Peer-Review Record

Can age be a modifiable risk factor? the impact of dietary patterns on the molecular mechanisms that underlie cardiovascular aging

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Reviewer 1: Anonymous

Reviewer 2: Anonymous

Round 1

Reviewer 1 Report

This review integrates what is known about mechanisms of vascular aging, mostly in the context of atherosclerosis development, and the beneficial or detrimental effects of diet. The review is well-written, easy to follow and formatted in a consistent manner, making navigation of the text easy. There are a number of recent reviews of vascular aging (e.g. Clayton et al. PMID: 36337728) in 2022. A focused review on MDP and aging (not just vascular aging) was also recently published (PMID: 36029811). Another 2022 review was on plant-based diets and vascular disease (PMID: 35807796). Finally in 2021 a review of dietary choices and premature vascular aging was published (PMID: 34393048). There is significant overlap between these and the current review. However the organization of the current review separating inflammation from ROS, considering IF and inclusion of epigenetic factors provide sufficient rationale for this review filling a void in the literature. There are several suggestions for revision.

Specific comments:

1. The Central Illustration could be improved to include some specificity. The cartoon does not indicate which stimuli (ox stress, epigenetics, inflammation) contribute to which components of aging. For example, substantial data link oxidative stress mechanistically to endothelial dysfunction and this may be responsible for hypertension and/or atherosclerosis Thus another layer of signalling might be warranted in the figure. Fig 1 gets at this but nonspecifically links every aging promotor box's content with every other box's

content (characteristics of each aging promotor). For example it could be gleaned from fig 1 that IR causes noncoding RNA levels to change and vice versa. The figure is too generic and some specificity is needed. Much of the damage from epigenetics and inflammation arises from underlying excess oxidative stress, but this is not evident in Fig.1.

2. The section on ROS is somewhat superficial. Rather than talking only about oxidative stress and ROS, the text should be more specific as to the species of ROS involved (e.g. peroxynitrite, h2o2, superoxide) and location of ROS generation felt to be important in aging vs disease (NADPH oxidase, mitochondria, xanthine oxidase, loss of SOD or catalase or glutathione).

3. Very little attention was paid to the concept that vascular aging and dietary interventions might result from changes in gut microbial flora. More discussion is needed. What in the diet is causing the change in gut flora? How does the effect occur (is it due to metabolism of food or beneficial substances released from gut flora or something else)?

4. Figure 2: The most significant effect of inflammation in the process leading to atherosclerosis is loss of endothelial NO. This leads to a vascular proliferative state with cytokines and adhesion molecule upregulation. The inflammatory elevation in ROS may be what stimulates inflammation in the liver but this concept is lacking in the figure.

Other comments

5.Be sure to refer to original articles and not to other reviews when possible (e.g. reference 21 cited on line 176 on page 9).

6. Each section of the paper should have a synthesizing paragraph interpreting the body of literature cited, helping the reader to navigate conflicting data, describing new concepts or ideas prompted by existing literature. This is done well in some sections (IF treatment of inflammation) but not in others (MDP and oxidative stress). This is particularly needed following the discussion of IF on lines 513-522 where the reader is left hanging after reading results of several articles.

7. All of the discussed aging mediators (inflammation, oxidative stress, and epigenetics) lead to atherosclerosis. Are these three factors all part of the same signaling pathway working through a common mechanism or are they part of parallel independent pathways or something else. A paragraph outlining or speculating about these relationships is needed.

8. How do you differentiate aging-induced changes vs. age-unrelated development of atherosclerosis?

9. The epigenetics section has a nice introductory discourse but does not do a good job of linking specific methylation or acetylation sites or specific mIRs with their respective effects on regulation of intracellular pathways involved in atherosclerosis, endothelial dysfunction, and arterial stiffness.

10. Do the intermediate endpoints of arterial stiffness, diastolic dysfunction and endothelial function change similarly in MDP and PBP and IF or are there differences?

11. What is known about clinical trials of MDP on hard outcomes like MACE, rather than on biomarkers of atherosclerosis?

12. If data exist, please add a paragraph commenting on whether there is additive effect of IF on either MDP or PBP

13. In Figure 3 the vertical blue 'down' arrow should be a blocking arrow not a stimulating one since the 4 effectors of a healthy diet REDUCE ox stress in aging. Furthermore the title should be something like "beneficial dietary patterns" since western diets would not produce these effects.

14. It might be mentioned that epigenetic modifications occur in the fetus of mothers with stress, leading to heightened sensitivity to cv risk factors early in life and early onset HTN and CVD in the offspring. This may in part explain hereditary components of atherosclerosis arterial stiffness and CV risk factors like HTN.

Author Response

1. The Central Illustration could be improved to include some specificity. The cartoon does not indicate which stimuli (ox stress, epigenetics, inflammation) contribute to which components of aging. For example, substantial data link oxidative stress mechanistically to endothelial dysfunction and this may be responsible for hypertension and/or atherosclerosis Thus another layer of signalling might be warranted in the figure. Fig 1 gets at this but nonspecifically links every aging promotor box's content with every other box's content (characteristics of each aging promotor). For example it could be gleaned from fig 1 that IR causes noncoding RNA levels to change and vice versa. The figure is too generic and some specificity is needed. Much of the damage from epigenetics and inflammation arises from underlying excess oxidative stress, but this is not evident in Fig.1.

We thank the Reviewer for the comments, which have now substantially improved our Central Illustration. We have now adjusted the path diagram to more accurately represent the pathobiology of cardiovascular aging. Importantly and per the Reviewer comments, we have now shown how insulin resistance can induce epigenetic changes. Additionally, we have added illustrative annotation to the Central Illustration, which may benefit the readers.

2. The section on ROS is somewhat superficial. Rather than talking only about oxidative stress and ROS, the text should be more specific as to the species of ROS involved (e.g. peroxynitrite, h2o2, superoxide) and location of ROS generation felt to be important in aging vs disease (NADPH oxidase, mitochondria, xanthine oxidase, loss of SOD or catalase or glutathione).

We thank the Reviewer for this comment. We added a paragraph describing specific ROS and the location of their generation. The most studied ROS species in aging and disease are hydrogen peroxide and superoxide radical, and the most relevant locations were mitochondria and peroxisome (with beta oxidation of lipids).

"Oxidative stress refers to the imbalance between production and neutralization or elimination of reactive oxygen species (ROS). ROS include both free radicals and molecules that have the potential to become free radicals. Free radicals are molecules that contain unpaired electrons in their outer orbit, rendering them unstable and prone to react with other molecules by exchange of electrons. Non-free radical ROS can be reduced to form free radicals. Free radicals include superoxide, hydroxyl, peroxyl, hydroperoxyl and alkoxyl radicals. Other species that can generate free radicals include hydrogen peroxide, hydroxide ion, and peroxides(79). Exchanging of electrons by free radicals alters the structure of important molecules including DNA, proteins, and lipids(80). Some ROS-induced changes are necessary for certain biological processes, such as signaling. However, they can also cause damage to these molecules which ultimately can accumulate and lead to further cellular damage (81, 82).

The primary location of ROS generation is the mitochondria, by electron leakage from the electron transport chain (ETC). This occurs when NADH or FADH donate electrons to oxygen or water, producing hydrogen peroxide or superoxide (83, 84). However, additional processes in the cell can generate ROS, including beta oxidation of fatty acids which produces hydrogen peroxide which occurs in the peroxisome (85)."

3. Very little attention was paid to the concept that vascular aging and dietary interventions might result from changes in gut microbial flora. More discussion is needed. What in the diet is causing the change in gut flora? How does the effect occur (is it due to metabolism of food or beneficial substances released from gut flora or something else?)

We thank the Reviewer for the comment which will add more depth to this discussion. The literature available suggests that this topic is still under investigation and the exact mechanisms are evolving. We have added a brief paragraph providing more detail regarding the mechanism by which dietary patterns, specifically the MDP, may alter gut microbial flora.

"It is further suggested that following a MDP can alter the microbiota of the gut, leading to lower levels of cholesterol and increased short-chain fatty acids that contribute to reduced cardiovascular risk (42, 43). There is ongoing investigation to determine the exact mechanisms by which dietary patterns such as MDP have a dynamic and beneficial effect on gastrointestinal flora composition. One proposed hypothesis suggests that MDP may select against bacteria that utilize simple sugars and oligosaccharides while promoting growth of bacteria that utilize polyphenols, polysaccharides, and soluble fibers (42, 44)."

4. Figure 2: The most significant effect of inflammation in the process leading to atherosclerosis is loss of endothelial NO. This leads to a vascular proliferative state with

cytokines and adhesion molecule upregulation. The inflammatory elevation in ROS may be what stimulates inflammation in the liver but this concept is lacking in the figure.

We thank the Reviewer for the comment. We have now modified Figure 2 to show that loss of nitric oxide is the primary component of endothelial dysfunction. Additionally, we have adjusted the wording to show that both cytokines and reactive oxygen species induce protein expression and release of inflammatory mediators via the liver.

5. Be sure to refer to original articles and not to other reviews when possible (e.g. reference 21 cited on line 176 on page 9).

We have cited primary sources as much as possible.

6. Each section of the paper should have a synthesizing paragraph interpreting the body of literature cited, helping the reader to navigate conflicting data, describing new concepts or ideas prompted by existing literature. This is done well in some sections (IF treatment of inflammation) but not in others (MDP and oxidative stress). This is particularly needed following the discussion of IF on lines 513-522 where the reader is left hanging after reading results of several articles.

We thank the Reviewer for the comments. We have added paragraphs to assist the reader with synthesizing the information presented throughout the review.

"This data is promising and suggests that the MDP decreases the level of inflammatory modulators that contribute to hypertension and atherosclerosis. The mechanisms for this anti-inflammatory effect are most likely due to increased consumption of polyphenols and long-chain fatty acids, direct attenuation of immune cell pathways, anti-platelet effects, and alteration of GI flora. The above studies underscore the culmination of this anti-inflammatory effect, with a decrease in molecules such as CRP, IL-6, TNF-a, IL-8, and MCP-1 that are associated with atherosclerotic disease. In addition to these microbiological effects, there is substantial evidence to support benefits of the MDP on inflammatory conditions such as hypertension, hyperlipidemia, and diabetes mellitus which can lead to improved cardiovascular outcomes (46-52)."

"PBP does appear to decrease levels of inflammatory molecules such as CRP. This is likely a result of increased consumption of anti-inflammatory nutrients as well as decreased exposure to inflammatory, catalytic molecules derived from processed carbohydrates and animal products that lead to increased inflammation and oxidate stress. This anti-inflammatory effect appears to be stronger over a long-term duration. However, there is a need for additional clinical studies to better understand the effect on other inflammatory cytokines."

"A western dietary pattern clearly contributes to the epigenetics changes leading to enhanced cardiovascular risk and premature cardiovascular aging. However, some of the evidence reviewed shows that simple modifications to the traditional western diet pattern including limiting dietary fat and salt can help mitigate and potentially reverse some of the

adverse effects of the western diet. It is important to characterize the negative epigenetic effects of the western diet, as it may yield diagnostic and therapeutic targets in future studies. Additional investigation is needed to characterize further epigenetic mechanisms through which the western dietary pattern leads to cardiovascular aging."

"Dietary patterns cause alterations in levels of oxidative stress, ultimately leading to differing effects on cardiovascular aging. Increased oxidative stress has been shown to lead to accelerated atherosclerosis, endothelial dysfunction, increased arterial stiffness, and diastolic dysfunction. CR, IF, and the MDP have all been shown to decrease levels of oxidative stress while the western dietary pattern increases levels of oxidative stress. Both the MDP and CR have been extensively studied and have significant support for their effects on reducing oxidative stress. Research on IF is scarce, but some studies have demonstrated promise towards the improvement of oxidative stress in humans although it is unclear if this is the main mechanism of its benefit."

7. All of the discussed aging mediators (inflammation, oxidative stress, and epigenetics) lead to atherosclerosis. Are these three factors all part of the same signaling pathway working through a common mechanism or are they part of parallel independent pathways or something else. A paragraph outlining or speculating about these relationships is needed.

We thank the Reviewer for the comment and now have now provided a paragraph below to denote the interplay of inflammation, epigenetics, and oxidative stress.

"Inflammation may occupy a central role in modifying the epigenome and vice versa. For example, cytokines have the potential to induce changes in chromatin; however, cytokines themselves are under epigenetic regulation1. On the per-person level, individuals with clinical CVD and high C-reactive protein levels have been found to have DNA hypermethylation in peripheral blood leukocytes2. There have been several epigenetic modifiers, including TET2, DNMT3A, HDAC3, HDAC9, JMJD3, and KDM4A that have been found to be important in the epigenetic modeling, via methylation and histone modification, of macrophages which are a primary cell line involved in oxidative stress reactions3. Thus, inflammation, oxidative stress, and epigenetics seem to have co-interdependent pathways that underly the initiation, progression, and regression of atherosclerosis."

(1). Wierda RJ, Geutskens SB, Jukema JW, Quax PHA, van den Elsen PJ. Epigenetics in atherosclerosis and inflammation. *J. Cell. Mol. Med.* 2010;14(6 A).
(2). Stenvinkel P, Karimi M, Johansson S, et al. Impact of inflammation on epigenetic DNA methylation - A novel risk factor for cardiovascular disease? *J Intern Med* 2007;261(5).
(3). Jin F, Li J, Guo J, et al. Targeting epigenetic modifiers to reprogramme macrophages in non-resolving inflammation-driven atherosclerosis. *Eur Hear J Open* 2021;1(2).

8. How do you differentiate aging-induced changes vs. age-unrelated development of atherosclerosis?

We thank the authors for this comment. Although age is one of the primary drivers of atherosclerosis, we wanted to elucidate the mechanisms that overlap between age and

atherosclerosis thereby making a connection between the mechanism and atherosclerosis development. That distinction, we hope, will address this comment.

9. The epigenetics section has a nice introductory discourse but does not do a good job of linking specific methylation or acetylation sites or specific mIRs with their respective effects on regulation of intracellular pathways involved in atherosclerosis, endothelial dysfunction, and arterial stiffness.

We thank the Reviewer for the comment and have now provided an additional information within the sections highlighting DNA methylation and histone modification on specific pathways leading to mechanisms cardiovascular aging including atherosclerosis and ventricular dysfunction

DNA Methylation:

"Genes affected included several homeobox genes previously implicated in vascular diseases and TBX20, which was associated with upregulation of PPAR-ϒ and endothelial protection (147). Chronic inflammation is involved in development of atherosclerosis, and DNA methylation is associated with regulation of inflammatory signal transduction pathways and expression of molecules such as TNF-a, IL-4, IL-6 and ICAM-1 (145, 148, 149)."

"Expression of both DNMTs and TETs are likely key regulators in this process, and have been shown to be implicated in expression of genes implicated in cardiac hypertrophy such as Myh6 and Xirp2 (152, 153)."

Histone Modification and Chromatin Remodeling:

"HDACs plays a key role in the development of vascular hypertrophy and pathogenesis of hypertension, with one example being the facilitation of vascular smooth muscle proliferation by interaction of GATA-binding factor 6 and HDAC4 (159). HDACs also play a key role in the pathogenesis of LV hypertrophy and impaired myocardial relaxation, a process which was able to be lessened by a novel HDAC inhibitor in a murine model (160)."

"The role of histone methylation in this setting is quite complex and can promote or inhibit cardiac hypertrophy. Increased activity of JMJD2A, a demethylase of trimethylated H3K9, is associated with cardiac hypertrophy in both human and mice models (161). There is also evidence to suggest that additional histone modifications such as phosphorylation play key roles in cardiac hypertrophy, but more research is needed to characterize the mechanism of these changes (152)."

10. Do the intermediate endpoints of arterial stiffness, diastolic dysfunction and endothelial function change similarly in MDP and PBP and IF or are there differences?

We thank the Reviewer for the comment and have now provided an additional section succinctly addressing the effect of different dietary patterns on intermediate endpoints.

"Intermediate Endpoints
It is important to consider intermediate endpoints associated with the molecular mechanisms of cardiovascular aging. While there is limited data directly comparing the three dietary patterns discussed, there is literature available to assess the effects of these dietary patterns on arterial stiffness, blood pressure, and diastolic dysfunction.

There are a number of studies which demonstrate a decrease in arterial stiffness with higher compliance to MDP, PBP, or IF (69-71). However, the design of the studies are heterogenous, making it difficult to create a direct comparison among the dietary patterns.

Similarly, there have been studies assessing the impact of these three dietary patterns on left ventricular systolic and diastolic function. One cross-sectional study showed that higher levels of adherence to the MDP can lead to improved LV structure and function compared to lower adherence (72). A RCT completed during the PREDIMED trial revealed decreased levels of N-terminal pro-brain natriuretic peptide compared to low-fat diet, which may also suggest improvement in diastolic function (73). A different cross-sectional study of 133 individuals over the age of 60 noted a lower prevalence of diastolic dysfunction in individuals who adhered to a PBP compared to a non-PBP (74). There are limited studies on the effect of IF on left ventricular function; however, animal studies suggest it could improve systolic function and decrease left ventricular remodeling (75).

Meta-analyses have also revealed modest improvement in systolic and diastolic blood pressure in patients following a PBP, with one study noting an average decrease in systolic blood pressure of 2.66 mmHg and 1.69 mmHg decrease in diastolic blood pressure (76). A comparative study showed a larger decrease in systolic and diastolic blood pressure in the Mediterranean diet group (-9.3 and -7.3 mmHg respectively) compared to the PBP group (-3.4 and -4.1 mmHg) (77). A separate study followed 1422 individuals who adhered to IF for one year and found that the average systolic blood pressure decreased from 131.6 mmHg to 120.7 ($P < 0.001$), and the average diastolic blood pressure decreased from 83.7 to 77.9 ($P < 0.001$) (78).

This data suggests all three dietary patterns may have a beneficial effect on these intermediate endpoints. It further suggests that MDP and IF may provide a larger decrease in blood pressure when compared to PBP. However, there is less data available directly comparing the effect of these dietary patterns on arterial stiffness and diastolic dysfunction. Moreover, the indices used to compare these two endpoints are variable, which further complicates a comparative analysis. This could provide a basis for future research trials, as more RCT's directly comparing the effect of these dietary patterns on arterial stiffness and diastolic dysfunction may provide valuable insight."

11. What is known about clinical trials of MDP on hard outcomes like MACE, rather than on biomarkers of atherosclerosis?

We thank the Reviewer for the comment and have now provided a paragraph to address the clinical effects of MDP on MACE.

"Given the reduction in inflammatory factors and risk of atherosclerosis, studies have further sought to quantify the effect of MDP on major adverse cardiac events (MACE). The PREDIMED trial noted a relative decrease in risk of major cardiac events such as myocardial infarction, stroke, and death from heart disease by 30% in the MDP supplemented with olive oil or tree nuts when compared to the control group (45). In another trial, 605 individuals who previously suffered a myocardial infarction consumed either MDP or a Western diet for 4 years. The group following a MDP had significantly fewer cardiac deaths, cardiovascular events, and hospitalizations (46). Moreover, the REGARDS trial showed a trend toward lower risk of SCD in a large sample of individuals who followed a Mediterranean diet when compared to other dietary patterns (47)"

12. If data exist, please add a paragraph commenting on whether there is additive effect of IF on either MDP or PBP.

To our knowledge, this data exists in the literature about ketogenic diet but we did not encounter data that talked about these dietary patterns and additive effects.

13. In Figure 3 the vertical blue 'down' arrow should be a blocking arrow not a stimulating one since the 4 effectors of a healthy diet REDUCE ox stress in aging. Furthermore the title should be something like "beneficial dietary patterns" since western diets would not produce these effects.

Changes made to Figure 3.

14. It might be mentioned that epigenetic modifications occur in the fetus of mothers with stress, leading to heightened sensitivity to cv risk factors early in life and early onset HTN and CVD in the offspring. This may in part explain hereditary components of atherosclerosis arterial stiffness and CV risk factors like HTN.

We thank the Reviewer for the comment and have now provided commentary on the impact of maternal-fetal epigenetics and impact on cardiovascular risk, particularly as an area of future research.

"Maternal diet and nutrient supplementation can lead to direct epigenetic changes in offspring predisposing to cardiovascular risk factors and disease (138, 139)."

"Lastly, additional research is needed to assess the impact of maternal diet on epigenetics and cardiovascular disease in offspring. Maternal exposure to both famine and overnutrition have been shown to alter cardiovascular risk in offspring, but there is little data assessing the epigenetic mechanisms of this phenomenon or if any of the beneficial dietary patterns discussed would provide a cardioprotective benefit to subsequent generations."

Reviewer 2 Report

The review article entitled "Can Age Be a Modifiable Risk Factor? The Impact of Dietary Patterns on the Molecular Mechanisms that Underlie Cardiovascular Aging" is very interesting. However, it has some minor concerns.

1. All the figures need to be well presented and improved.

2. Dietary pattern and Cardiovascular Aging from different countries can be discussed more in the manuscript.