Oxidatively generated DNA/RNA damage in psychological stress states

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1. BACKGROUND

1.1. INTRODUCTION

Both mental disorders and non-pathological psychological stress states are associated with molecular, cellular and clinical signs of accelerated aging. These include telomere shortening, atrophic changes in the brain, and an increased prevalence of age-related somatic diseases [1-3]. Oxidative stress on nucleic acids is a critical component of cellular and organismal aging [4], a regulator of telomere length [5], and a suggested pathogenic mechanism in age-related somatic disorders [6,7].

Based on these observations, the oxidatively generated damage to nucleic acids, which is the focal point of this thesis, could constitute a molecular-level link between psychological stress and negative somatic influences. In the present studies, we aimed to investigate the relation between psychological stress states, stress hormone secretion, and the urinary excretion of specific markers of systemic DNA/RNA damage from oxidation, 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) and 8-oxo-7,8-dihydroguanosine (8-oxoGuo), respectively. This overall framework was applied in studies of healthy, elderly humans, of patients suffering from a severe mental disorder (schizophrenia), and in an animal model of prolonged psychological stress.

1.2. BASIC CONCEPTS OF PSYCHOLOGICAL STRESS AND THE PHYSIOLOGICAL STRESS RESPONSE

There is no general consensus on the definition of psychological stress. For the purpose of this thesis, psychological stress refers to a subjective state elicited by the perception of threat, damage or overwhelming environmental demands, which subsequently lead to a physiological stress response, executed by stress “mediators” [1,8]. A stress mediator could, for example, be the peripheral release of a stress hormone such as cortisol [9]. The adaptive condition elicited by the stress response is often referred to as allostasis, and the damaging, maladaptive effects of prolonged, uninterrupted allostasis as allostatic load [10]. When the context allows it, “stress” and “psychological stress” will be used interchangeably.

The brain regions primarily involved in forming the psychological experience of stress, and in the launch of a stress response, are the hippocampus, critically involved in learning and memory, the amygdala, involved in fear responses and arousal, as well as the medial prefrontal cortex, involved in cognition and fear extinction. From these interconnected regions, projections to hypothalamic and brain stem nuclei mediates the activation of the two major hormonal effector systems of the stress response: The hypothalamic-pituitary-adrenal (HPA)-axis and the sympathetic-adrenomedullary system, respectively [8].

Activation of the HPA-axis results in the peripheral release of cortisol. Upon stimulation of neurons in the paraventricular nucleus of the hypothalamus, corticotropin-releasing hormone is released and acts on the anterior pituitary to secrete adrenocorticotropic hormone (ACTH) [11]. ACTH is released to the blood stream, and stimulates the adrenal cortex to secrete cortisol (or, in rodents, corticosterone). Through the binding to glucocorticoid

receptors (GR), cortisol exerts a negative feedback on HPA-axis activity, thus playing a central role in shutting down the stress response when the stressor is no longer present. This GR mediated feed-back inhibition occurs at both the cortical, hippocampal, hypothalamic and pituitary level [11].

Cortisol exerts its effects in virtually every organ of the body. The overall peripheral effects of cortisol are energy mobilization and conservation through increases in gluconeogenesis, inhibition of muscle and fat glucose uptake, and inhibition of the immune system. In the central nervous system, cortisol participates in the regulation of mood, sleep and cognition [12].

Age, gender, genetic predisposition and environmental context all have an influence on the neurohormonal stress processing, and thus these factors contribute to the large interindividual variation in stress sensitivity and resilience [13]. Furthermore, it is important to emphasize that while some human stress states such as depression are associated with increased HPA-axis activity [14], others, such as the post-traumatic stress syndrome, are associated with hypocortisolism [15].

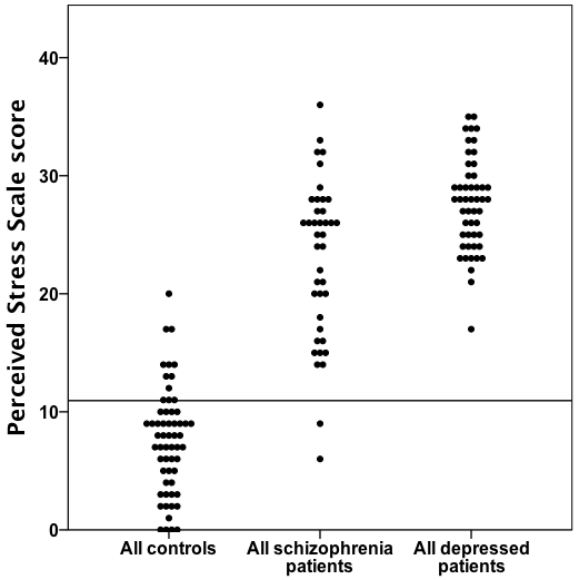
1.3. EVIDENCE FOR ACCELERATED AGING IN PSYCHOLOGICAL STRESS AND MENTAL DISORDERS

1.3.1. Somatic morbidity and mortality

Suffering from a mental illness is a highly stressful state (Figure 1) [16-19]. Both non-pathological psychological stress and mental disorders such as schizophrenia and affective disorders have been identified as a risk factor for several types of somatic morbidity. These tend to be diseases of aging, such as cardiovascular disorders [20], type-2-diabetes [21,22], and Alzheimer's disease [23,24]. Chronic elevations of cortisol are suspected to be a central mediator of these negative health effects [25,26]. With respect to cancer, which has an intimate relationship with the molecular biology of aging [7], stress per se does not seem to influence cancer incidence [27]. Correspondingly, the evidence for an influence of both schizophrenia [28,29] and depression [30,31] on cancer risk and mortality has been inconsistent. Collectively, the evidence suggests a susceptibility for metabolic and neurodegenerative disease, rather than malignant disorders, in human stress states and mental disorders.

While there is insufficient evidence to conclude that stress increases mortality in humans, some prospective epidemiological studies have indicated that baseline stress markers such as high levels of subjective stress or the loss of a child are associated with excess natural-cause mortality [32,33]. It has repeatedly been observed that both schizophrenia and affective disorders are associated with a severely elevated prevalence of - and mortality from – somatic disorders such as cardiovascular disease and type 2 diabetes [22,34-37]. Life expectancy for people with serious mental disorders is reduced with as much as 15-20 years, which is mainly due to death from natural causes, i.e. not a result of suicides [38]. Lifestyle, diet, inadequate somatic care and side effects of psychotropics may all partly account for these observations. However, given that stress in itself has a negative influence on somatic health, it is also possible that the prolonged stress state of being mentally ill contributes to these poor health outcomes.

FIGURE 1:



Perceived Stress Scale (PSS) scores in 68 healthy controls, 40 schizophrenia patients (Total PANSS score 64-124 points) and 55 depressed patients (HDRS score 14-35 points) who filled out the PSS during the PhD course. 28 healthy controls and 26 moderately depressed patients from a study conducted by Jesper Krogh, M.D. at Bispebjerg Hospital are included (used with permission). The horizontal line indicates the mean score of the general Danish population, as obtained in the study of Nielsen et al (2008). All groups are significantly different from each other (P<0.001, one-way ANOVA with post-hoc Tukey test).

1.3.2. Stress effects in the brain

Early evidence suggested that fatal stress in primates caused neuronal death in the CA3 area of the hippocampus [39]. However, wide-spread cell death is not observed in human stress states such as depression, or in animal models of psychological stress [40]. Rather, stress is associated with a potentially reversible remodeling of hippocampal dendrites that leads to a reduced branching of the dendritic tree, and thereby a reduction of the total number of synapses. [41,42]. Similar remodeling occurs in the prefrontal cortex, where it was recently found that advancing age negatively affect the neuronal ability to recover from stress-induced morphological changes [43]. The hippocampal volume reduction seen after chronic stress has been observed in many psychiatric disorders, including depression and schizophrenia [44].

1.3.3. Telomere shortening

The increased age-related morbidity and atrophic brain changes observed in psychological stress and mental disorders have lead to the hypothesis that stress may accelerate inherent cellular aging processes [45]. Telomeres are nucleotide repeats that cap chromosomal ends, thereby protecting the end of the DNA strand from being recognized as a strand break. In most cells, telomeres shorten with every cell division, and this attrition may be causally involved in organismal aging [46,47]. Oxidative stress is a key determinant of telomere length and stability [5,48-50].

In an influential study from 2004, Epel and coworkers measured telomere length in circulating mononuclear cells of mothers giving care to seriously ill children (N = 39) and a control group of mothers with healthy children (N = 19) [2]. Although telomere

length did not differ between the groups, there was a negative correlation between telomere length and both the chronicity of caregiving and perceived stress levels. Furthermore, perceived stress was positively correlated with an index of oxidative stress. Subsequently, telomere length has been found to be reduced after early social deprivation [51], in mood disorders [52], and in schizophrenia [53].

1.4. OXIDATIVELY GENERATED NUCLEIC ACID DAMAGE IN AGING, PSYCHOLOGICAL STRESS AND IN SCHIZOPHRENIA

1.4.1. Basic biochemistry of oxidative stress

During mitochondrial respiration, Reactive Oxygen Species (ROS) are formed due to the leakage of electrons from the electron transport chain, which may reduce oxygen to form superoxide ($O_2^{\bullet-}$). Superoxide is subsequently converted into other ROS, including hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\bullet}OH$). The mitochondria are the main endogenous source of ROS, and perturbations in mitochondrial function may lead to increased ROS formation [54].

The intracellular redox balance plays a key role in cellular homeostasis, e.g. by regulating signal transduction pathways, some of which bear relevance to aging by their involvement in cell cycle or apoptosis, such as p53 [55,56]. However, ROS also have the potential to directly damage a variety of redox-sensitive molecules, including lipids, proteins and DNA/RNA. To avoid the toxicity of ROS, a wide range of antioxidant mechanisms are present in all aerobic organisms. Oxidative stress is defined as a state in which the production of ROS exceeds the antioxidant potential of a given biological system [57].

1.4.2. Oxidatively generated nucleic acid damage in aging

Several independent lines of evidence support a central role of oxidatively generated nucleic acid damage in aging. Firstly, DNA damage from oxidation accumulates with age, in particular in tissues with limited cell proliferation, such as the brain [58]. A reduced ability to repair DNA damage from oxidation or other insults leads to a progeric phenotype in both animals [59] and humans [60]. Down-stream effects of genomic damage include cell senescence and programmed cell death (apoptosis) mediated by the activation of signaling factors such as ATM, p53, bcl-2 and the caspase enzyme families. These events are activated by oxidatively generated damage to DNA, as well as other genotoxic insults such as telomere shortening, and are thought to underlie the lack of tissue renewal and loss of function inherent to aging [6,46,56].

Recently, an increasing attention towards the neurodegenerative and age-advancing potential of RNA oxidation has emerged. RNA is single-stranded, not protected by specific proteins, and located in the proximity of the mitochondria. Thereby, RNA may be at higher risk of being oxidatively modified than DNA [61]. The oxidation of mRNA may cause translation errors and reduced protein synthesis [62,63], and the oxidation of non-coding RNA species could affect the regulation of protein translation, gene expression, and neuronal synaptic plasticity [64].

Oxidative stress has been implicated as a pathogenetic mechanism in several diseases of aging, including atherosclerosis and hypertension [65,66], type 2 diabetes and late diabetic complications [67], as well as neurodegenerative disorders such as Alzheimer's disease [68-73]. Furthermore, genotoxic stress from oxidation is thought to play a central role in carcinogenesis, in that the continuously generated oxidative modifications of DNA, including 8-oxodG, have a mutagenic potential [6]. A number of lifestyle and demographical factors are associated with increased oxidative stress, some of which are known age-advancing factors. These include smoking [74,75], obesity [76] and male gender [77].

1.4.3. Oxidatively generated nucleic acid damage induced by stress hormones and psychological stress

Both in vitro and in vivo studies indicate that prolonged elevations of glucocorticoids and restraint stress may negatively influence mitochondrial function [78-80]. Furthermore, short-term in vitro exposure of murine fibroblasts to both cortisol and catecholamines were found to increase DNA strand breaks, decrease total DNA repair capacity, and regulate a number of genes involved in DNA damage signaling pathways [81]. Graded corticosterone treatment dose-dependently increased the levels of 8-oxodG in mitochondrial DNA in the liver of rats [82].

In two early studies, a significant increase of 8-oxodG in nuclear DNA was found after acute stress in the liver and the frontal cortex, respectively [83,84]. More recently, increased DNA strand breaks were found in the frontal cortex, amygdala and hippocampus after acute restraint stress, and in the hippocampus after subchronic (7 days) stress exposure [85].

A few studies have attempted to estimate oxidatively generated DNA damage in non-pathological human stress states. Irie et al measured leukocyte levels of 8-oxodG in 54 healthy Japanese workers, and found a positive correlation to perceived workload and psychological stress among the female participants [86]. Similarly, Inoue et al found that certain high stress job models correlated to the excretion of urinary 8-oxodG [87]. In a study of healthy medical students, an increase in blood cell nucleotide excision repair capacity was observed during the exam period [88].

1.4.4. Oxidatively generated nucleic acid damage in schizophrenia

Oxidative stress has been implicated as a potential pathogenic mechanism in a range of psychiatric disorders [89]. Schizophrenia is a severe mental illness of unknown etiology, with symptoms including disturbances of thought, self-experience and emotion, as well as hallucinations and delusions [90]. In schizophrenia, evidence for peripheral and cerebral mitochondrial dysfunction [91-93], decreases in antioxidant defenses [94-96] and increases in a range of oxidative stress markers [97-99] have been reported. Furthermore, some evidence suggests a beneficial effect on psychopathology of antioxidant therapy (e.g. vitamin C or the glutathione precursor n-acetyl cysteine) [100,101].

Only a few studies have addressed the possibility of genotoxic stress from oxidation in schizophrenia. Psimadas and coworkers measured lymphocyte levels of DNA damage, as measured by the comet assay, and repair efficiency after challenge in 20 medicated schizophrenia patients and 20 healthy controls. DNA damage did not differ at baseline. After treatment with increasing doses of either hydrogen peroxide or gamma irradiation, similar levels of DNA damage were observed both immediately after treatment and 2h post-treatment, thus indicating a similar ability to repair DNA [102]. Likewise, Young and coworkers found no difference in lymphocyte DNA damage in 16 medicated schizophrenia patients

and 17 controls, neither at baseline nor after a hydrogen peroxide challenge [103].

In a post-mortem study, Nishioka and Arnold used immunostaining for 8-oxodG in the hippocampus of elderly patients with poor-outcome schizophrenia and non-psychiatric controls. There was a tenfold increase in 8-oxodG positive neurons in patients, and a similar increase in the cell cycle activation marker Ki-67. The two markers were correlated in the schizophrenia group. There were no neuropathological signs of Alzheimer's disease (neurofibrillary tangles or amyloid beta plaques) [104]. In a more recent study, Che and coworkers used a monoclonal antibody that detects the 8-oxoguanine lesion in both DNA and RNA for staining of the dentate gyrus, CA1 and CA3 areas of the hippocampus in controls and patients suffering from major depression, bipolar disorder and schizophrenia. All patients had increased antibody binding, with the schizophrenia group showing the highest levels in all regions. Staining was predominant in the cytoplasm compared to the nucleus, indicating a relatively higher amount of oxidatively damaged RNA than DNA [105].

A range of studies have examined the effect of antipsychotic medication on oxidative stress markers, although none have used nucleic acid oxidation markers. The evidence suggest a difference between first vs. second generation antipsychotics, in that first generation antipsychotics appear to be substantially more prone to induce cerebral and peripheral oxidative stress in animals and humans [106,107], perhaps in association to the occurrence of extrapyramidal side-effects [108].

1.5. URINARY 8-OXO-7,8-DIHYDRO-2'-DEOXYGUANOSINE AND 8-OXO-7,8-DIHYDROGUANOSINE AS MARKERS OF SYSTEMIC OXIDATIVELY GENERATED DAMAGE TO DNA AND RNA

Urinary 8-oxodG is a widely used biomarker of oxidative stress. A number of experiments support the notion that urinary 8-oxodG is in fact a marker of systemic oxidative stress on DNA. The induction of wide-spread DNA damage by the exogenous carcinogen 2-nitropropane lead to the transient accumulation of 8-oxodG in nuclear DNA of the bone marrow, liver and kidneys, and a corresponding increase in urinary 8-oxodG excretion [109]. Furthermore, a dose of venously injected 8-oxodG was completely recovered in urine within 4 hours in pigs [110]. Although similar experimental data on 8-oxoGuo are not available, the two markers are usually closely correlated, and thus probably reflect the same whole-body oxidative stress on nucleic acids.

It is widely agreed that urinary 8-oxodG stems from enzymatic repair mechanisms, because the rate of which it is generated would otherwise result in a cellular accumulation of damaged nucleotides incompatible with life, and because the contribution from other sources such as diet or cell turnover is negligible. Furthermore, 8-oxodG is not generated from spurious oxidation in urine [111]. However, it is presently unknown which repair mechanism is primarily responsible for the release of free 8-oxodG. The base excision repair (BER) enzyme 8-oxoguanine DNA glycosylase (OGG1) excises only the nucleobase, yielding an abasic site with an intact deoxyribose backbone, and the free compound generated from this reaction will consequently be 8-oxoguanine, and not 8-oxodG [112]. Other suggested mechanisms are the nucleotide excision repair pathway or the action of the Nudix (nucleoside diphosphate linked moiety X)-type motif 1 (NUDT1) enzyme, which sanitizes the nucleotide pool for oxidatively damaged nucleotides, thus preventing their incorporation into DNA [111].

8-oxo-7,8-dihydroguanosine (8-oxoGuo) is the ribonucleoside analogue of 8-oxodG, and is therefore considered a marker of RNA oxidation [113]. The background for the release of 8-oxoGuo is even less well characterized than 8-oxodG, and it may be due to both specific degradation or active repair of oxidatively damaged RNA [114]. In steady state, it is argued that the formed oxidative modifications of nucleic acids will be continuously repaired following first-order kinetics, and thus the urinary excretion of 8-oxodG/8-oxoGuo reflects the whole-body oxidative stress on nucleic acids, rather than changes in repair activity [113,115]. The intra-individual 8-oxodG excretion has a negligible diurnal [116] and day-to-day variation [117,118].

It is not possible to determine the anatomical origin of 8-oxodG/8-oxoGuo from the urinary excretion of the markers. In humans, the brain consumes 20% of the oxygen, but only constitutes 2% of the total body weight. Furthermore, the brain has relatively low antioxidant defenses [119]. Finally, the urinary excretion of 8-oxodG is increased in neurodegenerative diseases [70,72] and after experimental induction of cerebral oxidative damage [120], strongly suggesting that 8-oxodG crosses the blood-brain barrier. Hence, from a theoretical point of view, the brain would be expected to contribute substantially to the urinary levels of 8-oxodG/8-oxoGuo.

2. AIM AND HYPOTHESES

2.1. OVERALL AIM AND HYPOTHESIS

The overall aim of the PhD project was to investigate the relation between psychopathology, psychological stress, cortisol secretion and oxidatively generated DNA and RNA damage. The main hypothesis was that psychological stress states are associated with increased DNA/RNA damage from oxidation, as measured by the urinary excretion of markers of systemic DNA/RNA oxidation (8-oxodG and 8-oxoGuo, respectively). This hypothesis was tested within the context of a major class of psychiatric morbidity, namely schizophrenia, and in an animal model of prolonged psychological stress. Finally, the association between the 24 hour urinary excretion of cortisol and 8-oxodG/8-oxoGuo was investigated in a cohort of elderly, healthy individuals.

2.2. SPECIFIC HYPOTHESES

Paper I

- 24h urinary excretion of cortisol is positively correlated to the excretion of 8-oxodG/8-oxoGuo measured in the same samples from a cohort of elderly, mostly healthy individuals.

Paper II

- Schizophrenia patients excrete higher amounts of 8-oxodG/8-oxoGuo than healthy controls.
- The excretion of 8-oxodG/8-oxoGuo is positively correlated to levels of perceived stress and measures of cortisol secretion in patients and controls.

Paper III

- Chronic restraint stress in rats increases urinary corticosterone and 8-oxodG/8-oxoGuo, as well as DNA damage from oxidation in the hippocampus and frontal cortex.

- Chronic restraint stress causes an induction of cerebral DNA repair enzymes.
- Cerebral levels of DNA damage from oxidation are positively correlated to the urinary 8-oxodG/8-oxoGuo excretion.

3. METHODS

3.1. STUDY DESIGN, RECRUITMENT AND PRACTICAL PROCEDURES IN THE HUMAN STUDIES

3.1.1. InChianti study (Paper I)

InChianti (Invechiarre in Chianti, Aging in the Chianti Region) is an epidemiological study of approximately 1500 randomly selected citizens of >65 years of age from two small towns in Tuscany, Italy, included from 1998 through 2000. The primary aim of the study was to determine risk factors for the declining ability to walk in late life [121]. As a part of the original study, participants made a 24h urine collection, and urinary cortisol was assayed. Researchers at the Department of Clinical Pharmacology, Copenhagen, analyzed a subsample of the participants (N=220) for the content of 8-oxodG/8-oxoGuo in the same urinary samples, in order to study the association between low-grade inflammation and oxidative stress on nucleic acids. The 220 participants were selected from the upper and lower tertiles of selected inflammation markers (CRP and IL-6). The two groups did not differ in 8-oxodG/8-oxoGuo excretion, as described elsewhere [122]. We used the same subsample to cross-sectionally investigate the relation between 24h cortisol and 8-oxodG/8-oxoGuo excretion. The potential pitfalls of this approach are discussed in detail in the paper.

3.1.2. Schizophrenia study (Paper II)

The schizophrenia study was the primary study of the PhD course. The design was an observational, cross-sectional study of patients vs. healthy controls, with an additional follow-up investigation of the patients after 4 months. Patients were recruited consecutively from September 2008 through April 2011 by referral from doctors at the Psychiatric Centre Copenhagen, as well as by active screening for potential participants at the wards and outpatient clinics. The staff at Psychiatric Centre Copenhagen was informed about the project and received regular reminders about referral. AJ performed all the clinical ratings of patients, biological sampling and post-sample handling, as described in Paper II. Other research assistants at the centre performed the screening of the healthy controls for lifetime psychiatric morbidity (see below for details).

AJ and other research assistants recruited the healthy controls from the blood donation corps at Rigshospitalet. They lived in roughly the same urban area as the patients and were group-matched for age and gender, but differed on a number of lifestyle and metabolic variables. The control group was also used for two other research projects at the centre. The specific in- and exclusion criteria, as well as all the baseline characteristics, for patients and controls are presented in the paper.

3.2. RATINGS AND QUESTIONNAIRES (PAPER II)

3.2.1. Diagnosis and psychopathology

The diagnosis of schizophrenia was obtained by the comprehensive structured diagnostic interview Schedules of Clinical Assessment in Neuropsychiatry (SCAN) [123]. SCAN was also used to screen the healthy controls for present or lifetime psychiatric morbidity. The interview covers a wide and detailed range of psychiatric symptoms. Screening questions are used to identify the psychopathological domains affected. We used the computerized version of SCAN, where a software algorithm providing fulfilled ICD-10 or DSM-IV diagnoses is available. Formal training and joint SCAN ratings with other research assistants were performed before and during the first year of the study. If the referring physician considered the diagnosis of schizophrenia at referral to be uncertain, the SCAN-interview was made on a separate day to allow for a more thorough psychopathological assessment.

The severity of psychopathology was measured by the Positive and Negative Syndrome Scale (PANSS) [124]. PANSS is a widely used scale based on a semi-structured interview. It was chosen for its level of detail and its distinction between positive (e.g. delusions and hallucinations), negative (e.g. social withdrawal and poor emotional contact), and general (e.g. anxiety, depression) symptomatology. The scale contains 30 items that are rated from 1-7 points, yielding a score range of 30-210 points. Formal training and joint ratings with an expert PANSS rater (AFJ) were performed throughout the duration of the study. The intraclass correlation coefficient between AJ and AFJ was 0.91 for positive items, 0.76 for negative items, 0.79 for general items and 0.83 for all items, indicating very good agreement across all subscales.

3.2.2. Perceived psychological stress

For the assessment of subjective psychological stress, we chose the Perceived Stress Scale, which was also used in the telomere study by Epel et al [2]. The scale has been extensively validated in non-psychiatric populations [125,126], and scores for the general Danish population are available [127]. In 10 simple questions, the scale covers feelings of loss of control, being overwhelmed, irritability, nervousness etc. Thereby, the scale addresses core features of psychological stress. The answers are translated into scores of 0-4, yielding a range of 0-40 points.

For the present study, we made a slightly modified translation of the existing Danish version in order to reduce the amount of figurative language, which is unsuitable in psychiatric patients who may suffer from thought disturbances with concrete interpretations. The author of the scale Dr. Sheldon Cohen (Carnegie Mellon University, USA) approved the re-translation into English of the revised version. The usual timeframe of the scale is 4 weeks, but we chose to limit the period to 2 weeks, in order to better capture acute alterations in perceived stress. The variation of PSS scores in healthy controls and psychiatric patients who filled out the questionnaire during the PhD course is presented in Figure 1.

3.3. CHRONIC RESTRAINT STRESS (CRS) IN METABOLISM CAGES (PAPER III)

CRS is one of the most frequently applied animal models of chronic stress. It reliably generates a range of the biological hallmarks of stress: Increased glucocorticoid output and relative adrenal gland weight, reduced expression of the cerebral glucocorticoid

receptor, as well as dendritic retractions in both the prefrontal cortex and the hippocampus. Furthermore, the model is associated with depressive behavioral changes (as measured by e.g. the Forced Swim Test). These effects are reversible with antidepressant treatment such as selective serotonin reuptake inhibitors and electroconvulsive stimulations [42,128-130].

In order to reliably collect urine for 8-oxodG/8-oxoGuo detection, we performed the full 3-week restraint stress paradigm in metabolism cages (MC). 24 Sprague-Dawley rats were matched by weight to either MC + stress (MCS) or MC + handling/weighing (MC controls, MCC). To our knowledge, CRS has not previously been performed in MC. In MC, the animals are housed singly and no forms of enrichment other than a small shelter are available. The cages have a grid floor to allow for the passage of excretions into the collection system. Due to concerns that this form of housing would be stressful in it self and thereby interfere with the validity of the model, we included a second control group (N = 12), which was housed in standard cages with a littermate (Group Cage Controls, GCC). These control animals were handled and weighed daily.

The urinary excretion of corticosterone was measured in all 24h urinary samples, whereas the content of 8-oxodG/8-oxoGuo was determined on day -3 (the first day in the MC), and day 2, 8 and 21 after stress. After sacrifice, frontal cortex and hippocampal levels of DNA damage, expression of selected DNA repair enzymes and inflammation markers were determined (see below).

3.4. URINARY CONTENT OF 8-OXODG/8-OXOGUO BY ULTRAPERFORMANCE LIQUID CHROMATOGRAPHY WITH TANDEM MASS SPECTROMETRIC DETECTION (UPLC-MS/MS)

The determination of the urinary content of oxidized nucleosides is challenging due to the biochemically heterogeneous composition of urine and the low concentrations of the nucleosides, making the demands for both specificity and sensitivity very high. Two major approaches have been applied: An immunobased technique using commercially available antibodies (ELISA), and gas- or liquid chromatographic separation with subsequent electrochemical or mass spectrometric detection. The chromatographic methods have been validated in an international collaboration [131], and are superior to ELISA in linearity and accuracy [132].

The UPLC-MS/MS is a highly specific and sensitive technique. The method established at the Department of Clinical Pharmacology in Copenhagen has a lower detection limit for urinary 8-oxodG/8-oxoGuo of 1 nM. The method involves an automated sample handling, allowing for the fast processing of large numbers of samples. After a brief centrifugation of the urine, the samples are mixed with a buffer containing isotope-labeled internal standards of 8-oxodG/8-oxoGuo. The otherwise unprocessed samples are injected into the UPLC column. After chromatographic separation, the eluent fraction containing the analytes is injected into the mass spectrometer. Here, the analytes are ionized, and after a further selection of 8-oxodG/8-oxoGuo, the nucleosides are split into two ion fragments in a collision chamber. The “quantifier” ion is used to quantify the concentration of the analyte as the ratio to the corresponding internal standard. The concomitant determination of the “qualifier” ion serves to control for the absence of interfering molecules, and can be used for back-up quantification in the case of interference [113].

In the InChianti and CRS studies, 24h urine samples were available. Due to the difficulty of obtaining 24h urine samples from psychiatric patients, spot urine samples were used, and 8-oxodG/8-oxoGuo were normalized to urinary creatinine levels. This procedure is widely applied, and reference values for healthy individuals have been obtained [133].

3.5. OXIDATIVELY GENERATED DNA DAMAGE IN BRAIN TISSUE BY THE SINGLE CELL GEL ELECTROPHORESIS (COMET) ASSAY

When measuring cellular levels of DNA damage, the major concern is the spurious oxidation of the non-oxidized nucleotides, which are present in much higher numbers than the oxidized lesions, and which will readily become oxidized under the process of isolating the DNA. The comet assay provides an indirect method for the detection of DNA oxidation products, where less purification steps are needed. In general, the comet assay detects lower levels of DNA oxidation than HPLC-based techniques. Single cells are isolated from the tissue, placed in a agarose gel and lysed. Under electropheresis, the DNA migrates through the gel and generates a “comet”, which differs in size as a reflection of the amount of strand breaks in the DNA. After staining, 100 randomly selected nuclei are scored by a standardized visual rating system (other quantification principles also exist). When additionally pre-treating the cells with repair enzymes that break DNA at specific oxidatively damaged sites, additional strand breaks will be formed. By subtracting the comet score of untreated cells from the score of treated cells, a measure of the levels of different oxidative modifications (i.e. corresponding to the specific repair activity of the enzyme) is obtained. A calibration curve individualized to the investigator is generated from reference samples with known levels of lesions per 106 base pairs, allowing for a translation of the comet score into lesions per 106 base pairs [134].

3.6. GENE EXPRESSION BY REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR)

RT-PCR is a widely used methodology for the measurement of gene expression. The method is based on the amplification and quantification of gene products as they accumulate in real time during the polymerase chain reaction. After the isolation of RNA and DNAse treatment, cDNA is synthesized by the reverse transcriptase enzyme. The cDNA is amplified by incubating a polymerase, gene-specific primers and a probe containing a fluorescent reporter and a quencher dye. Through the exonuclease activity of the Taq DNA polymerase enzyme, the reporter dye is released from the quencher dye, thus allowing emission of fluorescence that can be continuously detected during the reaction. A threshold for the detection of fluorescence is set slightly above the background (noise) level. The number of PCR cycles that a given gene product needs to enter exponential amplification and exceed the threshold is termed the Ct value. The lower the Ct value, the more copies of the gene are present in the sample. Quantification can either be performed by calculating the exact number of gene copies using a standard curve (absolute quantification), or by calculating the difference between the Ct values for the gene of interest and a housekeeping gene (relative quantification). Because we were interested in relative changes in gene expression across experimental groups and tissue samples, rather than absolute copy numbers, the latter method was applied.
The comet assay and RT-PCR were used in Paper III. Lab technicians at the Institute of Public Health, University of Copenhagen, Denmark, performed the analyses.

3.7. CORTISOL AND CORTICOSTERONE

Details of the collection procedures for plasma, saliva and urine samples for the detection of cortisol/corticosterone are described in the individual papers. Cortisol in plasma and saliva was analyzed by an electrochemiluminescence immunoassay at the Department of Clinical Biochemistry, Rigshospitalet, Denmark, as described in Paper II. The method is used for the day-to-day analysis of cortisol at Righospitalet, and is under continuous quality control. Corticosterone was analyzed by a commercially available ELISA kit, performed by a lab technician at the Department of Experimental Medicine, Faculty of Health Sciences, University of Copenhagen, Denmark.

3.8. POWER CALCULATION

A power calculation was done for the schizophrenia study. At a difference of 10% in 8-oxodG between patients and controls, an estimated variation of 13%, a two-sided $\alpha = 0.05$ and power $(1-\beta) = 0.90$, N = 36, i.e. there would need to be included at least 36 patients and 36 controls. In the final study, 40 healthy controls and 40 schizophrenia patients were included. Thirty-two of the patients completed the study at follow-up.

3.9. ETHICS

All studies and procedures were approved by the relevant authorities: The Italian National Institute of Research and Care on Aging Ethical Committee, the Regional Committee on Research Ethics (Capital Region of Denmark), the Danish National Data Protection Agency and the Danish Animal Experiments Inspectorate.

4. RESULTS

4.1. PAPER I

The main finding of the InChianti study was a positive correlation between urinary 24h excretion of cortisol and both 8-oxodG and 8-oxoGuo excretion measured in the same samples ($R2 = 0.15$, $P < 0.001$ for both markers). This association was robust for the adjustment for a range of confounders of oxidative stress in multiple regression analysis (age, sex, BMI, serum ferritin, blood glucose, insulin, inflammation status (± low-grade inflammation), smoking status and previous diagnosis of cancer). The difference in 8-oxodG and 8-oxoGuo excretion between the lower and the upper cortisol excretion quartile were 57% and 61%, respectively. All analyses were run with and without participants with a previous diagnosis of cancer, which did not affect the results.

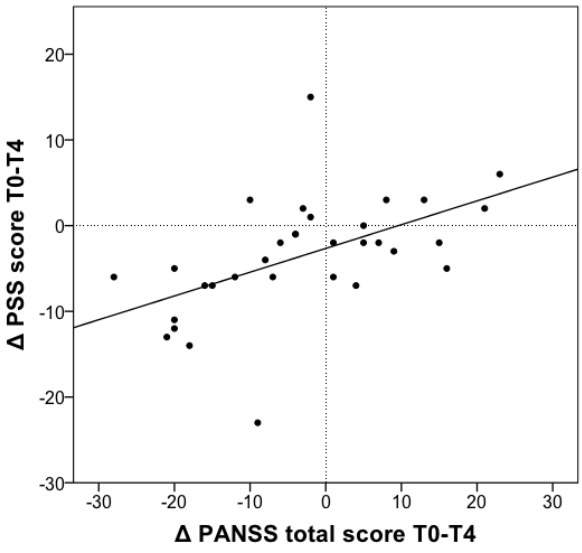
4.2. PAPER II

In support of our primary hypothesis, the main finding of the schizophrenia study was a 20% increase in the median urinary excretion of both 8-oxodG and 8-oxoGuo in schizophrenia patients vs. healthy controls ($P = 0.003$ and $P < 0.001$, respectively). This difference persisted after a comprehensive adjustment for multiple demographic, lifestyle, and metabolic variables differing between the groups, as well as two plasma antioxidants (vitamin C and uric acid) in multiple regression analysis. Furthermore, the marker excretion did not correlate to any measure of medication load (number of antipsychotics used, the WHO Defined Daily Dose of antipsychotics, or the Defined Daily Dose of all medications). The 8-oxodG and 8-oxoGuo excretion at 4-month follow-up continued to be elevated compared to the baseline levels of the healthy controls. There was an indication of a specific susceptibility to nucleic acid oxidation in schizophrenia, in that a plasma marker of lipid peroxidation, malondialdehyde, did not differ between the groups.

In contrast to our secondary hypothesis, 8-oxodG/8-oxoGuo excretion was not correlated to perceived stress or any of the two measures of cortisol secretion (9AM fasting plasma cortisol and the area under the curve for day-time salivary cortisol output) in schizophrenia patients, neither at baseline nor in relation to changes at follow-up. However, in the healthy controls we found a positive correlation between both markers and 9AM plasma cortisol, thus partially replicating the finding from Paper I.

In the patients, individual changes in PSS scores correlated significantly with changes in psychopathology (Figure 2). Interestingly there was no cross-sectional correlation between PSS score and psychopathology at baseline, indicating that the same objective severity of psychopathology is associated with very different levels of perceived stress among individuals. This may reflect that the PSS would be expected to be sensitive not only to the stress associated directly with psychopathology, but also stress from any other source. The PSS scores obtained in the present study are in good agreement with those obtained in other studies of schizophrenia patients [16,19].

FIGURE 2:



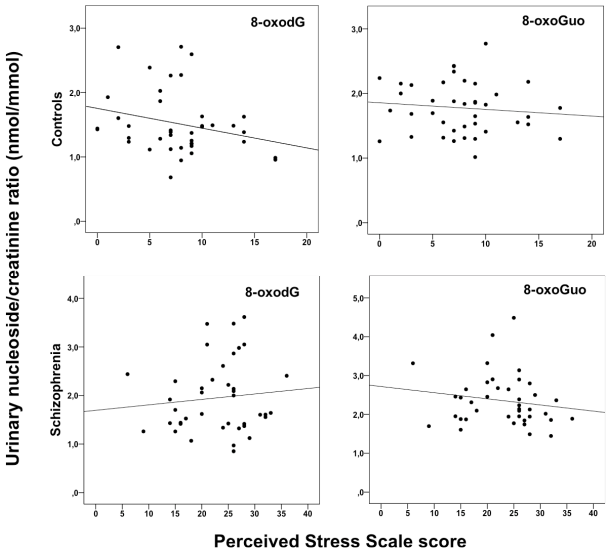
The association between the T0-T4 difference in Perceived Stress Scale (PSS) score vs. T0-T4 difference in psychopathology in schizophrenia patients. Pearson's r = 0.44, P = 0.01.

4.3. PAPER III

In the CRS study, MC housing did not appear to be a significant psychological stressor in itself. Weight gain and relative adrenal gland weight were similar in the group cage and MC cage control animals, and the urinary corticosterone excretion in the MC control animals was stable. Rats subjected to restraint stress showed a 2.5 fold increase in corticosterone excretion from day 2 after stress, which was sustained throughout the study. This contrasts the finding of others that singular blood determinations of corticosterone exhibits normalization after repeated restraint [130,135]. In spite of this substantial increase, urinary levels of 8-

oxodG/8-oxoGuo did not differ between the groups. Likewise, there were no differences between the groups in neither frontal cortex or hippocampal levels of oxidatively generated DNA damage as measured by the comet assay. However, we found a general trend towards an induction of the DNA/nucleotide pool repair enzymes (MCS > GCC/MCC), which was significant for hippocampal Nudt1. Stress did not significantly influence the expression of neither of the inflammation markers.

FIGURE 3:



Urinary 8-oxodG/8-oxoGuo excretion vs. Perceived Stress Scale scores in controls (upper panel) and schizophrenia patients (lower panel). None of the correlations are statistically significant.

4.4. PRELIMINARY RESULTS FROM A STUDY OF SEVERELY DEPRESSED PATIENTS RECEIVING ELECTROCONVULSIVE THERAPY

To further extend on the findings from the animal study, we investigated 8-oxodG excretion in patients receiving electroconvulsive therapy (ECT). The results are highly preliminary and therefore not presented in manuscript form.

ECT results in a rapid and substantial improvement of depressive symptoms in the majority of patients [136]. Therefore, patients receiving ECT are an attractive population for the prospective study of the relation between stressful human mental states and nucleic acid damage from oxidation. Urinary samples were obtained from 29 severely depressed patients at the Psychiatric Centre Copenhagen. The patients were originally included in two ongoing studies on the effect of ECT on magnetic resonance scan outcomes, and as a part of these studies, patients gave a urine sample before and one week after a series of treatments. Patients were rated for psychopathology (Hamilton Depression Rating Scale, HDRS [137]) and perceived stress (PSS). Inclusion criteria were: Age 18-70, fulfilling ICD-10 criteria for either unipolar or bipolar severe depression, and eligible for ECT (as assessed by the patients usual physician). Exclusion criteria were significant somatic disease (e.g. heart disease, neurodegenerative disorders, cancer), alcohol or drug abuse, and coercion.

One week after the completion of the ECT series, there was a highly significant reduction in both HDRS (pre-treatment 26.9 (± 4.5) points, post-treatment 11.7 (± 6.8), P < 0.001) and PSS score (pre-treatment 29.1 (± 3.7), post-treatment 19.2 (± 9.6), P < 0.001). 64% responded to treatment (post-ECT HDRS <50% of pre-treatment value), and 33% achieved remission (HDRS ≤ 7). In spite of these dramatic changes in psychopathology and perceived stress, we found no change in the urinary excretion of 8-oxodG after treatment (mean difference = 0.01 (95% CI -0.16 – 0.13) µmol/nmol creatinine, P = 0.85, paired samples t-test). Post-treatment changes in 8-oxodG excretion where not related to treatment response (P = 0.66) or remission status (P = 0.49).

5. DISCUSSION

5.1. SYNTHESIS OF THE FINDINGS

The overall hypothesis of an association between psychological stress and oxidatively generated DNA/RNA damage was not supported by these studies (Figure 3). 8-oxodG/8-oxoGuo was elevated in schizophrenia patients, and this difference was robust for multiple adjustments, stable over time, and not correlated to medication load. Thereby the finding is not inconsistent with the possibility of a fundamental mitochondrial dysfunction in schizophrenia, and it provides a possible molecular-level link between schizophrenia and the signs of accelerated aging associated with the disorder [53,138,139].

We found no correlations, neither at baseline nor at follow-up, to perceived stress or measures of cortisol secretion. Preliminary evidence from a prospective study of severely depressed patients recovering with ECT treatment added further support to the conclusion that 8-oxodG excretion is not sensitive to changes in psychopathology or perceived stress.

We found significant correlations between measures of cortisol secretion and 8-oxodG/8-oxoGuo in healthy controls in both Paper I and II, providing a possible causal link between hypercortisolemic states and age-related disease. However, a restraint stress-induced massive and prolonged increase in corticosterone did not lead to changes in the urinary oxidation markers in rats. This could be due to compensatory mechanisms such as a stress-induced reduced calorie intake, as discussed in the paper. Alternatively, it is not cortisol/corticosterone that directly induce oxidative stress on nucleic acids, but rather the long-term metabolic changes induced by glucocorticoids [140]. Finally, it is possible that the young animals used in the study are relatively resilient to pro-oxidant effects of glucocorticoids, whereas elderly individuals such as the human participants from the InChianti study would be more sensitive. These issues could be approached by studies of urinary 8-oxodG/8-oxoGuo excretion during restraint stress and exogenous corticosterone administration in young and aged animals, respectively.

5.2. STRENGTHS AND LIMITATIONS

While the specific strengths and limitations of the studies are mentioned in the papers, a few general remarks should be made. In any cross-sectional, observational study of a given biomarker, the influence of confounding factors is unavoidable, and the risk of not taking an important confounder into account is present. To partly overcome this problem, a prospective analysis of intraindividual differences can be applied, and this was attempted in the schizophrenia study. However, confounding effects may still play a role.

The methodological golden standard for the elimination of confounding effects is the randomized trial. However, a randomi-

zation of humans to either a control situation or experimentally induced, prolonged psychological stress is not ethically feasible. Therefore, the translational approach of studying the same biological phenomenon in an animal model of stress seems appropriate. We consider it the major strength of the studies as a whole, that human and animal investigations were combined. The use of animal experiments allowed for further exploring the cerebral DNA oxidation and repair.

We further consider it a strength that rigorous clinical ratings were combined with a state-of-the-art determination of nucleic acid oxidation markers, and that the same UPLC-MS/MS technique was applied across studies.

5.2.1. Paper I

The major strength of the InChianti study was the determination of both cortisol and 8-oxodG/8-oxoGuo in the same 24h urinary samples, which had not previously been done. The study was based on data that had been obtained for a different purpose, and this approach has a number of limitations. For example, a measure of perceived stress was not available. Because the study was based on a relatively small subsample, we did not exploit the survival data available from the InChianti cohort. Determination of the urinary 8-oxodG/8-oxoGuo of the entire cohort would allow for survival analysis, and this is a planned future study.

5.2.2. Paper II

It could be argued that the schizophrenia study was not properly designed for the detection of stress-associated changes in 8-oxodG/8-oxoGuo, because patients did not differ sufficiently in psychopathology and perceived stress from T0-T4. Patients were recruited from both in- and outpatients clinics, and some of them had a very stable psychopathology. The inclusion of only hospital-admitted, acutely ill patients might have yielded larger differences over time, but at the expense of a reduced generalizability to the general schizophrenia population. Hence, while the study was sufficiently powered for the comparison to healthy controls, it may have been underpowered for the detection of a correlation between changes in perceived stress and 8-oxodG/8-oxoGuo excretion.

Given that the Psychiatric Centre Copenhagen provides mental health services for more than 1,200 patients with schizophrenia, a large number of potential participants have not been screened for participation. Because the study was hospital-based, more patients requiring hospitalization than stable outpatients were included. Furthermore, patients with severely disorganised thinking and patients subjected to coercion were excluded for ethical reasons. On the other hand, the included patients did exhibit a wide range of symptom severity as measured by the PANSS scores. However, these factors will give rise to selection bias, and this should be taken into consideration when interpreting the results, which cannot necessarily be extrapolated to milder or very severe forms of the disease.

We only applied one measure of psychological stress, namely PSS. The scale was chosen for its brevity, its previously shown association to telomere shortening, and the fact that it measures current, subjective, short-term perceived stress levels, and thereby were relevant to our hypothesis of a connection between a current mental state and a biological marker. However, other measures of stress exposure such as lifetime or recent stressful events could perhaps have contributed to the findings.

The patients were all medicated, and although we did not find an association between 8-oxodG/8-oxoGuo and medication load, an influence of medication cannot be ruled out. The finding should be replicated in drug-naïve patients.

While many known or potential confounders of oxidative stress where taken into consideration in the statistical analysis, it could be argued that the control group recruited at the local blood donation corps was “too healthy” to be a relevant comparison to psychiatric patients. It is difficult to obtain an otherwise healthy control group with a metabolic and lifestyle profile similar to that of schizophrenia patients. However, a comparison to psychiatric patients from different diagnostic categories could be performed. The collection of samples is ongoing, and therefore such an analysis might be possible in future studies.

5.2.3. Paper III

We used a well-established model of chronic stress, and the biological markers of stress-induction (corticosterone, relative adrenal gland weight) indicated that the model was valid, even when performed in MC. To our knowledge, CRS has not previously been performed in MC, and this variation of the model opens new opportunities for the research in the metabolic effects of stress.

However, it should be noted that several of the oxidative stress related parameters measured in the study were affected in the MC controls. For example, urinary 8-oxodG and 8-oxoGuo tended to increase more in the MCC than the MCS group at day 2 after the initiation of stress. A possible explanation of this observation is a generally elevated metabolism due to the lack of cold protection provided by the litter and the littermate. Due to technical difficulties we limited the time points for 8-oxodG/8-oxoGuo determination to 4, thus substantially reducing the temporal resolution with which we were able to monitor the marker variation.

6. CONCLUSION AND IMPLICATIONS

In the present studies, we have addressed various aspects of the relation between psychopathology, psychological stress, stress hormones secretion, and oxidatively generated damage to nucleic acids. The results from Paper I, and to a certain extent also from Paper II, indicate an association between cortisol levels and oxidative stress on nucleic acids in healthy humans, providing a possible explanation for how hypercortisolemic states lead to age-related disease.

We found that systemic oxidatively generated nucleic acid damage is increased in schizophrenia, in a manner that appeared to be independent of a range of lifestyle and metabolic parameters, plasma antioxidant status and medication load, but with no associations to perceived stress or cortisol secretion. Given that the UPLC-MS/MS is a highly validated method that allows for a high throughput analysis of samples obtained non-invasively, urinary 8-oxodG/8-oxoGuo could be useful biomarkers in larger-scale prospective studies of morbidity and mortality in schizophrenia, and perhaps also in other mental disorders.

In the CRS-MC study, we found that the experimental induction of psychological stress and HPA-axis activation did not lead to increased urinary excretion of the oxidation markers, or to increased levels of frontal cortex and hippocampal DNA damage from oxidation after 3 weeks of restraint. This finding was supported by preliminary data from a study of patients recovering

from severe depression with ECT treatment. However, gene expression studies indicated that in the same brain regions, DNA repair enzymes were induced by stress, providing a possible aspect of allostatic adaptation that has not, to our knowledge, previously been addressed. This finding should be replicated and extended upon in a separate study.

In conclusion, we could not confirm our hypothesis of an association between psychological stress and 8-oxodG/8-oxoGuo excretion, both in a human study of healthy controls and schizophrenia patients, and in an animal experiment of chronic restraint stress. As discussed, a number of methodological and biological issues may underlie this finding, and a number of future experiments have been suggested. The studies have provided new insights into the association between glucocorticoids and systemic oxidative stress on nucleic acids, as well as the adaptive biological countermeasures initiated under stressful conditions. Finally, urinary 8-oxodG/8-oxoGuo could prove to be useful as specific markers of systemic nucleic acid damage in larger-scale prospective studies of mental disorders and their associated, severely elevated somatic health risks.

7. SUMMARY

Both non-pathological psychological stress states and mental disorders are associated with molecular, cellular and epidemiological signs of accelerated aging. Oxidative stress on nucleic acids is a critical component of cellular and organismal aging, and a suggested pathogenic mechanism in several age-related somatic disorders.

The overall aim of the PhD project was to investigate the relation between psychopathology, psychological stress, stress hormone secretion and oxidatively generated DNA and RNA damage, as measured by the urinary excretion of markers of whole-body DNA/RNA oxidation (8-oxodG and 8-oxoGuo, respectively). The main hypothesis was that psychological stress states are associated with increased DNA/RNA damage from oxidation.

In a study of 40 schizophrenia patients and 40 healthy controls matched for age and gender, we found that 8-oxodG/8-oxoGuo excretion was increased in schizophrenia patients, providing a possible molecular link between schizophrenia and its associated signs of accelerated aging. We found no association between psychopathology, perceived stress or cortisol secretion and 8-oxodG/8-oxoGuo excretion in the patients. In the controls, there were positive correlations between 8-oxodG/8-ocoGuo excretion and 9AM plasma cortisol, but no associations to perceived stress.

In an animal study of experimentally induced chronic stress performed in metabolism cages, we found no increase in urinary 8-oxodG/8-oxoGuo or cerebral (hippocampal and frontal cortex) levels of oxidatively generated nucleic acid damage. However, there was a trend towards an increased expression of genes involved in DNA repair, possibly reflecting a compensatory mechanism.

In a study of 220 elderly, mostly healthy individuals from the Italian InChianti cohort, we found a significant association between the 24h urinary cortisol excretion and the excretion of 8-oxodG/8-oxoGuo, determined in the same samples.

Collectively, the studies could not confirm an association between psychological stress and oxidative stress on nucleic acids. Systemic oxidatively generated DNA/RNA damage was increased in schizophrenia, and linked to cortisol levels in healthy humans. Finally, the cerebral repair of DNA may be an aspect of the adaptation to stress that, to our knowledge, has not previously been addressed.

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