Oxidative stress in chronic diseases: causal inference from observational studies

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CHAPTER 10

Main findings and future perspectives

SUMMARY OF MAIN FINDINGS

The overall aim of this thesis was to disentangle the role of oxidative stress in chronic disease, with a focus on cardiovascular disease (CVD) and related cardiometabolic risk factors. The results derived from the studies described in this thesis addressed two main questions with the application of state-of-the-art epidemiological research methods: 1) at a population level, whether mitochondrial dysfunction is a causal risk factor in the development of atherosclerotic CVD and related intermediate risk factors (**Part II**), and 2) whether dietary antioxidants impose any clinical-relevant benefits in the prevention of atherosclerotic CVD (**Part III**). In addition, this thesis also sheds light on the role of inflammation in neurological diseases (**Part IV**). Furthermore, the implications of the work comprised in this thesis for future research are discussed in this part.

In **Chapter 2**, we reviewed the current biological knowledge of oxidative stress and its relation to ageing and age-related diseases in experimental and epidemiological studies. Despite reactive oxygen species being closely involved in the maintenance of cell function via a diverse array of signaling pathways, their overproduction leads to oxidative-related macromolecule damage and mitochondrial dysfunction which further result in altered lifespan and the manifestation of multiple age-related diseases. However, the associations between oxidative stress with diseases, such as CVD, are yet inconclusive, especially based on the findings from different antioxidants in observational studies and randomized clinical trials (RCTs). Nevertheless, several weaknesses in the study design and the concept of antioxidative capacity should be considered when interpreting those conflicting results. Moreover, novel and specific biomarkers of oxidative damage are warranted to monitor the effect of antioxidants supplementation.

In the general population, changes in leukocyte mitochondrial DNA copy number (mtDNA-CN) have been proposed to be a proxy for mitochondrial function and mtDNA-CN is becoming an attractive biomarker due to its relative ease of measurement. In **Chapter 3 and 4**, in line with the principles of triangulation in etiological studies, we examined the role of mtDNA-CN in CVD and metabolic traits using a combination of a prospective cohort design in the UK biobank followed by a Mendelian randomization (MR) approach. In **Chapter 3**, observational analyses among participants free of CVD at study inclusion showed that low mtDNA-CN was a risk factor of both incident coronary artery disease and heart failure. Using MR with summary-level data from the currently largest genetic consortia and biobanks, these associations were validated for coronary artery disease, but not for heart failure likely, which might be due to the phenotypic heterogeneity of heart failure. In **Chapter 4**, we further explored the association between mtDNA-CN with 168 blood-derived metabolomic measures of predominantly lipids and lipoproteins (sub)particles measured by using the Nightingale NMR-based platform. We observed associations between low mtDNA-CN and an atherogenic metabolomic profile, characterized by high levels of most lipids. These findings suggest that mitochondrial dysfunction, as proxied by mtDNA-CN, may have an influence on lipid dysregulation, a well-documented risk factor for atherosclerosis and a causal risk factor in the pathogenesis of atherosclerotic

CVD. Further studies are needed to test its validity in patients' risk classification and disease prevention.

In **Chapter 5**, we investigated the potential causal association between multiple circulating antioxidants and coronary heart disease using an MR design, including up to 768,121 participants with 93,230 cases from three large genetic consortia. We provided evidence that genetically predicted and thus lifelong higher circulating antioxidants levels, either as authentic circulating levels that are similar in magnitude to those achieved by dietary supplements or concentrations of corresponding metabolites, are unlikely to reduce the risk of coronary heart disease. However, the circulating antioxidant levels may not be equivalent to the authentic functional levels, particularly in the case of vitamin E with distinct catabolism upon oxidative modification. This highlights the need to link the markers that are specific for antioxidant capacity, i.e. antioxidants' functional levels, to CVD and related risk factors.

Vitamin E can be catabolized via either hepatic enzymatic pathways or oxidized in periphery[1]. In the hepatic pathway, vitamin E is enzymatically converted to a spectrum of enzymatic metabolites with successive shortening of the phytyl side chain. Alternatively, vitamin E acts as peroxyl lipid radicals scavenger and forms oxidized metabolites with the opening of the chromanol ring. These metabolites are predominantly excreted from the body via urine. In **Chapter 6 and 7**, we focused on the cross-sectional associations between circulating vitamin E, urinary enzymatic and oxidized metabolites, and cardiometabolic traits in approximately 500 middle-aged healthy individuals from the Netherlands Epidemiology of Obesity (NEO) Study. In **Chapter 6**, we specifically found that higher urinary levels of oxidized metabolites, but neither circulating vitamin E nor urinary enzymatic metabolites, were associated with lower insulin resistance. Similarly, in **Chapter 7**, we found that the associations of 147 NMR-based metabolomic measures, mostly consisting of lipids and lipoprotein (sub)particles, with enzymatic metabolites, have directions similar to those with circulating vitamin E. However, associations of metabolomic measures with oxidized metabolites were markedly different from those with both circulating vitamin E and enzymatic metabolites. These findings highlight that circulating vitamin E may be representative of the enzymatic catabolism but not the antioxidative function of vitamin E.

Inflammation is inextricably linked to oxidative stress. While the association between inflammation and atherosclerotic CVD is well-established, its role in neurological diseases is not clear. In **Chapter 8**, we investigated the bidirectional association between inflammatory bowel disease (IBD), as a disease model of sustained chronic inflammation, and depression using MR study in a combined sample size of 693 183 individuals (36 507 cases) for IBD and 534 635 individuals (71 466 cases) for depression, respectively. No association was observed between genetically influenced IBD and risk of depression, whereas genetically predicted depression was associated with a higher risk of IBD. In **Chapter 9**, we explored the causal effect of 41 systemic inflammatory markers on cognitive function and brain atrophy measures using an MR design. Similarly, no significant association after correcting for multiple testing was observed between 40 out of 41 inflammatory markers and any of the brain outcomes. These results however could not refute the role of inflammation in neurological diseases. In contrast, it

may indicate that rather than being a cause, an excessive inflammatory response is likely a consequence of neurological diseases.

FUTURE PERSPECTIVES

Antioxidants in chronic disease

The benefits of antioxidants have been widely hyped by the media and food industries. Their easy access largely facilitates the popularity of antioxidants supplementation in the general population. Based on the results derived from this thesis, some clinical experts now suggest stopping exploring the assumed protective effect of individual antioxidants in RCTs for atherosclerotic CVD[2]. However, from our perspective, it is still not the end of antioxidant supplementation as an easy and cheap method for chronic diseases prevention.

Antioxidant supplementation is often aimed at increasing circulating levels of antioxidants. However, the circulating levels are not necessarily corresponding to their functional levels in most of the cases due to several influential factors such as genetic background, individual health status, and the levels of other antioxidants in the circulation. For example, vitamin E shares common mechanisms for cellular uptake and efflux with cholesterol in many different cell types that are tightly regulated by genes[3]. Once taken up by cells, the intracellular distribution of antioxidants to organelles is regulated by different transport proteins binding to vitamin E, such as α-tocopherol transfer protein. In addition, vitamin C regenerates vitamin E by reducing vitamin E radicals formed during scavenging radicals. Hence, it could happen that an individual with higher supplementation with subsequently higher circulating levels has lower functional levels in certain target locations, e.g., mitochondria, to counteract overwhelming local production of oxidants. Due to the differences in antioxidant capacity among individuals, even identical supplementation strategies may possibly not lead to the same health outcomes. Therefore, monitoring the effect after supplementation with plausible biomarkers is of great interest. The ideal approach to monitor the supplementation effect is to measure the direct change of scavenging antioxidants. Although this is not possible for many antioxidants, it might be feasible in the case of vitamin E, where its urinary oxidized metabolites but not enzymatic ones are associated with CVD risk factors and thus represent the functional levels.

Furthermore, the selection of a proper population for supplementation is also critical in addition to rigorous monitoring strategies. Factors that could alter the total antioxidative capacity in the body should be considered. For example, metabolic syndrome patients had approximately 12% greater oxidation reduction potential and 59% lower total amount of readily oxidizable molecule reserves compared with healthy adults[4]. The bioavailability of vitamin E was shown to be reduced and the elimination delayed in these patients who have increased lipid peroxidation, independent of the co-ingested dairy fat amount[5]. Likewise, individuals with hyperlipidemia have been found to have reduced uptake of the newly absorbed vitamin E into blood[6]. Therefore, supplementation should be administrated to a population who are most likely to benefit. This group of population should be selected based on certain features (e.g., genotype, nutritional or health conditions, etc.) which could induce significant heterogeneous responses, for example, participants with different haptoglobin genotype. Haptoglobin binds hemoglobin with high affinity and stability, avoiding the release of heme iron from hemolysis into circulation, consequently preventing the production of

hydroxyl radical. Vitamin E supplementation has been shown to be associated with an approximately 35% reduction in CVD specifically in individuals with both diabetes and haptoglobin 2-2[7]. Furthermore, as mitochondria are among the most important vulnerable sites to oxidative stress, mitochondrial dysfunction may serve as the biomarker of oxidative damage for population selection for supplementation. Particularly, mtDNA-CN might be a proxy of mitochondrial dysfunction due to its possible causal role in atherosclerotic CVD. Indeed, in a previous study, adding mtDNA-CN to the 2013 American College of Cardiology/American Heart Association assessment tool further improves discrimination for CVD events that is mainly driven by the improvement for coronary heart disease risk, as well as improves sensitivity and specificity on initiating statin therapy for primary prevention of atherosclerotic CVD[8].

Notwithstanding, chronic diseases are multifactorial with heterogeneous pathophysiology and do not fit with the "one cause – one mechanism – one disease – one therapy" paradigm. A single strategy that only targets oxidative stress might not be sufficient to show significant pathophysiological effects on the complex pathways. Alternatively, "multifactor – multi-treatments" could be more effective since there are possible synergistic benefits from two or more agents with acceptable safety and efficacy. A combination of treatments, for example, traditional treatment plus antioxidants supplementation as adjuvant therapy, may achieve better outcomes than antioxidants supplementation only. For instance, middle-aged type 2 diabetic patients who received metformin treatment plus vitamin E and/or vitamin C had significant improvement of glucose measures as well as lipid profiles compared to patients with metformin treatment[9].

Collectively, further exploration of antioxidants in the prevention and treatment of CVD will remain important given their clear role in disease pathogenesis. Well-selected participants for supplementation and better biomarkers to monitor supplementation effects in trials can provide further insight into the role of antioxidants in CVD.

Inflammation in neurological diseases

Inflammation has emerged as an important mechanism in almost all neurological diseases, including depression and neurodegenerative diseases, such as Alzheimer's disease (AD)[10,11]. Inflammation contributes to disease pathogenesis via both the periphery and central nervous system, the latter of which is often referred to as neuroinflammation that is characterized by microglial activation and the presence of infiltrating leukocytes in the brain parenchyma. Genetic and epidemiological studies have shown robust associations between inflammation and neurological disease, however, therapeutic strategies against inflammation have been proven not very successful. This will be discussed taking AD as an example in this section.

In humans, several genetic variants associated with AD have been discovered in genome-wide association studies (GWAS) and are mapped to genes that are involved in regulating the innate immune response both within the central nervous system and in the blood. For example, genes that encode proteins involved in the regulation of complement activation and phagocytic function

of myeloid cells have been associated with AD, such as Triggering Receptor Expressed On Myeloid Cells (TREM2)[12,13], CD33[14], clusterin (CLU[15]), and complement component (3b/4b) receptor 1 (CR1)[15,16]. Similarly, in epidemiological case-control studies, peripheral inflammation, characterized by high levels of pro-inflammatory proteins such as interleukin-6 and C-reactive protein (CRP) in blood, is related to cognitive decline and neurodegeneration[17]. Moreover, long-term use of non-steroidal anti-inflammatory drugs also reduces the risk for developing AD in large prospective cohorts[18-20]. Nevertheless, in RCTs, both steroid and non-steroidal anti-inflammatory drugs generally failed to show any beneficial effect on cognition or overall AD severity[21,22].

Importantly, it is worth noting that there might be a dual role of inflammation in the progression of diseases, similar to the role of oxidative stress in chronic diseases. The inflammation dynamics frames the AD progress within a lifelong perspective of adaption to inflammatory insults induced by debris and misfolded proteins. Indeed, several studies have shown that the functions of microglia are stage-specific and change dynamically with AD progression. At the preclinical or early stages of AD, microglia can be neuroprotective assisting the clearance of accumulated amyloid beta protein, a major pathological hallmark of AD, whereas at the advanced stages of AD, microglia lose their protective function and shift to a more proinflammatory state via releasing numerous cytokines and chemoattractants, exacerbating neuroinflammation[23]. Similarly, blood monocytes that are capable to work in the blood and to infiltrate into the brain to clear amyloid-beta, share the same dynamics as microglia in the progression of AD[24,25]. This may partly explain the null effects in anti-inflammatory related trials in AD, where suppression of inflammation in patients at the early stages of AD would hypothetically unintendedly inhibit immune cell-mediated phagocytosis, in addition to other heterogeneities in RCTs such as drug dosage, administration time, and duration of the follow-up. Therefore, a too low systemic inflammatory response would probably lead to a less efficient clearance of waste products thus being associated with a high risk of AD.

Nevertheless, current evidence has been predominantly focused on a high inflammatory response in diseases, and few studies have explored the associations between a low inflammatory response and risk of neurological diseases including AD. Not surprisingly, in longitudinal studies in the general population, low levels of circulating CRP[26], complement component 3 (C3)[27], and apolipoprotein E[28,29] were observed to be associated with a higher risk of developing AD and all-cause dementia. This highlights the importance to consider the dynamics of inflammation in the development and progression of neurological diseases, particularly the shift from a beneficial to a harmful role. An in-depth understanding of the dual and complex role of inflammation may provide promising opportunities to identify novel biomarkers in the early detection of disease and facilitate trial designs for drug development.

Mendelian randomization in genetic epidemiology

The rapid technological development and decreased costs in genetic epidemiology have facilitated us to investigate the genetic makeup of multiple com-

plex traits. Notably, the sample size of the datasets for genetic associations has expanded largely in the last few years for the discovery and replication of genome-wide association studies (GWAS) findings, and more and more genetic variants are discovered. This covers a broad range of diseases, lifestyle-related phenotypes, and molecules measured using -omics platforms, including metabolomics[30,31] and proteomics[32,33]. On the one hand, tremendous summary-level data from GWAS are publicly available from most of the large genetic consortia. On the other hand, established mega-biobanks provide another fertile source to conduct research with well-genotyped and high-quality phenotypic data, such as UK Biobank[34], Estonian Biobank[35], China Kadoorie Biobank[36], Million Veteran Program[37], and FinnGen with longitudinal population-based cohort design. Importantly, individual-level data from biobanks with open access upon requirement offers opportunities for in-depth analyses, for example, generating summary statistics with desired covariates adjusted to avoid potential collider bias, conducting subgroup analyses, and investigating non-linear causal effects between exposure and outcome.

The unprecedented availability of these types of data leads to the extreme popularity of MR in research with relatively little effort and expense. Alongside, methodologies related to MR analyses have been considerably advanced over the past ten years. Various novel research methods and extensions of the basic MR design have been proposed in MR analyses attempting to account for possible violations of the instrumental variable assumptions and to overcome potential pitfalls[38]. These methodological approaches include sensitivity analyses of weighted-median estimator[39] and MR-Egger[40] to handle pleiotropy, bidirectional-MR to evaluate causal directions of effect between two traits[41], two-step MR[42] and network MR[43] to assess mediation, multivariable MR to deal with genetically correlated exposures[44], multifactorial MR to account for exposure interaction[45], and MR-clust to cluster genetic variants with similar causal effects[46], among others. These developments substantially raise the implementation and transparency with relative ease to perform MR analyses, especially using the two-sample approach[47]. In the last decade, there has been an exponential acceleration in the number of publications on MR, both in methodology and application, from 61 hits in 2010 to about 1300 in 2021 retrieved from PubMed.

Although available methods allow for rigorous analysis and robust causal inference with different situations, almost all methods assume a linear relationship between the exposure and outcome. It is worth emphasizing that the associations between oxidative stress with CVD are unlikely to be linear[48]. The contributions to a large proportion of CVD from oxidative stress are predominantly among individuals who have excessive amounts of oxidants or insufficient amounts of antioxidants. It is thus important to be aware that any of the relationships are more likely to be detected in specific groups that have a low antioxidants concentration or high oxidative damage. An example goes with the vitamin D debate: high circulating 25-hydroxyvitamin (25[OH]D) are associated with a decreased risk of several diseases and mortality in epidemiological observational studies, whereas RCTs of vitamin D supplementation or two-sample MR approach using summary statistics showed null findings. Interestingly, stratified MR analyses based on individual-level data according to baseline 25(OH)D concentrations suggest a causal relationship between 25(OH)D concentrations and mortality

for individuals with low vitamin D status[49]. Therefore, the inclusion of the overall population in MR studies might be inappropriate and will bias the estimate towards null unless a clear linear association has been shown. The use of proper data and methods[50,51] could identify causal non-linear relationships that might otherwise be undetected.

Furthermore, despite the identified risk factors that could be used for risk classification using prediction, the translation of the effects obtained from MR to clinical interventions should be done with caution. The MR estimates are the effects of genetically predicted risk factors on outcomes, thus representing the effects of lifelong differences in the level of the investigated risk factors. In the real-world studies, therapeutically induced changes in the risk factors by interventions in RCTs normally last only for the duration of the trial, thus representing a short- to medium- period effect of the differences in the risk factor. However, most causal risk factors seem to have cumulative effects on the outcome over time. Consequently, the anticipated effects of RCTs evaluating therapies targeted causal risk factors might be smaller than those from MR studies, especially in chronic diseases with a long development time. Notably, lifelong exposure to per unit lower plasma low-density lipoprotein showed a 3-fold greater reduction in the risk of coronary heart disease than that observed in RCTs with statin treatment started later in life[52].

Taken together, implementation of the best practical MR methodology is needed to infer causality between risk factors with chronic diseases. A cautious application of the results to intervention with optimal strategies will lead to potential targets for the prevention and treatment of chronic diseases.

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