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## **Oxidants and antioxidants as targets for cardiovascular disease prevention: evidence from observational and causal inference studies**

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# 1

## GENERAL INTRODUCTION

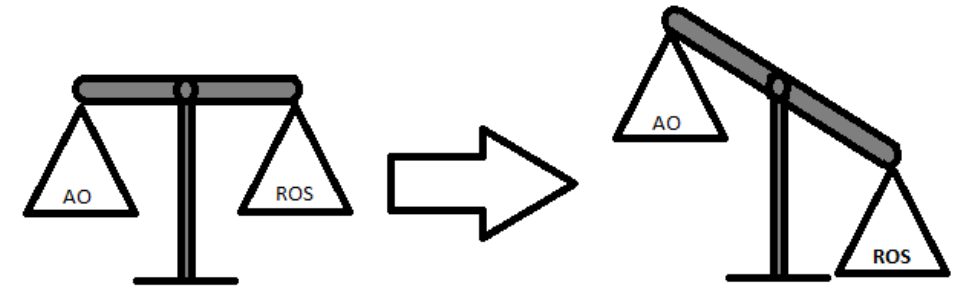


# INTRODUCTION

Developments in science, medicine and society, particularly throughout the last few centuries, have led to an increasing ability to treat and cure many previously deadly diseases. Although life expectancy has steadily increased from the 1800s onwards, in part due to better treatment of infectious diseases, different major causes of death emerged. The prevalence of non-communicable, age-related diseases such as cardiovascular disease (CVD) have greatly increased and this increase is expected to continue as our society further ages [1]. Although CVD has been studied for decades, and successful interventions have been identified and implemented, CVD remains a leading cause of death worldwide. CVD accounted for an estimated 18.6 million deaths worldwide in 2019 [2], and simultaneously accounted for a significant reduction in quality of life in those individuals that suffered a non-fatal event. For this reason, it is crucial to further expand our understanding of the mechanisms leading to CVD, to identify novel strategies that further reduce CVD disease risk.

Advancing age is a primary risk factor for many major chronic diseases [3]. Ageing is generally defined as broad, time-dependent functional decline. The cellular and molecular ageing processes have been separated into the 9 hallmarks of ageing: Genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication [3]. These 9 hallmarks are thus processes generally thought to contribute to the ageing process and together determine the ageing phenotype.

Mitochondrial dysfunction is one of the hallmarks that may underly various adverse effects observed in ageing. Mitochondria are an important source of Reactive Oxygen Species (ROS), as an inevitable byproduct of their essential role in energy production [4]. ROS are generated during the process of oxidative phosphorylation and under physiological conditions efficiently scavenged. Although normal cellular function requires some degree of ROS (e.g., for intracellular communication purposes), pathological processes such as ageing may result in a disbalance between ROS production and scavenging (**Figure 1**). This disbalance is characterized by insufficient scavenging capacity to effectively eliminate ROS. As ROS are a highly reactive chemical, they could damage surrounding cellular components by reacting with them, ultimately causing cell damage or cell death [5]. To prevent ROS from inflicting cellular damage, different compounds collectively named antioxidants act as scavengers of these free radicals aiming to maintain appropriate ROS levels. Therefore, both optimal ROS production and sufficient antioxidant activity is pivotal to prevent oxidative damage [6].



**Figure 1.** When either ROS production is increased or antioxidant activity is decreased, this may result in a disbalance. This disruption could lead to cellular damage or even cell death.

Oxidative stress is shown to play an important role in the pathophysiology of stroke, one of the main types of CVD [7, 8]. Increased ROS levels could lead to direct or indirect damage to the vascular wall via altered platelet aggregation, endothelial dysfunction, dysfunctional vasodilation, and/or disturbed vascular permeability [8]. These changes could lead to local lesions and might therefore increase the risk of clinical manifestations such as (ischemic) stroke. Lipid peroxidation by ROS is one of the oxidative processes that might contribute to the development of CVD. Peroxidized lipids can subsequently induce proinflammatory responses in the vascular system which play a crucial role in the initiation and progression of CVD [9-11].

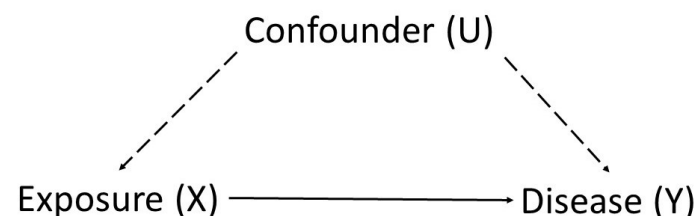
Several studies have shown that decreased blood antioxidant levels are associated with increased CVD incidence [7, 12-15]. However, intervention studies exploring antioxidant supplementation have been unable to show reduction in the risk of developing clinical outcomes related to CVD [16, 17]. This lack of effectiveness of the studied antioxidant compounds thus far does not provide definitive proof for the role of ROS in CVD risk.

Although Randomized Controlled Trials (RCTs) are generally considered the gold standard of evidence, the effects of an altered oxidative stress balance might not be visible in the relatively short time period of an RCT. Additionally, the pathogenesis of atherosclerosis, a process where lesions are formed in the arteries that could lead to the buildup of plaque and eventually could result in CAD and stroke, often takes decades [18]. In other words, it might be improbable to demonstrate the effect of an altered oxidative stress balance on the development of CVD using RCTs. Thus, whether oxidative stress should ultimately be seen only as a marker of CVD risk might still require further investigation.

## Epidemiology

Epidemiological research, while simple in principle, can be prone to oversimplification. To present a famous example: When studying lung cancer, one would probably establish that lung cancer patients have an increased chance of suffering from yellow stained

fingers. However, common sense would tell us that yellow fingers do not cause lung cancer. Simultaneously, lung cancer does not result in yellow fingers. The crux is that both phenotypes are caused by a third factor, smoking. Smoking in this case can be considered a *confounding* factor. Confounding is a common problem affecting most, if not all, epidemiological studies. The concept of confounding is visualized in **Figure 2**. A variable is considered a confounder if it can affect both the exposure and the outcome of the study. Knowing what potential confounders could be, and how to correct for them, is what makes epidemiological research complex.



**Figure 2.** The concept of confounding. Both the exposure (X) and the outcome (Y) are influenced by the confounder (U). Therefore, the assumed association could be caused by the confounder.

This thesis aims to build on the basic premise of epidemiology, while simultaneously taking a more sub-group specific approach. Where risk factors and their effect are generally determined at a population level, the focus shifts towards the investigation of the difference in individual effect that these risk factors have on CVD, dependent on additional factors. This could potentially lead to a better understanding of the multidimensionality of CVD, and how risk factors affect the disease risk of other factors.

### Reverse causation

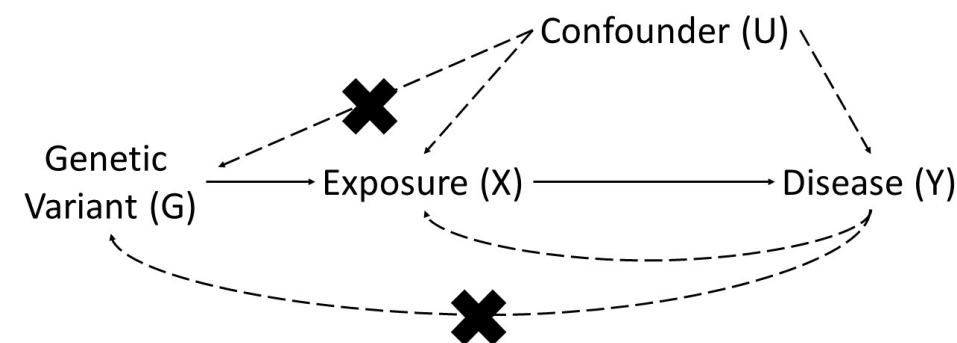
Another problem within epidemiology is the concept of causality. Although observational research can link two occurrences together, the certainty that one conditions leads to another is not guaranteed. On top of that, the directionality of the relation cannot be assured. In theory, instead of an increased risk factor causing the observed outcome, the outcome could cause an increase in risk factor. This is what we call reverse causation. It is important to understand direction in a relationship, as identifying a causal variable is crucial in the eventual prevention of the outcome.

### Mendelian Randomization

RCTs are the gold standard to assess whether an observational association is also a causal relation. However, RCTs are often costly, may be impossible for lack of intervention tools or may even be unethical. [19] An alternative approach one can use to assess

causal relationships is the Mendelian Randomization (MR) method [20, 21]. MR uses independent genetic variants associated with the exposure as instrumental variable (**Figure 3**) [22]. Since the genetic make-up of an individual is unlikely to be affected by outside factors, when performing an analysis using genetic variants as a proxy for the determinants, the potential association is not susceptible to confounding or reverse causation and therefore allows us to approximate the causal association between the exposure and the outcome.

The genetic variants associated with the exposure are often SNPs obtained from Genome-Wide Association Studies (GWAS) on the exposure. Since the size and availability of genetic data has increased substantially over the last decade, more genetic instruments have become obtainable for use and thus more MR studies can be done on many exposures in large sample sizes that are implausible for RCTs. However, it is important to note that in order to be able to perform a Mendelian Randomization study and interpret causality, several assumptions are made. First, the genetic variants used as instrument variables for the exposure are associated with the exposure. By performing a GWAS on the exposure you ensure that this assumption is not violated. Second, there is no confounding of genetic variants with the outcome. Third, the genetic variants are only associated with the outcome through the exposure. When genetic variants are directly associated with the outcome, you are no longer able to discern the lone effect of the exposure [23]. These assumptions are often tested during a study, and although not all three of them can definitively be proven to be fulfilled, it is possible to assess the likelihood of an assumption being violated. Finally, it should be noted that MR assesses the assumed lifelong exposure of the determinant, compared with the limited exposure of an RCT. However, as MR attempts to assess the lifelong exposure, it is important to realize that MR assumes the effect of the genetic variants do not change over time, which might not always be true.



**Figure 3.** Mendelian Randomization: The genetic variants are associated with the exposure and used as a proxy. As the genotype is independent of confounders and unaffected by reverse causation, a found association would be causal.

### Interplay of risk factors

For certain risk factors for CVD, causality has already been established. It is known, among others, that obesity, high blood pressure and high LDL cholesterol increase the risk on the development of CVD, and that interventions targeting these risk factors lower the risk substantially [2, 24-26]. However, CVD is a multifactorial, dynamic disease construct. Consequently, research should take into account the interplay of risk factors when studying CVD. Aside from the sum of effects of relevant risk factors, their potential interactive effect should also be taken into account. In other words, the presence of one risk factor might influence another risk factor in such a way it alters their association with CVD.

## OUTLINE OF THIS THESIS

The general aim of this thesis is to address the phenotypical causes of mitochondrial dysfunction, resulting in oxidative imbalance, and the cerebrovascular consequences in the general population.

Possible risk factors will be studied for causality and established risk factors will be studied for causality differences in subgroups, with the ambition to increase the understanding of the impact of these known risk factors on individual cardiovascular disease risk. Therefore, a proof-of-concept study will be performed to investigate how causal relations between cardiovascular risk factors and coronary artery disease can be altered by socioeconomic status. The studies of this thesis will be performed in several unique cohorts. The Netherlands Epidemiology of Obesity (NEO) study is a population-based, prospective cohort study designed to study obesity-related pathways and diseases [27]. Although the main cohort is oversampled with individuals with overweight or obesity, this study will use a subsample from Leiderdorp, with no BMI selection for participation. The UK Biobank is a UK-based prospective cohort study recruited from the general population [28].

In **Chapter 2**, I will measure serum  $\alpha$ -TOH and vitamin E metabolites, as a reflection of an individual's vitamin E status, and study their association with the lifestyle factors smoking, sleep, physical activity, and food and alcohol intake. This study will be performed in the NEO study.

**Chapter 3** will focus on the causal relation between several antioxidants and cerebrovascular disease risk. Here, I look for a possible causal relationship between genetically-influenced diet-derived antioxidants with ischemic stroke using Mendelian Randomization (MR). This study will be done using summary level data from three large databanks: the UK Biobank, MEGASTROKE, and FinnGen, resulting in a total of 1.1 million participants.

**Chapter 4** will study the association between mitochondrial DNA copy numbers

(mtDNA-cn), as a marker for mitochondrial dysfunction which drives oxidative stress levels, and stroke using both an MR as well as a survival-analysis approach using the UK Biobank. The triangulation of causal inference as described by Lawlor et al. [29] will be adopted to increase the credibility of our results.

**Chapter 5** is centered around the effect of individual characteristics on cardiovascular disease (CVD) risk factors. This study will be performed in the UK Biobank and is specifically focused on how socio-demographic characteristics could modify the causal association between classical CVD risk factors and coronary artery disease (CAD). In this study, I hypothesize that atherogenic cardiovascular disease is a multidimensional disease where different combinations of risk factors could alter the individual impact of said risk factors on CAD incidence.

In the final part of this thesis (**Chapter 6**), the main study findings and the future perspective of this research field will be discussed.

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