and executive function and regulates the inflammatory responses, is altered in

persons experiencing complicated grief (Bonanno et al., 2002; Hek et al., 2011; Lovallo 2005; Parkes, 1998).The finding implies that persons with complicated grief may be more vulnerable to developing cognitive problems (Maciejewski et al., 2007) depression, and medical conditions than are persons with normal grief. Follow-up studies are needed, however, to demonstrate the clinical consequences of these observations.

Grief and Sleep Quality in Older Adults

In chapter 4.2, I explored the cross-sectional and longitudinal associations of grief and complicated grief with sleep quality in older adults. Cross-sectional findings showed that normal and complicated grief were associated with shorter sleep duration and lower sleep quality. Complicated grief can be regarded as a bereavement situation in which sleep duration is likely to be affected (Doi, Minowa, Okawa, & Uchiyama, 2000; Monk, Germain, et al., 2008). Sleep disturbances are particularly prevalent in depressed bereaved persons; even bereaved persons who fail to meet a formal diagnosis of depression have measurable sleep impairment (Reynolds et al., 1992). Indeed, the cross-sectional analysis showed that the association between grief and complicated grief with sleep indicators was largely explained by depressive symptoms. Prospectively, however, I did not find an association between grief and sleep parameters. The results may be explained by the mechanisms of adaptive coping (Stroebe & Schut, 1999), which the grieving participants develop during prolonged exposure to grief. Participants who were evaluated in a follow-up assessment around six years after their initial loss demonstrated that grieving persons may reach a "stable state" in which they experience no further change in sleep quality. Also, another explanation for these findings may be that sleep quality had deteriorated before the onset of complicated grief. We did not conduct pre-bereavement sleep assessment in this study, so it was not possible to evaluate the bi-directional effects in the cross-sectional analysis, i.e., whether bereavement triggered the decline of sleep duration and quality or whether sleep impairment preceded the grief reaction. Further studies should be performed applying a bi-directional analysis to distinguish whether causal effects are presents.

Bereavement and Quality of Life

In chapter 4.3 we explored the association between bereavement and QoL. The death of a loved one leads to grief characterized by yearning and longing, decreased interest in ongoing activities, and frequent thoughts of the deceased which can hamper the quality of life (QoL). Therefore, I aimed to synthesize all available evidence on various forms of grief in relation to QoL. Overall, I found that different types of bereavement and bereavement severity are associated with lower QoL. Subjects with complicated grief, a disorder characterized by persistently high levels of bereavement, experience persistent deficits in QoL. Additionally, these findings suggest that bereavement may be associated with QoL in a gender-specific manner, with female grievers having lower QoL. These gender differences might also be explained by multiple adaptive mechanisms, relation or compliance, and differences in male/female social support/network, (Caetano, Silva, & Vettore, 2013) each of which merits further investigation. Also, our study indicated that QoL in bereaved family members was significantly lower than in controls. Our study is the first attempt to systematically review and critically appraise the literature on the subject and pool the existing studies that evaluated the associations of bereavement with overall QoL, but the evidence is limited and hampered by the scarcity and suboptimal quality of

the studies in the topic. Therefore no firm conclusion can be drawn and the findings we reported do not carry any implications on causality because all the included studies are observational.

Determinants and Predictors of Grief Severity and Persistence

After 6 to 12 months, most bereaved persons adapt to the loss. The demonstrate a reduction in grief intensity and a return to a different but meaningful and satisfying life without the deceased (Bonanno et al., 2002). However, for some bereaved individuals, the adaptation might be complicated, slowed, or halted, leading to persistence of grief (Boelen & van den Bout, 2008; Shear et al., 2007). Persistent grief is associated with lower QoL and increased risk of chronic disease, including cardiovascular disease and cancer (Zisook & Shear, 2009). It is important to identify factors associated with persistent grief in order to identify the population at risk of developing prolonged or complicated grief to provide them with support and adequate treatment. In chapter 5.1, I investigated the factors related to grief severity in cross-sectional analyses. I also investigated the determinants of grief cessation in longitudinal analyses, within a population-based study of middle-aged and elderly persons. Our cross-sectional results show that female sex, loss of a child, lower education, higher depressive symptoms, and difficulties in daily activities were associated with grief severity. However, in the longitudinal analysis, grief severity was the only predictor significantly associated with grief persistence. We hypothesize that the baseline severity of grief captures psychological features that predict timing of grief cessation, such as poor coping, anger and detachment from others. Bereaved individuals with good coping abilities might change their lifestyle leading to empowerment of self, focusing on existing and new relationships, while preserving their health and well-being (Neimeyer, 2006). Further longitudinal studies are needed to confirm and further explore these findings and to identify whether more potential predictors of grief severity and duration exist.

METHODOLOGICAL CONSIDERATIONS

Assessment of Grief Severity within Limitations of the Current Instruments and Terminology

Considering the nature of complicated grief and its impact on health, concerns about the definition of complicated grief and how it should be defined have been raised in several studies and by different authors (Beroud, Ferry, Henzen, & Sentissi, 2014; Lombardo et al., 2014; Maciejewski, Maercker, Boelen, & Prigerson, 2016). In the literature, there is a range of terms used to describe variations in normal grief, as well as operationalizing and distinguishing complicated grief. Uncertainties in terminology and conceptual confusion make it difficult for researchers and health care providers to interpret the data on grief and implement new clinical findings into practice. Healthcare professionals use a vast spectrum of terms to describe complicated grief, including prolonged grief disorder, persistent complex bereavement disorder, complicated grief disorder (CGD), pathological grief, traumatic grief, atypical grief, and delayed grief. All these terms are synonymous and introduce difficulties in interpreting the results across studies. However, in recent years there has been an increase in consistency in defining the condition of complicated grief and identifying consistent measures to assess the condition (Lobb et al., 2010).

In the studies presented in this thesis, I have defined the state of complicated grief and how it differs from normal and persistent grief with precision (Chapter 4). We diagnosed complicated grief based on a modified Dutch version of the 19-item Inventory of Complicated Grief (ICG) (Prigerson et al., 1995). To be diagnosed with complicated grief, participants underwent face-to-face interviews. They were asked if in the past two years they had lost someone over whom they were still grieving. If yes, these participants were classified as grievers and were further assessed with the ICG to measure grief severity. Complicated grief symptoms were assessed as present amongst participants who scored equal to or greater than 22 on the ICG score and who grieved for longer than 6 months (Newson et al, 2011; Saavedra Perez et al, 2015). The use of a slightly modified version of the original ICG might have introduced some issues in defining complicated grief and its validity. However, in a pilot study, we found that the measure has high internal consistency and convergent and criterion validity, demonstrating that this tool can be widely used in a population-based and observational setting as ours (Newson et al., 2011). The screening/preliminary question that determined which participants would take the ICG, functionally allowing participants to identify themselves as grievers, may have resulted in an over- or underestimation of the number of grievers. However, the opportunity to self-define a griever should not affect the number of cases diagnosed with complicated grief. On the other hand, a limitation of our study was the lack of tool to assess grieving status in a normal manner. Moreover, as with any questionnaire, and considering the time that measurements were undertaken and the use of self-report data, recall bias, measurement error and possibly non-differential misclassification in grief status might be present. However, this is likely to bias results towards the null.

Limitations of Sleep Assessment

In the study presented in Chapter 4 I investigated the association between grief status and sleep parameters. I did not have available objective measures of sleep duration nor of overall sleep quality. Sleep duration was self-reported and assessed by the following question: "During the past month, on average, how many hours of actual sleep did you get at night?" This question is extracted from the PSQI interview, while the subjective sleep quality was assessed using the whole Pittsburgh Sleep Quality Index (PSQI). The PSQI is a self-rating questionnaire that measures sleep quality and disturbance retrospectively over a 1-month period, resulting in a global score between 0 and 21. Higher values of the PSQI score indicate poorer sleep quality. These data were gathered based on the patients' recall of how many times they woke up or how long they slept during the night, which could have introduced recall bias and misclassification of sleep outcomes. While questionnaires are easy to perform and are not costly, the optimal methodology to assess sleep would be to use polysomnographic measurements of sleep. However, polysomnographic measurements for more than one night are hardly feasible in large studies. Unfortunately, polysomnographic measurements in the Rotterdam Study were not performed at the time of our research, and now they are available only on a limited number of participants (N=1000), who do not overlap with assessments of the Rotterdam Study we used for our analysis (Luik, Zuurbier, Whitmore, Hofman, & Tiemeier, 2015). Alternatively, despite the fact that it does not measure sleep quality, we could have used actigraphy to obtain objective sleep duration. Actigraphy is a method that infers wakefulness and sleep from the presence or absence of limb movement. It estimates sleep duration more accurately than do sleep diaries and agrees reasonably with polysomnography((NIAAA), 2006). In a pilot study using Rotterdam Study

data, we found substantial discrepancies between self-reported and actigraphic sleep duration. We found that the differences between the two assessment types are not random, and, rather, depend on age, sex, depressive symptoms, cognitive function and functional disability (Van den Berg et al., 2008). As these determinants are likely to be associated with grief status as we show in this thesis, this phenomenon may bias the results. Therefore, in our study on grief and sleep presented in Chapter 4, the subjective measurements we used to assess sleep duration and quality might be the reason for the spurious associations we found cross-sectionally. Alternatively, the subjective measurements may have obscured the true associations that we did not find in the longitudinal assessment. Therefore, use of multiple measures of sleep duration and quality can help to examine the consistency of the results over assessment methods.

Residual Confounding

A confounder is a factor (variable) that influences both the dependent variable and independent variable, and is not an intermediate in the causal pathway between the exposure and outcome. In observational studies, when a confounding factor is not taken into account, biased estimates may be obtained. In order to rule out possible confounding bias, in all our analyses we adjusted for multiple potential confounders, which were selected based upon previous studies. Nevertheless, since our studies are observational, the problem of residual confounding might still be present. Some of the confounders that were not considered in the studies based on original data and presented in Chapter 3 and 4 might be some psychiatric diagnoses (such as Generalised Anxiety Disorder, Post Traumatic Stress Disorder or Panic Attacks), somatic diagnosis as cancer, physical activity, and frailty index at the time when grief status was assessed. Therefore, the results in these studies might be explained by residual confounding. Residual confounding, depending on how the unmeasured factor related to exposure and outcome can lead either to overestimation or underestimation of the observed effect estimate.

Analysis of Longitudinal Data

Power

Sample size and statistical power estimate are fundamental for designing and conducting a study because they determine the strength of the association between the exposure and outcome of interest. As in most observational studies, we do not have access to the entire population of interest, so it is difficult to obtain optimal sample size and statistical power. To have more robust estimates of the outcomes in question, a large sample size is needed so that sampling error will be reasonably small. Studies characterized by small sample size will result in effect estimates that are too imprecise to be of much use outside the very specific sample. In longitudinal studies, power analysis is more challenging and complex because several factors such as number of repeated measurements and levels of missing data can affect the estimates of the required sample size. In this thesis, the studies presented in Chapters 4 and 5 have a smaller sample size compared to our cross-sectional analysis due to low response rate or loss of follow-up. Therefore, the studies in Chapters 4 and 5 may have limited power to detect an association. However, based on our power analysis, we had sufficient power to show consistent longitudinal effects. Including more participants with grief and complicated grief in the study presented in Chapter 5 might have strengthened the findings.

Reverse Causality

Longitudinal analysis, in contrast to cross-sectional design, reduces the risk of reverse causality. Reverse causality happens when the outcome causes changes in exposure. In our studies, for example, we cannot rule out that existing sleep problems or alterations in hypothalamic–pituitary–adrenocortical axis activity and cortisol levels make individuals vulnerable to more severe or prolonged grief or complicated grief. Indeed, as Boelen and Lancee (Boelen & Lancee, 2013) have pointed out, poor sleep quality is a known risk factor for many different forms of a psychopathology, including depression and PTSD. However, we performed sensitivity analyses in our studies to examine whether reverse causality could influence our results. For instance, in the longitudinal analysis investigating the association between grief and sleep parameters we excluded people who had poor sleep at baseline in order to explore the possibility of reverse causality. This sensitivity analyses provided no evidence of reverse causality.

Missing Data and Selection Bias

Issues stemming from missing data in observational studies are unavoidable, particularly in prospective studies. Data may be missing due to loss to follow-up, low response rate, or because participants were not invited to follow-up assessments. In the studies presented in chapters 4 and 5, we identified some missing data on exposure and/or outcomes, which might have introduced selection bias and might have influenced the validity of our results. The participation rate in all of our original studies was not 100%, but was always satisfactory. However, in the longitudinal analysis on grief and sleep selection bias is unlikely because (non) participation was neither associated with the self-reported grief status, nor with sleep duration/quality. In our longitudinal analysis on determinants of grief cessation, the non-response analysis showed that older persons and less healthy participants were more likely to be lost to follow-up. We might carefully infer that unhealthy persons were less likely to participate in the follow-up study, compared to the healthier individuals. Selection effects may have led to the inclusion of more grievers with healthy coping style whose loss events occurred long ago. It has been shown that using a selected source population for a cohort study usually leads to bias towards the null, but may affect the generalizability of our results to older and frailer populations (Brunner, Stallone, Juneja, Bingham, & Marmot, 2001).

Bias and Heterogeneity in Systematic Reviews

Bias might be present in all studies, and systematic reviews are subject to the same biases as those present in the original studies included in the review, as well as all biases related to the systematic reviewing process. Biases included publication bias, language bias, and biases that arise from poorly defined methodology can be present when performing a systematic review. However, in the systematic review included in this thesis, we followed an a priori protocol with clearly defined inclusion and exclusion criteria. Further, we tried to minimize the impact of publication bias by employing a thorough search strategy in six databases in which no language or date restrictions were applied, with the help of an experienced librarian. Also, we checked reference lists of identified studies and contacted experts in the field. An unavoidable problem in systematic reviews is the heterogeneity of the included studies. Study heterogeneity can arise when performing a systematic review and can limit the opportunity to undertake a meta-analysis. It is unavoidable that a systematic review of studies will include heteroge-

neous studies, comprised of different design, interventions, population characteristics, and exposure and outcome definitions. Study heterogeneity must be considered, evaluated and reported in studies that summarize the evidence. In the reviews included in this thesis, it is possible that effect estimates from individual studies cannot be combined, due to the high identified heterogeneity. Heterogeneity in the input parameters (e.g. exposure and outcome assessments) made pooling of the existing data unfeasible. Further, the generally small number of studies limited our opportunity to obtain summary estimates or to use subgroup analysis involving various study-level characteristics (ethnicity, age, etc.) to extensively explore the potential sources of heterogeneity that could be observed when pooling results from different studies. Lastly, the results inferred in the systematic reviews we performed should be interpreted with caution since they are limited by the quality of the individual published studies included in the review.

FUTURE RESEARCH

The Role of DNA Methylation and Histone Modifications in Neurodegenerative Diseases

Our systematic review reported epigenetic regulation of 31 genes (including cell communication, apoptosis, and neurogenesis genes in blood and brain tissue) and their relationship to AD and PD. Although there have been some promising results in the fields of epigenetics and neurodegenerative diseases, challenges related to study design, harmonized methodology of assessment, tissue heterogeneity and others are still present. Due to the mostly cross-sectional design of the included studies, inadequate adjustment for relevant confounders, and lack of replication in the case of new findings, many questions remain about the temporal relationship between epigenetic modifications and neurological diseases, as well as the significance of the findings in disease pathology. Therefore, studies in larger cohorts with longitudinal design and repeated measurements of epigenetic marks, including proper adjustment for multiple confounders may help to identify epigenetic changes that have clinical significance and could lead to strategies for intervening in neurological diseases. Further, to establish a causal direction between epigenetic marks and disease, Mendelian Randomization (MR) methods can be helpful. MR is considered to be a 'natural' randomized control trial since it uses common genetic polymorphisms as instrumental variables for the exposure of interest. Additionally, the distribution of genetic variation is thought to be unrelated to confounders, which are a common source of false positives in epidemiological studies. Currently, varied methods are being used to assess epigenetic marks. The use of similar methylation assessment methods in future studies is important so different studies' results can be compared effectively. Combination with eQTL mapping may also have a more precise overview on which epigenetic marks determine differential expression and thus disorder symptoms. Finally, future studies should explore further areas of epigenetic regulatory mechanisms beyond DNA methylation, including histone modifications, which remain very poorly characterized in the context of neurodegenerative diseases.

The Functions of Estrogen Receptor Beta in the Female Brain

Our research showed that ERβ has multiple functions in the female brain. Nevertheless, because the current evidence on this topic comes from animal studies, one should be cautious in extrapolating those findings to humans. To establish potential therapeutic and preventive strategies targeting ERβ and to further our understanding of the importance of ERβ in women's mental health, future studies should be conducted in humans. Longitudinal studies in humans should examine whether levels of ERβ in blood relate to improved mental health in women, including lower levels of depression, anxiety, and sleep disorders, all of which are common symptoms in women during the menopausal transition. Also, clinical trials should be designed and implemented to investigate whether ERβ ligands can confer the neuroprotective effects of estradiol in postmenopausal women, avoiding its specific adverse effects on other tissues such as those of uterus and breast, as well as on risk of diabetes and cardiovascular disease.

Menopausal Transition and Alcohol Use Disorders

I aimed to evaluate whether women have an increased risk of alcohol abuse during the menopausal transition and postmenopausal period (chapter 3 .2). However, there is a lack of studies that investigate women-specific treatment strategies, so further studies about gender differences in alcohol intake are necessary. Clinical studies should focus more on identifying and confirming some predictors of treatment retention in women. Some known predictors are pre-treatment characteristics, such as referral source, psychological functioning, personal stability, and number of children. These pre-treatment characteristics may be important predictors of length of stay or treatment completion. Future research should also aim to provide more insight into Naltrexone (Vivitrol) based treatments and explore the role of a "polypragmatic" approach in pharmacotherapy by identifying additional harm or benefits from sedatives and SSSRI medications.

The Impact of Complicated Grief on diurnal cortisol levels, sleep quality and overall quality of life

During the lifespan, people are likely to experience the loss of a close person. Evidence suggests that several factors that are associated with bereavement can cause poor health, including adverse cardiometabolic health and increased risk of mortality. Losing a dear person can be very stressful, so the first stages of bereavement are often accompanied by an acute stress-type response. Thus, we were interested to see how grief status is related to levels of the stress hormone cortisol. In our study, we did not find an association between grief and cortisol levels. However, cortisol findings were based on saliva samples, so we suggest that future research might further explore how grief status relates to serum levels of cortisol that were not available in this study. Additionally, future research in this area might be more informative if they use repeated measurements of cortisol levels, in order to investigate longitudinal effects of grief process on cortisol. Cortisol, which is balanced by the hormone dehydroepiandrosterone (DHEA), weakens the immune system as it has stimulatory effect on the immune function (Khorram, Vu, & Yen, 1997). Levels of DHEA sharply decrease around the age of 30, and thus leave the immune system more vulnerable to cortisol's influence in times of stress. Future studies should also investigate the association between DHEA and cortisol, as well as how grief could impact their respective levels. It may also be interesting to examine whether DHEA and cortisol

play a role in the severity of grief and grief cessation. Similarly, research on grief and sleep should use more accurate and objective measures of sleep duration and quality, such as polysomnographic measurements, as well as repeated measurements of sleep parameters. These changes in study design would help to determine the longitudinal and directional effects: whether bereavement triggered the change in hormone levels, or vice versa.

Our review on bereavement and QoL shows that different types of bereavement and bereavement severity are associated with QoL, and that women might experience lower QoL than men. However, the magnitude of the effect on the association between bereavement and QoL is difficult to assess because of the scarcity of evidence, the low quality of the studies available, and the large heterogeneity in bereavement assessment, type of bereavement and QoL measures used, study design and quality. Factors such as recall bias and the challenges of objective interpretation of emotional status might hamper the quality of recording grieving states and require consideration in future study design. Objective interpretation of emotional status in particular is difficult because severe bereavement may be under-reported and should be assessed and re-assessed by healthcare professionals.

Beyond the caution needed in interpretation of grief length and severity, future studies should also consider other social, emotional, and health-related factors associated with grief. For instance, most studies define the state of grief as "present" or "absent" after the loss event, not taking into account the severity of grief and its duration (complicated grief or prolonged grief disorder). The continuation or persistence of degrees of severity in bereavement need more research in order to harmonize the definitions and distinct different bereavement status. Moreover, it is necessary to harmonize bereavement and QoL assessment, and to design better studies that adjust for a broad range of confounders and select a representative sample. Prospective studies are required before any level of causality can be inferred from the existing findings, and residual confounding may explain the associations observed. Taking into account all these methodological considerations, future research may provide a more reliable assessment of the association between bereavement and QoL.

Determinants and Predictors of Grief Severity and Persistence

Our cross-sectional results show that female sex, loss of a child, lower education, higher depressive symptoms, and difficulties in completing daily activities were associated with grief severity. However, in the longitudinal analysis, only grief severity predicted grief persistence (chapter 5.1). Therefore, analysis in future studies might include more potential predictors such as physical activity, frailty index, nutrition, socio-economic status, ethnicity and other comorbidities (e.g.CA, GAD and panic attacks), as well as hormone levels and different biomarkers. Moreover, larger cohort and longitudinal studies with sufficient power are necessary to identify other potential predictors of grief severity and persistence.

IMPLICATIONS FOR TREATMENT AND PREVENTION

We reported that several epigenetic marks in brain and blood tissue were associated with AD and PD. Methylation profiling in peripheral blood has great potential to identify neurological disorders-related methylated regions. Methylation profiling has potential clinical utility since it may allow clinicians to

identify high-risk individuals who may benefit from preventive and therapeutic interventions. Also, increasing our knowledge of environmental-related epigenetic marks may be used for early detection and primary prevention of the global burden of neurodegenerative diseases. Implementing reliable tools to track lifetime exposure at both population and individual levels will allow scientists to dissect and understand epigenetic variations and how they may affect the risk of neurodegenerative disease and treatment efficacy. Epigenetic modifications, unlike genetic changes, are usually reversible. Therefore, they may represent an important target for therapeutic intervention in neurodegenerative diseases.

Overall, our results on ERβ show abundant functions of ERβ in the female brain and support the notion that future therapies targeting ERβ could constitute a novel preventive strategy and treatment for neurological diseases in females. ERβ agonists might mimic the actions of 17β-estradiol in the brain without causing other physiological responses mediated by other estrogen receptors. In view of the high burden of depressive disorders and neurodegenerative disease, and despite advances in their prevention and treatment, transfer of these novel therapies into the field of neurological diseases could constitute a suitable alternative in the future.

So far, achievements in alcohol abuse treatments are based on the outdated and debunked "alcoholism" model that observes alcoholism as a singular disease, without variation related to age, gender or setting. Women who are perimenopausal, menopausal, and postmenopausal alcohol users have a higher risk of severe comorbidities and alcohol abuse. As every subpopulation has its own specificities in overall health status and behavioral/cultural patterns in alcohol consumption, future behavioral and pharmaceutical treatment should be gender and age-targeted. Specific treatment programming may enhance treatment retention among certain subgroups of women.

Finally, as aging populations might be increasingly exposed to the loss of a dear person, clinical settings should target the early diagnosis of complicated grief. Even more important, behavioral and pharmaceutical prevention treatments should be based on initial severity of grief. In line with this theory, our results on the determinants of grief indicate that targeting patients with an ICG score over 22 in acute grief phase (latest at 6 months after a loss) may prevent the development of complicated grief and reduced quality of life. Also, if our findings on the association between bereavement and QoL are replicated in future studies, it may imply that QoL and bereavement are two different constructs, and thus QoL could be assessed as an additional intervention/treatment outcome. For example, researchers and providers evaluating bereavement care interventions might consider including QoL outcome measures in their projects.

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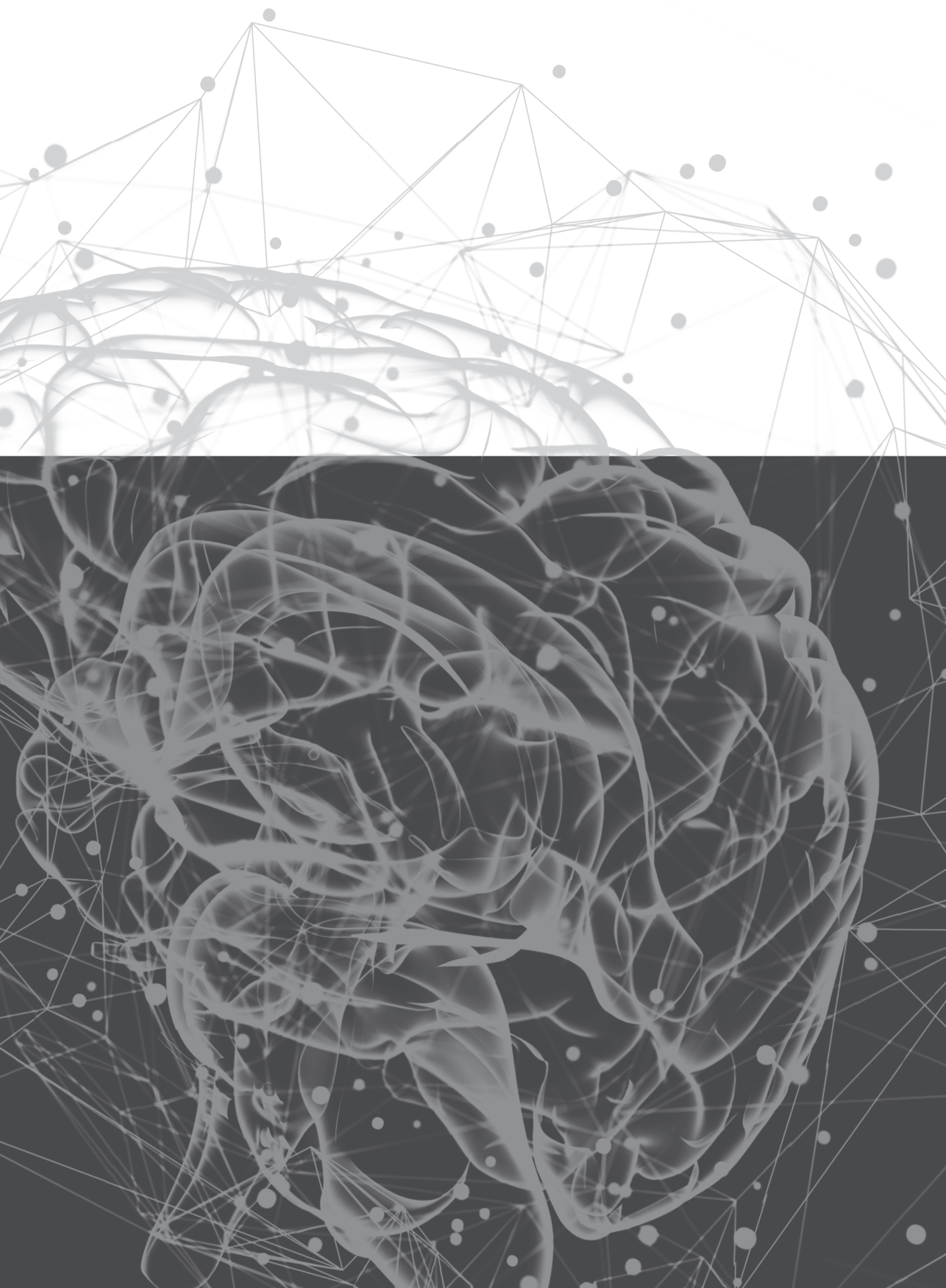
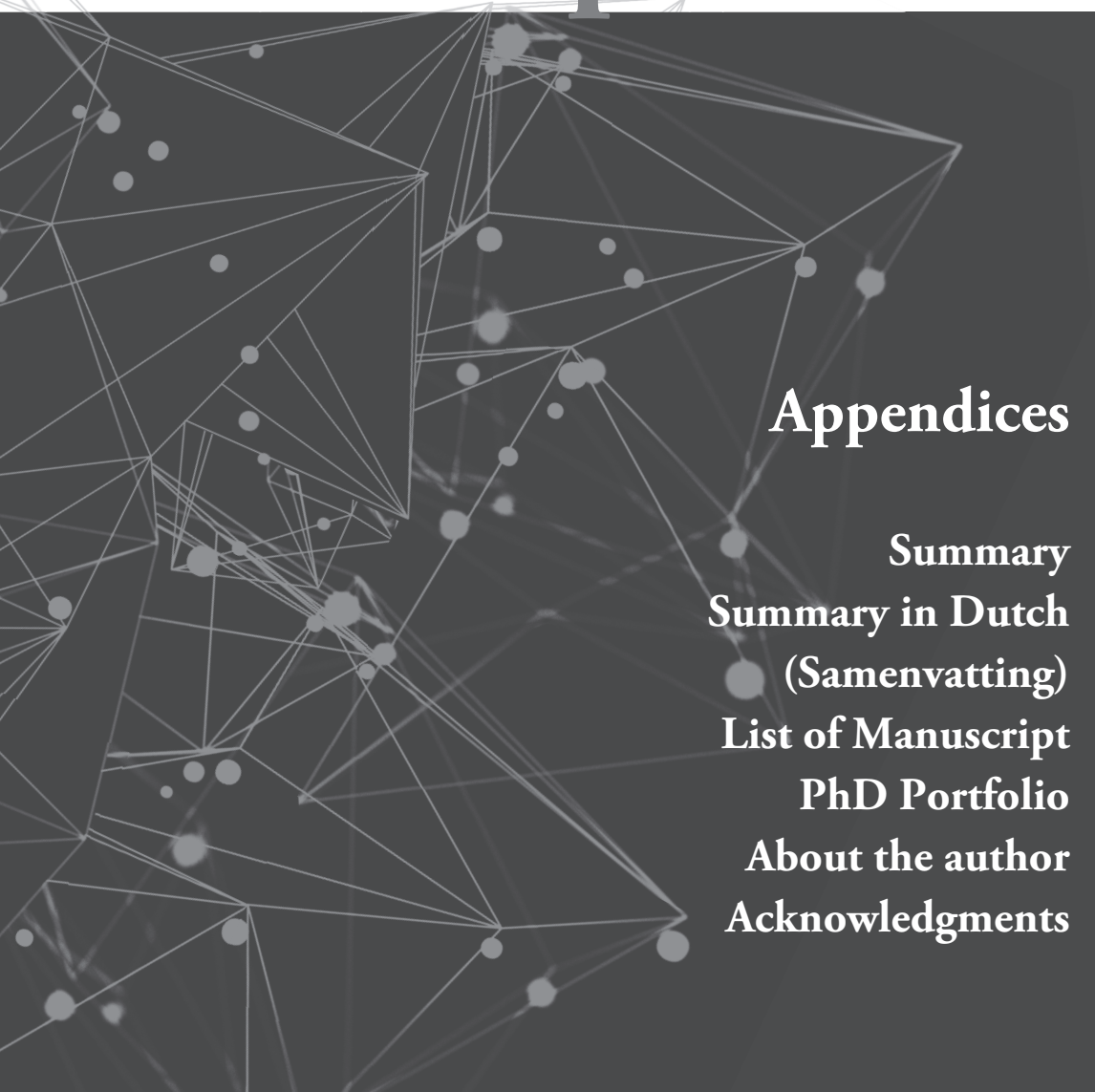


Chapter 7



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7.1

Summary

Ageing is becoming a significant global challenge which will require economic and social adjustments. Aging causes morphological changes in the brain associated with increased prevalence and incidence of neuropsychiatric diseases, mostly depression, Alzheimer's Disease (AD) and Parkinson's disease (PD). Neuropsychiatric diseases impair quality of life and pose a high economic burden to individuals and society. Thus, in order to better face these challenging diseases, it is of importance to understand factors that could contribute in their pathophysiology. It is now widely accepted that there is a strong genetic component in the development of neurodegenerative diseases, whereas emerging evidence is indicating also epigenetic mechanisms such as DNA methylation and histone acetylation as key players in the development of AD and PD. Also, women due to longer lifespans and unique risk factors, face the highest burden of these diseases, especially in menopausal years where major hormonal changes, such as a sharp decrease in estradiol, occur. To improve women's neuropsychiatric health after menopause, estrogen receptorβ has been suggested as a novel target therapy for prevention and treatment of neuropsychiatric diseases. Further, alcohol misuse among elderly is on the rise and elderly women are particularly vulnerable to the adverse effects of alcohol, and alcohol use disorders in this subgroup are often overlooked or misdiagnosed. Also, elderly person has an increasing chance of a traumatic death of a loved one. An estimated 10-20% of bereaved people continue to grieve for a prolonged period. This complex condition is termed as Prolonged Grief Disorder (PGD), which can have a severe influence on the quality of life as it impairs daily functioning and sleep and may increase the risk of certain diseases. Our insight of the association between the dynamics of grieving process and overall health outcomes is still insufficient.

The aim of the thesis was to identify factors associated with neuropsychiatric disorders among the elderly. The first objective of the thesis was to identify epigenetic as well as women-specific factors that can play a role in the development of neuropsychiatric outcomes with emphasize on neurodegenerative diseases and alcoholism. A second objective was to identify the impact of grief and complicated grief on cortisol secretion, sleep pattern, and overall quality of life, as well as to identify factors associated with grief persistence.

In chapter 2 of this thesis, we summarize all available evidence in humans assessing the association of DNA methylation and histone modifications with AD and PD. We identified 75 articles meeting our eligibility criteria and were included in this review. Overall, 11453 individuals were included within the systematic review, with a total of 2640 for AD and 2368 for PD outcomes. The findings of this study indicate significant epigenetic differences between patients with neurodegenerative diseases and healthy individuals. Furthermore, candidate gene studies have shown that some genes known to play a role in maintenance and function of neurological tissues are differentially methylated in diseased individuals. In addition, a number of these genes are similarly methylated in blood and brain tissue. Therefore, methylation profiling in peripheral blood to identify neurological disorders-related methylated regions has a high potential clinical utility. It may allow clinicians to identify high-risk individuals who may benefit from preventive and therapeutic interventions. Also, given the reversible nature of epigenetic aberrations, targeting the epigenome can be a novel preventive strategy and treatment for AD and PD.

In chapter 3 we focus on women's health, examining factors in women that might play a role in the development of neuropsychiatric outcomes. The chapter 3.1 presents a systematic review of all the available evidence evaluating the function of ERβ in the female brain and the role of age and meno-

pause on ERβ actions. Summarizing the evidence from 49 studies based on animal models, we show that ERβ effects neurological functions via messenger proteins and regulation of protein expression. It also promotes neurogenesis, modulates the neuroendocrine regulation of stress response, grants neuroprotective role against ischemia and inflammation, and reduce anxiety- and depression-like behaviors. Considering the increasing cases of depressive disorders and neurodegenerative diseases in women, our results support ERβ targeted treatments as novel therapy in improving women's health. Most importantly, future studies should be conducted in humans to further our understanding of the importance of ERβ in women's mental health.

In chapter 3.2 we provide an overview of prevalence of drinking patterns and alcohol dependence, risk factors, health impacts and treatment challenges for women as they progress through middle and older age. We explored the phenomena of female vulnerability to alcohol's harmful effects, alcohol-related diseases and other consequences of drinking earlier in life (compared to men). We also covered the menopausal transition and specificity of mental and somatic health burdens in postmenopausal period in association to alcohol abuse. . We concluded that due to social stigmas, women tend to have more difficulty gaining access to treatment and recovering from alcohol dependence than do men. Therefore current studies have limited knowledge on this topic and further research needs to be done.

In chapter 4, we present original data analyses and systematic reviews of the literature to examine the impact of grieving process on health. The cross-sectional study presented in Chapter 4.1 examined the association of complicated grief and normal grief with the diurnal cortisol patterns in 2084 persons aged older than 55 years within the Rotterdam Study. The study showed that compared to normal grievers, participants with complicated grief had lower levels of morning cortisol, and lower levels of overall diurnal cortisol. Participants with complicated grief also showed lower levels of morning cortisol than the non-grievers. However, cortisol secretion patterns did not differ between persons with normal grief and non-grieving controls.

In chapter 4.2 we determined cross-sectional and longitudinal relations of grief and complicated grief with sleep duration and quality in the general population of elderly adults. We included 5,421 men and women from the prospective population-based Rotterdam Study. Our findings showed that complicated grief was cross-sectionally associated with shorter sleep duration and lower sleep quality. These associations were explained by the presence of depressive symptoms. The prospective analyses showed that sleep duration and sleep quality did not decline further during follow-up of persons who experienced grief or complicated grief. In chapter 4.3 we explored whether bereavement is associated with quality of life (QoL). Fourteen studies (5 studies were cross-sectional 4 were longitudinal studies (3 prospective and 1 retrospective) and 5 case–control studies) were included with data on 1,604 middle-age and elderly participants. Overall, bereavement, its types and grief severity were associated with lower QoL, especially in women. Nevertheless, the evidence on grief and QoL is limited and hampered by the suboptimal quality of the studies in the topic.

In chapter 5.1 we investigated correlates and predictors of bereavement severity and persistence (triggered by "loss of a loved one"; referent group partner loss) in the Rotterdam Study . Our findings showed that cross-sectional, female sex, child-lost, higher depressive symptoms, lower education, and difficulties in daily activities were independently associated with a higher bereavement severity. Prospectively, the baseline value of the grief severity was the single predictor significantly associated

with grief persistence. These results suggest that only grief severity is independently associated with grief persistence. Further studies are needed to confirm our findings.

Lastly, Chapter 6 provides a general discussion of the work presented in this thesis. We therefore, summarize our principal findings and discuss the main methodological consideration. Further, we reflect upon the findings and potential implications our results might have. The general discussion was concluded with a section that proposes directions for future research.

7.2

Samenvatting

Vergrijzing is een significante, mondiale uitdaging, die economische en sociale aanpassingen zal vergen. Ouder worden gaat gepaard met morfologische veranderingen in de hersenen, die worden geassocieerd met toenemende prevalentie en incidentie van neuropsychiatrische ziekten, voornamelijk depressie, de ziektes van Alzheimer (AD) en Parkinson (PD). Neuropsychiatrische ziekten beperken de kwaliteit van het leven en leggen een zware economische last op individuen en de gemeenschap. Om deze uitdagende ziekten beter het hoofd te kunnen bieden, is het belangrijk om de factoren die zouden kunnen bijdragen aan hun pathofysiologie te begrijpen. Het is tegenwoordig algemeen geaccepteerd dat er een sterke genetisch component is in de ontwikkeling van neurodegeneratieve ziekten, terwijl nieuw bewijs aantoont dat epigenetische mechanismes zoals DNA methylatie en histon acetylatie een belangrijke speler zijn in de ontwikkeling van AD en PD. Daarbij dragen vrouwen de meeste last van deze ziekten door een hogere levensverwachting en unieke risicofactoren Vooral in de menopauzale jaren, door belangrijke hormonale veranderingen, zoals een scherpe afname van estradiol. Om de neuropsychiatrische gezondheid van vrouwen na de menopauze te verbeteren wordt estrogeen receptor β gesuggereerd als nieuwe target therapie voor de preventie en behandeling van neuropsychiatrische ziekten. Verder neemt alcoholmisbruik onder ouderen toe en oudere vrouwen zijn extra vatbaar voor de negatieve effecten van alcohol. Alcoholmisbruik stoornissen in deze subgroep worden vaak niet erkend of verkeerd gediagnostiseerd. Ouderen lopen verder ook een verhoogd risico op de traumatische dood van een geliefde. Naar schatting, 10-20% van de rouwenden, rouwt gedurende een langere periode. Deze complexe conditie wordt Prolonged Grief Disorder (PGD) genoemd en kan grote invloed hebben op de kwaliteit van het leven, omdat het dagelijks functioneren en de slaap wordt beïnvloed en de kans op bepaalde ziekten wordt vergroot. Onze kennis van de associatie tussen de dynamiek van het rouwproces en de algemene gezondheid is ontoereikend.

Het doel van dit proefschrift was het identificeren van factoren die geassocieerd zijn met neuropsychiatrische stoornissen bij ouderen. Het eerste doel hierbij was het identificeren van epigenetische en vrouwspecifieke factoren die een rol kunnen spelen in de ontwikkeling van neuropsychiatrische eigenschappen met nadruk op neurodegeneratieve ziekten en alcoholisme. Het tweede doel was het identificeren van de invloed van rouw en pathologische rouw op cortisol afgifte, slaappatroon en algehele kwaliteit van leven, evenals het identificeren van factoren die geassocieerd zijn met aanhoudende rouw.

In hoofdstuk 2 van dit proefschrift vatten we al het beschikbare bewijs samen die de associatie van DNA methylatie en histon modificatie met AD en PD in mensen beoordeelt. We identificeerden 75 artikelen die aan onze criteria voldeden waarna zij werden opgenomen in onze review. In totaal zijn 11453 individuen in deze systematische review opgenomen, waarvan 2640 met AD en 2368 met PD . De resultaten van deze studie wijzen op significante epigenetische verschillen tussen patiënten met neurodegeneratieve ziekten en gezonde individuen. Geselecteerde genetische studies hebben bovendien uitgewezen dat sommige genen, die een rol spelen in het onderhoud en functioneren van neurologisch weefsel, anders worden gemethyleerd in zieke individuen. Een deel van deze genen wordt bovendien op vergelijkbare wijze gemethyleerd in bloed en hersenweefsel. Hierdoor heeft DNA methylatie profilering in perifeer bloed, om neurologische aandoening gerelateerde veranderingen in DNA methylatie te identificeren, grote klinische potentie. Het zou clinici de mogelijkheid kunnen geven individuen met een verhoogd risico te identificeren, die baat zouden kunnen hebben van pre-

ventieve en therapeutische interventies. Vanwege de omkeerbare natuur van epigenetische aberraties, kan het richten op het epigenoom, een nieuwe preventieve strategie zijn bij het behandelen van AD en PD.

In hoofdstuk 3 focussen we op de gezondheid van vrouwen door factoren te onderzoeken die een rol kunnen spelen bij de ontwikkeling van neuropsychiatrische resultaten. Hoofdstuk 3.1 toont een systematische review van al het beschikbare bewijs, dat de functie van Erβ in het vrouwelijk brein, de rol van leeftijd en menopauze op Erβ evalueert. Door de resultaten van 49 onderzoeken op basis van diermodellen samen te vatten, laten we zien dat Erβ neurologische functies beïnvloedt via messenger eiwitten en de regulatie van eiwit expressie. Bovendien bevordert Erβ ook de neurogenese, moduleert het de neuro-endocrine regulatie van stressrespons, speelt het een neuroprotectieve rol tegen ischemie en ontstekingen en verlaagt het angst- en depressie-achtig gedrag. Het toenemend aantal gevallen van depressieve aandoeningen en neurodegeneratieve ziekten in vrouwen in acht nemende, ondersteunen onze resultaten Erβ-gerichte behandelingen als nieuwe therapie ter bevordering van de gezondheid van vrouwen. Het belangrijkste is dat er onderzoek gedaan zou moeten worden met mensen om ons begrip van het belang van Erβ voor de geestelijke gezondheid van vrouwen te bevorderen.

In hoofdstuk 3.2 geven we een overzicht van de prevalentie van drinkpatronen en alcoholverslaving, risicofactoren, invloed op de gezondheid en uitdagingen in de behandeling voor vrouwen op middelbare en oudere leeftijd. We onderzochten de vrouwelijke kwetsbaarheid voor de negatieve effecten van alcohol, alcohol gerelateerde ziekten en andere gevolgen van drankgebruik in eerdere fasen van het leven (in vergelijking met mannen). We keken ook naar de overgang naar de menopauze en de specifieke mentale en somatische gezondheidsproblemen die in de postmenopauzale periode geassocieerd zijn met alcoholmisbruik. We concludeerden dat vrouwen, door sociale stigmata, meer moeite ondervinden bij het toegang krijgen tot behandeling en het herstellen van alcoholverslaving dan mannen. Bestaand onderzoek heeft daardoor beperkte kennis over dit onderwerp en verder onderzoek is nodig.

In hoofdstuk 4 presenteren we originele data analyses en systematische reviews van de literatuur om de invloed van het rouwproces op de gezondheid te onderzoeken. Het transversale onderzoek in hoofdstuk 4.1 onderzocht de associatie van pathologische rouw en gewone rouw met het dagelijkse cortisol patroon van 2084 personen van 55 jaar en ouder binnen de Rotterdam Studie. Het onderzoek toonde aan dat in vergelijking met gewone rouwenden, deelnemers met pathologische rouw lagere waarden hadden voor ochtend cortisol en lagere waarden van de totale dagelijkse cortisol. Deelnemers met pathologische rouw hadden ook lagere waarden voor ochtendcortisol dan niet-rouwenden, maar cortisol afgifte verschilde niet tussen personen met gewone rouw en de niet-rouwende controlegroep.

In hoofdstuk 4.2 stelden we transversale en longitudinale relaties van rouw en pathologische rouw met slaaplengte en -kwaliteit in de algemene populatie van ouderen vast. Het onderzoek omvatte 5421 mannen en vrouwen uit het prospectieve bevolkingsonderzoek Rotterdam Studie. Onze resultaten lieten zien dat pathologische rouw transversaal was geassocieerd met kortere slaap en lagere slaapkwaliteit. Deze associaties werden verklaard door de aanwezigheid van depressieve symptomen. De prospectieve analyses toonden aan dat slaaplengte en -kwaliteit niet verder achteruit gingen bij vervolgonderzoek van personen met rouw of pathologische rouw.

In hoofdstuk 4.3 onderzochten we of verlies geassocieerd is met kwaliteit van leven (QoL). Viertien studies (5 studies waren transversaal, 4 longitudinale studies (3 prospectief en 1 retrospectief) en

5 case-control studies) werden meegenomen, met data over 1604 middelbare en oudere deelnemers. Over het algemeen was een sterfgeval, de types en de rouwintensiteit geassocieerd met lagere QoL, vooral in vrouwen. Het bewijs over rouw en QoL is echter beperkt door de suboptimale kwaliteit van studies over het onderwerp.

In hoofdstuk 5.1 onderzochten we correlaties en predictoren van rouwintensiteit en rouwvolharding (veroorzaakt door "verlies van een geliefde"; referentiegroep verloren partner) in de Rotterdam Studie. Onze resultaten lieten zien dat, transversaal, vrouwelijk geslacht, kind verlies, zwaardere depressieve symptomen, lager onderwijsniveau en moeite met dagelijkse activiteiten onafhankelijk geassocieerd waren met een hogere rouwintensiteit . De basiswaarde van rouwintensiteit was prospectief de enige predictor die significant geassocieerd was met rouwvolharding. Deze resultaten suggereren dat alleen rouwintensiteit onafhankelijk geassocieerd is met rouwvolharding. Verder onderzoek is nodig om onze resultaten te bevestigen.

Hoofdstuk 6 bevat een algemene discussie van het werk in dit proefschrift. We vatten daarin onze belangrijkste resultaten samen en bediscussiëren de belangrijkste methodologische overwegingen. Verder reflecteren we op de bevindingen en mogelijke implicaties die onze resultaten kunnen hebben. De algemene discussie wordt afgesloten met een sectie die richtingen voor toekomstig onderzoek voorstelt.

7.3

List of manuscripts

Bramer, W. M., **Milic, J**., & Mast, F. (2017). Reviewing retrieved references for inclusion in systematic reviews using EndNote. Journal of the Medical Library Association : *JMLA*, 105(1), 84–87. http://doi.org/10.5195/jmla.2017.111

Wen, K-x*, **Milic, J***., El-Khodor, B., Dhana K., Nano, J., Pulido, T., Kraja, B., Zaciragic, A., Bramer, W.M.,Troup, J., Chowdhury, R., Ikram, M.A., Dehghan, A., Muka, T., Franco, O.H. (2016) The Role of DNA Methylation and Histone Modifications in Neurodegenerative Diseases: A Systematic Review. *PLoS ONE* 11(12): e0167201. https://doi.org/10.1371/journal.pone.0167201

Vargas, K. G*., **Milic, J***., Zaciragic, A., Wen, K.-x., Jaspers, L., Nano, J., Franco, O. H. (2016). The functions of estrogen receptor beta in the female brain: A systematic review. Maturitas, 93 (Supplement C), 41-57. doi:https://doi.org/10.1016/j.*Maturitas.*2016.05.014

Saavedra Perez, H. C., Direk, N., **Milic, J**., Ikram, M. A., Hofman, A., & Tiemeier, H. (2017). The Impact of Complicated Grief on Diurnal Cortisol Levels Two Years After Loss: A Population-Based Study. *Psychosomatic Medicine,* 79(4), 426-433. DOI: 10.1097/PSY.0000000000000422

Milic, J., Saavedra Perez, H., Zuurbier, L. A., Boelen, P. A., Rietjens, J. A., Hofman, A., & Tiemeier, H. (2017). The Longitudinal and Cross-Sectional Associations of Grief and Complicated Grief With Sleep Quality in Older Adults. *Behavioral Sleep Medicine*, 1-12. doi:10.1080/15402002.2016.1276016

Milic, J., Muka, T., Ikram, M. A., Franco, O. H., & Tiemeier, H. (2017). Determinants and Predictors of Grief Severity and Persistence: The Rotterdam Study. *Journal of Aging and Health,* 0898264317720715. doi:10.1177/0898264317720715

Muka, T., Asllanaj, E., Avazverdi, N., Jaspers, L., Stringa, N., Milic, J. et al. (2017). Age at natural menopause and risk of type 2 diabetes: a prospective cohort study. Diabetologia. 2017 Jul 18. doi: 10.1007/s00125-017-4346-8

Milic J., Glisic M.,.Asllanaj E., Troup J., Kiefte J. C., Pletsch Borba L., Voortman T., Rojas L. Z., van Beeck E. F., Muka, T., Franco, O. H. (2017) Menopause, ageing and alcohol use disorders in women. *Accepted for publication in Maturitas.*

Milic, J., Rojas L. Z., Tiemeier, H., Grabe, H., Voelzke, H., Bramer, W. M., Franco, O. H., van Beeck, E., Muka, T. Quality of Life and Bereavement: A Systematic Review. *Manuscript in progress.*

Jabbarian L., **Milic, J.** Anticipatory Grief in Women after a Cancer Diagnosis: Clinical Correspondence. *Submitted for publication.*

Milic J., Ahmadizar F., van der Wel, N. P. A., van Beeck, E.F. Geriatric drinking Pharmacotherapy Options, Issues and Considerations. *Submitted for Publication.*

Glisic, M., Asllanaj E., Kastrati, N., **Milic, J.**, Portilla Fernandez, E., Nano, J., Ochoa-Rosales, C., Kraja, B., Bano A., Bramer W.M., Danser J., Roks A., Franco O.H., Muka T. Phytoestrogen supplementation and body composition in postmenopausal women: systematic review and meta-analysis of randomized controlled trials. *Manuscript in preparation.*

Farajzadegan, Z., Khalil, N., Jafari, N., Milic, J., Mokarian, F. Quality of Life with Women with Breast Cancer in the Structural Equation Model Approach. *Manuscript in preparation.*

Milic, J., Alcaz, S., van Beeck. Treatments of combined addictions to alcohol, narcotics and gambling. *Manuscript in preparation*

7.4

About the author

Jelena Milic was born on November 18th 1975 in Belgrade, Serbia. She graduated at Faculty of Medicine of Belgrade University in 2004. During her studies Jelena worked as a visiting researcher at Kennedy Lab for Neurobiology, CALTECH Institute, Los Angeles, USA. After graduation she was granted a scholarship on behalf of European Union commission and continued with an research internship and master at the Emergency care unit of Faculty of Medicine, La Sapienza University in Rome, Italy. She was focusing on admissions and treatment of psychiatric and neuropsychiatric patients. After she graduated in 2006, Jelena continued with a Master of Science Program in Public Health at Faculty of Medicine University of Belgrade, Serbia. This program, helped her to understand how health issues and policies are represented, experienced and understood by people. Also she learned how research evidence is used to improve population health. In 2012, Jelena finished her specialization in Family supportive psychotherapy with focus on addictive diseases treatment at the Institute of Mental Health of University of Belgrade, after which she became a licensed psychotherapist. In order to have a better understanding of the quantitative approach in clinical research and clinical practice, with a focus on the patient and mental health, she decided to do the Program in Clinical Epidemiology and a PhD in the Netherlands Institute of Health Sciences (NIHES) and Erasmus MC, Rotterdam the Netherlands. For her PhD studies, Jelena was granted Erasmus Mundus - ERAWEB scholarship on behalf of European Commission. Jelena is also employed at National Institute of Public health of Serbia and plans to further co-develop the collaboration between her home institute and NIHES.

7.5

PhD portfolio

SUMMARY

Name PhD student: Jelena Milic
Erasmus MC department: Epidemiology
Research School: Netherlands Institute for Health Sciences (NIHES)
PhD period: October 2012- November 2017
Promotor: Prof.dr. Oscar H. Franco
Co- promotors: Dr. Taulant Muka and Ass. Prof.dr. Ed van Beeck

Training	Year
Master of Science in Epidemiology	2012-2013

Core courses
- Study design
- Biostatistical methods I: basic principles I
- Clinical Epidemiology
- Methodologic thopics in Epidemiologic Research
- Biostatistical Methods II: Classical Regression Models

Advanced and Skill courses
- Psychiatric Epidemiology
- Medical Demography
- Quality of Iife Measurement
- From problem to Solution in Public health
- English language
- Introduction to Medical Writing
- Courses for the quantitative researcher

Erasmus Summer Program
- Principles of Research In Medicine
- Methods of Public health Research
- Health Economics
- Cohort Studies
- Case-Control Studies
- Introduction of the Global Public Health
- Primary and Secondary Prevention Research
- History of Epidemiologic Ideas
- Social Epidemiology
- Markers and Prognostic Research
- Logistic Regression

Seminars and meetings

ErasmusAGE research meetings	2015-2017
Seminars at the department of Epidemiology	2013-2017
2020 meetings	2013-2017
Epi Psychiatry meetings	2013-2015
Erasmus MC PhD days	2015-2018

Psychotherapeutic collaborations and activities

Peer-consultation and lectures about handling terminally ill patients and their caregivers, Awali hospital, Southern governorate, Bahrein	2015-2017
Supervising the support group for expats mental health problems, Rotterdam	2016-2017

Other

Peer review of articles for scientific journals	2017-2018

Scholarships

Caltech's Summer Undergraduate Research Fellowships (SURF) program- neuroscience	2000
La Sapienza University, Rome, Italy & European Commission Master scholarship	2005
ERAWEB master and PhD scholarship grant for PhD studies	2012
Erasmus Trustfonds – conference participation grant	2016

7.6

Acknowledgments

"One day, in retrospect, the years of struggle will strike you as the most beautiful."

Sigmund Freud

I am pleased to have the opportunity to convey my genuine gratitude for the intellectual and personal assistance I have received in completing my research.

Doing this research has been stimulating hard work and a great experience.

I would like to thank my supervisory team and professors with whom I cooperated the most. I would never have made it without them. Prof. dr. O.H. Franco, thank you for the support and the encouragement, for teaching me how to be a team player and how to get things done. Thank you for your support during the process. Dr. T. Muka, thank you for all the hard work that you contributed to get me to this point, and for never losing trust in me. I am sure life will bring us many more challenges and I am looking forward to them. Dr. E.F. van Beeck, thank you very much for the patience and kindness you have shown, I hope that this thesis is not the end but just the beginning of many nice collaborations to come. Prof. dr. H. Tiemeier, I am happy to have been part of Psychiatric-epidemiology group. Hopefully the future will bring even more exciting collaborations. Prof. dr. M.A. Ikram, thank you for all your understanding and support throughout my PhD trajectory, I am very grateful that you agreed to be the secretary of my small commission and to judge the quality of my thesis.

Further, I express my sincere gratitude to the other members of the small committee, prof. dr. A. Burdorf and prof. dr. M. Rees, both for accepting the invitation to be on the committee and for evaluating this thesis. To the members of the large doctoral committee, thank you very much for participating in my PhD defense. It is a great honor for me to have you as members of my doctoral committee.

Prof. dr. A. Burdorf thank you for your support, advice, referrals, teaching me how to reach out and seek further engagement. I am hoping that we can collaborate in the future.

My gratitude goes out to the participants in the Rotterdam Study on which part of this thesis is based, as well as my Erasmus colleagues who work in the research centers and on data management of the Rotterdam Study project. They provided the essential basis for my research.

I kindly thank all my coauthors (too many to mention you all by name) for your work and cooperation. I appreciate all your efforts and investments. Most of all, thanks to W.M. Bramer who collaborated with me on the systematic reviews. I am so grateful to you and I am looking forward to the possibility that we will cooperate more. Dr. R. Freak-Poli your enthusiasm, empathy, happiness, motivation, genuine support and friendship will always be in my heart. I am very happy that we are in contact and I will always remember how you kept me going (Afterword).

My collaboration with Prof. dr. H. Völzke and Prof. dr. H. Grabe from Greifswald Medical University in Germany made my PhD trajectory more interesting and fruitful. Mutual visits and presentations, meeting the team members of their departments, and learning about current projects brought us into a collaboration that makes me feel very enthusiastic. In this process I developed a new interest in the assessment and monitoring of the health of communities and populations at risk and I am looking forward to further collaboration. I am very grateful for all your support.

I was lucky to have team of great colleagues who edited and proofread my manuscripts who always provided constructive feedback: J. Troup, MPH, Erasmus MC; D. Mennie, combined BA program in Philosophy and Psychology, University of Lethbridge, Alberta, Canada; and., E. Louise Rosefire Goddard, from the units of childcare and development, Sir Gar college, Carmarthenshire, South West Wales.

Dr. M. Kavousi thank you for your creative idea that inspired me to design the layout of the thesis.

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My special appreciation goes to Erasmus senior researchers: dr. T. Voortman, dr. J.C. Kiefte-de Jong and dr. M. Ghanbari. Whenever I needed advice, help or just a friend, you were there. You were my role models and my motivation. Thank you all so much!

I express my special gratitude to Paranymphs S. Maas and C. Ochoa-Rosales. I am happy to have you by my side, hoping this friendship will last.

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All my colleagues from the Psychiatric-epidemiology and ErasmusAGE groups will always be part of my network. Thank you for the interesting meetings and exchanges of ideas and the good times we've had over the last few years. I will always cherish your friendship and support. Dr. K. Braun and Dr. C.M. Koolhaas, you have provided me with a reality check and you have been great collaborators in the last part of my final sprint towards defense. Thank you, Kim for organizing the group for thesis printing organizing everything for us, and for leading us through the administration and fund raising processes. Dear Chantal, your presentation at the 2020 meeting gave me the exact idea for what I wanted to emphasize in my presentation. I would like to thank our flawless social committee for

all the cookie breaks, gatherings, stories and laughs: S. Maas, A. Nhi Nguyen, C. Ochoa-Rosales, E. Aribas, H. Aliahmad – thank you for all your effort and good will!

Out of the many roommates I have had over the past several years, I would like to thank the ones I have spent the most time with: E. Loehrer Guicherit., Dr. L. Jaspers and L.Z. Rojas. My dear Elizabeth (greetings to all in the US), I am so happy to have had the chance to share an office and part of my life with you. I miss you a lot and think about you and your family every day! I was so happy that we had a reunion when you came to Rotterdam last year, and I hope we will meet again. Dear Loes, the experience we shared when you went through the thesis preparation and final defense helped me tremendously to understand better what is ahead of me. Dear Lyda, thank you for being my friend, my support, and my coauthor. The joy that Oscar and you brought to my life will always be highly appreciated. Thank you for introducing me to your lovely family, for all the gatherings, going to the cinema, dining, and all the nice things we did together. The same goes to Fadila and Amra, as we often spent time together. Arash, thank you for your kind help with graphs and formatting, for nice coffee brakes and your kindness. Dear Hoyen, I am happy for you in your new position and I hope you will finish your manuscripts and complete your thesis soon. I value our friendship and I think you are an amazing person with great interests and fabulous taste in movies and arts. Thank you all for the talks, cinemas, trips, music, dining, visits and quality time we spent together.

I will always be thankful to dr. M. van der Gaast, the colleague who provided all the help and support at my time of need, and without whom this thesis would not have been possible to complete. Dear Mark, I am most sincerely thankful. I cannot imagine anyone else being so considerate and kind and full of trust and empathy. You made me a better person by teaching me that, at the end of the day, only our actions can speak for us: "The proof of the pudding is in the eating". Also, you taught me not to panic by a simple rule: "Keep breathing".

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My honest gratitude to dr. P.H. Wismans and dr. B.J. Krenning for their kind help and experience with situations and states that accompany the completion of a PhD process.

My life and wellbeing, balance and strength were always under the supervision of Praktijk Buckley: dr. M. Buckley, dr. L. de Jong (I am tremendously grateful for all your care and attention), dr. M. de Groen, doktersassistente M. Adams - I am extremely grateful for everything. I will always cherish the support and understanding they provided, as well as specific bonds we developed. Also to FysioHolland – Schieweg, to physiotherapist and my personal trainer T. Verstelle. Luckily enough, this team of wonderful colleagues empowered me and remain my big support.

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Dear Ms M. Wiersma and Mr M.Blok, thank you for making this PhD possible.

At my home Institute of Public Health of Serbia, I am grateful to the Former director dr D. Ilic, acting director dr. V. Jovanovic, and the chief of our Department dr I. Ivanovic. I am thankful for all the understanding, support, and for following my progress.

Dear Director, dear dr. Verica, thank you for teaching me a valuable lesson when in a difficult moment in our phone conversation, you helped me regain self-control. I will never forget your vivid voice saying authoritatively: "Wake up and makeup, love and trust yourself, relax, get out there and do it! ". Also, I will never forget all your kind support, the correspondence and talks. This helped me to feel in touch with my home institution and colleagues.

Dear dr. Ivan, when I first came to our Center and expressed some doubts about finding the right place and fitting in, you gave me the most valuable lesson: "If you cannot make it here, why would you expect to make it anywhere else? Generally, people think it is about the setting. The art of survival does not depend upon the setting but on your own survival skills". I am tremendously thankful for all the lessons I learned and for the best chief of the center – ever!

To all my dear psychiatrist, psychotherapist, psychologist and social worker colleagues I am very grateful for the years of clinical cooperation that I am now trying to transfer to my research.

Dr. B. Farajzadegan, my dear roommate was of a great help and brought a lot of love to my life. Dear Ziba, you became the part of my family. We shared food, love, knowledge, life, we shared it all. I will never forget your kindness and hospitality. I am looking forward to seeing you!

I am afraid that I might have left many people out and I offer my sincere apologies, I hope you all know you are in my heart.

I would like to shortly reflect on the chain of events that brought me to this point.

Firstly, I would like to express my thankfulness to the Caltec Institute, Pasadena, U.S, for selecting me for neurobiology research program grant. Thanks Prof. Dr. M. B. Kennedy for giving me the opportunity to learn about gel electrophoresis (SDS-PAGE). Dear Marry in your lab I studied about biochemical signal transduction systems in central nervous system synapses. We focused on a complex of signaling proteins, called the postsynaptic density, located just underneath excitatory receptors in the central nervous system. This experience developed my further interest in memory and lately in rumination and cognitive decline which further inspired my PhD research.

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Looking forward to the future!

7.7

Afterword

Motivation letter

Written to the author by dr. Rosanne Freak-Poli,*

from Melbourne, Australia, August, 2015

Dear Jelena,

I think about you a lot and I hope I will mediate your reflections.

In this letter I would like to address to specific issues regarding PhD studies, as it might be helpful to consider options for progress in one's trajectory.

Personally, I believe that I have a good relationship and collaboration with my supervisors and that this is essential. Of course, we sometimes have different points of view, but these mainly stem from having different background knowledge and once we discuss things we come to a resolution on how to proceed.

Unfortunately having difference in mind between the colleagues and supervisors is part of work! I wish it were not the case, but it is very common and causes much stress.

I try very hard not to let personal differences affect me as usually there is nothing I can do differently. Sometimes getting upset "adds fuel to the fire" (provokes the creativity in the situation). When people see that someone is upset, they can get defensive and it makes the situation worse.

My advice to all is not to worry about your relationship with potential co-authors. However, as mentioned before, your relationship with your supervisor(s) is important.

First, it is important to clarify the situation when starting the project. Perhaps making a suggestion, not a definitive decision on a proposal is a good idea? Initial results may change the course. That would take some time and then the situation may be different, and more beneficial?

Do not take in account anything expressed verbally (if there is nothing on paper). It would always help to get your plan of projects and progress clarified on paper and see exactly what is suggested. That way you would know the specifics of your mentors expectations?

In Australia it is common to have a first and second supervisor, and these can change during the PhD duration. I know that the rules have recently changed at Erasmus, and this is a great opportunity leading to favorable outcomes.

Regarding topic, it is common for supervisors to only be interested in papers that interest them. That is actually the best approach to getting them completed. They are less likely to help if they are not interested in the topic of the paper.

Therefore, should you have a particular field of interest you would like to research, and none of the mentors have exact click, take initiative, take the lead and perform hard work. Mentors would not oppose, but they would by rule of thumb, get more reluctant and less helpful in the process of the research.

At some moments , many wonder about the investments, pros and cons, and overall cost-benefits of doing the PhD.

It makes me feel sad to see that these thoughts are common among the students.

In case you are in doubt, you are very much worthy of doing a PhD - I do not think that is in question in my mind! I think that you are a hard worker and P in PhD is for persistence, h is for hard work and D is for determination!

What I also know is that PhDs can take a very, VERY long time. I have met highly intelligent people who have spent 10 years completing their PhDs for various reasons. What you need to think about is whether you can spend 3 years, 5 years, 8 years, etc. working on it? Is it worth it?

After you complete the first paper, it should go faster - but maybe it won't. PhD also often overlaps with time to create a family, especially if one is at the start or half way of the trajectory a bit later in the career.

Perhaps private-family life is more important at the moment than a career? Perhaps, all can be combined with good will, relations that provide support and teamwork.

These decisions are life changing and cannot be taken lightly. No one will question your decision either way - and I definitely hope that no one questions me taking a year off for maternity leave!

I am really sorry that you are struggling with introspection. This is only the sign of intelligence. Unfortunately you can't change other people but you can change how you deal with this situation. I trust in you!

I am sending love.

Rosanne

* *Dr. Rosanne Freak Poli is researcher in active ageing focusing on the relation between physical health and psychological health. She completed her undergraduate studies at the University of Adelaide with majors in Statistics, Psychology and Public Health. Rosanne's PhD in Epidemiology, undertaken at Monash University, evaluated a workplace physical activity health program. She investigates the relationship between happiness and health. The first stage of her research was undertaken at Erasmus Medical Centre in The Netherlands utilizing The Rotterdam Study. Rosanne continues to use her longitudinal statistical analytical skills to investigate aspects of active ageing in the Australian population. Rosanne's key interests in active ageing include: social isolation, loneliness and depression, well-being and resilience and loss of independence. Rosanne is affiliated to Erasmus Mc and open to collaborations.*

"Everything will turn out right, the world is built on that."

Mikhail Bulgakov