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

Martens, L.G.

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GENERAL DISCUSSION

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Despite successful prevention and treatment options, cardiovascular diseases (CVD) remain a leading cause of death worldwide. This thesis investigated potential mechanisms leading to CVD in order to characterize existing and identify novel targets for future preventive strategies to reduce CVD incidence and associated disease burden. For this aim, the causes of mitochondrial dysfunction, resulting in oxidative stress, and the cerebrovascular consequences in the general population were assessed. Additionally, the causality of risk factors for CVD were studied and possible differences in subgroups of the population were investigated to determine the possibility of more personalized cardiovascular disease risk prediction. In the following paragraphs, the main findings as well as their implications and future perspectives will be discussed.

MAIN FINDINGS

Antioxidants

High levels of reactive oxygen species (ROS) have previously been associated with higher risk of developing CVD [1-3]. As antioxidants are the natural scavengers of ROS, in **Chapter 2** we studied how lifestyle factors are associated with antioxidant levels, and its derivatives, in blood and urine. In a study sample of about 500 participants from the Netherlands Epidemiology of Obesity study [4], we measured serum vitamin E levels and urinary vitamin E metabolite levels as a reflection of antioxidant status. We observed that some lifestyle factors associate differently with vitamin E serum levels as compared to urinary vitamin E metabolite levels. For example, smoking was associated with higher urinary vitamin E metabolite levels, but not with serum vitamin E levels. These findings can be interpreted in the context of higher oxidative stress, which has been associated with smoking behavior [5-7], resulting in the increased turnover of serum vitamin E to urinary metabolites. However, oxidative stress apparently does not result in lower serum vitamin E levels given the null association between smoking and vitamin E levels in serum. Other research in the same study sample showed similar results, where urinary vitamin E metabolites were associated with lower insulin resistance, but not serum vitamin E [8]. Together, these findings indicate that measuring only serum vitamin E levels is not an accurate reflection of antioxidant activity. Since serum vitamin E levels are apparently controlled differently compared with secreted vitamin E metabolites, this might also explain why increasing serum vitamin E levels through supplementation does not significantly reduce disease risk [9, 10]. These results raise the hypothesis that higher urinary vitamin E levels might be a better reflection of oxidative stress. However, this remains to be investigated in large prospective cohort studies in combination with large-scale causal inference studies.

As smoking is one of the major risk factors associated with (any) stroke [11, 12], our observed association between smoking and higher urinary vitamin E metabolite levels could speculate towards the hypothesis that smoking affects stroke pathology, at least partly, via increased oxidative stress levels. Several other studies have also linked smoking behavior to increased oxidative stress levels [5-7], but used different measures as reflection of increased oxidative stress levels. Although higher antioxidant intake has been associated with lower stroke risk in observational cohort studies [3, 13-15], the results of **Chapter 3** show that these associations are likely not causal. When studying disease risk, it is vital to provide evidence whether a risk factor is also a causal factor. However, studying causal factors for cardiovascular disease in an RCT setting is challenging due to the generally long duration of disease development and associated exceedingly high economic costs. In Mendelian Randomization studies, genetic variants are used as instrumental variables to approximate a causal association between an exposure and an outcome. This provides a cheaper, more practical alternative approach. In **Chapter 3**, we investigated both circulating antioxidant blood and metabolite levels and their association with ischemic stroke risk using MR. We found that none of 5 genetically-influenced diet-derived antioxidants (Vitamin E, Carotene, Lycopene, Ascorbate, and Retinol) were associated with ischemic stroke risk. In other words, no evidence was found for a possible causal association between diet-derived antioxidant levels and ischemic stroke. These results are in agreement with the findings that increasing circulating levels of antioxidants via supplementation do not reduce the risk of ischemic stroke in RCTs [16, 17]. A similar study investigating antioxidant levels and coronary heart disease using MR also found no evidence for a causal association [18], as having (partly) overlapping pathophysiological mechanisms. Recent reviews covering oxidative stress mechanisms have suggested that circulating antioxidant levels are indeed not reflective of actual antioxidant capacity [9, 10]. This indicates that increasing antioxidant levels via supplementation is clinically irrelevant for CVD risk reduction, at least in the general population.

Commonly suggested methods to reduce stroke risk are physical exercise and smoking cessation [19]. Although these methods do not increase circulating antioxidant levels, **chapter 2** of this thesis has shown they are associated with altered antioxidant metabolites. This relation again highlights the difference between serum antioxidant levels and antioxidant capacity and might reveal why habitual exercise reduces stroke risk but antioxidant supplementation does not.

Oxidants

To investigate both sides of the oxidative stress mechanism and its relation to CVD, we shifted our focus from antioxidants to oxidants, in the form of ROS. Mitochondria are a major source of ROS production and have evolved elaborate means to scavenge these ROS. Mitochondrial dysfunction may thus result in increased ROS production,

and is therefore considered to be a driver and hallmark of the ageing process [20]. Mitochondrial dysfunction can be proxied using the mtDNA copy number (mtDNA-CN). MtDNA-CN can be assessed by estimating the mtDNA abundance relative to genomic DNA. Therefore, we aimed to study mtDNA abundance, as a proxy for mitochondrial dysfunction, and the effect it might have on the development of cardiovascular diseases such as stroke (**Chapter 4**).

In our prospective analyses, after correcting for potential confounders, we observed no association between mtDNA abundance and stroke. Although extensive sensitivity analyses provided weak evidence for lower mtDNA abundance as a causal factor for ischemic stroke, these results were not in line with the prospective multivariable-adjusted cross sectional analyses. Furthermore, the evidence favoring a possible relationship was only found after the exclusion of pleiotropic SNPs associated with platelet count, which were half of the originally included SNPs. Therefore, caution should be taken when interpreting these results. Further studies will have to shed light on the nature of this type of pleiotropy. One study did report an association between low mtDNA-CN and increased risk of incident stroke [21]. However, we could not replicate these results, while using a much larger sample size and applying triangulation by adding MR analyses in over 1 million participants.

Although mitochondrial dysfunction is thought to increase ROS production, which could damage surrounding cell structures [22], it is questionable whether the effect of increased mitochondrial dysfunction as measured in leukocytes reflects damage induced by ROS that could lead to stroke. For instance, mitochondrial dysfunction in endothelial cells might result in ROS-induced cell damage that lies within the direct causal pathway of stroke development. One study found great variation in mtDNA abundance between liver, kidney, brain, lung, muscle and heart samples, which was interpreted as a reflection of tissue-specific differences in mitochondrial activity [23]. However, another study has shown that there is a correlation between leukocyte mitochondrial dysfunction and metabolic health of other, different tissues [24]. In this paper, mitochondrial dysfunction measured in blood was shown to be predictive for neurodegenerative disease incidence [24], indicating that leukocyte mtDNA abundance could be used to study cerebrovascular disease onset. Importantly, tissue-specific mtDNA abundance itself could be a marker for other health conditions. Obesity causes increased mitochondrial dysfunction in multiple tissues [25, 26], which may be reflected by lower mtDNA abundance. However, a recent study has shown that there was no causal association between genetically-influenced BMI and mtDNA-CN in a large sample from European-ancestry participants from UK Biobank. [27]. Thus, future research will have to disentangle the exact causes of mitochondrial dysfunction, and how mitochondrial dysfunction is linked to adverse health outcomes. In order to continue studying the potential contribution of mitochondrial dysfunction to stroke risk, investigating relevant measurement data, such as mitochondrial dysfunction, from endothelial cells might be the next logical step. However, as blood samples tend to be more readily available, discovering and analyzing blood biomarkers that reflect endothelial dysfunction might be a more practical approach.

RISK MODIFICATION IN SUB-POPULATIONS

In **Chapter 5**, we studied whether established risk factors of CVD exert different effects in specific sub-populations. It is known that inhabitants of lower SES neighborhoods have an increased risk of CVD development [28-31]. Our results showed that the increased risk for CAD attributable to increased BMI can be modified by SES. In other words, an increased genetically-influenced BMI resulted in a higher risk on CAD in low SES groups when compared with the same genetically-influenced BMI increase in high SES groups. Therefore, when tackling obesity as a strategy for CAD prevention, a sub-group specific approach taking SES into account might lead to a proportionally higher reduction in CAD cases and should be considered. However, this approach has some drawbacks. From a social perspective, there might be some ethical considerations. Placing individuals, or city areas, into groups of high or low SES and targeting them with different programs might be stigmatizing [32, 33]. Additionally, as BMI was still associated with increased stroke risk in all SES groups, a separate approach may wrongly convey that the high SES group is devoid from disease risk. Nonetheless, from a scientific point of view it might be relevant to continue to study known associations in greater detail by investigating subgroup specific effects. In the end, by optimizing correct risk allocation, the results of these studies could aid in future individualized treatment options.

REFLECTION ON MAIN FINDINGS

Study population characteristics are one of the main considerations to be taken into account when reflecting on the results of this thesis. Since the research in this thesis used European-ancestry cohorts, it may not be generalizable to other (non-European) populations. Therefore, it is relevant to replicate these studies in datasets of non-European ancestry. In addition, the results from **Chapter 5** demonstrated that individuals of low SES have an increased CVD risk. This group is generally underrepresented in population research, including in the UK Biobank. This indicates that the highest risk group in these studies is simultaneously the least accurately represented in the study population [34, 35]. Therefore, it is possible that our results are an underestimation of the difference between SES groups, as the lowest SES group of the general population might not be fully represented by the lowest SES group of our study population. Furthermore, participants of the UK biobank are thought to be relatively healthy [34]. Participants were between 40 and 70 years of age at inclusion of the study. Although the

UK biobank started in 2005, this can still be considered relatively young to study stroke incidence. This is also reflected in our relatively low number of cases (2%), compared with the general life-time stroke incidence of 25% [36]. As a consequence, a number of participants currently in the control group might become cases when more follow-up years are completed.

Since traditional observational association studies cannot determine causal effects, we combined multivariable-adjusted analyses with MR. However, MR has several underlying assumptions. One of these is that MR assesses the association using the assumed life-long exposure of the determinant in its genetic instrument, whereas the data from the studied population reflects only the outcomes up until the point of data collection. There could be uncertainty as to whether outcomes such as disease occurrence are altered in the future. Increasing follow-up years of the study cohort should decrease the likelihood of this possibility, although the final conclusion might not be drawn for years to come. Additionally, the genetic instruments used in these studies accounted for a relatively low amount of variation of the outcome. Possibly a future GWAS could provide stronger instruments. Alternatively, it is imaginable that genetically derived oxidative stress related to stroke incidence is only activated in combination with adverse lifestyle and environmental factors, which can be assessed by future studies of gene-environment interactions.

CONCLUSIONS AND FUTURE PERSPECTIVES

In this thesis, we observed that lifestyle factors smoking and physical exercise are associated with urinary vitamin E metabolite levels, which we hypothesize to be a marker of antioxidant activity. However, we did not find evidence supporting a causal association between either antioxidant levels or mtDNA abundance and stroke risk. Adiposity and glucose homeostasis were found to be associated with urinary vitamin E metabolites, but not with circulating vitamin E blood levels [8, 37]. Combined with the lack of success of vitamin E supplementation in clinical trials [38, 39], these results support the hypothesis that vitamin E levels do not reflect antioxidant status or capacity. In other words, the rate limiting step in the scavenging process of antioxidants such as vitamin E is not the circulating blood levels but rather a downstream process. Discovering this rate limiting step will be crucial in order to study the real effect of antioxidants on cardiovascular disease risk.

A partial contributor to the observed null-findings could be the studied population. Therefore, if these studies were to be repeated, and replicated, in the future, an older study population might provide more accurate results. Nevertheless, the current associations are still representative for this study population, taking into account age group and follow-up duration. Additionally, the study populations used in this thesis were very large. Even after studying up to 1 million participants, no association between determinants and outcomes was observed. Although by increasing the study population statistical significance could be achieved, the found effect would be so small that clinical (and biological) relevance is highly unlikely. Furthermore, our results show that lower SES groups have worse CVD outcomes with identical risk factors. Future studies would have to take into account that their results might be biased towards a healthier volunteer population. Generally, this would lead to an underestimation of the real population risk factor prevalence, as those with on average worse health variables will be underrepresented [40, 41]. However, even with having a bias towards a healthier population, any found correlation within the study population is likely to be confounded by bias. A study has shown that within-subject analyses are not affected by so-called non-response bias [41]. Nonetheless, new recruitment should focus on obtaining a participant distribution to better reflect the general population.

The scientific field continues to evolve and novel ways to accurately measure oxidative stress are being developed. Therefore, it would be premature to state that the results from this thesis entirely answer the question whether oxidative stress is causally associated with stroke. However, similar studies using the same study population, but investigating different phenotypes reflective of higher oxidative stress levels, found similar results. For instance, genetically determined antioxidant levels were not causally associated with coronary heart disease [18]. Additionally, no evidence was found for the causal association between low mtDNA copy numbers and type 2 diabetes risk [27].

As science becomes increasingly data-driven and larger datasets become (publicly) available, future studies should be able to carry out more in-depth research, increasing the potential to provide conclusive evidence of the relation between oxidative stress and cardiovascular disease. A higher number of participants would not only increase the number of cases, but additionally allow for the investigation of subgroup specific effects. Although the current studied population in itself was considerably large, our results in **Chapter 5** have shown that there could be relevant differences in risk effect between two subgroups within a specific population. A previous study has presented similar results where CAD risk attenuated with age [42]. Therefore, it would be naïve to treat such a case group as homogeneous. Future studies could focus on true personalization of risk by further disentangling sub-group specific effects. In turn, this would open up possibilities for individualized medicine and prevention strategies.

This thesis provides novel insights in the relationship between oxidative stress and CVD using both biological and genetic data. Although associations were established, no conclusive evidence supporting a causal association between oxidative stress and CVD was found. However, as CVD is thought to be a complex and heterogenous disease, it is important to keep in mind that there are likely multiple pathways that together affect the progression of this disease. Our results already showed a complex combined relationship between genetically-influenced BMI, low SES, and CVD. When taking into

account the long onset of CVD, future research demands a more sophisticated approach in order to explain the underlying mechanisms of CVD development. For example, data on risk factors should be gathered throughout a person's lifespan, starting from a relatively young age. This would result in a better visualization of the true exposure per risk factor for each participant. This could be combined with individual genetically determined risk factors. Individuals who are at increased risk would then be invited for periodic screening. Although this method might be initially expensive, if effective, the reduction of CVD cases and patients would ultimately lower the burden on our healthcare system [43, 44]. Consequently, in order to truly combat the global healthcare burden caused by CVD, interventions might have to aim for a younger target population and continuous monitoring of risk factors over the life course.

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