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## Modifiable cardiovascular risk, hematopoiesis and innate immunity

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### Abstract

Unhealthy diet, lack of exercise, psychosocial stress and insufficient sleep are increasingly prevalent modifiable risk factors for cardiovascular disease. Accumulating evidence indicates that these risk factors may fuel chronic inflammatory processes that are active in atherosclerosis and lead to myocardial infarction and stroke. In concert with hyperlipidemia, maladaptive immune system activities can contribute to disease progression and increase the probability of adverse events. In this review, we discuss recent insight into how the above modifiable risk factors influence innate immunity. Specifically, we focus on pathways that raise systemic myeloid cell numbers and modulate immune cell phenotypes, reviewing hematopoiesis, leukocyte trafficking and innate immune cell accumulation in cardiovascular organs. Often, relevant mechanisms that begin with lifestyle choices and lead to cardiovascular events span multiple organ systems, including the central nervous, endocrine, metabolic, hematopoietic, immune and, finally, the cardiovascular system. We argue that deciphering such pathways provides not only support for preventive interventions but also opportunities to develop biomimetic immunomodulatory therapeutics that mitigate cardiovascular inflammation.

### Keywords

atherosclerosis; hematopoiesis; macrophages; lifestyle; arteriosclerosis; inflammation; sleep; stress; metabolism; bone marrow

### Subject Terms:

Atherosclerosis; Lifestyle; Risk Factors

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## Introduction

Our highly dynamic understanding of atherosclerosis has generated certainty: the involvement of an overactive and maladaptive immune system is no longer debated<sup>1-4</sup>. Indeed, the Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS), which reduced cardiovascular events by targeting the inflammatory cytokine interleukin-1 $\beta$ <sup>5, 6</sup>, marks the end of the era during which the role of inflammation was emerging. With the notion that dampening inflammation may reduce cardiovascular events now widely accepted, the field is entering a new phase of discovery, with the goal of translating this insight into immunomodulatory cardiovascular therapeutics with acceptable safety profiles. A key challenge is to find therapeutic targets that reduce harmful inflammation without compromising host defense and immune system function. It may take considerable time and resources to meet this and other challenges, but with a growing understanding of the complexity and heterogeneity of the immune system and rapidly improving research tools, we are now identifying previously unknown mechanisms and pathways linking inflammation and atherosclerosis. The motivation for this work could not be more clear: atherosclerosis and its complications are still the leading cause of mortality world-wide<sup>7</sup>.

It has long been evident that lifestyle vastly affects cardiovascular health<sup>8-15</sup>. Five years ago, we argued that it may be worthwhile to probe how lifestyle affects cardiovascular inflammation<sup>16</sup>. Meanwhile, the strong connections between lifestyle-related risk factors and the immune system have become increasingly clear, as has these connections' influence on cardiovascular health. We here revisit this argument to review new data, discuss emerging opportunities and highlight promising areas that require further investigation. Specifically, we discuss how diet, sleep, psychosocial stress and physical activity influence systemic numbers and phenotypes of innate immune cells, as well as their production and supply to atherosclerotic lesions.

## Risk factors and importance of lifestyle for atherosclerosis

The growing understanding of how specific cardiovascular risk factors lead to the development and progression of atherosclerosis has enhanced our ability to assess each individual patient's risk more precisely. Epidemiological studies have identified the relative importance of atherosclerotic risk factors, which can be categorized into non-modifiable and modifiable. Non-modifiable factors include age, sex, ethnicity and hereditary factors. Modifiable risk factors include smoking, hypertension, obesity, diabetes, high levels of low-density lipoprotein, low levels of high-density lipoprotein and increased cholesterol and triglycerides<sup>17</sup>. The prevalence and potency of these risk factors vary. Often, risk factors act in concert to promote atherosclerosis, which is a multifactorial disease. Delving into risk factors' precise contributions to and mechanistic roles in the pathogenesis of atherosclerosis has fueled important therapeutical advances<sup>18</sup>, such as HMG-CoA reductase inhibitors that lower low-density lipoprotein cholesterol<sup>19, 20</sup> and antihypertensive drugs that reduce blood pressure<sup>21-24</sup>, both of which potently inhibit atherosclerotic progression and reduce its

complications. A lifestyle-related example is smoking, which was estimated to contribute to approximately one third of coronary deaths in the 1980s<sup>25</sup>.

In addition to these well-established risk factors, we recognize that certain lifestyle choices such as a sedentary behavior<sup>26</sup> and exposure to psychosocial stress<sup>27, 28</sup>, air pollution<sup>29</sup> and environmental noise<sup>30</sup> associate with increased CVD risk. Several large-scale cohorts, including a prospective observational study in 20,721 Swedish men, demonstrated that adhering to a healthy lifestyle associated with a 79% reduction (95% CI: 34% to 93%) in the incidence of myocardial infarction<sup>31–33</sup>, and another study with 461,211 participants reported a hazard ratio of 0.61 (95% CI: 0.56 to 0.66) for ischemic stroke<sup>12, 34</sup>. Such health benefits likely also extend to high-quality sleep<sup>35, 36</sup>, regular physical activity<sup>14</sup> and a healthy diet<sup>37–39</sup>. Mechanisms by which lifestyle exerts beneficial effects are likely manifold and remain incompletely understood; we argue that protective mechanisms likely involve the immune and hematopoietic systems.

## Hematopoiesis in CVD

Hematopoiesis is the process by which blood cells are continuously replenished. This life-long renewal process is well regulated and able to respond to increased demands, as when blood cells are lost due to bleeding or infection. Hematopoietic stem cells (HSCs) form the apex of the differentiation cascade and are unique in their ability to self-renew and diversify into any blood cell. They reside almost exclusively in the bone marrow and are relatively rare and mostly quiescent<sup>40–43</sup>. HSC give rise to various downstream hematopoietic stem and progenitor cells (HSPC) that proliferate at higher frequencies than HSC and are more restricted to distinct lineage differentiation potentials<sup>44</sup>. This process of differentiation and self-renewal is tightly regulated by a complex interplay of cell-intrinsic and microenvironmental factors in order to maintain hematopoietic homeostasis<sup>45</sup>. Within the bone marrow, there are special localized areas with a highly regulated microenvironment referred to as the ‘stem cell niche’. These niches are formed by several stromal cells, including endothelial cells, osteoblasts, macrophages and various mesenchymal stromal cells, which secrete messengers known as ‘niche factors’. In concert with growth factors (e.g., M-CSF, GM-CSF), niche factors such as the cytokines Cxcl12 and Scf1 regulate HSPC proliferation and maintenance. Other molecules, e.g. angiopoietin-1, thrombopoietin and osteopontin, contribute to HSPC maintenance by binding to receptors expressed by these cells<sup>46–50</sup>. Additionally, adhesion molecules like Vcam-1 and E-selectin retain HSPC and leukocytes in the bone marrow<sup>51</sup>. Once committed, myeloid progenitor cells differentiate into monocytes and neutrophils. Their release into circulation is rigidly controlled by various signals, including the Ccr2 ligand Ccl2 and the Cxcr4 ligand Cxcl12. Hematopoiesis and leukocyte trafficking between tissues and circulation are influenced by circadian rhythms<sup>52–58</sup>. For further reading on how the circadian rhythm controls hematopoiesis, we refer to several excellent reviews<sup>54, 59–61</sup>.

The hematopoietic system responds sensitively to cardiovascular events such as myocardial infarction, after which bone marrow myeloid progenitors and monocytes expand<sup>62</sup> and circulating leukocytes are recruited to the ischemic myocardium<sup>63, 64</sup>. After stroke, myelopoiesis increases while the hypothalamic-pituitary-adrenal axis mediates B

lymphopoiesis defects<sup>65–70</sup>. Myeloid cells recruited to the injured brain may contribute to neural recovery but also to reperfusion injury<sup>71, 72</sup>. Atherosclerosis leads to low-grade, systems-wide chronic inflammation during which, as described in ApoE<sup>-/-</sup> mice with atherosclerosis, blood monocyte levels increase progressively<sup>73–75</sup>.

The increased bone marrow activity in CVD centers on myelopoiesis and is elicited by an expanding number of signals. Hyperlipidemia is prominently among these, acting directly on HSPC and leukocytes via cholesterol efflux pathways<sup>76, 77</sup>. For example, ABCA1, ABCG1 and HDL, which clear cholesterol from macrophages, inhibit HSPC proliferation, reducing the production of atherogenic leukocytes<sup>77</sup>. Experimentally, *Abca1*<sup>-/-</sup> and *Abcg1*<sup>-/-</sup> –deficient mice exhibit leukocytosis and increased numbers of stem and progenitor cells, thus exaggerating atherogenesis<sup>77</sup>. The mechanistic links connecting hypercholesterolemia with hematopoiesis were clarified by studies reporting that apoA-I binding protein (AIBP), which mediates cholesterol efflux, controls HSPC activity<sup>76, 77</sup>. AIBP activates Srebp2, which is a transcription factor relevant for cholesterol synthesis<sup>76</sup>. Srebp2 up-regulates Notch signaling, thereby enhancing hematopoietic stem cell expansion. In human blood, LDL cholesterol correlates with HPSC numbers and Srebp2, and HPSC from individuals with high LDL cholesterol had higher Notch gene expression than HSPC from individuals with low LDL cholesterol<sup>76</sup>. In mice, Srebp2 inhibition can prevent HSPC emergence. These studies<sup>76, 77</sup> link hypercholesterolemia with hematopoiesis in atherosclerosis and identify cholesterol efflux pathway genes as key regulators of hematopoiesis. For a more detailed overview of how atherosclerosis influences hematopoiesis and the bone marrow niche, we refer to a related review<sup>78</sup>.

## Immune cells in cardiovascular pathology

Macrophages, white blood cells that are part of the innate immune system, are found in large numbers in all healthy organs, where they closely interact with their surroundings<sup>79, 80</sup>. During homeostasis, macrophages self-renew through local proliferation, with only a small proportion arising from monocyte recruitment. Macrophages' primary functions involve removing debris and pathogens. Over the past decade, it has become clear that macrophages pursue many additional functions, which depend on their origins, microenvironment and phenotype. For example, macrophages support cardiac conduction by interacting with cardiomyocytes<sup>81</sup>. Yolk sac-derived embryonic macrophages promote coronary artery development<sup>82</sup> and aortic LYVE-1+ macrophages moderate steady-state arterial tone by interfacing with smooth muscle cells and collagen<sup>83</sup>. These tasks may be disrupted during inflammatory conditions such as myocardial infarction and atherosclerosis, when local resident macrophages die<sup>84</sup> and inflammatory, monocyte-derived macrophages engage with surrounding stromal and immune cells, secreting proinflammatory molecules that contribute to tissue repair or destruction<sup>85</sup>. After myocardial infarction, tissue-resident macrophage numbers and phenotypes also shift in remote, uninjured organs such as the lung, liver, brain and kidney<sup>86</sup>. Other systemic inflammatory conditions, particularly sepsis, likewise evoke systemic macrophage adaptations that may affect cardiovascular health<sup>86</sup>. It is largely unknown how lifestyle factors shape resident leukocytes in tissue.

The pathogenesis and progression of atherosclerosis are closely linked to inflammatory processes within the arterial wall, where modified lipoproteins are rendered proinflammatory and activate the overlying endothelium<sup>87</sup>. The consequence is a chronic, low-grade immune response that recruits additional leukocytes, including monocyte-derived phagocytes, into the subendothelial space. During atherosclerosis development, increased levels of circulating LDL cholesterol lead to vascular wall deposits, which are taken up by macrophages that then become foam cells. These cholesterol-laden plaque macrophages do not readily remove the lipid material from the vascular wall. Rather, these cells accumulate locally, enhance inflammatory processes and eventually die, contributing to the formation of the necrotic core often seen in ruptured plaques. In healthy and diseased mice, the arterial macrophage population is heterogeneous and consists of several subtypes that vary in function and gene expression profile<sup>2, 87–89</sup>. Although some data suggest some lesional macrophage-like cells may arise from smooth muscle cells<sup>90, 91</sup>, most macrophages stem from hematopoietic progenitors in the bone marrow and spleen<sup>73–75</sup>. Plaque macrophages' comparatively short four-week lifespans, measured in mice with atherosclerosis<sup>92</sup>, necessitate continuous replenishment. Macrophages in early atherosclerotic lesions almost all derive from recruited monocytes, while in later lesions most arise from local macrophage proliferation, though their local ancestry still descends from monocytes<sup>92</sup>. In the lesion, macrophages accumulate progressively and adopt phenotypes that fuel inflammatory processes, leading to tissue destruction<sup>93, 94</sup>. These inflammatory macrophages can activate their surroundings, include tissue-resident stromal and other immune cells, and release inflammatory chemokines and cytokines, which recruit more leukocytes to the lesion. In the long run, macrophages contribute to plaque destabilization by amplifying local inflammation and secreting various proteases that destabilize fibrous components of the plaque.

Other immune cells, including neutrophils and lymphocytes, also inhabit the atherosclerotic plaque and contribute to its growth and destabilization. Because lesional neutrophils are relatively rare, their contributions were long underestimated<sup>95</sup>. Recent studies, however, have demonstrated their presence and role in the pathogenesis of atherosclerosis<sup>96–98</sup>. For instance, neutrophils can deposit granule proteins (e.g., proteinase-3, azurocidin, cathepsin G, neutrophil elastase) on the endothelium, impairing endothelial function, inducing adhesion and promoting inflammatory monocyte recruitment<sup>99–103</sup>. The cells promote erosion of the endothelium<sup>104</sup> via neutrophil extracellular traps. Lesional neutrophils may activate plaque macrophages and shift their phenotype towards a more inflammatory state<sup>105, 106</sup>. Additionally, neutrophils can render the plaque more vulnerable by exhibiting myeloperoxidase-dependent oxidative stress<sup>107, 108</sup> and by secreting matrix metalloproteinases (e.g., MMP2, MMP8 MMP9) that degrade endothelial layers<sup>109–111</sup>. The presence and function of lymphocytes in atherosclerotic lesions have been well studied<sup>112–114</sup>, and we refer the interested reader to reviews on that subject<sup>115–117</sup>. Despite our detailed understanding of these processes, translational success in the form of clinical immunotherapy targeting plaque leukocyte numbers, phenotypes or functions in atherosclerosis has not yet been realized. We believe that a better understanding how risk and lifestyle factors modulate cellular immunity may provide new therapeutic points of attack, hopefully with a similar impact as the research on hypercholesterolemia which led to the development of statin therapy.

## Diet, metabolism and obesity influence innate immunity

In the U.S., obesity is increasingly prevalent in both adults and children and is now a major public health concern<sup>118, 119</sup>. Obesity contributes to dyslipidemia, insulin resistance, diabetes, hypertension, sleep apnea and metabolic syndrome, all of which increase the risk of ischemic events<sup>120, 121</sup>. Abdominal obesity, in which excessive fat accumulates in the visceral area, has particularly strong links to cardiovascular disease<sup>122</sup>. For further reading on the connections between obesity and cardiovascular disease, we refer to several excellent reviews<sup>123–125</sup>.

Healthy diets, such as the Mediterranean diet, may associate with reduced cardiovascular events<sup>38</sup>. A recent randomized multi-center trial of 7,447 participants revealed that the hazard ratio for major cardiovascular events was 0.69 (95% confidence interval, 0.53 to 0.91) for participants consuming a Mediterranean diet with extra-virgin olive oil supplementation as compared to a control diet<sup>38</sup>. In contrast, consuming red meat may promote the progression of atherosclerosis<sup>126, 127</sup>. Saturated fat, sodium and preservatives in red meat have been attributed to elevated blood pressure and cholesterol levels. Bacterial metabolism of dietary L-carnitine, a trimethylamine abundant in red meat, produces trimethylamine N-oxide (TMAO). This microbiotic metabolite impairs reverse cholesterol transport in macrophages<sup>126, 127</sup> and expands inflammatory monocyte numbers<sup>128</sup>, thus aggravating atherosclerosis.

Intermittent fasting, among other health-promoting diets, are currently receiving attention for their potential beneficial effects on cardiovascular health. Specifically, intermittent fasting lowers blood glucose<sup>129, 130</sup>, triglycerides<sup>131</sup>, cholesterol<sup>132, 133</sup>, blood pressure<sup>131, 134, 135</sup> and circulating proinflammatory cytokines<sup>136</sup>. Short-term fasting reduces circulating monocyte numbers and their metabolic and inflammatory activity<sup>137</sup>. Healthy humans and mice exposed to 4h and 20h of fasting had fewer circulating monocytes while neutrophils remained unchanged. These effects were mediated by dietary carbohydrate and protein levels but not triglycerides, highlighting the importance of specific dietary components. During fasting, circulating monocytes are retained in the bone marrow, while their cell death and production rates appeared to be unaffected. Blocking glycolysis similarly decreased circulating monocyte numbers, suggesting this metabolic pathway is one driving factor. Indeed, fasting in mice with hepatocyte-specific loss of AMPK or PPAR $\alpha$ , both involved in nutrient sensing, did not reduce circulating monocyte numbers. Fasting in humans and mice also lead to reduced Ccl2 plasma levels. The chemokine Ccl2 is a major regulator of monocyte migration from the bone marrow to inflammatory sites<sup>138</sup>. Intriguingly, fasting in PPAR $\alpha$ -deficient mice did not curtail circulating Ccl2, thereby highlighting the mechanistic role of hepatic metabolic pathways. In the liver, fasting affected cytokines and chemokines that regulate Ccl2 production, revealing that hepatic metabolic pathways have a previously unknown function in regulating inflammation. Re-feeding mice after hours of fasting normalized circulating monocyte numbers, indicating that diet-induced shifts in monocytes are only temporary. This study<sup>137</sup> directly links dietary glucose intake with innate immune cell trafficking and demonstrates the interconnections among hematopoietic organs, metabolism and systemic inflammation. The authors also clarified that fasting does not compromise monocyte emergency mobilization during acute infection;



however, it remains unclear if fasting affects emergency hematopoiesis, i.e. leukocyte production during host defense or tissue repair.

Other work demonstrated that during caloric restriction, defined as 50% reduced food intake, memory T cells depart from secondary lymphoid organs and accumulate within the bone marrow in a process coordinated by glucocorticoids and bone marrow adipocytes<sup>139</sup>. During caloric restriction, circulating glucocorticoids levels increased, promoting memory T cell death in peripheral lymphoid organs, blood and adipose tissue. T cells accumulated in the bone marrow and assumed a state of energy conservation, resulting in protection against cell death. The authors observed that caloric restriction triggered bone marrow remodeling, as reflected by increased numbers of T cell-recruiting factors, erythrocytes and adipocytes<sup>139</sup>. These mechanisms could be interpreted as strategies to maintain immune function during times of food restriction by redistributing immune cells to different compartments in which they are protected from diet-induced stress.

Both the studies detailed above reveal that caloric intake controls tissue immune tone and link dietary habits to immunity. This research demonstrates the close interplay between metabolism and immunity<sup>140</sup> and should motivate further efforts to explore how the metabolic-immune axis controls immune cell trafficking and production. It is tempting to speculate that chronic intermittent fasting affects the magnitude of immune cell responses. A number of interesting questions arise: What happens to the immune system during fasting in obese individuals? Does fasting positively affect atherosclerotic lesion size or phenotype by reducing monocytes and lesional macrophage numbers? Does fasting affect the bone marrow niche and hematopoiesis?

There is growing interest in understanding how organ-specific metabolic sensors affect systemic metabolic processes, especially those relevant to obesity's development and complications. A recent study described that T cells may serve as such sensors<sup>141</sup>. Integrin  $\beta 7$ + natural gut intraepithelial T lymphocytes (natural IELs) can fine-tune systemic metabolism. *Integrin  $\beta 7$ -/-* mice, which are deficient in natural IELs, are metabolically hyperactive and protected against obesity, hypercholesterolemia, hypertension and diabetes when fed a high-fat diet. Further, *Integrin  $\beta 7$ -/-* mice developed smaller atherosclerotic lesions in the aorta, with reduced plaque macrophage numbers, due to an overall improved metabolic state. Mechanistically, the study showed that these protective properties were driven by IELs' ability to control the availability of GLP-1, a potent incretin hormone<sup>141</sup>. This study describes an immuno-metabolic checkpoint that functions as a nutrient sensor in the gut and mediates systemic metabolic activity, thereby affecting cardiovascular health.

Obesity is known to be associated with Ccl2-dependent infiltration of monocytes and macrophages into adipose tissue<sup>142-144</sup>, a phenomenon that contributes to the development of insulin resistance and diabetes<sup>142, 143</sup>. Unbiased cell profiling of leukocytes in adipose tissue maps the heterogeneous immune cell landscape with increasing resolution, identifying macrophage subsets that regulate adipocyte size and function<sup>145</sup>. In mice and humans, obesity leads to monocytes and neutrophilia with increased numbers of bone marrow myeloid progenitors<sup>146-148</sup>. Transplantation experiments of adipose tissue into lean recipient mice confirmed an adipose tissue-driven elevation in bone marrow myeloid progenitor

numbers<sup>147</sup>. Weight loss in both mice and humans reduced monocytosis and neutrophilia<sup>147</sup>. Mechanistically, adipose tissue-derived S100A8/A9 promoted tissue macrophages to express Tlr4 and produce Il-1 $\beta$ , which signaled to Il-1 receptor-expressing bone marrow myeloid progenitor cells and increased neutrophil and monocyte production<sup>147</sup>. Within human bone marrow, adipocytes are among the most abundant stromal cells<sup>149, 150</sup> and evidence of their putative roles in regulating hematopoiesis is currently emerging. Recent studies have shown that bone marrow adipocytes are a relevant source of stem cell factor (Scf) and that adipocyte-specific Scf deletion reduces HSC numbers<sup>151, 152</sup>. Interestingly, in mice, *Scf*-deletion in adipocytes more potently affected hematopoiesis in the tail vertebrae than in long bones, such as the femur, which contain fewer adipocytes<sup>152</sup>. This supports the interesting yet not well studied concept of bone marrow heterogeneity and should be investigated further.

Another rapidly growing area of study that connects dietary habits and cardiovascular disease focuses on trained immunity, i.e. the process by which epigenetic reprogramming of innate immune cells induces memory within the innate immune system<sup>153</sup>. A recent study<sup>146</sup> confirmed previous reports<sup>77, 154</sup> that hyperlipidemia engenders systemic inflammation via increased hematopoiesis, adding an intriguing mechanistic angle related to trained immunity in HSPC<sup>146</sup>. Western diet feeding of *Ldlr*<sup>-/-</sup> mice created long-lasting epigenetic reprogramming in granulocyte-monocyte progenitors (GMP), changes that affected their function even four weeks after terminating high-fat diet. This reprogramming was NLRP3-dependent, as high-fat diet in *Nlrp3*<sup>-/-</sup>/*Ldlr*<sup>-/-</sup> mice did not induce leukocytosis or GMP activation and resulted in smaller atherosclerotic lesions compared to *Ldlr*<sup>-/-</sup> mice<sup>146</sup>. This study identified the NLRP3 inflammasome as a key mediator of high-fat diet-induced systemic inflammation and expanded epigenetic reprogramming in the context of trained immunity to hematopoietic progenitors.

These studies are specific examples and in no way represent the entire wealth of work on how diet and metabolism affect the immune and hematopoietic systems (please see a recent, more comprehensive position paper<sup>140</sup>). However, these studies converge on the idea that diet profoundly affects immunity and thus inflammatory diseases such as atherosclerosis.

## Sleep

Although sleep is an essential part of life, epidemiological studies show that more than half of adults in the United States sleep fewer than the recommended eight hours a day<sup>155</sup>. Sleep can be impaired by either insufficient duration, representing quantity, or insufficient depth, representing quality. Fewer than seven hours of sleep are considered insufficient, and the consequences of acute sleep deprivation include reduced general alertness and cognitive performance, impaired short- and long-term memory<sup>156</sup> and diminished decision-making abilities. However, in healthy middle-aged men, one night of quality sleep was able to restore cognitive performance deficits resulting from insufficient acute or chronic sleep<sup>157</sup>. Long-term lack of sleep has detrimental effects on health and associates with increased risk of obesity<sup>158</sup>, diabetes and impaired glucose tolerance<sup>159</sup>, cancer<sup>160</sup>, cardiovascular disease<sup>35, 161–163</sup> and anxiety and depression<sup>163, 164</sup>. A cross-sectional study including 30,397 participants revealed that participants who sleep <5 hours per night had an odds ratio



of 2.2 (95% confidence interval, 1.78–2.71) for developing cardiovascular diseases<sup>162</sup>. Participants sleeping > 9 hours also had increased risk, with an odds ratio of 1.57 (95% confidence interval, 1.31–1.89)<sup>162</sup>.

Despite mounting evidence for links between lack of sleep and disease, little is known about the mechanisms underlying this connection. Studies investigating the effects of sleep deprivation on cardiovascular health<sup>36</sup> have been done in patients with sleep apnea, a chronic condition that affects about 4% of the population in the United States<sup>165</sup>. Sleep apnea is characterized by frequently interrupted breathing, mostly due to upper airway collapse during sleep<sup>166–168</sup>. Patients suffer from multiple arousals per night, resulting in fragmented sleep. This is associated with higher coronary<sup>169, 170</sup> and cerebrovascular<sup>171</sup> morbidity and mortality. Sleep apnea patients have elevated plasma levels of inflammatory cytokines including TNF- $\alpha$ <sup>172</sup>, IL-6<sup>173</sup>, IL-8<sup>172</sup> and C-reactive protein<sup>173</sup> compared with either an obese-only control group or healthy individuals. Individuals with sleep apnea also have higher circulating leukocyte counts<sup>174–176</sup>, however, whether this is due to increased hematopoiesis remains unclear. Another potentially important mechanistic factor in sleep apnea is that during arrested breathing, blood oxygen drops, resulting in multiple hypoxic events that stimulate the sympathetic nervous system. This complex scenario makes it challenging to distinguish the discrete contributions of sleep fragmentation, recurrent hypoxia and stress to the pro-inflammatory conditions observed in sleep apnea patients.

A recent study on sleep fragmentation's effect on innate immunity and atherosclerosis provides some clarity (Figure 2)<sup>177</sup>. Mice subjected to sleep fragmentation had higher bone marrow hematopoietic stem cell numbers and more circulating myeloid cells. Hypocretin, a neuropeptide that regulates arousal, wakefulness and appetite, is produced in the lateral hypothalamus and was reduced in the plasma and bone marrow of mice subjected to sleep fragmentation. Circulating hypocretin suppressed hematopoiesis and atherosclerosis, as *Hcrt*<sup>-/-</sup> mice had more hematopoietic stem cells in their bone marrow and larger atherosclerotic lesions. Parabiosis of wildtype and *Hcrt*<sup>-/-</sup> mice did not result in increased hematopoiesis in *Hcrt*<sup>-/-</sup> parabionts, as circulating wildtype hypocretin sufficiently suppressed hematopoiesis. Mechanistically, circulating hypocretin signaled to hypocretin receptor-expressing pre-neutrophils in the bone marrow, controlling their CSF1 production and, consequently, HSPC proliferation. Bone marrow transplant experiments revealed that hematopoietic knockout of *Hcrt1*, including in pre-neutrophils, elevated hematopoiesis by impairing hypocretin signaling suppression. Sleep-fragmented *Ldlr*<sup>-/-</sup> mice fed a high-fat diet over 12 weeks had larger atherosclerotic plaques with higher lesion macrophage numbers than *Ldlr*<sup>-/-</sup> mice with uninterrupted sleep. Finally, sleep-fragmented mice with hematopoietic *Csf1* deletion did not show increased hematopoiesis or atherosclerosis. Intriguingly, hypocretin supplementation in mice was able to counteract sleep fragmentation-induced hematopoiesis and atherosclerosis, possibly offering novel therapy options<sup>177</sup>. This study described a neuro-immune axis in which sufficient sleep sustains circulating hypocretin levels that constrain hematopoiesis, circulating innate immune cells and thus atherosclerosis.

Circadian rhythms also affect the hematopoietic and immune systems, and therefore cardiovascular health. Sleep is a major entrainment factor for circadian rhythm, and irregular

sleeping habits, e.g. those experienced by shift workers, lead to misalignment with the central clock, rendering individuals at higher risk for cardiovascular events<sup>178</sup>. It has long been observed that cardiovascular events, such as stroke and myocardial infarction, exhibit circadian incidences and peak in the early morning hours<sup>179, 180</sup>. Indeed, myocardial infarction during that time of day correlates with worsened outcomes<sup>137, 181, 182</sup>. Animal studies have demonstrated that an overactive immune response at the beginning of the active phase impairs cardiac wound healing and function after myocardial infarction<sup>57, 58, 183</sup>. Although the development of atherosclerosis is a chronic process, a recent study suggests that diurnal myeloid cell invasion into the arterial wall could perpetuate atherosclerosis progression<sup>184</sup>. The authors show in atherosclerotic mice that monocyte and neutrophil recruitment to plaque lesions varies by time of day, peaking during the transition from active to resting phase. This circadian recruitment pattern depends on Ccl2 production by neutrophils, monocytes, B cells and T cells. In support of this mechanistic link, systemically blocking Ccl2 or genetically deleting *Ccr2* abrogated the temporal aspect of leukocyte recruitment to atherosclerotic lesions. Intriguingly, daily application of a *Ccr2* antagonist specifically at the beginning of the active phase reduced monocyte and neutrophil adhesion to the microcirculatory endothelium, consequently decreasing plaque size and lesional macrophage numbers<sup>184</sup>. This study indicates that daily leukocyte shifts between circulation and arteries contribute to atherosclerotic growth, and anti-inflammatory strategies applied at a specific time of day may prevent atherosclerosis progression.

During steady state, the circadian rhythm regulates bone marrow hematopoiesis through adrenergic signals transmitted to Nestin+ stromal cells by the  $\beta$ 3-adrenergic receptor, leading to rapidly regulated Cxcl12 levels<sup>53, 54, 185</sup>. In humans and mice, circulating leukocytes, particularly Ly6C<sup>high</sup> monocytes<sup>55, 58</sup>, oscillate throughout the day and traffic between tissues and blood in a process regulated by chemokines and adhesion molecules expressed by leukocytes and tissue endothelial cells respectively<sup>55–58, 186–188</sup>. During the resting phase (i.e. daytime as mice are active at night), aged Cxcr4<sup>high</sup> neutrophils return to the bone marrow, where they are cleared by macrophages that secrete feedback signals to the bone marrow niche, thereby regulating hematopoiesis and leukocyte egress<sup>189</sup>. These pathways are likely affected by lack of sleep and sleep fragmentation, including experiences such as shift work and jet lag. Conversely, sufficient and uninterrupted sleep will restore these biological rhythms.

## Psychosocial stress

Acute and chronic stress are well-recognized risk factors for the development of obesity, diabetes and hypertension, all of which give rise to cardiovascular diseases, particularly atherosclerosis<sup>190–193</sup>. Acute stress, as is experienced during earthquakes, the World Cup soccer championships or short-term experimental stressors, increases the risk of myocardial infarction and pulmonary embolism<sup>27, 194</sup>. Chronic stressors, such as post traumatic stress disorder in veterans<sup>195</sup>, job loss or divorce, lead to increased risk for cardiovascular events. Chronic psychosocial stress also often overlaps with confounding unhealthy lifestyle choices including smoking, alcohol consumption, irregular sleeping habits and a sedentary lifestyle<sup>196</sup>. Stress has multiple, interdependent impacts on health.

Recent work has shed light on how stress affects immunity and hematopoiesis. There are two major pathways through which stress activates immunity: the limbic-hypothalamic-pituitary-adrenal axis and the sympathetic-adreno medullar axis<sup>197</sup>. Behavioral and physical stress activate the limbic-hypothalamic-pituitary-adrenal axis, initiating a hormone release cascade that includes glucocorticoid secretion from the adrenal glands<sup>197</sup>. Fear and other stressors are processed by the amygdala and hypothalamus, triggering the sympathetic nervous system and raising levels of systemic catecholamines (e.g. epinephrine, norepinephrine)<sup>198</sup>. Circulating catecholamines, especially noradrenaline, signal through  $\alpha$ - and  $\beta$ -adrenergic receptors expressed by bone marrow stromal<sup>53, 185</sup> and most immune cells<sup>199</sup>, including lymphocytes, myeloid and hematopoietic progenitors.

In the bone marrow, sympathetic nerve fibers along the arteriolar vasculature innervate Nestin+ stromal cells, which are part of the hematopoietic niche and express  $\beta$ 3-adrenergic receptors. In steady state, sympathetic nerve fibers secrete norepinephrine, which decreases Cxcl12 production in Nestin+ stromal cells<sup>53, 54, 185, 200</sup>. Cxcl12 is a cytokine that retains stem and progenitor cells and Cxcr4-expressing neutrophils within the marrow and promotes HSC quiescence, among many other functions. Stress-related mechanisms of sympathetic bone marrow innervation are not always harmful, as recently described by a study demonstrating that sympathetic bone marrow signaling counteracts the declining hematopoietic differentiation potential that comes with hematopoietic aging<sup>201</sup>. The authors showed that loss of sympathetic nerves or adrenergic  $\beta$ 3-receptor signaling in the bone marrow leads to premature hematopoietic aging in young mice. Intriguingly, treatment with a sympathomimetic drug that selectively activates the adrenergic  $\beta$ 3-receptor was able to reverse hematopoietic stem cell aging in old mice<sup>201</sup>. These findings reveal that the sympathetic nervous systems plays a previously unidentified beneficial role in maintaining bone marrow niche function.

Chronic psychosocial stress over several weeks produces monocytosis and neutrophilia in mice and humans<sup>202, 203</sup>. Stress and stress-induced hormones raise leukocyte levels across other species including fish, hen, rat and cattle<sup>204–208</sup>. Administering 5-fluorouracil, which eliminates proliferating progenitors, completely neutralized stress-induced leukocytosis in mice<sup>202</sup>, thus linking stress-induced leukocytosis with enhanced progenitor proliferation. Indeed, several murine studies have shown that stress increases hemopoietic stem and progenitor cell proliferation in and release from the bone marrow into the circulation, thereby accelerating atherosclerosis<sup>202, 209–212</sup>. Mechanistically, stress causes bone marrow sympathetic nerve fibers to release noradrenaline, which reduces Cxcl12 production by  $\beta$ 3-expressing bone marrow stromal cells<sup>202</sup>. Treating stressed mice with  $\beta$ 3-adrenergic receptor blocker abolished the stress-induced drop in Cxcl12 and curtailed hematopoiesis. *ApoE*<sup>-/-</sup> mice subjected to chronic stress over several weeks showed accelerated hematopoiesis leading to higher output of disease-propagating neutrophils and inflammatory monocytes, which promoted fibrotic cap thinning and plaque inflammation<sup>202</sup>. Taken together, these studies characterize a stress-sensitive neuro-immune axis between the sympathetic nervous system and bone marrow stromal cells that regulates hematopoiesis and affects cardiovascular health.

In support of these mechanistic data, a recent study linked activity in the amygdala, a brain region involved in processing emotions such as stress and fear, with cardiovascular disease risk<sup>213</sup>. The longitudinal study included 293 patients, 22 of whom had a cardiovascular event within the 3.7-year followup period. PET imaging revealed that enhanced amygdalar <sup>18</sup>F-fluorodeoxyglucose activity correlated with increased bone marrow and arterial <sup>18</sup>F-fluorodeoxyglucose signal and associated with elevated cardiovascular disease risk, with a hazard ratio of 1.59 (95% confidence interval, 1.27–1.98). Patients with high amygdalar PET signal had significantly decreased event-free survival compared to patients with low amygdalar PET activity. These observations align well with the concept of a neuro-immune axis in which the amygdala is a key structure linking fear and stress to immunity and cardiovascular disease.

The work summarized above indicates the need to enhance our mechanistic understanding of how the central nervous system interacts with the immune and hematopoietic systems. In particular, there is growing interest in resilience mechanisms, i.e. how the pro-inflammatory effects of psychosocial stress, which is often unavoidable, may be mitigated, especially with respect to inflammatory and cardiovascular pathways. To this end, it would be interesting, for instance, to modulate amygdalar activity in an experimental setting and decipher specific stress hormones' effects on select leukocyte subsets, and their consequences for cardiovascular health. There are numerous additional questions that merit investigation: Which autonomic signals and hormones regulate leukocyte, progenitor and bone marrow niche function when the sympathetic nervous system is not activated by stress? How do lifestyle factors such as sleep, diet and exercise affect this balance, which likely influences hematopoiesis, immunity and cardiovascular health? Do exposure to pollution and environmental noise act on immune cells and their progenitors via stress-related or alternative pathways?

## Physical inactivity and exercise

The beneficial effects of physical activity on health, particularly cardiovascular health, have been extensively studied. A sedentary lifestyle distinctly affects the immune system and raises atherosclerotic risk<sup>26, 214–216</sup>. A large-scale study including 55,137 participants investigated if regular physical activity in the form of leisure-time running was associated with lower cardiovascular mortality<sup>215</sup>. Indeed, the study found that individuals who ran as little as 51–80min per week had lower hazard ratios for cardiovascular mortality (0.56, 95% CI: 0.43 to 0.73)<sup>215</sup>. The potentially protective effects of regular exercise involve metabolic sequelae such as improving glucose tolerance<sup>217</sup>, insulin sensitivity<sup>218–221</sup> and decreasing blood lipid concentrations<sup>222</sup>, thereby guarding against obesity<sup>214</sup>. Regular exercise can induce anti-inflammatory pathways<sup>223</sup>; however, the precise mechanisms of this effect remain unclear. Several studies have observed that regular exercise associates with lower circulating monocyte and neutrophil numbers in humans and mice<sup>224–226</sup>. Mice fed a high-fat diet over several weeks and exposed to regular exercise had fewer skeletal muscle inflammatory cytokines, lower macrophage muscle infiltration<sup>227</sup> and less inflammatory macrophage phenotypes<sup>228</sup>. A human study investigating how 12 weeks of exercise affect inflammatory markers in the skeletal muscle tissue of elderly patients paralleled these animal studies, showing that exercise resulted in 50% less IL-6 and TNF- $\alpha$  mRNA<sup>229</sup>. In

contrast to research documenting regular exercise's anti-inflammatory effects, several human and animal studies indicate that strenuous bouts of exercise lead to increased inflammatory cytokine levels, leukocytosis and bone marrow cell release into the circulation, immediately after the physical activity<sup>230–233</sup>. These sudden effects may not be solely mediated by the metabolic shifts described above. Exercise activates both the somatomotor cortex and the autonomic nervous system, enabling skeletal muscle contraction, movement pattern coordination and adjustments in cardiovascular physiology according to system demand<sup>234, 235</sup>. Strenuous exercise modulates stress-related brain regions, including the amygdala<sup>236</sup> and hypothalamic-pituitary adrenal axis, and increases peripheral sympathetic tone triggering leukocytosis<sup>237, 238</sup> and progenitor release<sup>239–241</sup>. Supporting the observation that strenuous exercise leads to stress-like immune responses, injecting epinephrine or noradrenaline into rats resulted in elevated blood leukocyte levels similar to those produced by acute strenuous exercise<sup>242, 243</sup>. Of note, higher blood pressure and heart rate during exercise may raise the risk of acute ischemic events in the setting of existing CVD<sup>244, 245</sup>. Caution should thus be used when evaluating research studies on exercise and immunity, as some animal studies do not use voluntary exercise. In some experimental settings, mice are forced to swim or forced to run on a treadmill motivated by mild electric shocks. Both of these forced exercise models likely induce psychological stress and may hence result in a mixed phenotype that reflects more than the positive effects of physical activity. Precise mechanisms by which voluntary exercise may affect short-term immune activation or bone marrow cell release have not yet been described, although stress hormones and sympathetic nervous signaling likely contribute, as they do for humans under stress<sup>202, 203</sup>. It would be of great interest to experimentally address how the bone marrow niche and hematopoietic factors respond during and shortly after voluntary physical activity, especially beyond stress-induced mechanisms.

A physically active lifestyle associates with better cardiovascular health, perhaps by improving energy consumption and protecting against obesity. Interestingly, long-term regular physical activity in humans has immunosuppressive effects that lower circulating monocyte and lymphocyte numbers<sup>246–250</sup>. A recent study of individuals with increased cardiovascular risk showed that 16 weeks of daily walking, 1–2 hours per day, did not significantly lower circulating cytokines or oxidative stress markers but did curtail peripheral blood mononuclear cells' ability to produce cytokines upon stimulation<sup>247</sup>. Individuals with the longest walking times showed the strongest reduction in cytokine production capacity, suggesting an inverse correlation between physical activity and immunity in this setting. Similar to previous observations on exercise and metabolism, this study revealed that walking decreased peripheral blood mononuclear cells' oxygen consumption and lactate production, thereby positing enhanced cellular metabolism as one potential mechanism driving these cells' attenuated immune response after physical activity, but this explanation lacks mechanistic proof.

One possible explanation for the lower circulating leukocyte counts after long-term physical activity is dampened hematopoiesis. Indeed, recent work demonstrated that regular voluntary exercise in mice calms the bone marrow niche and promotes hematopoietic quiescence (Figure 3)<sup>224</sup>. In this study, mice were given permanent access to a treadmill in their cage for 6 weeks and voluntarily ran, on average, 11.6 km per day. Compared to

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sedentary mice, running mice had reduced bone marrow HSPC numbers and less proliferation, resulting in lower circulating leukocyte counts for all subsets except erythrocytes. Running decreased leptin production in adipose tissue, consequently lowering the levels in circulation and the bone marrow. Reduced leptin signaling to leptin-expressing stromal cells in the bone marrow niche of running mice elevated HSC quiescence-promoting niche factors, including Cxcl12. Giving mice access to running wheels for 6 weeks followed by 3 weeks of running wheel withdrawal revealed that although leptin levels quickly reversed, circulating leukocytes and their production remained lower even weeks after exercise termination. ATAC sequencing of hematopoietic progenitors sorted from these mice showed that running induces long-term epigenetic changes impacting chromatin accessibility and affecting progenitor proliferation weeks after exercise ends. Specifically deleting the leptin receptor in leptin-expressing stromal bone marrow niche cells, using *Prrx1-creERT2; Lepr<sup>fl/fl</sup>* mice, mimicked the exercise-induced dampening effect on hematopoiesis and improved outcomes after MI and in atherosclerosis. To test whether the reduced leukocyte levels in physically active mice would impair host defense and bacterial clearing, running mice and sedentary controls were subjected to two different models of sepsis, intraperitoneally injected lipopolysaccharides and cecal ligation and puncture. Interestingly, running mice responded to sepsis with elevated bone marrow HSPC activity and higher circulating leukocyte numbers, resulting in improved survival compared to sedentary mice. These findings indicate that physical activity in mice dampens hematopoiesis via modulating the bone marrow niche, consequently improving cardiovascular disease outcomes without impairing host defense mechanisms that are crucial for surviving infections. These data support previous studies that show physical activity has anti-inflammatory effects in humans and mice<sup>225, 226, 247, 251, 252</sup>. These studies also raise the concern that nearly all mice in research facilities are kept in cages without access to running wheels. Rather than being classified as neutral controls, these mice should be considered exposed to sedentary lifestyle, and the possible effects on experimental research should be anticipated.

Several questions remain unanswered: Does exercise affect extramedullary hematopoiesis in the spleen? Is the type, timing and intensity of exercise important, as some authors<sup>232, 253, 254</sup> suggest exercise has a u-shaped effect on immunity? What cell-cell interactions are influenced by sedentary behavior, in particular, are there additional local and long-distance adipocyte-, stromal- and neural-immune cell interactions? Future research efforts aimed at clarifying these questions will help us understand how a physically active lifestyle can benefit health and guide physicians in making specific exercise recommendations to their patients. Given the increasing prevalence of obesity and diabetes, propagating physical activity is a highly cost-effective health intervention that is currently underused. Societal development has reduced the necessity of physical activity in employment and transportation, probably contributing to the obesity epidemic. Despite its low entry costs, exercise is now a leisure activity and is becoming a luxury for many people who work long hours sitting at desks, preceded and followed by sedentary commutes. Solving this problem requires creative prevention strategies built around behavior. For example, why are elevators typically centrally located in the lobby, beautiful and air conditioned while staircases are often hidden and less attractive?



## Conclusion

Epidemiology research established a strong correlation between modifiable risk factors and cardiovascular outcomes. Motivated by these clinical data, and by the insight that atherosclerosis is not only a lipid storage but also an inflammatory disease, the field has begun to investigate how lifestyle-related behavior influences pathways that involve immunity. While these studies clarify that leukocyte production and phenotype are shaped by exposure to stress, diet choice and sleep hygiene, to date the uncovered mechanisms have relied on cellular and molecular components well known from work in neuroscience, metabolism, hematology and immunology. Connecting these fields in interdisciplinary teams reveals those cross-cutting pathways. However, the currently known mechanisms are unlikely to be the only important players; rather, they establish a proof-of-principle. The experimental platforms described herein, including sleep disruption, chronic mild stress and voluntary exercise are robust, have been used in their respective fields for multiple years and are straightforward to implement in any laboratory. We posit that these tools can serve as discovery platforms in the search for currently unknown pathways and targets that build resilience against cardiovascular inflammation. One promising avenue for such work is to investigate the microbiome, metabolome, proteome, transcriptome and epigenome, perhaps even in meaningful combinations, with unbiased assays. Such work would give rise to large data sets to help discover currently unknown health-promoting pathways and related drug targets. Importantly, drug targets discovered with these platforms may have desirable toxicity profiles. For example, mice with access to running wheels produced fewer leukocytes but survived peritonitis better, an observation that suggests voluntary running may serve as a discovery platform for anti-inflammatory therapeutics that do not compromise host defense. Thus, the currently available and quickly growing body of data serves as a motivation to focus on unbiased discovery work into how health-promoting behavior reduces modifiable cardiovascular risk.

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## Non-standard Abbreviations and Acronyms:

<b>CSF</b>	colony stimulating factor
<b>CVD</b>	cardiovascular disease
<b>HSC</b>	hematopoietic stem cells
<b>HSPC</b>	hematopoietic stem and progenitor cells
<b>MMP</b>	matrix metalloproteinase

<b>SCF</b>	stem cell factor
<b>PET</b>	positron emission tomography

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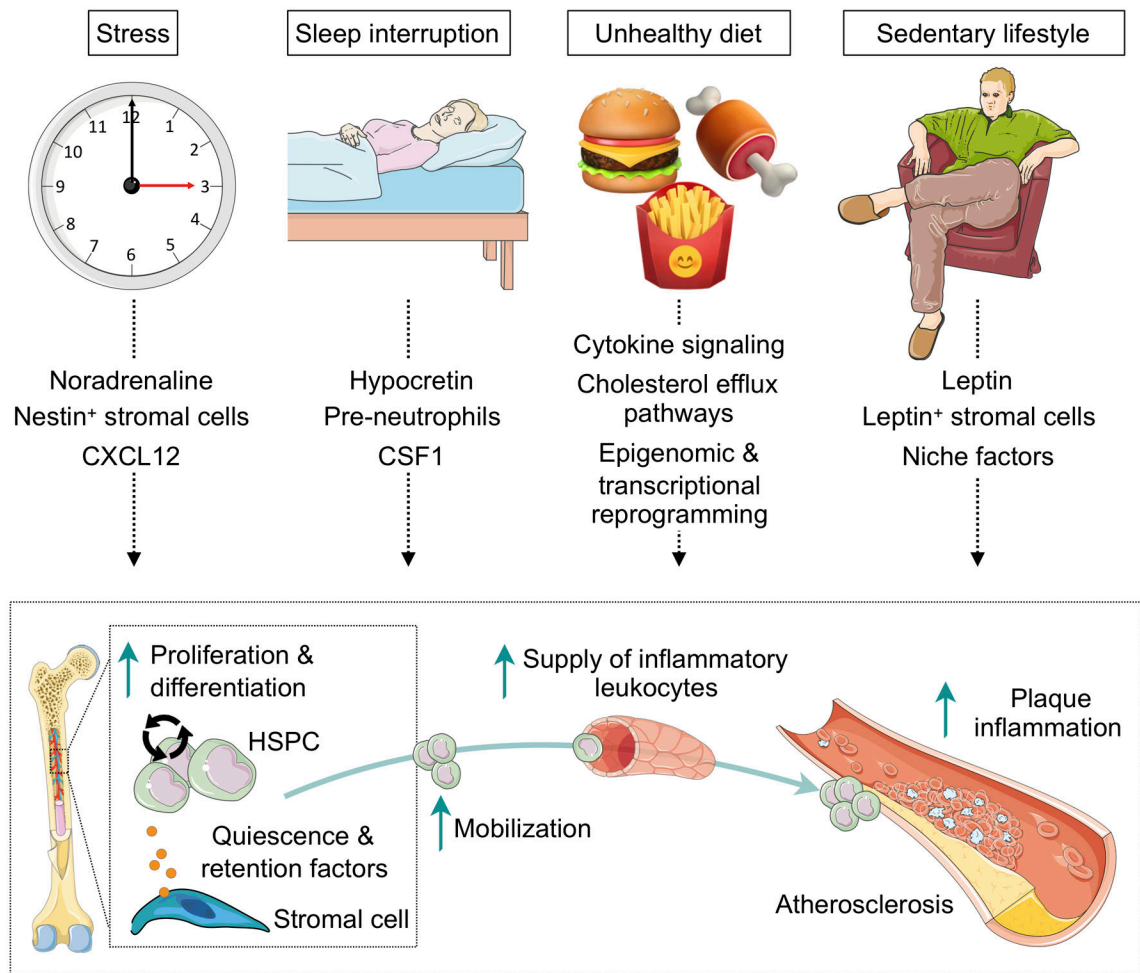
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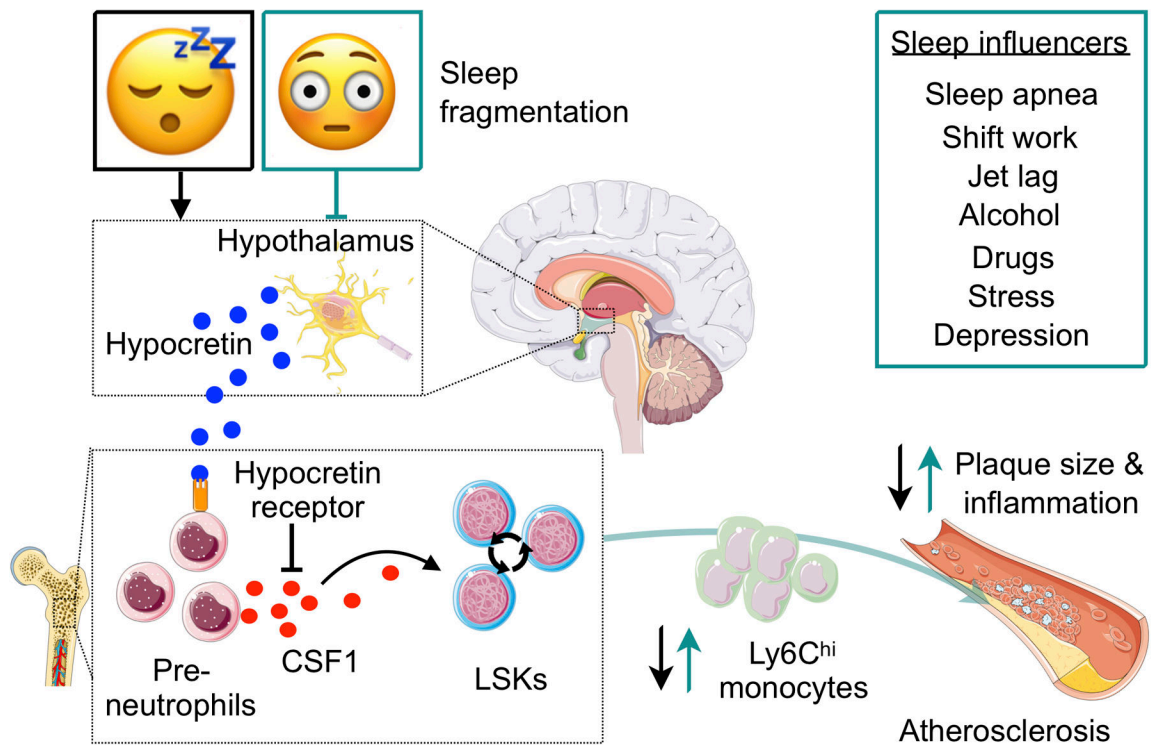
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**Fig. 1. Lifestyle factors affect hematopoiesis in cardiovascular disease.**

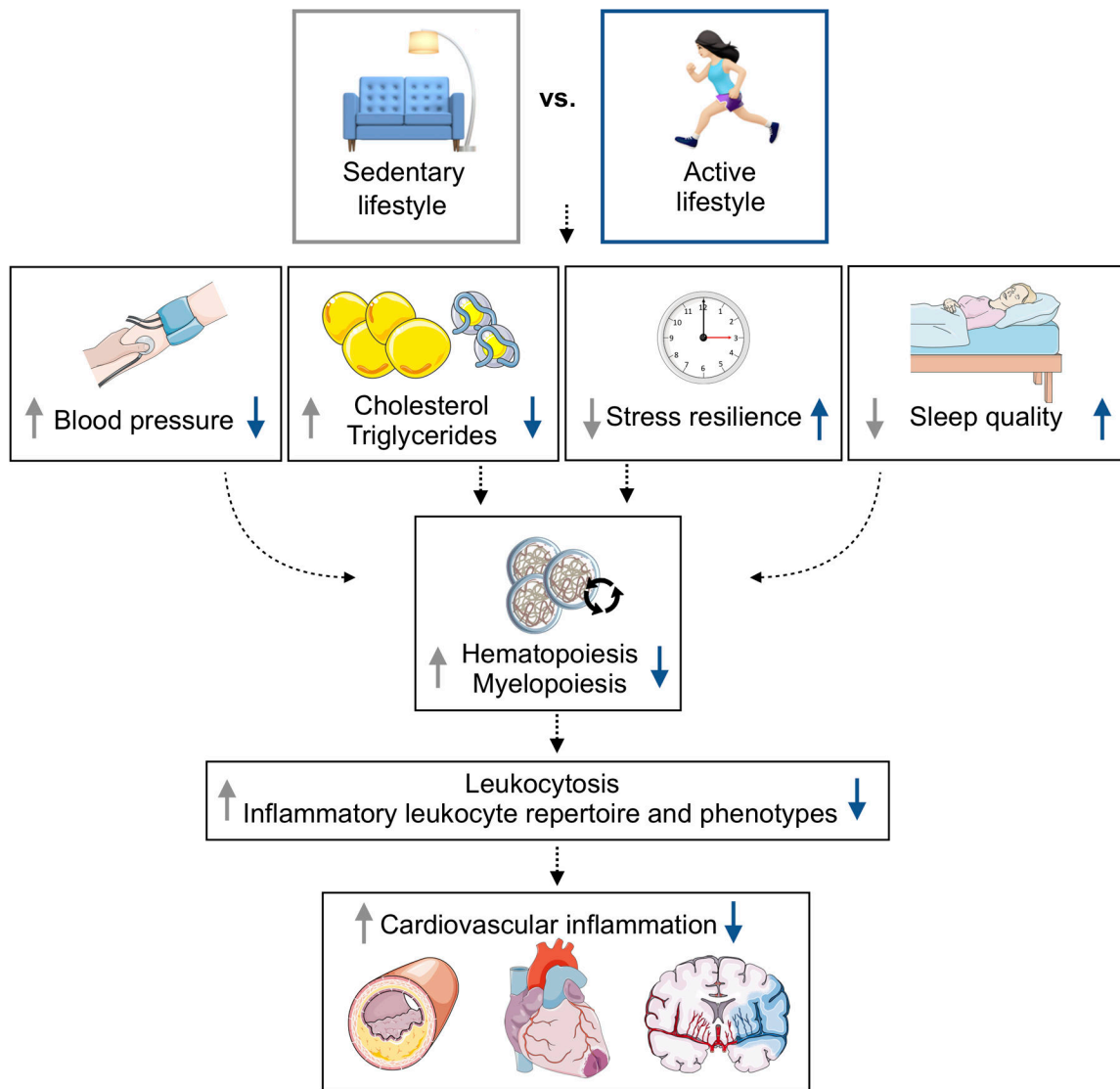
The cartoon illustrates the various pathways how lifestyle factors influence hematopoiesis and increase inflammatory leukocyte supply to the atherosclerotic lesion, accelerating its progression. CXCL indicates C-x-c chemokine ligand; CSF1, colony stimulating factor 1; HSPC, hematopoietic stem and progenitor cell. Cartoon summarizes data in references<sup>76, 77, 146, 177, 202, 224, 255</sup>.





**Fig. 2. Sleep controls hematopoiesis and protects against atherosclerosis.**

The cartoon illustrates how sleep influences hematopoiesis and moderates leukocyte supply to the atherosclerotic lesions. Sleep induces hypocretin release from the lateral hypothalamus, which circulates to the bone marrow and reduces CSF1 secretion from hypocretinreceptor-expressing pre-neutrophils. Sleep fragmentation leads to decreased release of hypocretin, heightening bone marrow CSF1 levels. CSF1 stimulates hematopoietic stem and progenitor cell proliferation, resulting in increased supply of inflammatory leukocytes. The cartoon was adapted from McAlpine et al. (ref<sup>177</sup>). CSF1 indicates colony stimulating factor 1.



**Fig. 3. Interrelated pathways and modifiable risk factors for cardiovascular disease.** The cartoon depicts how physical activity may moderate innate immunity through multiple organs systems, ultimately affecting cardiovascular disease inception and progression.