Oxidative stress in chronic diseases: causal inference from observational studies

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CHAPTER 1

General introduction, study population, and thesis outline

INTRODUCTION

According to WHO's 2019 Global Health Estimates, chronic diseases, also known as non-communicable diseases, make up seven of the top ten causes of death, accounting for 71% of all deaths globally. Among those, cardiovascular disease (CVD) remains the most common cause of mortality[1] and neurological disorders are the leading cause of disability and the second leading cause of death[2] worldwide. The Global Disease Burden study estimated 17.8 million deaths attributable to CVD in 2017, which represents an increase of 21% in population mortality from CVD and an increase of 15% in years of life lost in the decade before 2017[1]. Similarly, approximately 3 million deaths and 26% increment in years of life lost are attributed to neurological disorders[1]. A set of traditional risk factors have been well acknowledged for chronic diseases, including but not limited to, tobacco use, obesity, hypertension, hyperlipidemia[3]. These have been combined into multiple risk assessment tools to estimate individuals' risk for developing diseases such as CVD[4-6] and dementia[7]. Pharmacological and lifestyle interventions targeting several risk factors can also substantially reduce CVD risk[8-11]. Nevertheless, most of these main causes, if not all, share a common pathological mechanism in the development of chronic diseases, and that is oxidative stress[12]. Therefore, oxidative stress is fundamental among the pathophysiological mechanisms leading to chronic diseases, and a better understanding of their associations may provide early opportunities as a preventive and therapeutic target.

Oxidative stress

Oxidative stress was first formulated in 1985 by Helmut Sies and refined afterwards[13-15]. It refers to conditions whenever the generation of reactive oxygen species (ROS) exceeds endogenous antioxidant capacity, leading to disruption of redox signaling and molecular damage. ROS are the by-products of aerobic metabolism predominantly generated in mitochondria through the electron transport chain during oxidative phosphorylation. The heart and brain are high oxygen-consuming organs to produce energy continuously, with large amounts of mitochondria constituting up to one-quarter of cardiomyocyte volume[16]. This inevitably increases the susceptibility of the heart and vasculature and the neural system to oxidative stress and associated damage. Notably, ROS in low to modest levels regulates multiple redox-dependent vascular wall signaling processes[17], whereas maladaptive excessively high ROS levels mediate irreversible and nonspecific macromolecular damage to cellular membranes, proteins, and DNA[18], and induction of inflammation[19] and dysregulation of vascular functions[20], particularly endothelial dysfunction by disrupting the nitric oxide signaling cascade[20,21]. Collectively, high levels of oxidative stress promote the development of atherosclerosis and further CVD, as well as other chronic diseases.

Mitochondrial dysfunction

ROS generated in mitochondria contributes to mitochondrial dysfunction, which in turn stimulate mitochondrial ROS overproduction, forming a vicious circle. Mitochondrial dysfunction has been widely seen as a hallmark of the ageing

process[22], with disruption of energy transduction, and perturbed calcium and redox homeostasis[23,24], and plays a role in the etiology of multiple age-related diseases. Mitochondria have their own circular genome, the cytoplasmic mitochondrial DNA (mtDNA), consisting of 37 genes, 13 of which encode multi-subunit enzymatic components of the electron transport chain. Individual mitochondria may contain two to ten copies of the mitochondrial genome, known as mtDNA copy number (mtDNA-CN). Therefore, considerable variations exist across cells, tissues, and individuals. The alterations of mtDNA-CN per nucleated cell might be indicative of aberrant mitochondrial health and are associated with perturbations of bioenergetics, mitochondrial membrane potential, and oxidative stress[25], and therefore could roughly serve as an easily accessible and minimally invasive surrogate biomarker of mitochondrial dysfunction[26].

In epidemiological cohort studies, lower peripheral leukocyte mtDNA-CN has been associated with several CVDs and CVD-related risk factors. For example, in the prospective Atherosclerosis Risk in Communities (ARIC) study comprising about 20,000 participants with no history of CVD during a follow-up of up to more than 20 years, a low mtDNA-CN was associated with a 1.29- and 1.11- times higher risk of coronary artery disease and stroke, respectively[27]. A low level of mtDNA-CN was cross-sectionally associated with several cardiovascular risk factors such as obesity, hypertension, hyperglycemia, and diabetes in 408,361 participants[28].

Antioxidants

Antioxidants are substances that neutralize oxidants and their actions to maintain a biological redox steady state. Given the crucial role of ROS in the pathogenesis of chronic diseases, particularly CVD, it has been of particular interest for decades to investigate the protective role of antioxidants in CVD. Of those, dietary-derived modifiable ones have been central for a long time, including β-carotene, vitamin C, and E, among others. However, an important paradox of antioxidants exists.

Since the late 1980s, multiple epidemiological studies have been carried out to investigate the role of antioxidants in CVD. In prospective epidemiological cohort studies, high intake of dietary antioxidants at baseline, either as dietary components or supplements, or high blood concentration of antioxidants including vitamin C and E, have been consistently associated with a lower CVD risk[29-35]. For example, two major studies from 1993 concluded that high consumption of vitamin E was associated with a reduced risk of coronary heart disease[36,37]. These promising findings were widely advocated in the mass news media and led to a significant increase in supplement use in the general public. Not surprisingly, a considerable proportion of the population used dietary antioxidative supplements over the last few decades, particularly in the United States[38]. Notwithstanding, the translation of these findings into evidence-based interventions has not been so straightforward. Randomized clinical trials (RCTs) and their meta-analyses generally failed to demonstrate a significant beneficial effect of antioxidants supplementation, including β-carotene, vitamin C, E, B12, B6, and folic acid, on CVD outcomes and related risk factors[39-45]. Currently, there is inadequate evidence on the benefits of several supplementations in the prevention of CVD[46].

Myths or facts

This vitamin paradox raises the importance of correct causal inference to contribute to our etiological understanding of the role of antioxidants in CVD and in public health. Although the associations between oxidative stress, proxied by mitochondrial dysfunction or low levels of antioxidants in the blood, and CVD have been identified in several observational studies, several limitations challenge the causal inference of the original research findings.

Conventional observational epidemiological studies are often plagued by confounding, reverse causation, and other forms of bias that can limit the validity of the findings from these studies. The confounding factors that causally influence both a risk factor and an outcome induce an spurious association between the risk factor and the outcome. Adjustment for known confounders is possible in statistical analyses, but residual confounding may remain, either due to confounders not being measured or measurement errors in the confounder. Reverse causation is the situation in which the observed association between the risk factor and an outcome is because the outcome causally affects the risk factor. For example, people with early symptoms of CVD may adjust their lifestyle to be healthier accordingly, with higher dietary intake of antioxidants and earlier initiation of supplementation than those without symptoms. Therefore, an interpretation of these spurious associations as causality would be misleading.

RCTs are regarded as the "gold standard" to establish causal effects for all intervention studies because imbalances of participants' characteristics are eliminated by randomization and any differences in outcome can therefore only be attributed to the intervention. However, there are substantial challenges to examine antioxidants supplementation in RCTs, and several mechanisms could explain the null findings in RCTs. First, administration strategies are highly heterogenous, such as timing, monotherapy or not, dose, and treatment duration. For example, supplementation started later than irreversible ROS damage could have a negligible clinical beneficial effect. Moreover, a clear limit in circulating levels is reached by antioxidant supplementation that might be below the therapeutic levels[47]. Also, the circulating antioxidants from supplementation may not access the required target sites to scavenge ROS, especially the mitochondria, and could not represent the functional levels. For instance, vitamin E undergoes different catabolism depending on whether radical-dependent or not and generates distinct metabolites that can be detected in the urine[48]. Furthermore, the form of the antioxidants, natural or synthetic, may exert an influence, such as lower bioactivity of synthetic vitamin E than the natural ones, characterized by preferentially non-oxidation metabolites in urine[49].

Therefore, there is an urgent demand to explore innovative and rigorous research designs for examining the effects of antioxidants taking those complexities in antioxidants and administration into account.

Mendelian randomization in causal inference

Mendelian randomization (MR) has emerged in recent years as a powerful study design in causal inference. It uses genetic variants associated with the exposure of interest to investigate the causal effect of modifiable risk factors on outcomes.

Genetic variants inherited from parents to offspring at conception are randomly assorted and segregated at meiosis and are used as proxies of the exposure levels. As a result, individuals are divided into two comparable groups. Those who carry the effect allele (e.g. with an increased level of exposure) are assigned to the group with higher levels of the exposure of interest whereas those who carry the alternative allele are assigned to the group with lower levels of exposure. Through this process of random allocation, genetic variants are independent of environmental and other genetic factors, except for those variants that are in linkage disequilibrium of the variant of interest. Hence, genetic variants will not be related to any potential confounding factors that affect the exposure and the outcome and any difference in outcomes between genetically defined groups can be directly attributed to the exposure. Consequently, the MR design is considered as a "natural experiment", mimicking RCTs, where individuals are randomized to carry a genetic variant rather than to an intervention. In addition, the earlier disposition of genetic variants than the exposure measurements ensures the correct temporal order to eliminate the possibility of reverse causation. Therefore, MR can be used to estimate the causal relationship between exposures and outcomes largely free from confounding, in contrast to conventional epidemiological approaches.

The massively increasing availability of genotyped and high-quality phenotypic data in large study samples and mega biobanks during the past decade facilitates us to apply MR designs and to generate valid and well-powered approximated causal associations between exposures and outcomes. Indeed, the past decade has witnessed a considerable increase of publications using the MR approach50. To obtain reliable causal estimates of the effect of an exposure on an outcome from MR studies, the following three principal assumptions should be fulfilled by the genetic variants, namely that they 1) are associated with the exposure in genetic studies (relevance); 2) are not related to any observed or unobserved confounding factors (independence); 3) are associated with the outcome exclusively through its effect on exposure (exclusion restriction), as illustrated in **Figure 1**.

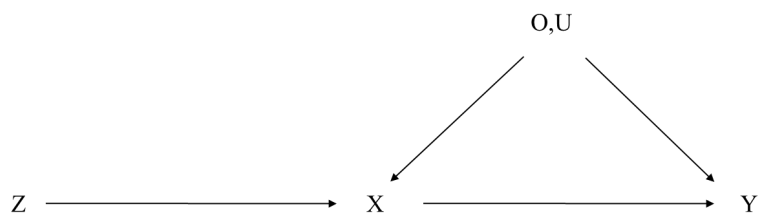

Figure 1 A graphical presentation for causal inference using Mendelian randomization.

Z represents the genetic instruments. To conclude that exposure of interest (X) is a causal risk factor for the outcome (Y), three assumptions need to be satisfied. First, relevance is implied by the arrow from Z to X (Z→X), where Z is causally associated with the exposure. Second, independence is that Z is independent of any observed (O) or unobserved (U) confounding factors. Third, exclusion

restrictions signify that Z is independent of Y conditioning on exposure and confounding factors, namely no path from Z to Y other than via X (Z→X→Y), i.e., no horizontal pleiotropy.

OUTLINE OF THIS THESIS

This thesis aims to provide extensive insights into the role of oxidative stress in the onset of chronic diseases with an emphasis on CVD and related cardiometabolic risk factors via applying conventional epidemiological approaches in combination with Mendelian randomization (MR) designs. The thesis is structured in five. In **Part I** we provide an overview of oxidative stress and its role in ageing and age-related diseases on the progress beyond state-of-the-art. In **part II** we investigate the associations between mitochondrial dysfunction and CVD and lipid profiles. In **Part III** we focus on the role of antioxidants in CVD and cardiometabolic traits. In **Part IV** we expand the scope of this thesis by examining the association of inflammation, which is inextricably interrelated to oxidative stress, with neurological diseases. **Part V** contains a summary of the main findings from this thesis and a general discussion.

Part I: General aspects

Oxidative stress has been put forward for decades, the role of oxidative stress in ageing and age-related diseases is however controversial. In **Chapter 2**, we provide a detailed overview of the generation of reactive oxygen species and their role in redox signaling and oxidative damage. In addition, we review the current evidence regarding the association of oxidative stress and ageing and age-related diseases as CVD and neurodegenerative disease. We then summarize the possible reasons for the inconsistency and put forward some remarks.

Part II: Mitochondrial dysfunction in cardiovascular disease

"Triangulation" in etiological epidemiology could be helpful to strengthen causal inference by integrating several different approaches that are assumed to have different and largely unrelated sources of potential bias[51]. When estimates obtained from different approaches converge on a similar effect, evidence favors that the relationship between exposure and outcome is causal. Hence, we combine a prospective cohort study design using data from the general population from the UK biobank and a Mendelian randomization framework exploiting publicly available summary-level data from the large consortia and mega-biobanks to infer causality different exposures and outcomes. We specifically look into the association between mitochondrial DNA copy number and incident coronary artery disease and heart failure in **Chapter 3** and metabolomic profiles (mostly lipids and lipoprotein fractions) in **Chapter 4**.

Part III: Antioxidants in cardiovascular disease

We still do not know the causal nature of the associations between antioxidants and CVD because of the contradictory results from observational studies and RCTs and considering the limitations of both designs. Here, we focus on dietary-derived antioxidants, predominantly on the most appealing yet debatable well-known chain-breaking antioxidant: vitamin E. In **Chapter 5**, using a Mendelian randomization design, we investigate the associations between genetically predicted circulating antioxidants levels (vitamins E and C, retinol,

β-carotene, and lycopene), both as absolute levels and their metabolites, and the risk of coronary heart disease, using publicly available genetic consortium. Since vitamin E is partly catabolized in a radical-dependent way, even the circulating levels are not representative of the functional levels, which might be the most plausible explanation for the null findings between circulating vitamin E and CVD. Accordingly, there are two forms of vitamin E metabolites in the urine, the oxidized metabolites being indicative of its antioxidative capacity, and enzymatic metabolites. We subsequently link these two metabolites to cardiovascular risk factors and cardiometabolic traits, including glucose metabolism in **Chapter 6** and lipoprotein profiles in **Chapter 7** in the Netherlands Epidemiology of Obesity study (NEO) using an observational study design.

Part IV: Inflammation in neuropsychiatric diseases

Inflammation and oxidative stress are inextricably linked. Inflammation, the "host defense" against pathogens and chemical and physical challenges towards tissue integrity to restore tissue homeostasis through inducing various repair mechanisms, involves an enhanced release and accumulation of ROS by activated immune cells. Evidence has emerged that ROS play a critical role in the pathophysiology of inflammation. Inflammation has been well-acknowledged in atherosclerotic CVD but its association with neurological diseases is less known. For this reason, we expand the scope of this thesis by examining also neurological diseases as additional outcomes with large relevance to population health. In **Chapter 8**, we investigate the associations between inflammatory bowel disease that is characterized by chronic inflammation and depression using a bidirectional Mendelian randomization approach. In **Chapter 9**, we examine the role of multiple systemic inflammatory markers in cognitive function and brain atrophy measures.

Part V: Summary

Finally, **Chapter 10** summarizes the main findings of this thesis and discusses some future perspectives in the field.

MAIN STUDY POPULATIONS

The Netherlands Epidemiology of Obesity study (NEO)

The NEO study is used in **Chapters 6 and 7**. This population-based prospective cohort study started in 2008 and includes 6,671 individuals aged 45-65 years, with an oversampling of individuals with a self-reported body mass index (BMI) of 27 kg/m^2 or higher. All inhabitants aged between 45 to 65 years from the municipality of Leiderdorp were invited irrespective of their BMI. The study was approved by the medical ethical committee of the Leiden University Medical Center (LUMC), and all participants gave written informed consent. Participants were invited to come to the NEO study center of LUMC for one baseline study visit after an overnight fast. Prior to this study visit, participants collected their urine over 24 hours and completed a general questionnaire at home in terms of their demographic, lifestyle, and clinical data in addition to specific questionnaires on diet and physical activity. Patients were asked to bring their medication use within one month prior to the visit and relevant information was recorded by research nurses. Fasting blood samples were drawn for biochemical measurements.

UK biobank

Individual and summary-level data of the UK biobank are used in **Chapters 3, 4, 5, and 8**. The UK Biobank is a prospective cohort with 502,628 participants between the age of 40 and 69 years recruited from the general population at multiple assessment centers across the UK between 2006 and 2010. Invitation letters were sent to eligible adults registered to the National Health Services (NHS) and living within a 25 miles distance from one of the assessment centers. Participants provided information on their lifestyle and medical history through touch-screen questionnaires and physical measurements. Blood samples were collected for genotyping. The study was approved by the North-West Multi-center Research Ethics Committee (MREC). Access for information to invite participants was approved by the Patient Information Advisory Group (PIAG) from England and Wales. All participants provided electronically written informed consent for the study.

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