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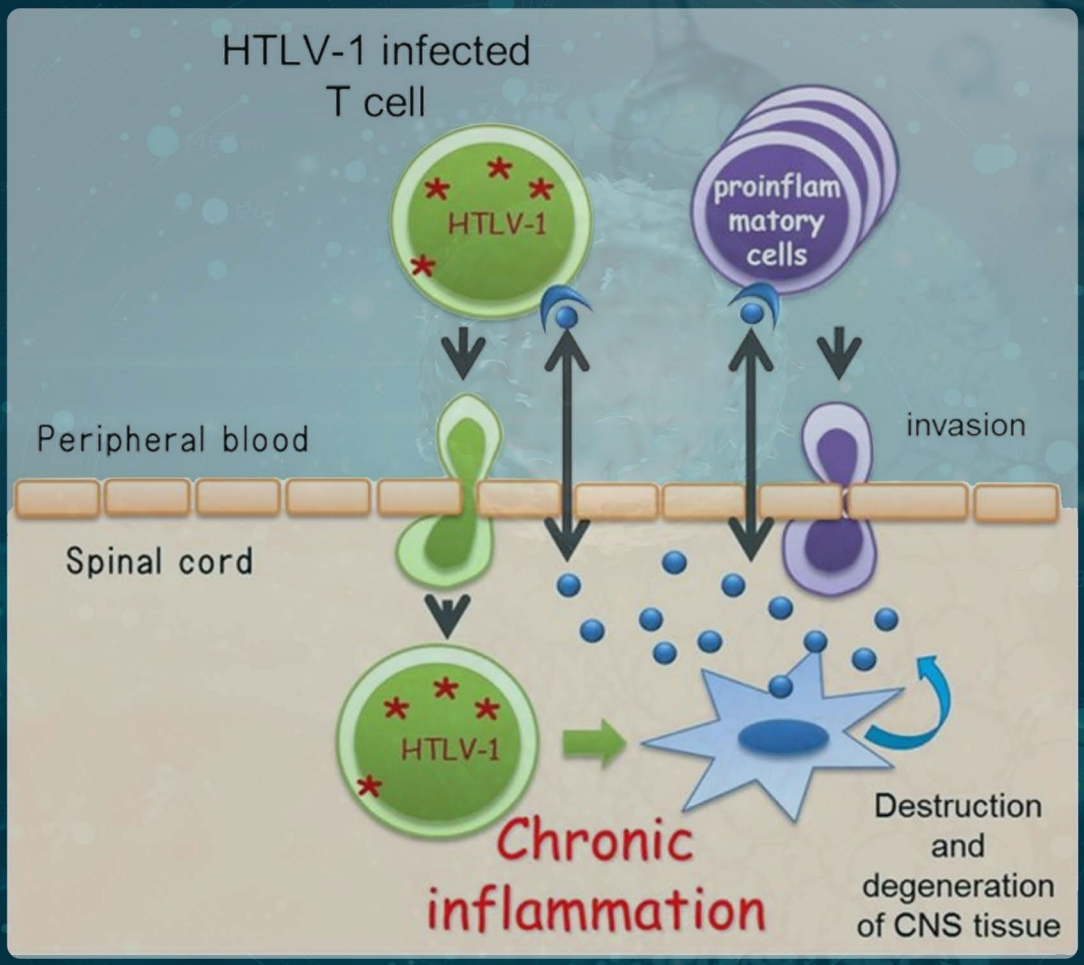


## Inflammatory and anti-inflammatory responses in human T-lymphotropic virus Type 1 infection

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REVIEW ARTICLE

# The multifaceted functions of mineralocorticoid receptor in cardiometabolic disease

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## Abstract

Cardiometabolic diseases (CMDs), which cause 31% of all global deaths, are one of the greatest public health challenges. Mineralocorticoid receptor (MR), as a key nuclear transcription factor, is an important drug target for the treatment of CMDs. It is known that MR is expressed in almost all tissues and organs involved in cardiovascular homeostasis, including immune tissue, adipose tissue, brain, heart, kidney, and blood vessels. In the pathophysiology of CMDs, MR exerts different functions in different tissues and cells. This review summarizes the roles of MR in various cell types and discusses the molecular mechanisms through which MR exerts it functions in CMDs.

***Keywords:*** Cardiometabolic diseases; Mineralocorticoid receptor; Mineralocorticoid receptor antagonist

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## 1. Introduction

Cardiometabolic diseases (CMDs) remain the leading cause of morbidity and mortality worldwide[1]. CMDs contain a constellation of cardiovascular diseases (CVDs), including coronary heart disease, stroke, hypertension, and associated metabolic disorders, such as obesity and diabetes[1,2]. The incidence of classical risk factors for CMDs such as smoking, high blood pressure, and hypercholesterolemia has decreased over the past few decades[2]. However, the rate of obesity and the prevalence of type 2 diabetes have markedly increased[2,3]. This changing disease pattern poses a huge challenge to the treatment of CMDs. Although there is a growing number of drugs that target classical cardiovascular risk factors, such as hypertension and hyperlipidemia, current treatment regimens do not prevent most cardiovascular events[4]. Therefore, it is necessary to further study the pathophysiological mechanism of CMDs to identify new targets for personalized therapy.

Mineralocorticoid receptor (MR) is a member of the nuclear receptor superfamily[5]. MR is involved in transcriptional regulation and plays important roles in CMDs[5-7]. The steroid hormone aldosterone is a classical MR ligand that regulates blood pressure and promotes sodium retention in the kidney[8,9]. An increasing number of clinical

trials have shown that the activation of aldosterone/MR signaling increases the risk and adverse clinical outcomes in patients with hypertension, myocardial infarction (MI), heart failure (HF), and stroke[10,11]. Experimental evidence has demonstrated that MR activation by aldosterone induces oxidative stress, inflammation, and fibrosis, all of which contribute to the progression of CMDs[12,13]. However, aldosterone is not the only ligand for MR. Glucocorticoids have similar affinity and specificity for MR[14]. In certain cells, 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) restricts the binding of glucocorticoids to MR by converting endogenous glucocorticoids to metabolites that have poor affinity for MR[7]. Conversely, the enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) converts the inactive dehydrogenated form of glucocorticoids into active form[7,15]. The ligand-independent activation of MR has also been investigated. Rac1, a small GTPase belonging to the Rho family, can activate MR, leading to nuclear translocation in renal and cardiac diseases[16,17]. It has been reported that high glucose activates the transcriptional activity of MR through protein kinase C β signaling[18] and high salt causes renal MR activation through the induction of oxidative stress and exacerbates renal injury[19].

It has been clearly established that MR is expressed and functional in extra-renal tissues, such as brain, liver, lung, colon, bone, heart, vasculature, and immune system[20,21]. The role of MR varies in different cell types. For example, monocyte/macrophage MR deficiency affects macrophage polarization and vascular remodeling[22,23]. The deletion of vascular smooth muscle MR ameliorates aging- or angiotensin (Ang) II-induced hypertension in mice[24]. Although the overexpression of MR in endothelial cells promotes vasoconstriction and leads to hypertension[25], the deletion of MR has no effect on blood pressure[25,26]. MR knockout in T cells alleviates renal and vascular lesions and reduces Ang II-induced hypertension[27]. Therefore, elucidating the regulatory mechanisms of MR in different cell types may provide new strategies to treat CMDs.

This review summarizes the effects of MR in different cell types on the pathological process of CMDs and specifically highlights the expression and function of MR in immune cells, adipocytes, vascular cells, and myocardial cells. We also discuss the application and challenges of MR antagonists in the treatment of CMDs, to highlight promising novel therapeutic strategies.

## 2. Immune cell MR and CMDs

Inflammation and immune cells are closely related to the pathogenesis of CMDs[5,28-30]. The previous studies have shown that aldosterone activates the immune system to promote the expression of inflammatory cytokines and the recruitment of immune cells, both of which contribute to tissue damage and impaired healing in CMDs[30]. MR is expressed in most immune cells, including monocytes/macrophages, dendritic cells (DCs), T cells, and B cells[30]. Immune cell MR is involved in the pathological processes of many cardiovascular and metabolic diseases.

### 2.1. Macrophage MR and CMDs

#### *2.1.1. Role of macrophage MR in atherosclerosis*

Macrophages are the main immune cells in atherosclerotic plaques and are closely related to the pathological process of atherosclerosis[31]. MR activation by aldosterone increases atherosclerosis plaque size and macrophage numbers in the plaques[32]. Conversely, MR inactivation by eplerenone decreases macrophage oxidative stress and improves atherosclerosis[33]. Recent studies have suggested that macrophage MR plays a major role in atherosclerosis[34,35]. MR deficiency in macrophages decreases plaque size in early- and late-stage atherosclerosis through different mechanisms. In the early stage of atherogenesis, MR deficiency downregulates the expression of P-selectin glycoprotein ligand-1 (a critical mediator of leukocyte rolling) in macrophages and suppresses leukocyte trafficking to reduce inflammation in atherosclerotic plaques[35]. Macrophage-specific MR deletion inhibits macrophage foam cell formation and increases the phagocytic and efferocytosis capacities of macrophages in a mouse model of late-stage atherosclerosis[34].

#### *2.1.2. Role of macrophage MR in MI*

Macrophage MR deficiency has been shown to improve cardiac function and decrease the size of infarct scar following MI, with enhanced infarct neovascularization and scar maturation[36]. Mechanistically, MR deletion in macrophages promotes post-MI cardiac repair by enhancing neutrophil efferocytosis, suppressing free radical formation, and regulating fibroblast activation status[36]. Targeting macrophages with eplerenone-containing liposome protects against cardiac dysfunction and adverse cardiac remodeling following MI, thus suggesting that the targeted delivery of MR antagonists to macrophages post-MI could be a novel strategy to prevent the side effects of MR antagonists on electrolytes[36].

#### *2.1.3. Role of macrophage MR in hypertension*

The role of macrophage MR has also been investigated in different models of hypertension[22,37,38]. In deoxycorticosterone acetate (DOCA)/salt- and low-dose treatment of $N^G$-nitro-L-arginine methyl ester (L-NAME)/salt-induced hypertension models, the deletion of macrophage MR reduces systolic blood pressure, diminishes cardiac fibrosis, and inhibits the expression of pro-

inflammatory factors, with no change in the recruitment of macrophages to the heart[37,38]. In an L-NAME/AngII-induced hypertension model, MR deficiency in macrophages not only protects against cardiac hypertrophy, fibrosis, and vascular damage, but also decreases macrophage recruitment to the heart[22]. Intriguingly, in this model, the blood pressure of macrophage MR deficient mice was slightly elevated[22]. Mechanistically, MR deficiency in macrophages inhibits the expression of M1 markers and increases the expression of alternatively activated M2 markers[22]. In addition, MR deficiency synergizes with interleukin (IL)-4 to facilitate the polarization of macrophages to M2 phenotype signaling[22] (Figure 1).

### 2.1.4. Role of macrophage MR in arterial injury

Macrophage MR is also involved in the repair process of arterial injury (AI). MR deficiency in macrophages inhibits AI-induced neointimal hyperplasia by inhibiting macrophage accumulation and vascular inflammation[23]. At the molecular level, MR deletion promotes the polarization of macrophages to an anti-inflammatory phenotype by suppressing activator protein 1 (AP-1)/nuclear factor κB (NF-κB) signaling pathways in macrophages[23] (Figure 1). MR deficiency also inhibits the migration and proliferation of macrophages both *in vivo* and *in vitro*[23]. However, further research is required to determine the underlying mechanism.

### 2.1.5. Role of macrophage MR in obesity and diabetes

Macrophages are closely related to obesity and type 2 diabetes mellitus (T2DM)[39]. MR antagonists have been shown to improve hepatic steatosis and insulin resistance in obese animal models[40,41]. A deficiency in macrophage MR improves glucose intolerance, insulin resistance, and hepatic steatosis in obese mice[42], implying that macrophage MR plays an important role in obesity and T2DM. Mechanistically, in the presence of estrogen, MR deletion directly upregulates estrogen receptor alpha (ERα) expression in macrophages and enhance the secretion of hepatocyte growth factor (HGF)[42]. Subsequently, the macrophage-secreted HGF phosphorylates and activates hepatocyte Met, which mediates a decrease in lipid accumulation and an increase in insulin signaling in hepatocytes, thus improving hepatic steatosis and insulin resistance in obese mice[42] (Figure 1). These results suggest that the MR/ERα/HGF/Met axis is a potentially important metabolic pathway linking macrophages to hepatocytes.

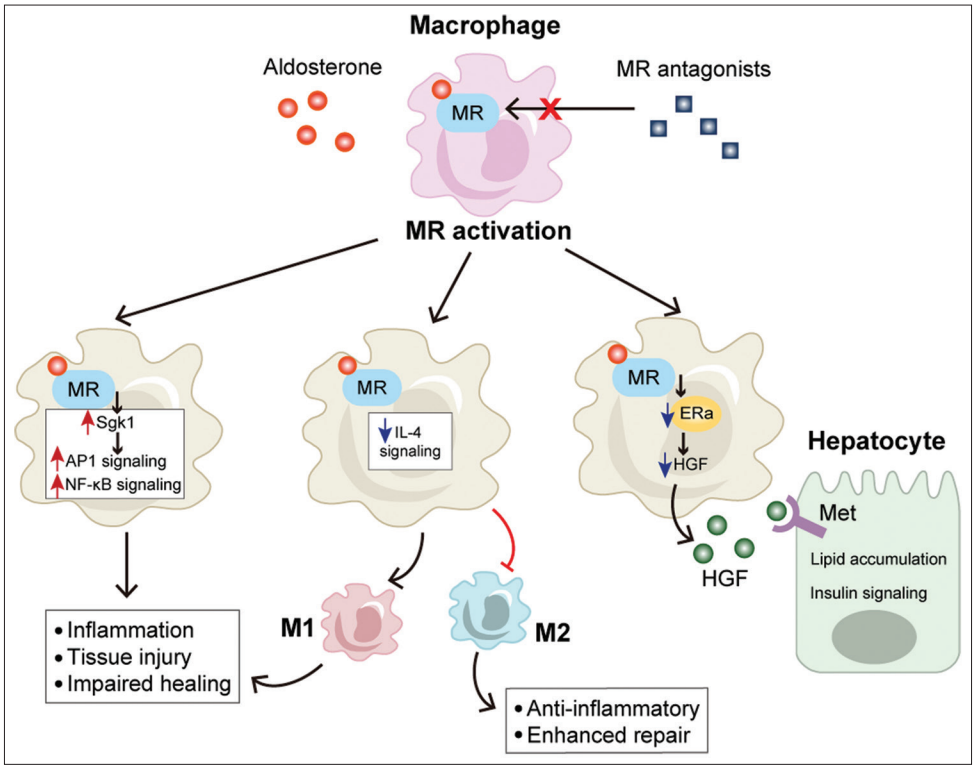


**Figure 1.** Role of mineralocorticoid receptor (MR) in macrophages. MR activation in macrophages promotes the polarization of macrophages to a proinflammatory phenotype by activating activator protein 1/nuclear factor κB signaling pathway, which leads to excessive inflammation, tissue injury, and impaired healing. In addition, MR activation suppresses interleukin-4 signaling and promotes macrophage polarization to M1 phenotype. In Kupffer cells, the resident macrophages in the liver, MR affects the secretion of hepatocyte growth factor (HGF) by regulating estrogen receptor alpha expression. HGF could phosphorylate and activate hepatocyte Met, which mediates lipid accumulation and insulin signaling in hepatocytes.

### 2.2. Role of DC MR in hypertension

DCs are activated and increased in the secondary lymphoid tissue of hypertensive mice treated with Ang II infusion or DOCA salt[43]. The specific ablation of DCs (CD11c-expressing cells) prevents the development of hypertension as a result of Ang II infusion[44,45]. MR is also expressed and functional in DCs[46]. MR activation in DCs promotes the differentiation of T cells into pro-inflammatory Th1 and Th17 phenotypes and decreases the proportion of regulatory T cells (Tregs) (Figure 2). This imbalance between T helper cells and Tregs contributes to the pathogenesis of hypertension and its associated complications[47,48]. Further experiments have shown that aldosterone pretreatment activates DCs, promotes the expression of DC maturation markers CD80 and CD86, and induces DCs to secrete cytokines such as IL-6 and IL-23, thereby activating $CD4^+$ and $CD8^+$ T cells[46]. These activated T cells then migrate to the kidney and vasculature, producing interferon gamma (IFNγ) and IL-17A and exacerbating hypertension[49] (Figure 2). Spironolactone, an MR antagonist, effectively inhibits DC activation, T cell immunity, and the development of hypertension[30,46].

Araos *et al.* have provided information concerning the downstream mechanisms of DC MR activation in a nephrectomy-aldosterone-salt model of hypertension[50]. MR stimulation in DCs favors neutrophil gelatinase-associated lipocalin (NGAL) and IL-23 expression, which are involved in the development of fibrosis and Th17 response, respectively[50] (Figure 2). NGAL has been identified as a specific target of MR in cardiovascular cells[50-52]. The specific knockout of DC NGAL can effectively inhibit MR-dependent T cell activation and inflammatory response[50].

### 2.3. T cell MR and CMDs

#### *2.3.1. Role of T cell MR in hypertension*

The previous animal models and clinical studies have demonstrated that T cells play a key role in mediating renal and vascular inflammation and hypertension[53,54]. The role of T cell MR in hypertension has been investigated. In Ang II-infused mice, the deletion of MR in T cells strikingly decreases both systolic and diastolic blood pressures and attenuates renal and vascular damages[27]. In contrast, the overexpression of MR in T cells increases blood pressure in response to Ang II infusion[27]. Mechanically, MR in T cells, particularly $CD8^+$ T cells, interacts with transcription factors nuclear factor of activated T cells 1 (NFAT1) and AP-1 to regulate IFNγ production, and ultimately influences blood pressure[27] (Figure 2). Consistently, eplerenone, which is also an MR antagonist, attenuates AngII-induced hypertension and decreases IFNγ expression in $CD8^+$ T cells[27]. Other studies have shown that T cell MR is involved in the regulation of renal fibrosis and blood pressure in DOCA/salt-induced hypertension model by regulating the expression of C-X-C chemokine receptor type 4 (CXCR4)[55,56]. As reported, mineralocorticoid excess stimulates the accumulation of T cells in the kidney, which is significantly blunted by CXCR4

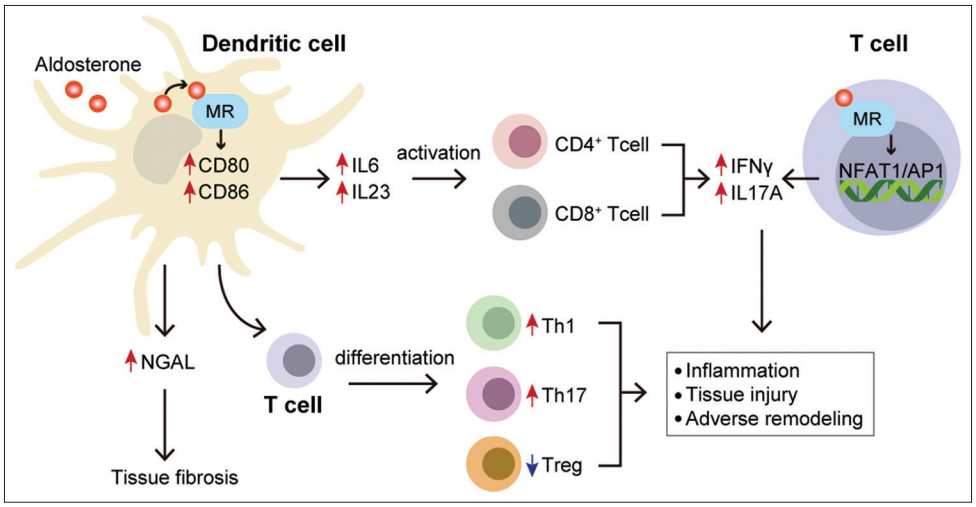


**Figure 2.** Role of mineralocorticoid receptor (MR) in dendritic cells (DCs) and T cells. MR activation in DCs promotes the differentiation of T cells into pro-inflammatory Th1 and Th17 phenotypes as well as decreases the proportion of regulatory T cells (Tregs). Moreover, MR activation enhances the expression of DC maturation markers CD80 and CD86 as well as induces DCs to secrete cytokines, such as interleukin (IL)-6 and IL-23, thereby activating $CD4^+$ and $CD8^+$ T cells. Activated T cells increase the expression of pro-inflammatory cytokines Interferon gamma (IFNγ) and IL-17A, both of which lead to tissue inflammation and injury. In addition, MR activation in DCs favors the expression of neutrophil gelatinase-associated lipocalin, which contributes to the development of fibrosis. In T cells, MR interacts with transcription factors nuclear factor of activated T cells 1 and activator protein 1 to regulate IFNγ production. The excessive inflammation eventually leads to tissue injury and adverse remodeling.

inhibition and MR antagonist[56]. These data suggest that T cell MR regulates the expression of chemokine receptors and inflammatory factors, affecting T cell aggregation and the level of inflammation, respectively, thus affecting blood pressure.

#### *2.3.2. Role of T cell MR in cardiac remodeling*

The previous study has shown that T cell MR is also involved in the pathological process of myocardial remodeling[17]. In a mouse model of abdominal aortic contraction (AAC)-induced cardiac hypertrophy, T cell MR deficiency reduces cardiac hypertrophy, fibrosis, inflammation, and dysfunction[57]. Consistently, MR antagonists inhibit cardiac hypertrophy and fibrosis, induced by AAC, and reduce the accumulation and activation of $CD4^+$ and $CD8^+$ T cells in mouse heart[57]. Mechanistically, T cell MR deletion could inhibit the expression of surface molecule CD44, CD69, and CD25, which are all important markers of T cell activation, suggesting that MR could directly regulate T cell activation[57]. Moreover, the deficiency of T cell MR suppresses the expression of IL-2 most likely by decreasing the amount of dephosphorylated NFATc2[57]. These results support that T cell MR contributes to cardiac remodeling and dysfunction following pressure load.

### 2.4. B cell MR

B cells are adaptive immune cells, and their main functions include antibody generation, antigen presentation, T cell co-stimulation, and cytokine production[58]. B cells have been progressively recognized as modulators of both adaptive and innate immune responses and for their role in the pathogenesis of CMDs, such as MI, HF, vascular disease, and obesity-associated metabolic disease[58,59]. MR expression has been found in B cells[30]. However, it is still unclear whether B cell MR plays a role in CMDs and whether MR regulates B cell function.

## 3. Vascular cell MR and CMDs

### 3.1. Role of MR in endothelial cells

#### *3.1.1. Role of endothelial cell MR in atherosclerosis*

Atherosclerosis, a chronic inflammatory disease, is a risk factor for many other CVDs[60]. Endothelial cells (ECs) play an important role in the pathological process of atherosclerosis[61]. In response to cardiovascular risk factors, injury to the vascular endothelium occurs, and a dysfunctional endothelium in turn promotes the adhesion and migration of white blood cells to vascular walls[61]. On the other hand, dysfunctional ECs promote vascular inflammation by expressing surface adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and endothelial selectin[62,63] (Figure 3). Caprio *et al.* have shown that aldosterone-activated MR increases ICAM-1 expression in primary human ECs and promotes leukocyte adhesion *in vitro*[64]. Further experiments have shown that ICAM-1 deletion in ECs alleviated vascular inflammation and plaque areas in a mouse model of aldosterone-induced atherosclerosis and MR directly regulated the gene expression of ICAM-1 in ECs[65], suggesting that MR plays a role in atherosclerosis disease through ICAM-1. Moss *et al.* have demonstrated that the deficiency of EC MR did not affect the plaque size or composition in a mouse atherosclerosis model[66]. However, plaque inflammation was significantly ameliorated in EC MR knockout mice[66]. These experimental results imply that EC MR mediates vascular inflammation in atherosclerosis.

#### *3.1.2. Role of endothelial cell MR in hypertension*

The role of vascular EC MR in blood pressure regulation has also been explored. Nguyen Dinh Cat *et al.* have demonstrated that the overexpression of MR in ECs elevates blood pressure at baseline and in response to endothelin 1 or Ang II infusion as well as enhances the contractile response of mesenteric arteries to vasoconstrictors without affecting the vascular morphology[25]. In contrast, MR deletion in ECs has no effect on blood pressure at baseline or in DOCA/salt- or Ang II-induced mouse models[67,68]. However, EC MR deficiency has shown to ameliorate cardiac remodeling by inhibiting the recruitment of macrophages and reducing the expression of myocardial pro-inflammatory factors in a mouse model of DOCA/salt-induced hypertension[67,68]. Meanwhile, EC MR deficiency has shown no effect on renal fibrosis, glomerular injury, proteinuria, or inflammation in a DOCA/salt-induced model[67-69]. Remarkably, MR deletion in ECs protects DOCA salt-induced aortic endothelial dysfunction but has no effect on the mesenteric artery[68]. In Ang II-induced hypertension, EC MR deletion improves the dilatation function of mesenteric (but not coronary) artery[26]. These data suggest that EC MR is involved in regulating vasoconstriction in a vascular bed-specific manner.

#### *3.1.3. Role of endothelial cell MR in angiogenesis*

EC MR also plays a role in angiogenesis. Zheng *et al.* have demonstrated that the deletion of EC MR promotes blood flow recovery and increases blood vessel density in ischemic limbs in a mouse model of hindlimb ischemia[70]. Moreover, MR knockout increases angiogenic potential, migration capacity, and EC proliferation *in vitro*[70]. Mechanistically, MR affects endothelial cell function by inhibiting signal transducer and activator of transcription 3 (STAT3) expression by binding to CCAAT enhancer-binding protein beta (C/EBPβ)[70] (Figure 3). Interestingly,

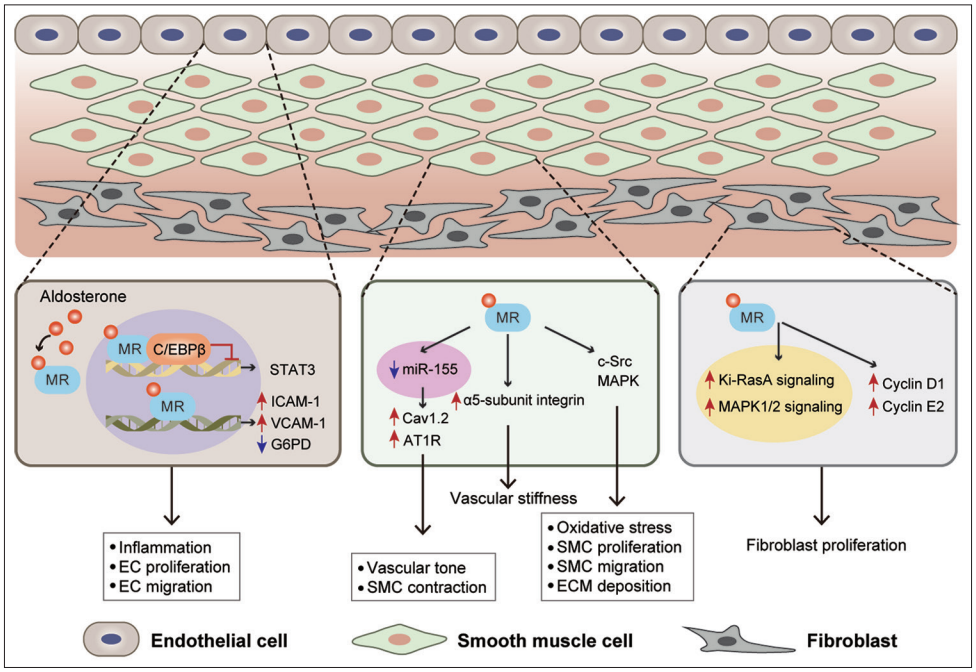


**Figure 3.** Role of mineralocorticoid receptor (MR) in vascular cells. In endothelial cells (ECs), MR activation promotes vascular inflammation by enhancing the expression of surface adhesion molecules, including intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and inhibiting the expression of glucose-6-phosphate dehydrogenase. In addition, MR affects endothelial cell proliferation and migration by inhibiting the expression of signal transducer and activator of transcription 3 by binding to CCAAT enhancer-binding protein beta. In smooth muscle cells (SMCs), MR regulates contraction by inhibiting the expression of microRNA-155 and increasing the expression of L-type calcium channel Cav1.2 and angiotensin type-1 receptor. Moreover, MR activation in SMCs promotes the expression of α5 subunit integrin and aggravates vascular stiffness. On the other hand, MR phosphorylates and activates non-receptor tyrosine kinases c-Src and mitogen-activated protein kinase (MAPK), thereby leading to vascular inflammation, oxidative stress, proliferation and migration of SMCs, and extracellular matrix deposition. In fibroblasts, MR activation promotes proliferation of fibroblasts by activating Ki-RasA and MAPK1/2 signaling as well as increasing cyclin D1 and E2 expression.

MR antagonists have opposite effects on angiogenesis in different models of diseases. Eplerenone, a selective MR antagonist, has shown to promote angiogenesis in rat hindlimb ischemia by improving the proliferation and function of endothelial progenitor cells[71]. However, Zhao *et al.* have reported that treatment with another MR antagonist spironolactone reduces symptoms of choroidal neovascularization (CNV) in patients with age-related macular degeneration (AMD)[72]. Furthermore, in a rodent model of AMD, MR antagonist or specific deletion of MR in ECs has shown to exert anti-angiogenic effects, partially through the upregulation of decorin expression[72]. These experimental results suggest that endothelial MR regulates angiogenesis differentially in different types of vessels and disease models.

### *3.1.4. Role of endothelial cell MR in obesity and diabetes*

Clinical studies have provided evidence to support a link between MR and vascular dysfunction in obese patients[73]. Hwang *et al.* have found that eplerenone improves vascular endothelial function in older adults, especially those with more total body fat and abdominal fat[73]. The previous studies have shown that EC-specific MR knockout does not affect glucose tolerance or inflammation in white adipose tissue but prevents endothelial dysfunction in obese animals[74]. Similarly, EC MR deficiency has shown to improve endothelial dysfunction, vascular stiffness, and cardiac diastolic dysfunction in western diet (WD)-induced obesity and T2DM female mice, without affecting the body composition or insulin sensitivity of mice fed WD[75,76]. Mechanistically, EC MR deletion ameliorates WD-induced aortic fibrosis and stiffness by inhibiting the endothelial expression of sodium channels, oxidative stress, and macrophage polarization, as well as enhancing the endothelial activation of nitric oxide (NO) synthase[76]. These results provide clear evidence that EC MR contributes to endothelial dysfunction and vascular stiffness in obesity and diabetes.

### *3.1.5. Role of endothelial cell MR in pulmonary arterial hypertension (PAH)*

PAH is a syndrome characterized by a progressive increase in pulmonary vascular load and significant pulmonary vascular remodeling[77]. Increased pulmonary artery resistance leads to right ventricle remodeling and failure[77,78]. The previous studies have reported that plasma

aldosterone levels are elevated in animal models and in patients with PAH, implying that MR signaling may be involved in the pathophysiological process of PAH[79-81]. Moreover, treatment with spironolactone or eplerenone attenuates pulmonary vascular remodeling without affecting the structure and function of the right ventricle in animal models of PAH[82,83], suggesting that the main protective role of MR antagonists is related to pulmonary vessels. In a recent study, Kowalski *et al.* corroborated previous observations that MR antagonists alleviate pulmonary arteriole remodeling and right ventricular hypertrophy through a mouse model of hypoxia-induced PAH[84]. Surprisingly, MR deletion in ECs attenuates hypoxia-induced PAH and right ventricular failure, but its deletion in smooth muscle cells (SMCs), fibroblasts, or myeloid cells does not have any significant effect[84]. Meanwhile, EC MR deficiency has shown to inhibit pulmonary arteriole remodeling and right ventricular hypertrophy, thus recapitulating the beneficial effects observed in eplerenone treatment[84]. Mechanistically, MR deletion in ECs attenuates the expression of genes related to blood pressure and Notch signaling pathway regulation in hypoxia-treated primary pulmonary ECs[84]. Of note, MR deficiency inhibits the upregulation of endothelin-converting enzyme 1 and endothelin receptor B, which are both elevated in response to hypoxic stimulation[84]. However, further investigation is needed to determine if the low expression of endothelin-converting enzyme 1 and endothelin receptor B in MR-deficient ECs improves pulmonary vascular remodeling. Moreover, SMCs, fibroblasts, and inflammatory cells are also involved in pulmonary arterial remodeling in PAH. However, only EC MR deletion can improve the PAH phenotype, suggesting the existence of a crosstalk between endothelial and other cell types. More studies are needed to understand the detailed molecular mechanisms.

### 3.2. Role of MR in SMCs

#### *3.2.1. Role of SMC MR in hypertension*

SMCs regulate blood pressure by modulating vasoconstriction[85]. It has been proposed that uncontrolled proliferating SMCs account for media thickening and vascular stiffness in the pathological process of hypertension[85]. The importance of MR in SMCs for blood pressure regulation has been widely reported[24,86,87]. Studies in mouse model have demonstrated that SMC-specific MR deficiency lowers blood pressure in both aged mice and high sodium-induced hypertensive mice[24,86]. Meanwhile, SMC-specific MR knockout mice have shown improved contractile function and anti-oxidative stress response to Ang II[24]. Mechanistically, MR regulates SMC contraction by inhibiting the expression of microRNA-155 and increasing the expression of L-type calcium channel Cav1.2 and angiotensin type-1 receptor[87] (Figure 3). On the other hand, SMC-specific MR deletion ameliorates vascular stiffness in aldosterone/salt-induced hypertensive mouse model with decreased expression of α5-subunit integrin[86] (Figure 3). Lu *et al.* have found that in SMCs, Ang II acts through angiotensin type 1 receptors to phosphorylate and activate the δ isoform of protein kinase C, which in turn activates the transcriptional activity of MR and subsequently promotes the expression of MR target genes as well as the proliferation of SMCs[88].

#### *3.2.2. Role of SMC MR in MI and HF*

The role of SMC MR in MI has also been investigated. It has been demonstrated that MR deletion in SMCs improves coronary and left ventricular (LV) dysfunction in mouse MI model[89]. Mechanistically, SMC-specific MR deficiency exerts a protective role in post-MI hearts by improving LV compliance and elastance as well as reducing interstitial fibrosis and oxidative stress[89]. However, the LV ejection fraction, LV mass, and heart infarct sizes have been shown to be similar between SMC MR knockout mice and control mice after MI. MR blockage by finerenone (nonsteroidal MR antagonists) has shown similar effects as well as[89]. These results support that SMC MR contributes to coronary and left LV dysfunction after MI. Recent animal experiments have suggested that SMC MR deletion exerts protective effect on transverse aortic constriction (TAC)-induced HF with markedly improved ejection fraction, cardiac stiffness, ventricular dimensions, intracardiac pressure, pulmonary edema, and exercise capacity[90]. Mechanistically, MR deletion in SMCs protects cardiac function and adverse cardiac remodeling by alleviating cardiomyocyte hypertrophy, inhibiting cardiac interstitial and perivascular fibrosis, reducing myocardial inflammation, as well as increasing cardiac capillary density and coronary blood flow reserve[90].

### 3.3. Role of MR in fibroblasts

Fibroblasts are the most important source of cardiac extracellular matrix (ECM), and they play an important role in the pathogenesis of MI, hypertension, and HF[91]. It has been reported that MR is expressed in fibroblasts[92]. *In vitro* studies have verified the direct role of MR in cardiac fibroblasts. Stockand and Meszaros have demonstrated that aldosterone is able to stimulate cardiac fibroblast proliferation through the activation of Ki-RasA and mitogen-activated protein kinase (MAPK)1/2 signaling[93] (Figure 3). This conclusion is supported by the findings of another experiment, showing that aldosterone promotes rat cardiac fibroblast proliferation by increasing cyclin D1 and cyclin E2 expression[94] (Figure 3). These findings suggest that aldosterone-induced MR activation promotes cardiac fibroblast proliferation *in vitro*. Whether

aldosterone promotes ECM production through the direct activation of fibroblasts remains a controversy. Fullerton *et al*. have shown that aldosterone has no direct effect on collagen synthesis in cultured rat cardiac fibroblasts[95]. On the contrary, Brilla *et al*. have demonstrated that low concentration of aldosterone increases collagen synthesis in cultured cardiac fibroblasts, and the effect of aldosterone can be inhibited by spironolactone[96]. Previous animal studies have shown that fibroblast-specific MR deficiency has no effect on cardiac function, cardiac hypertrophy, and fibrosis following TAC[97]. It has not been investigated whether fibroblast MR plays a role in other CVDs.

## 4. Cardiomyocyte MR and CMDs

### 4.1. Role of cardiomyocyte MR in MI and HF

Cardiomyocyte MR is known to be involved in cardiac remodeling processes following ischemia[98]. Cardiomyocyte-specific MR deficiency exerts a protective role in MI by improving infarct healing and cardiac function[99]. Mechanistically, cardiomyocyte MR deficiency inhibits oxidative stress, reduces cardiomyocyte apoptosis, enhances neovascularization, and prevents ECM deposition and cardiac hypertrophy following MI. At the molecular level, cardiomyocyte MR deletion reduces apoptosis and promotes healing by activating NF-κB signaling in the early stage of MI[99] (Figure 4). Cardiomyocyte MR is also crucial for determining acute cardiac functional recovery after ischemia-reperfusion injury[100]. Cardiomyocyte MR ablation can improve the recovery of cardiac systolic function and reduce arrhythmia post-ischemia/reperfusion[100]. The enhanced systolic function and decreased arrhythmia in the heart of cardiomyocyte MR knockout mice have been found to be associated with decreased calcium/calmodulin-dependent protein kinase II (CaMKII) activation and sodium-hydrogen exchanger-1 expression (NHE-1)[100] (Figure 4). CaMKII is involved in pathophysiological MR signaling in ischemic heart disease[101]. Moreover, the inhibition of NHE-1 could suppress ventricular arrhythmia in an ischemia-reperfusion model[102]. However, the ablation of MR in myocytes does not affect the development of cardiac hypertrophy, fibrosis, apoptosis, or inflammation in TAC mouse model despite its protective effects on cardiac dilation and dysfunction[97].

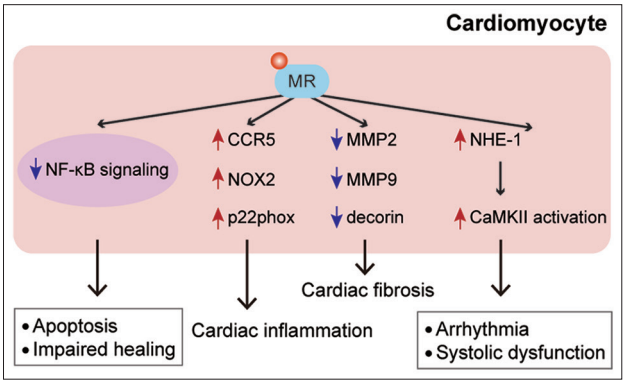


**Figure 4.** Role of mineralocorticoid receptor (MR) in cardiomyocytes. MR activation in cardiomyocytes affects the heart through different mechanisms. MR activation in cardiomyocytes (i) inhibits the early activation of nuclear factor κB (NF-κB) signaling, which is a key signaling component for early inflammatory activation and healing after myocardial infarction; (ii) promotes cardiac inflammatory responses by increasing the expression of T-cell chemoattractant CCR5, nicotinamide adenine dinucleotide phosphate oxidase subunit NOX2, and p22phox; (iii) enhances cardiac fibrosis by decreasing the expression of decorin and inhibiting matrix metalloproteinase 2/MMP9 activity; (iv) increases the expression of sodium-hydrogen exchanger-1, which in turn activates calcium/calmodulin-dependent protein kinase II, and ultimately leading to arrhythmia and systolic dysfunction.

### 4.2. Role of cardiomyocyte MR in non-ischemic cardiac injury

In a deoxycorticosterone/salt mouse model, cardiomyocyte MR deletion inhibits inflammatory responses by reducing the expression of T-cell chemoattractant CCR5, nicotinamide adenine dinucleotide phosphate oxidase subunit NOX2, and p22phox in the heart[103] (Figure 4). Moreover, cardiomyocyte-specific MR deficiency inhibits cardiac fibrosis by increasing the expression of decorin and matrix metalloproteinase 2 (MMP2)/MMP9 activity[103] (Figure 4). However, cardiomyocyte MR deletion does not affect cardiac function in this model[103]. On the other hand, cardiomyocyte MR plays an important role in cardiac arrhythmia[104]. Cardiac-specific overexpression of human MR leads to a high rate of sudden death without cardiac structural alteration[104]. The high mortality rate could be prevented by spironolactone[104]. In a study, surviving mice showed severe electrocardiography abnormalities, including prolonged ventricular repolarization and arrhythmias[104]. Mechanistically, cardiac MR overexpression causes ion channel remodeling, leading to an increase in action potential duration and calcium transient amplitude[104]. The role of cardiomyocyte MR in doxorubicin-induced cardiotoxicity has also been investigated[105]. Doxorubicin is widely used in cancer therapy, but its application is limited by cardiotoxicity[105]. The previous data have shown that both eplerenone and cardiomyocyte-specific MR deficiency attenuate doxorubicin-induced LV dysfunction[105]. These data suggest that the beneficial effect of eplerenone is related to the inhibition of cardiomyocyte MR.

## 5. Role of MR in adipocyte

### 5.1. Adipocytes

Adipose tissue, also known as "fat," is not only a metabolic organ but an endocrine organ with extraordinary plasticity and heterogeneity. Adipose tissue dysfunction

leads to abnormal responses to physiological signals and contributes to the progression of CMD, along with other pathological consequences. In the last decade, studies have revealed that MR plays an important role in adipose tissue, where MR is involved in regulating the pathophysiological process of adipocytes, including differentiation, autophagy, and adipokine secretion[106]. The regulatory role of MR in white and brown adipocytes is discussed below.

### 5.2. Function of the MR in white adipocyte

Several studies have demonstrated that MR has an essential role in regulating white adipocyte differentiation and adipogenesis *in vitro*[107-109]. However, Lee *et al.* have provided a contrary perspective that instead of MR, GR plays a dominant role in cortisol-mediated adipogenesis and adipokine production in human adipocytes[110]. Given the different conditions used in these studies, the individual and cooperative roles of MR and GR in regulating adipocyte differentiation require further investigation.

MR expression is increased in the adipose tissue of obese mouse model and obese patients[111,112]. Notably, the metabolic benefits of MR antagonists have been widely demonstrated by several animal studies[40,111,113,114]. MR blockade reverses obesity-induced white adipocyte dysfunction and insulin resistance as well as promotes the browning of white adipose tissue (WAT). Moreover, MR activation is involved in obesity-induced vascular dysfunction. On the contrary, MR blockade by potassium canrenoate improves adipose tissue senescence and vascular dysfunction in obesity[115]. Besides, adipocyte MR overexpression leads to impaired vascular contractility in non-obese mice[116]. Interestingly, a recent human study has shown that eplerenone inhibits interstitial fibrosis in subcutaneous adipose tissue in type 2 diabetes patients[117]. Another recent study has demonstrated that spironolactone ameliorates diet-induced hepatic steatosis and insulin resistance by improving WAT browning and inhibiting hepatic mitochondria dysfunction, oxidative stress, and inflammation[118]. However, the exact role of white adipocyte MR in obesity and its associated metabolic disorders remains uncertain. Several studies using genetic adipocyte-specific MR overexpression or knockout mice have revealed a deteriorative effect of adipocyte MR on the regulation of obesity-related metabolic disorders[112,119,120]. However, two other studies have shown that the depletion of MR in mature adipocytes exerts minor to modest improvements on obesity-associated glucose intolerance, insulin resistance, and hepatic steatosis[121,122]. In addition, another study has observed a relative resistance to diet-induced obesity in transgenic mice overexpressing human MR[123]. The discrepancy between adipocyte MR genetic manipulation and MR antagonists could be attributed to the heterogeneity of adipose tissue. The function of MR in other cell types should be taken into consideration in future work.

### 5.3. Function of MR in brown adipocyte

Several *in vitro* studies have shown that MR activation reduces the expression of uncoupling protein 1 (UCP-1), which is a thermogenic marker, during brown adipocyte differentiation[124,125]. Finerenone, a nonsteroidal MR antagonist, has been shown to improve the metabolic parameters of high-fat diet-induced obese mice *in vivo* through brown adipose tissue (BAT) activation, without affecting WAT expansion[126,127]. Notably, a recent clinical study has revealed that spironolactone increases human BAT activity in response to cold stimuli and food intake[128]. These studies suggest that brown adipocyte MR plays an important role in the control of energy expenditure in metabolic diseases. Further investigations are needed to determine the exact role of MR in brown adipocyte function and CMD.

## 6. Role of osteoblast MR in cardiac remodeling

Osteoblasts have endocrine functions and play important roles in homeostatic regulation of the internal environment of the body[129,130]. Osteoblast MR deletion diminishes atrial fibrosis in mutant transforming growth factor beta 1 (TGF-β1) transgenic mice by suppressing osteocalcin (OCN) expression[131]. In cultured atrial fibroblasts, OCN binds to G protein-coupled receptor family C group 6 member A (GPRC6A) and promotes the phosphorylation of protein kinase A (PKA) and cAMP-response element binding protein (CREB), thereby increasing fibroblast proliferation and migration[131]. Osteoblast MR deficiency has shown to improve cardiac function and inhibit adverse cardiac remodeling in a mouse MI model[132]. Mechanistically, calvaria ribonucleic acid (RNA) sequencing data have revealed that MR knockout decreases the expression of OCN, which works through GPRC6A to increase the phosphorylation level of ERK in cultured macrophages, thereby promoting macrophage proliferation and pro-inflammatory phenotypic differentiation[132]. Consistently, MR antagonists have been shown to inhibit the expression and secretion of OCN in post-MI mice and HF patients, which further suggests the existence of MR/OCN axis-mediated communication between osteoblast and the heart in pathological cardiac remodeling[132].

## 7. MR antagonists and clinical applications

Clinical trials have demonstrated that MR antagonists (MRAs) have beneficial effects in treating CVD[7].

MRAs can be categorized as steroidal and non-steroidal compounds based on their chemical class[133]. Steroidal MRAs include spironolactone and eplerenone, which have similar therapeutic effects but different pharmacological characteristics[7]. Finerenone is a third-generation, non-steroidal MRA with less side effects compared with steroidal compounds[134] (Table 1).

Spironolactone is a first-generation MRA, which has been used clinically for decades. It was originally approved as a diuretic for the treatment of edema, primary aldosteronism, and essential hypertension[135]. Subsequently, extensive studies have shown that spironolactone has significant benefits for severe HF, refractory hypertension, hypokalemia, and ascites secondary to cirrhosis[133]. The Randomized Aldactone Evaluation Study (RALES) trial has proven that spironolactone improves mortality and morbidity in patients with severe HF[136]. This trial further supports the use of spironolactone in HF patients. Spironolactone is a potent and competitive antagonist of MR. However, it also binds to androgen and progesterone receptors, causing endocrinal side effects, such as gynecomastia, impotence, and menstrual irregularities[136]. The major limitation of spironolactone in clinical application is the occurrence of hyperkalemia[137]. A pharmacoepidemiologic study has noted that there is a rapid increase in incidence of hyperkalemia following the online publication of the RALES trial. This is associated with an increase in spironolactone prescriptions for elderly patients with HF[138]. Subsequent clinical studies have suggested that the judicious use of spironolactone and closer patient monitoring could reduce the incidence of hyperkalemia[139,140]. Of note, it has been reported that 54% of hyperkalemia cases are associated with MRA treatments in MI or HF patients[141].

**Table 1. Properties and applications of MR antagonists**

| MRAs | Spironolactone | Eplerenone | Finerenone |
|---|---|---|---|
| Chemical composition | Steroidal compound | Steroidal compound | Non-steroidal compound |
| Affinity for MR | High[149] | Low[149] | High[149] |
| Tissue distribution | Kidney>heart[137] | Kidney>heart[137] | Balanced in kidney and heart[137] |
| Active metabolites | 7α-TMS Canrenone[150] | No active metabolites[150] | No active metabolites[150] |
| Half-life in humans | 7α-TMS: 13.8 h Canrenone: 16.5h[150] | 4–6 h[137] | 2–3 h[150] |
| Clinical applications | Edema[135] Primary aldosteronism[135] Hypertension[135] HF[132,135] | Hypertension[142,143] HF[142,143] | DKD[147,148] HF with T2DM[146] |
| Side effects | Gynecomastia[135] Impotence[135] Menstrual irregularities[135] Hyperkalemia[137] | Hyperkalemia[137,150] | Hyperkalemia (significantly lower incidence)[137] |

MRAs: Mineralocorticoid receptor antagonists; 7α-TMS: 7α-thiomethyl-spironolactone; HF: Heart failure; DKD: Diabetic kidney disease; T2DM: Type 2 diabetes mellitus; MR: Mineralocorticoid receptor

Eplerenone is a second-generation MRA with fewer side effects than spironolactone and a greater selectivity for MR[7]. However, it has lower affinity for MR than spironolactone, thus requiring a higher dose to achieve the same effect as the latter[135]. The results of Eplerenone Post-Acute **MI** HF Efficacy and Survival Study (EPHESUS) have revealed that eplerenone therapy reduces overall morbidity and mortality among patients with acute MI complicated by LV dysfunction and HF[142]. Similarly, the clinical benefits of eplerenone on mortality and morbidity in patients with systolic HF and mild symptoms have also been demonstrated in other clinical studies[143]. A randomized clinical trial has found that early eplerenone administration (within 3 – 7 days) post-acute MI improves outcomes in patients with LV systolic dysfunction and HF and this benefit is not evident when eplerenone is administered at a later stage (≥7 days)[144]. Furthermore, treatment with eplerenone during the acute phase of MI is safe and well-tolerated[145].

Finerenone is a novel, selective, nonsteroidal MRA, with the higher selectivity for MR than spironolactone and stronger MR-binding affinity compared to eplerenone[134]. Finerenone effectively blocks inflammation, fibrosis, adverse cardiovascular, and renal events mediated by MR overactivation[146]. The phase III Finerenone in Reducing Kidney Failure and Disease Progression in Diabetic Kidney Disease (FIDELIO-DKD) and Finerenone in Reducing Cardiovascular Mortality and Morbidity in Diabetic Kidney Disease (FIGARO-DKD) clinical trials have shown that finerenone slows the progression of kidney disease and reduces the risk of cardiovascular events and hospitalization for HF[147,148]. Finerenone confers beneficial effects on cardiovascular outcomes in patients with chronic kidney disease and type 2 diabetes and is well-tolerated in these patients[147,148]. The MR Antagonist Tolerability Study- HF (ARTS-HF) study has shown that finerenone is well-tolerated and decreases the levels of N-terminal (NT)-pro hormone B-type natriuretic peptide (NT-proBNP) to a similar extent compared with eplerenone in patients with worsening HF with reduced ejection fraction and chronic kidney disease and/or diabetes mellitus, thus suggesting the beneficial effects of finerenone[146]. The effects of finerenone in other CMD

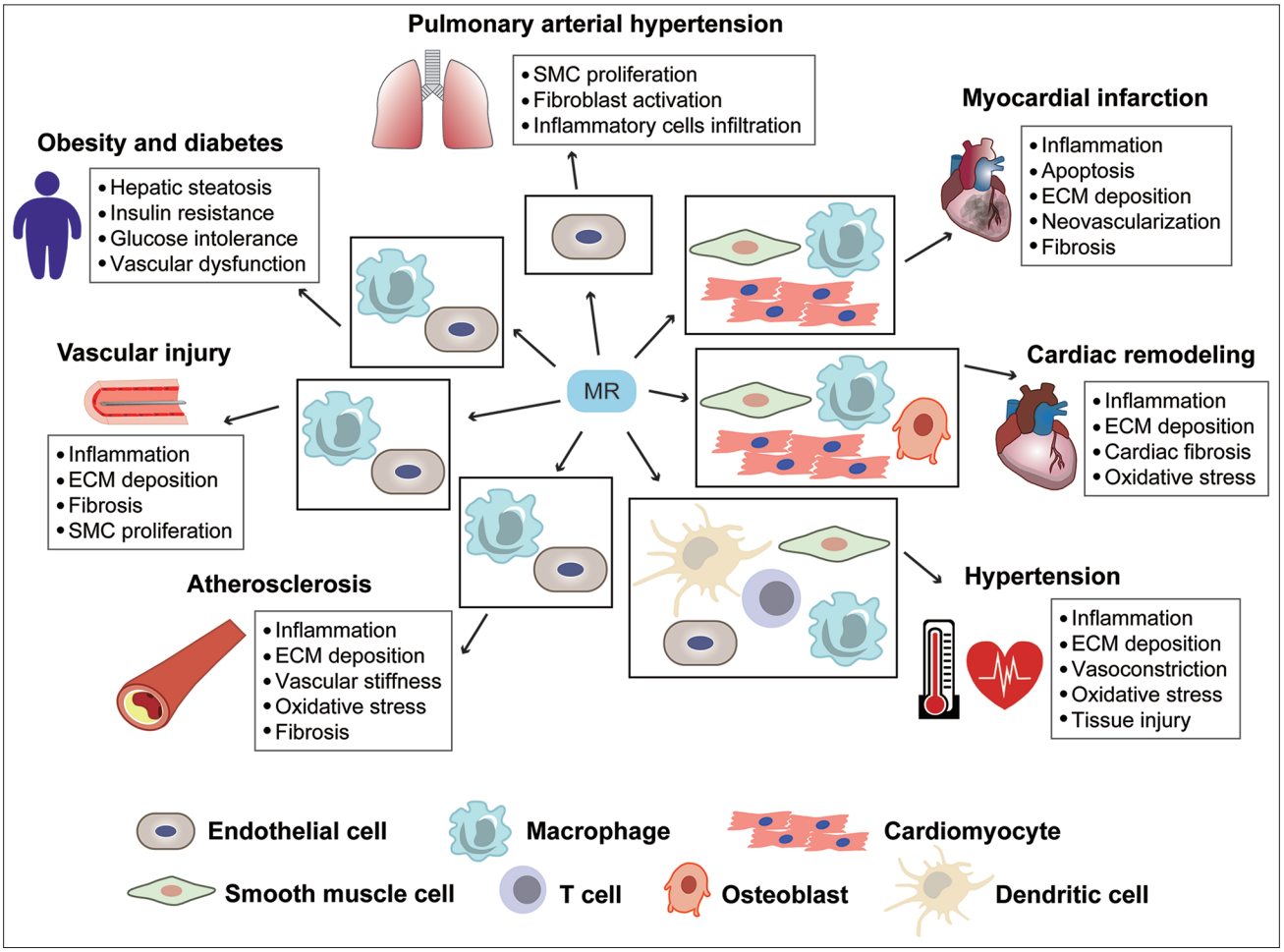


**Figure 5.** Role of mineralocorticoid receptor (MR) in the pathogenesis of cardiometabolic diseases. MR is expressed in a variety of cells and contributes to the pathological processes of cardiometabolic diseases. ECM: Extracellular matrix; SMC: Smooth muscle cell.

conditions such as hypertension, MI, and atherosclerosis, require further investigations.

## 8. Conclusions and perspectives

MR has been studied for more than 30 years, and substantial progress has been made in the understanding of the role of MR in CMDs. MR is expressed in almost all tissues and organs involved in cardiovascular homeostasis and participates in the pathophysiological process of CMDs (Figure 5). Inhibiting the functional activity of MR in these tissues may improve CMD outcomes. Although many animal experiments and clinical trials have described the function and role of MR in different tissues as comprehensively as possible, MR will continue to be an interesting and important research topic in CMDs, as many questions remain to be answered. The role of MR in some important tissues and cells has not been fully elucidated. For example, it is still not known whether B-cell MR plays a key role in the pathology of CMDs. The role of MR in adipose tissue has also not been fully explored. Elucidating the role of MR in these tissues will help to paint a more complete picture of the function of MR and will likely provide a more comprehensive basis for new therapeutic strategies.

MRAs, including steroidal and non-steroidal classes, are commonly used to treat CVDs in clinical practice. The novel non-steroidal MRA, finerenone, has a lower risk of hyperkalemia than steroidal MRAs due to differences in tissue distribution, receptor inactivation, drug half-life, and other factors. Recently, an increasing number of evidence has shown that finerenone could significantly improve renal prognosis and reduce the risk of CVD in adult patients with chronic kidney disease and T2DM. However, it still lacks direct evidence to support the application of finerenone in the treatment of CVDs, such as HF, MI, atherosclerosis, and hypertension. Illuminating the beneficial effects (and possibly side effects) of finerenone in CMDs will facilitate a broader application of this drug in clinical practice and inspire the development of the next generations of MRAs.

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## Conflict of interest

The authors declare that they have no competing interest.

## Author contributions

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*Writing – review & editing:* Jian-Yong Sun, Sheng-Zhong Duan.

## Ethics approval and consent to participate

Not applicable.

## Consent for publication

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## Availability of data

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ACCSCIENCE PUBLISHING

Global Translational Medicine

REVIEW ARTICLE

# Role of stem cells in aortic aneurysm

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## Abstract

In recent years, aortic aneurysms (AAs) have attracted increasing attention due to their asymptomatic onset and high mortality. In clinic, surgery and anti-hypertensive or lipid-lowering medicine is usually applied in the treatments of AA. However, AA is prone to relapse and sudden rupture may happen. Therefore, more effective prevention and treatment methods are urgently needed. Stem cells are believed to play a crucial role in vascular formation and regeneration of damaged tissues during vascular disease progression. With the development of single-cell RNA sequencing analysis, many populations of stem cells have been discovered in AA tissues. Recent studies have demonstrated that they may participate in the occurrence or development of AAs. Besides, there is a prospect in clinical treatment for AAs when regenerative medicine with stem cells comes into the picture. This review mainly discusses the latest findings on the crucial roles of stem cells in AAs as well as their potential therapeutic strategies of stem cells, which provides some references for the prevention, diagnosis, and treatment of AAs. Further studies are needed to explore the novel application of stem cell therapies for AAs.

***Keywords:*** Stem cell; Abdominal aortic aneurysm; Thoracic aortic aneurysm

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## 1. Introduction

Aortic aneurysm (AA) is a severe cardiovascular disease, which is usually asymptomatic but has a very high natural mortality rate. AA-related mortality rate is estimated to be approximately 200,000 people worldwide each year[1]. Until now, the exact etiology of the AA has not been fully clarified. Impairment of the structural and functional integrity of the aorta leads to aortic degeneration and biomechanical failure, which are main causes of the AA formation[2]. Recently, many studies have reported that many types of stem cells exist in the AA tissues and they can proliferate and differentiate into specific cell types. In adults, stem cells in vascular systems are mostly quiescent in their niches. But they can be activated in response to injury, such as AA, and participate in endothelial repair and vascular smooth muscle cell (VSMC) accumulation to form neointima[3]. The crucial roles of stem cells in the possible pathogenesis and treatments of the AA are outlined in the review.

## 2. Mechanism of AA formation

### 2.1. An overview of AA

AA is a permanent, local dilation of the aortic wall, usually >50% of the normal diameter of the aorta. AA is clinically termed according to the location. The one above the diaphragm in the upper aortic segment is defined as thoracic AA (TAA)[4] and the abdominal AA (AAA) is mainly located below the renal arteries[5].

Epidemiological studies have shown that male, elderly patients, smoking, hypertension, and atherosclerotic cardiovascular disease are risk factors for AAA[6]. TAA has a lower prevalence than AAA and is divided into three types: Syndromic, familial non-syndromic, and sporadic[7]. Syndromic TAA usually includes Marfan syndrome (*Fbn1* mutation), Ehlers-Danlos syndrome (collagen or collagen processing enzymes mutation) or Loeys Dietz syndrome (transforming growth factor beta [TGF-β] receptor mutation) and so on[1]. About 20% of TAA patients can be attributed to specific genetic variants, which are familial non-syndromic TAA. Among them, autosomal dominant inheritance is the most common type[7]. The risk factors of sporadic TAA are similar to AAA, such as smoking, hypertension, atherosclerotic cardiovascular disease, and so on[6].

Most AAAs expand slowly without symptoms. For every 0.5 cm increase in AAA diameter, the growth rate increases by 0.5 mm/year and the rupture rate doubles[8]. The growth rate of TAA is faster than that of AAA[9]. The mortality rate after the rupture of AA is as high as 60 – 90%[10]. As a result, it is essential to study the pathogenic mechanism and effective treatments of the AA.

Some of the AA, especially TAA, enlarge over time, until a tear forms in the intimal layer of the aortic wall typically above the sinotubular junction that leads to aortic dissection (AD)[11]. AD is a serious condition characterized by a split of the different layers of the aorta wall and a rush of blood through the dissection. It happens suddenly and causes nearly half of patients to die from aortic rupture[12]. To some extent, there are several similarities between the formation mechanism of AA and AD.

### 2.2. AAA

The mechanisms of AAA formation comprise VSMC injury and apoptosis, extracellular matrix (ECM) degradation, oxidative stress, and vascular inflammation[13].

VSMCs, as the major cell type in the medial aorta, play a key role in providing structural support, regulating vascular tone, and aiding in vascular remodeling[14]. Studies have shown that the damage of VSMC in AAA patients is mainly manifested in contractibility dysfunction. Normally, most VSMCs exhibit a contractile phenotype maintained by TGF-β signaling pathway[15]. Decrease of VSMC contractile phenotype markers (such as SM22-α and SMα-actin) expression but increase of inflammatory proteins release was observed in AAA tissues, which contributed to the AAA development[2]. Multi-lineage tracing of VSMCs and their progeny in a mouse AA model showed clonal expansion and the presence of phagocytic markers CD68 and lysosomal-associated membrane protein 2 (LAMP2). Thus, this is the activation of a switch of VSMC to the phagocyte-like phenotype in AA[16].

The ECM mainly produced by VSMCs in the arterial wall is composed of elastin, collagen, and proteoglycans[17]. The role of ECM degradation in AAA is mainly reflected in the destruction of collagen and elastin[18] and the accumulation of abnormal proteoglycans[19], which is facilitated by the formation of AAA. The ECM is primarily degraded by proteolytic enzymes, including matrix metalloproteinases (MMPs) and cysteine cathepsins[20]. Under conditions of aortic inflammation, MMPs can be increasingly secreted by neutrophils and macrophages. Among them, MMP1, MMP2, MMP9, MMP13, and MT1-MMP can destroy collagen, and MMP2, MMP9, and MMP12 can destroy elastin, leading to aortic ECM degradation and aneurysm generation[21] Cathepsin S/K/L can promote the formation of AAA, and a lack of that may reduce the inflammatory response in AAA lesions[20,22,23].

Oxidative stress refers to an imbalance in the production and elimination of ROS. An elevation of ROS level has been found in the tissues of AAA patients[24], and it is believed that ROS can upregulate proteolytic enzymes such as MMP to induce ECM degradation and promote VSMC damage as well[25]. The principle is that TGF-β signaling upregulates Nox4 to promote ROS production[26]. Compared with thick-walled AA, the expression of oxidative stress and proteolytic enzyme is significantly increased in thin-walled AA, which is considered to be the primary risk factor for AAA rupture, indicating the importance of oxidative stress in the AAA enhancement[26,27].

From the above, it can be inferred that the apoptosis of VSMC and the degradation of ECM are inseparable from the vascular inflammatory response, which proves the importance of inflammation in the pathogenesis of AA. It has been proven that inflammation is a common feature of AAA lesions, which is manifested by the widespread presence of inflammatory molecules, accumulation and infiltration of inflammatory cells, and changes in signal transduction[28]. Polarized macrophages with a pro-inflammatory phenotype (M1) aggregated in AAA tissue, triggering tissue degradation and promoting the aneurysm development in the $CaCl_2$ model[29]. In contrast, anti-inflammatory phenotype (M2) macrophages are

associated with regression of inflammation and tissue repair. In addition, a large number of B lymphocytes and T lymphocytes in AAA, with predominant $CD4^+$ T cells, promoted AAA development by accumulating macrophages and regulating ECM and protease synthesis[30]. In addition, neutrophils also contribute to the development of AAA. For example, IL-1β-induced neutrophil extracellular trap formation (NETosis) can lead to the formation of AAA[31].

### 2.3. TAA

Inherited diseases which mainly affect ECM and VSMC are common causes of TAA. Three major reasons of the formation of TAA and AAA are the same, but the specific mechanism is slightly different.

Unique transcriptome-regulated VSMCs were identified in AA tissues of adult Fbn1 $^{C1041G/+}$ (MFS) mice, and single-cell RNA sequencing of AA tissues from MFS patients confirmed similar VSMC regulation, mainly attributed to TGF-hanism is slight Krüppel-like factor 4 (Klf4) overexpression[32]. VSMC contractile dysfunction is also directly related to TAA. It was found that in the thoracic aorta of AAD patients, the VSMC inflammasome NLRP3-caspase-1 cascade can degrade contractile proteins, leading to VSMC contractile dysfunction[33]. A genetic imbalance of oxidative stress and ROS can trigger TAA. The mechanism of ROS imbalance is either insufficient ROS removal or increased ROS production due to the dysregulation of redox mediators[34]. In addition, oxidative stress inhibits autophagy in the MFS model, and the autophagy mechanism plays a key role in regulating VSMC death and endoplasmic reticulum stress-dependent inflammation, which has important effects on aortic wall homeostasis and repair[35].

In molecular genetics, mutations in genes encoding various components of the TGF-β signaling cascade are collectively referred to as TGF-β angiopathy (TGFβVs). Mutations in genes encoding components of the smooth muscle contractile proteins (ACTA2, MYH11, MYLK, and PRKG1) are thought to be the cause of smooth muscle vasoconstrictor disease[36]. Mutations of the FBN1 gene linking VSMC and ECM impair VSMC productivity, and ACTA2 and MYH11 mutations cause disruption of the ATP-dependent cyclic interaction, which, in turn, leads to VSMC productivity impairment. In addition, PRKG1 mutations result in kinase composition in the absence of cGMP. Constitutive activation drives VSMC relaxation. Mutations in the above genes all lead to the occurrence of TAA[37].

### 2.4. Cells in TAA and AAA

In past years, it was believed that the blood vessel wall was mainly composed of endothelial cells, VSMCs, and fibroblasts. Recently, many types of cells have been found by applying single-cell transcriptomics in AA tissues. In addition to endothelial cells, VSMCs and fibroblasts, it has been found that AA also includes dendritic cells, monocytes, mast cells, and neutrophils from the intima to the adventitia. Figure 1 shows the approximate distribution of those cells.

Although the etiology is different, cell damage, apoptosis and other inflammatory effects all contribute to the formation of AAA and TAA. Therefore, before studying the pathophysiological mechanism of AAA and TAA, it is necessary to understand the type of cells that are present and their basic characteristics. Single-cell RNA sequencing with AAA and TAA tissues reveals the presence of additional cell types, as shown in Table 1.

## 3. Stem cells

In addition to the cells mentioned above, stem cells are present in AA tissues as well. Stem cells have the potential to self-renew and differentiate into various cell types, which not only involve in embryonic development but also reside in adult tissue and can participate in tissue repair by cell proliferation, migration, and differentiation under various stimuli. Studies have shown that adult stem cells and progenitor cells exist in the adult cardiovascular system[49]. Stem cells are also significant in the treatment of AA.

### 3.1. Endothelial progenitor cells

Bone marrow-derived endothelial progenitor cells (EPCs) have the ability to differentiate into mature endothelial cells and are involved in angiogenesis during the embryonic period, as well as endothelial cell turnover and local angiogenesis after adult vascular injury[50]. Therefore, EPCs are important for the development and treatment of AA. Typical markers include CD157, EPCR, and $CD31^{low}$ $VEGFR2^{low}$ $IL33^+$ $Sox9^+$[51].

The number of peripheral circulating EPCs in AAA patients was reduced and their function was impaired[52], but was increased significantly in TAA patients[53]. Evidence also showed that the number of EPCs increases several days after AA repair, possibly contributing to the rapid endothelialization of blood vessels[54,55]. Current studies have shown that in a rat AAA model, overexpressed nuclear-enriched abundant transcript 1 (NEAT1) competitively binds to miR-204-5p and upregulates the expression of angiotensin (Ang) I in EPCs, thereby increasing the number of EPCs in peripheral blood to promote cell viability, migration, and tube formation in AAA[56]. Chemokine CXCL12 overexpression promotes the growth of smooth muscle-like cells and the expression of VEGF

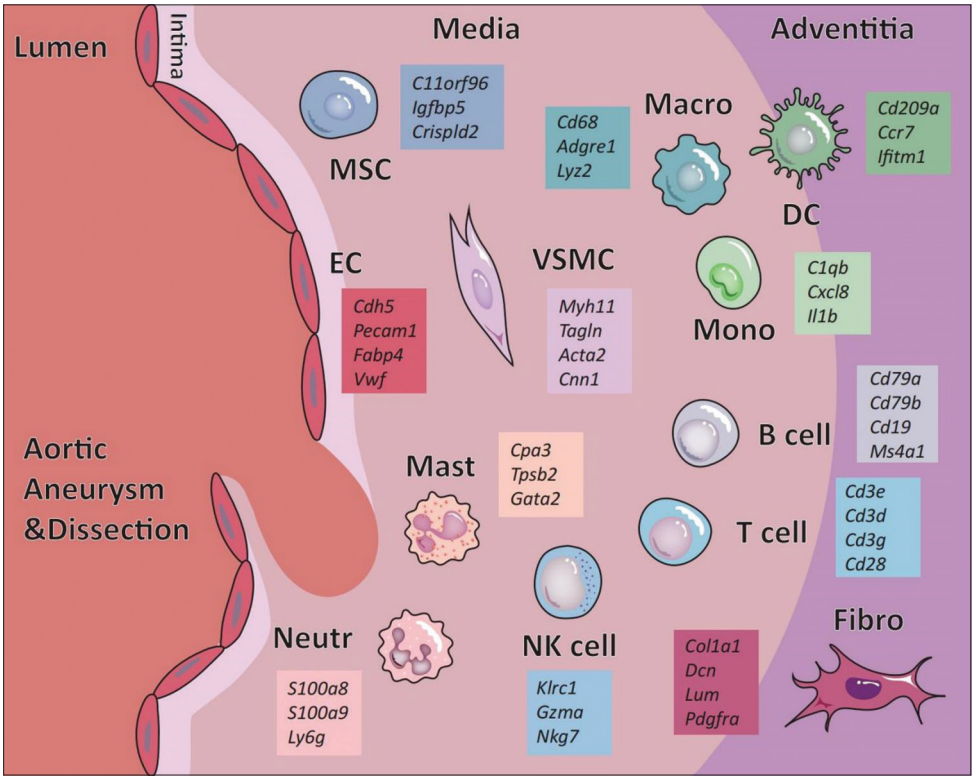


**Figure 1.** A schematic overview of cell types in aortic aneurysm and dissection. Various markers representing different cell types are listed in different layers of the aortic wall.
MSC: Mesenchymal stem cell; EC: Endothelial cell; Mast: Mast cells; Neutr: Neutrophils; VSMC: Vascular smooth muscle cell; Macro: Macrophages; Mono: Monocytes; DC: Dendritic cells; FibroL: Fibroblasts.

in the aneurysm lumen, while inhibiting the expression of MMP-2 and MMP-9 in aneurysm tissue *in vivo*[57].

So far, the effect of EPCs in AA has not been clearly concluded, but it can predict the occurrence, development and rupture of aneurysm at early stage, as well as the postoperative outcome.

### 3.2. Bone marrow-derived mesenchymal stem cells

Bone marrow-derived mesenchymal stem cells (BM-MSCs) are a class of adult stem cells with self-renewal and multi-directional differentiation potential, which have a wide range of roles in AA. Positive markers are found to be CD73, CD105, CD90, CD29, and STRO2[58]. Many studies have shown that mesenchymal stem cells (MSCs) have an effective therapeutic effect on AA, and the specific molecular mechanisms have been widely studied.

It is known that the pathogenesis of AA is closely related to vascular inflammation, and MSCs can reduce aortic inflammation to treat AA. The inhibitory effect of MSCs on dendritic cells, macrophages and T cells has long been demonstrated[59], and the specific mechanism of inhibition of inflammation in AA formation has recently been elucidated. MSCs also reduce inflammation and promote tissue repair by inducing a macrophage phenotype shift from M1 to M2, which may ultimately prevent the AA growth[60]. In the process of AAA formation, IL-17 produced by $CD4^+$ T cells can promote inflammation, and MSCs can inhibit its effect[61]. In addition to direct regulation, MSCs can also indirectly suppress aortic inflammation by modulating microRNAs to attenuate AA formation[62]. For example, miR-194 can inhibit the expression of BCL2 interacting protein 3 (BNIP3) through KDM3A, inhibiting the progression of AAA[63]. MSC-derived extracellular vesicles also contribute to the inhibition of AAA by regulating microRNA-147[64]. For the hydrolysis of elastin, adult BM-MSC-derived VSMCs can synthesize and assemble elastin to repair and regenerate the elastic matrix of AA[65].

Accordingly, MSCs have a good prospect in the treatment of AA, but many specific mechanisms are not fully understood.

### 3.3. Adipose-derived mesenchymal stem cells (ADSCs)

Adipose-derived mesenchymal stem cells (ADSCs) were also found to play an indispensable role in TAA and AAA.

**Table 1. Studies applying single-cell RNA sequencing that reveals cell population of the aortic aneurysm**

| Species | Diseases | Number of samples | Gender and genotype | Age (SD) | Induction method | Cell type | Marker | References |
|---|---|---|---|---|---|---|---|---|
| *Mus musculus* | AAA | $CaCl_2$-induced AAA and Sham, 4/group | Male C57BL/6J | 12 weeks | $CaCl_2$-induced mouse model | SMC | Myh11, Acta2 | [38] |
| | | | | | | EC | Cdh5, Pecam1 | |
| | | | | | | Fibro | Col1a1 | |
| | | | | | | Macro | Lyz2 | |
| | | | | | | Neutr | S100a8, S100a9 | |
| | | | | | | DC | Klrd1, Flt3 | |
| | | | | | | T cell and NK | Cd3g, Gzma | |
| | | | | | | B cell | Cd79a, Ms4a1 | |
| *Mus musculus* | AAA | Days 7 and 14 post elastase-induced AAA and Sham, 3/group | Male C57BL/6J | 10-week-old mice perfused for 7 or 14 days with elastase | Elastase-perfusion mouse model | SMC | Myh11, Tagln, Acta2 | [39] |
| | | | | | | Fibro | Dcn, Col1a1, Col3a1 | |
| | | | | | | EC | Cdh5, Pecam1, Fabp4 | |
| | | | | | | Mono/Macro | Cd68, Cdca3, C1qb | |
| | | | | | | B cell | Cd79a, Ly6d, Cd79b | |
| | | | | | | T cell | Cd3d, Cd3g, Cd28 | |
| | | | | | | DC | Cd209a, Ccr7, Ifitm1 | |
| | | | | | | Neural cell | Prnp, Mpz | |
| | | | | | | Eryth | Bpgm, Snca, Hba-a1 | |
| *Mus musculus* | AAA | 6 Ang II-induced AAA, 4 controls | Male $ApoE^{-/-}$ | 8-week-old mice perfused for 28 days with Ang II | Ang II-infusion mouse model | Fibro | Col1a1 | [40] |
| | | | | | | SMC | Acta2 | |
| | | | | | | EC | Pecam2 | |
| | | | | | | Macro | Cd68 | |
| | | | | | | B cell | Ms4a1 | |
| | | | | | | T cell | Cd3e | |
| | | | | | | Adipocyte | Adipoq | |
| | | | | | | Eryth | Alas2 | |
| *Mus musculus* | AAA | n=3 | $ApoE^{-/-}$ | 6–8-week-old mice perfused for 28 days with Ang II | Ang II-infusion mouse model | Macro | Cd11b, Cd68, Adgre1 | [41] |
| | | | | | | T cell | Cd3e | |
| | | | | | | B cell | Cd19 | |
| | | | | | | Neutr | Ly6g | |
| | | | | | | DC | Itgax | |
| *Mus musculus* | AAA | NA | Male $ApoE^{-/-}$ | 8-week-old mice perfused for 28 days with Ang II | Ang II-infusion mouse model | Fibro | Cd34, Pdgfra | [42] |
| | | | | | | Macro | Cd68, Ptprc | |
| | | | | | | SMC | Acta2 | |
| | | | | | | EC | Pecam1 | |
| | | | | | | T cell | Cd3e, Cd8a | |
| | | | | | | B cell | Cd19 | |
| | | | | | | Lymphatic EC | Lyve1, Pecam1 | |
| | | | | | | Proliferating cell | Top2a, Mki67 | |
| *Mus musculus* | AAA | NA | Male $ApoE^{-/-}$ | 12-week-old mice perfused for 4 weeks with Ang II | Ang II-infusion mouse model | Fibro | Col3a1, Col1a1, Dcn, Gsn | [43] |
| | | | | | | Mono/Macro | Cd68, Aif1, C1qb | |

*(Cont'd...)*

**Table 1.** *(Continued)*

| Species | Diseases | Number of samples | Gender and genotype | Age (SD) | Induction method | Cell type | Marker | References |
|---|---|---|---|---|---|---|---|---|
| | | | | | | EC | Cdh5, Pecam1, Fabp4 | |
| | | | | | | SMC | Myh11, Acta2, Tagln | |
| | | | | | | T cell | Cd3d, Cd3g, Cd28 | |
| | | | | | | B cell | Cd79a, Cd79b, Ly6d | |
| | | | | | | DC | Cd209a, Cd74 | |
| | | | | | | Granulocyte | S100a8, Ccr1, Ly6g | |
| *Mus musculus* | MFS | 4 $Fbn1^{C1041G/+}$ and 3 controls | 2 males and 2 females $Fbn1^{C1041G/+}$ and 2 males and 1 female controls | 4 weeks/24 weeks | $Fbn1^{C1041G/+}$ mouse MFS model | SMC | Acta2, Myl9, Myh11, Tpm2, Tagln | [32] |
| | | | | | | Modulated SMC | Fn1, Mgp, Nupr1, Eln, Tnfrsf11b, Igfbp2 | |
| | | | | | | Fibro 1 | Hsd11b1, Dpep1, Cygb, Gdf10, Smoc2 | |
| | | | | | | Fibro 2 | 1500015O10Rik, Prg4, Comp, Wif1, Thbs1 | |
| | | | | | | Ly6a+Fibro | Pi16, Ly6a, Clec3b, C3, Mfap5 | |
| | | | | | | EC | Pecam1, Cldn5, Ctla2a, Nts, Ccl21a | |
| | | | | | | Macro | Lyz2, Ccl4, C1qb, Cxcl2, Cd74 | |
| | | | | | | Pericardial | Upk3b, Msln, Myl7, Igfbp6, Igfbp5 | |
| *Mus musculus* | TAAD | BAPN-induced TAAD and control, 3/group | Male C57BL/6J | 3-week-old mice induced with BAPN for 28 days | BAPN-induced mouse model | SMC | Myh11, Cnn1 | [44] |
| | | | | | | Fibro | Pdgfra, Lum | |
| | | | | | | EC | Cdh5, Tie1 | |
| | | | | | | T cell | Cd3d, Cd3g | |
| | | | | | | B cell | Cd79a, Cd19 | |
| | | | | | | Macro | Itgam, Cd14 | |
| *Mus musculus* | AAD | WT and Sting-deficient mice unchallenged or challenged with HFD/Ang II, 3/group | Male and female WT C57BL/6J and Sting-deficient mice | 4-week-old mice with HFD for 5 weeks | Ang II-infusion mouse model | SMC | Acta2, Myh11, Mylk | [45] |
| | | | | | | Macro | Cd68, Adger1, F13a1 | |
| | | | | | | Fibro | NA | |
| | | | | | | EC | NA | |
| *Homo sapiens* | ATAA | 8 patients, 3 controls | 4 males and 4 females in patients and 1 male and 2 females in controls | 67.6 (8.1) years in patients and 62 (1) years in controls | / | T cell | Ccl5, Trbc2, Cd2, Cd3d, Trac, Cd52, Cxcr4, Il32, Gzmk, Il7r | [46] |
| | | | | | | Mono | Cxcl8, Il1b, Hla-dra, C1qa, C1qb, Cxcl3, Hla-dpa1, Cxcl2, C1qc, Cd74, Selenop | |
| | | | | | | SMC1 | Acta1, Myl9, Tagln, Tpm2, Cald1, Myh11, Dstn, Pln, Mfge8 | |
| | | | | | | NK | Xcl1, Xcl2, Klrd1, Gnly, Klrc1, Cd7, | |

*(Cont'd...)*

**Table 1.** *(Continued)*

| Species | Diseases | Number of samples | Gender and genotype | Age (SD) | Induction method | Cell type | Marker | References |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | Ctsw, Prf1, Cmc1 | |
| | | | | | | Fibro | Dcn, Lum, Fn1, Mgp, Cfh, Col1a2, Aebp1, Igfbp6, Gsn, C1r | |
| | | | | | | SMC2 | Myh10, Map1b, Igfbp2, Sparc | |
| | | | | | | EC | Vwf, Ifi27, Pecam1, Aqp1, Spry1, Sparcl1, Gng11, Igfbp4, Cavin2, Adgrl4 | |
| | | | | | | MSC | C11orf96, Igfbp5, Crispld2, Mt2a, Adirf, Mt1m, Plac9, Ndufa4l2 | |
| | | | | | | Plasma cell | Igha1, Mzb1, Ssr4, Prd×4, Fkbp11, Sec11c, Qpct, Lmf1 | |
| | | | | | | B cell | Ms4a1, Ly9, Bank1, Arhgap24, Cd79b, Ltb, Linc00926, Stag3 | |
| | | | | | | Mast | Gata2, Hpgds, Hpgd, Kit, Il1rl1, Laptm4a, Mlph, Vwa5a, Rgs13, Ltc4s | |
| *Homo sapiens* | AAA | 4 patients and 2 controls | 2 females and 2 males in patient and all males in controls | 67.2 (15.5) years in patients and 59.8 (2.1) years in controls | / | EC | NA | [47] |
| | | | | | | SMC | | |
| | | | | | | Fibro | | |
| | | | | | | Mono | | |
| | | | | | | Macro | | |
| | | | | | | B cell | | |
| | | | | | | T cell | | |
| | | | | | | NK | | |
| *Homo sapiens* | MFS | 1 patient | Male | 25 years | / | SMC | Carmn, Myh11, Cnn1 | [32] |
| | | | | | | Modulated SMC | Tnfrsf11b, Cytl1, Col8a1, Carmn, Myh11, Cnn1 | |
| | | | | | | Fibro 1 | Tnfrsf11b, Cytl1, Col8a1, Smoc1, Cdh19, Lum, Podn | |
| | | | | | | Fibro 2 | Cdh19, Lum, Pl16, Podn, Serpine2 | |
| | | | | | | EC | Mmrn2, So×18, Vwf | |
| | | | | | | Pericyte 1 | Co×4l2, Enpep, Notch3 | |
| | | | | | | Pericyte 2 | Carmn, Myh11, Cnn1, Co×4l2, Enpep, Notch3 | |
| | | | | | | Macro | Cd68, Fcer1g, C1qa | |

*(Cont'd...)*

**Table 1.** ***(Continued)***

| Species | Diseases | Number of samples | Gender and genotype | Age (SD) | Induction method | Cell type | Marker | References |
|---|---|---|---|---|---|---|---|---|
| *Homo sapiens* | MFS | 3 patients, 4 controls | 1 male and 2 females in patients and all males in controls | 33.7 (9.3) years in patients and 38 (10.7) years in controls | / | SMC | Smtn, Myh11 | [48] |
| | | | | | | Fibro | Col1a1, Lum, Dcn | |
| | | | | | | EC | Pecam11, Postn, Vwf | |
| | | | | | | Mono/Macro | Cd14, Cd68 | |
| | | | | | | CD8 T cell | Cd8a, Cd8b | |
| | | | | | | NK | Klrc1 | |
| | | | | | | Mast | Cpa3, Tpsb2 | |

AAA: Abdominal aortic aneurysm; ATAA: Ascending thoracic aortic aneurysm; MFS: Marfan syndrome; TAAD: Thoracic aortic aneurysm and dissection; AAD: Aortic aneurysm and dissection; F: Female; M: Male; WT: Wild type; NA: Not available; B APN: β-aminopropionitrile; Ang II: Angiotensin II; HFD: High-fat diet; MSC: Mesenchymal stem cell; VSMC: Vascular smooth muscle cell; EC: Endothelial cell; Fibro: Fibroblasts; Macro: Macrophages; Mono: Monocytes; DC: Dendritic cells; NK: Natural killer cells; Neutr: Neutrophils; Baso: Basophils; Eosin: Eosinophils; Mast: Mast cells; Eryth: Erythrocyte

CD90, CD44, CD29, CD105, CD13, CD34, CD73, CD166, CD10, CD49e, and CD59 are typical markers of ADSCs[66]. Earlier studies have shown that ADSCs positively regulate the transformation of macrophages to the M2 phenotype and inhibit the migration of neutrophils through paracrine factors, thereby inhibiting the elastase-induced expansion of mouse AAA.[67] Recently, it has been discovered that the mechanism is that ADSC-derived exosomes (ADSC-exos) and their microRNA-17-5p (miR-17-5p) inhibit AAA development[68].

At the same time, ADSCs of AAA patients showed signs of aging and decreased cellular function[69]. It is reasonable to speculate that the occurrence of AA is somehow related to the failure of ADSC inhibition. Therefore, recovery of ADSC function can be considered a method of AA therapy.

### 3.4. Vascular wall resident stem cells

There are many stem/progenitor cells presenting mainly in the adventitia. When vascular injury happens, vascular stem/progenitor cells are mobilized and attributed to vascular repair. These adventitial progenitors express the markers, such as stem cell antigen-1 (Sca-1), c-Kit, CD34, and Flk1[3].

The $Sca1^+$ cells, mostly residing in the aortic wall, produce growth factors and differentiate into fibroblasts and neural/glial antigen 2 ($NG2^+$) cells, which contribute to aortic repair and remodeling[70]. Bone marrow derived-$CD34^+$ stem cells, a type of vascular progenitor cell, are involved in the formation of AA due to altered blood flow[71].

It is found that cardiovascular progenitor cells can also differentiate into VSMCs through the SMAD3-dependent TGF-β signaling pathway[72]. Numerous reports have demonstrated that VSMCs can dedifferentiate, proliferate, migrate and transdifferentiate into other cell types[73], and have been extensively studied in vascular diseases such as atherosclerosis[74]. Similar recent studies on AA have found that during AA formation, the contractile medial VSMCs are reprogrammed into MSC-like cells such as osteoblasts, chondrocytes, adipocytes, and macrophages. It was also shown that VSMC reprogramming is driven, at least in part, by increased expression of KLF4, KLF2, and KLF5, and that both conditions of hypercholesterolemia and loss of TGFβ signaling are met[75]. VSMCs can also be reprogrammed to resident adventitial $Sca1^+$ progenitor cells (AdvSca1), namely, AdvSca1-SM cells, induced by KLF4. The cells mainly expressed genes related to hedgehog/WNT/beta-catenin signaling and ECM, promoting pathological vascular remodeling and fibrosis, and the depletion of KLF4 reduced AAA formation[76].

Multilineage differentiated persistent stress cells (Muse cell), one of the subgroups of MSCs, are a special kind of adult stem cells that exist in bone marrow, connective tissue and peripheral blood, express markers of pluripotent stem cells and have the ability to self-renew and differentiate into three germ layers. Compared with MSCs, Muse cells exhibited higher abilities of homing and migration to damaged sites[77].

### 3.5. Induced pluripotent stem cells (iPSCs)

Induction of four transcription factors (Oct3/4, Sox2, c-myc, and Klf4) by retroviral transfection reprograms single somatic cells (non-germ cells) to a pluripotent state similar to embryonic stem cells, that is, induced pluripotent stem cells (iPSCs). iPSCs can further proliferate and differentiate into many somatic cells, for example, vascular endothelial cells and VSMCs[78].

At present, iPSCs have been applied in the research of TAA. VSMCs differentiated from Marfan syndrome

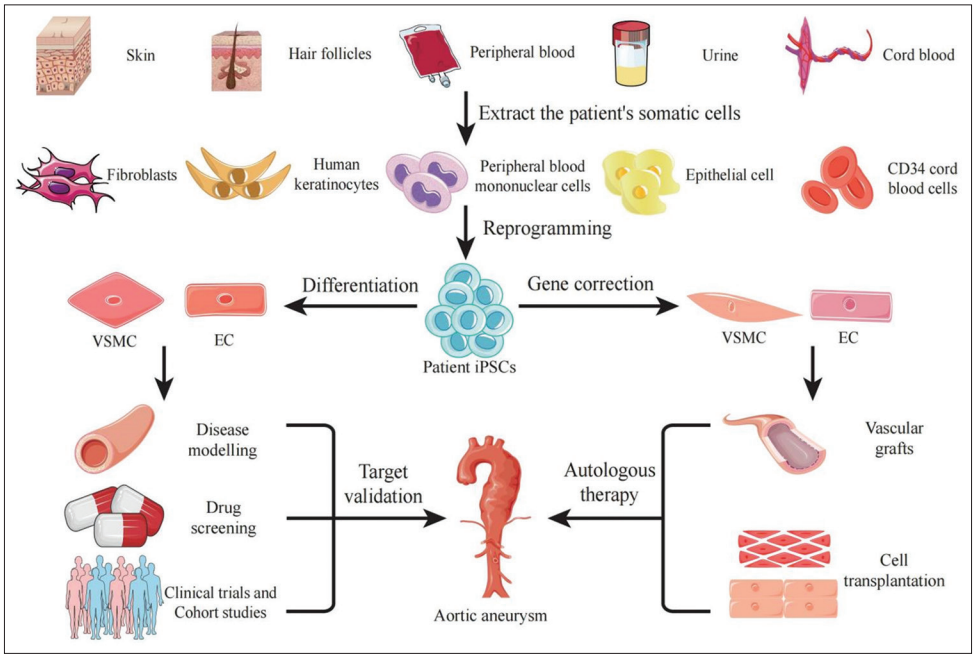


**Figure 2.** Potential applications of the patient somatic cell-derived hiPSC. The iPSC was derived from the patient's somatic cells. On the one hand, iPSC derived from the disease-affected cells could be directly differentiated into VSMC and EC for disease modeling, drug screening, clinical trials and cohort studies for targeted validation. On the other hand, iPSC after gene correction could be used for vascular transplantation and cell transplantation for autologous therapy.

patients-derived iPSCs, showing *Fbn1* mutation, TGF-β signaling activation, decreased contractility and increased apoptosis, was applied as a TAA disease model. The disease phenotype of MFS-iPSC-VSMCs was corrected by CRISPR/Cas9 gene editing, indicating that the *Fbn1* mutation is the main cause of Marfan syndrome. It is found that TGF-β inhibitor can inhibit the accumulation of fibrillin1 and the abnormal expression of MMPs[79].

iPSCs have also been shown to differentiate into a variety of cell types. To date, endothelial cells, EPCs and MSCs have been successfully differentiated from human iPSCs. And the MSCs generated from iPSC had stronger proliferation ability and telomerase activity[80]. Based on this, some researchers propose to combine iPSC technology with clinical research and pharmacogenetics to determine the best treatment method for each patient according to individual differences[81]. In addition to Marfan syndrome, other TAA syndromes such as Loeys Dietz syndrome can also be investigated using iPSC technology to study the disease mechanism[82]. At present, EPCs and vascular endothelial cells can also be differentiated from iPSCs, and further studied for mechanism research and therapeutic applications[83].

iPSCs have a wide range of clinical applications. Somatic cells are extracted from patient tissues and reprogrammed to obtain iPSCs. The iPSCs can be further differentiated into VSMCs and endothelial cells, which can be used for disease models, drug screening, and clinical trials of AA treatment. Gene modification is used for revascularization or cell transplantation in the treatment of AA. Figure 2 shows the origin, differentiation and application of iPSCs in patients.

## 4. Treatment and clinical application

If the AA is not treated promptly, the risk of progressive expansion, rupture, and even death is significantly high. Thus, large AA at risk is usually treated immediately with surgical treatment. Nonsurgical treatment like pharmacological treatment is beneficial for small AA[84]. Specific ways to treat AA are shown in Figure 3.

Due to their various types and functions, recent studies have reported that stem cells also have a certain application value in the treatment of AA. Researches on EPCs mainly focused on the mechanism of AA pathogenesis and recovery, but it still has great potential for clinical application. The number of EPCs doubled 14 days after internal aneurysm repair (EVAR), which may be used to predict AA development and evaluate post-treatment efficacy[55].

It has been demonstrated that intravenous or peripheral delivery of MSCs can inhibit elastase-induced AA expansion in animal models. MSC implantation inhibits Ang II-induced AA development in apoE$^{-/-}$ mice by

**Table 2. Animal models used in the treatment of AA involving stem cells**

| Cell type | Delivery | Injection time | Number of cells | AA model | Total time | Efficiency | References |
|---|---|---|---|---|---|---|---|
| BM-MSC | IV injection | Day 7771, 3, and 5 | $3\times10^6$/every time | Elastase-inducing mice AAA model | 14 days | Female MSC more strongly attenuate AAA growth, and MSCs of different sexes inhibit pro-inflammatory cytokines at varying levels | [100] |
| UC-MSC | IV injection | Day 1 and 5 | $1\times10^6$/every time | Elastase-inducing mice TAA model | 14 days | MSCs immunomodulate specific microRNAs associated with modulating hallmarks of aortic inflammation and vascular remodeling of AA. So, it has the potential to modulate TAA growth and development using targeted therapies designed with MSCs and miRNA | [62] |
| ADSC | IV injection | 2 h after inducing surgery | $1\times10^6$ | Elastase-inducing mice AAA model | 14 days | ADSCs ameliorate the progression of AAA by inducing a series of anti-inflammatory cell events mediated by parsecretory factors | [67] |
| UC-MSC | IV injection | Day 1 | $1\times10^6$ | Elastase-inducing mice AAA model | 14 days | MSCs attenuate NADPH oxidase-dependent HMGB1 production and inhibit AAA | [101] |
| Muse cells | IV injection | Day 0,7,14 | 20000/every time | Mice AA model by incubation of and elastase | 3 or 8 weeks | The significant attenuation of AA dilation was due to the spontaneous differentiation of Muse cells into VSMCs and ECs, and the preservation of elastic fibers | [92] |
| UC-MSC | IV injection | Day 1 | $1\times10^6$ | Elastase-inducing mice AAA model | 14 days | UC-MSC attenuates AA dilation, decreases elastin degradation and fragmentation, inhibits TNF-$\alpha$ and MMP expression levels during initiation and progression of AAA, and modulates the biological role of VSMC contractile phenotype | [88] |
| BM-MSC | Endovascular seeding | 14 days after xenograft implantation | A total of $1\times10^6$ | The xenograft AAA rats model | 4 weeks | BM-MSCs promote arterial regeneration and functional recovery. Endovascular seeding of BM-MSCs stabilizes the diameter of the already-dilated AAA | [102] |
| ASC | ASC-loaded RCP | Immediately after induction of AAA | / | Mice AAA model by incubation of and elastase | 2 weeks | ASC-loaded RCP suppresses development and progression of AAA | [103] |
| ADSC | Artery implantation | 4 weeks after the infiltration operation | $2\times10^6$ | AAA rats model by $CaCl_2$ infiltration | 28 days | ADSCs contribute to the reconstruction of elastic fibers by promoting the secretion and synthesis of elastin in SMCs | [104] |
| BM-MSC | IV injection | 28 days after the infusion operation | $1\times10^6$ | Ang II-infusion mouse model | 2,4,8 weeks | BM-MSCs reduce the incidence of AA, aortic diameter, the enzymatic activities of MMP-2 and -9, downregulate inflammatory cytokines, upregulate IGF-1 and TIMP-2, and preserve the construction of elastin | [105] |
| AD-MSC | Catheter | Day 5 | $1\times10^6$ | Elastase-inducing mice AAA model | 14 days | Periadventitial stem cell delivery ameliorates elastase-induced AAA by inhibiting aortic diameter dilatation and reducing fragmented elastin | [95] |
| MSC-EV | iv injection | Day 1 | $1\times10^6$ | Elastase-inducing mice AAA model | 14 days | MSC-EVs attenuate aortic inflammation and macrophage activation via | [64] |

*(Cont'd...)*

**Table 2.** ***(Continued)***

| Cell type | Delivery | Injection time | Number of cells | AA model | Total time | Efficiency | References |
|---|---|---|---|---|---|---|---|
| | | | | | | microRNA-147 during the initiation and development of AAA | |
| BM-MSC | IV injection | Week 0,1,2,3 | $1\times10^6$/every time | Ang II-infusion mice AA model | 4 weeks | Multiple intravenous administrations of BM-MSCs were effective to inhibit the inflammatory reactions in Ang II-induced AA in $apoE^{-/-}$ mice | [106] |
| MSCs-CM | IV injection | Day 0,3,6,9,12 | / | Ang II-induced mice model | 14 days | MSCs-CM regulates the polarization of M1/M2 macrophages to effectively alleviate Ang II-induced AA growth | [60] |
| BM-MSC | IV injection | Day 0 | $1\times10^6$ | Ang II-infusion mouse model | 2 weeks | BM-MSCs regress AA via regulation of the NF-κB, Smad3 and Akt signaling pathways | [107] |
| BM-MSC | Catheter | 14 days after xenograft implantation | / | The xenograft AAA rat model | 7 days | Cell therapy reconstructs the mechanical properties of the damaged abdominal aorta by improving tissue stiffness, stabilizes the geometry of AAA and reduces pressure changes in the artery wall | [108] |
| ADSC-exos | IV injection | Every 3 days | 100 μg/every time | Ang II-infusion mouse AAA model | 28 days | MiR-17-5p-rich ADSC-exos constraining AAA progression and inflammatory cytokines release, via the TXNIP-NLRP3 signaling pathway | [68] |
| Human Placental MSC | Tail vein injection | Day 1 | A total of $1\times10^6$ | Elastase-inducing mice AAA model | 14 days | MSCs significantly reduce AAA formation by immunomodulating IL-17-mediated inflammatory processes | [61] |

AA: Aortic aneurysm; TAA: Thoracic aortic aneurysms; AAA: Abdominal aortic aneurysm; MSC: Mesenchymal stem cell; BM-MSC: Bone marrow-derived MSC; UC-MSCs: Umbilical cord mesenchymal stem cells; ADSC: Adipose tissue-derived stromal cells; Muse cells: Multilineage-differentiating stress-enduring cells; EV: Extracellular vesicles; MSCs-CM: MSC–derived conditioned medium; ADSC-exos: ADSC-derived exosomes; IV: Intravenous; Ang Ⅱ: Angiotensin II; HMGB1: High mobility group box 1; VSMC: Vascular smooth muscle cells; EC: Endothelial cells; MMP: Matrix metalloproteinase; TNF-α: Tumor necrosis factor-α; ASC: Adipose tissue-derived stromal cells; RCP: A recombinant collagen-based patch; IGF-1: Insulin-like growth factor-1; TIMP-2: Tissue inhibitor of metalloproteinase-2; $apoE^{-/-}$: Apolipoprotein E-deficient; NF-κB: Nuclear factor kapp B; Smad: Sma- and Mad-related proteins; Akt: Protein kinase; miR-17-5p: microRNA-17-5p; TXNIP: Thioredoxin-interacting protein; NLRP3: NOD-like receptor thermal protein domain associated protein 3; IL-17: Interleukin-17

preserving elastin in the aortic wall. It can reduce MMPs and inflammatory cytokine levels[85], for example, reducing MCP-1, TNF-α, IL-6, and MMP-2/9 but increasing TIMP-1/2[86]. In terms of the delivery mode, the safety and feasibility of the NOGA (a company in USA) system for intravascular delivery of MSCs to the aortic wall in a AAA porcine model have been confirmed[87]. MSCs not only inhibit inflammation but also act on damaged VSMCs. Studies have shown that intravenous administration of human umbilical cord MSCs can maintain or restore the VSMC contractile phenotype in AAA[88]. VSMCs generated by MSC can stimulate the synthesis of aneurysm VSMCs to compensate for dead VSMCs and provide elastic power for aneurysm VSMCs[89]. For example, the use of PDGF + TGFβ1-induced BM-MSC or ADSC-derived VSMCs in tissue sites affected by vascular diseases such as AAA can achieve complete autologous therapy[90]. Mesenchymal stem cell-derived exosomes (MSC-exs) can also significantly attenuate the development of AAA in mice by inhibiting AAA formation and inhibiting inflammation and promoting ECM synthesis[91].

Muse cells were injected intravenously in a mouse AA model, and then they were found selectively migrated and integrated into the AA tissue. Subsequently, Muse cells can spontaneously differentiate into VSMCs and endothelial cells while retaining elastic fibers[92] and inhibiting inflammation[93]. Muse cells are characterized by significantly reducing the diameter of AA compared to non-Muse cells. Hence, muse cell is a promising cell type for AA treatment.

Recently, ADSC has also been studied in the field of treatment of AA. ADSCs have the characteristics of easy culture and expansion, pluripotency and differentiation ability, and the ability to produce trophic factors. Hence, ADSC has the potential for clinical application in the treatment of AA[94].

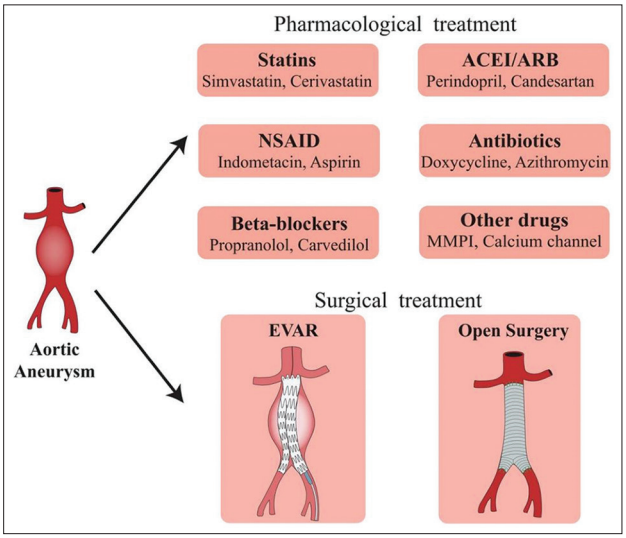


**Figure 3.** Pharmacological and surgical treatment of AA.
ACEI: Angiotensin-converting enzyme inhibitor; ARB: Angiotensin receptor blocker; NSAID: Non-steroidal Ant-inflammatory Drugs; EVAR: Endovascular aneurysm repair; MMPI: Matrix metalloproteinase inhibitor.

Recently, it has been practiced in a porcine model. In the aorta of animals treated with ADSC, the expression levels of vascular endothelial factor, MMP1 tissue inhibitor, and MMP3 tissue inhibitor were increased, and aortic dilatation was inhibited[95,96]. This new finding will facilitate further research on ADSC stem cell therapy for AA disease.

The application of iPSC in TAA treatment is widely studied. Cell lines of human induced pluripotent stem cells (hiPSC) have been generated for specific inherited vascular diseases such as Marfan syndrome[78,81]. There are also recent studies in the treatment of AAA that collagen scaffolds of induced pluripotent stem cell-derived smooth muscle progenitor cells (iPSC-SMPs) can be an effective carrier for the delivery of VSMCs to the AAA site[97]. iPSCs can also generate embryonic origin-specific VSMCs for research use[98]. iPSC technology for the treatment of AA has opportunities but also faces many challenges, such as the generation and quality of iPSCs, tumorigenicity risk, and immune rejection[99]. Therefore, the iPSC treatment technology is still in its infancy, and there are still numerous difficulties to overcome in practical clinical application. The above-mentioned applications of various stem cells in the treatment of AA are mainly based on research findings, and thus, more extensive studies are still needed. In recent years, studies have mainly focused on mesenchymal stem cells from various sources, as shown in Table 2.

## 5. Conclusion and outlook

AAA and TAA are severe and lethal diseases, and their pathogenesis has been extensively studied and elucidated at the cellular and molecular levels. However, there are several limitations and unresolved issues. For example, AAA is mostly induced by Ang II, which limits research on inflammatory mechanisms. Research of TAA is most focused on genetic models. In the occurrence, growth and rupture of AA, more studies have focused on the developmental stage rather than defining the entire disease process[7]. Therefore, new models and more innovative approaches are needed to study AA.

As for the role of stem cells in AA, the current research may suggest that they play a crucial role in the occurrence and development of AA. But more importantly, the clinical treatment of AA by utilizing the differentiation and proliferation ability of stem cells has a good prospect. EPC will be mainly used for the prediction and prognostic evaluation of AA, but at present, it is mainly used to study the mechanism of EPC for AA. A few randomized controlled clinical studies of MSCs in the treatment of AAA have been registered and implemented[109]. There is no follow-up literature so far, but there is a growing number of preclinical studies on the therapeutic potential of MSCs[61-64,85,88,89], focusing on the in-depth study of stem cell therapy for AA[86,87,90,91]. Currently, the main direction is to differentiate Muse cells into other cells for treatment[77,92,93]. Studies on ADSCs and MSCs are similar, mainly focusing on the inhibition of anti-inflammation in the development of AA[67-69,94-96]. The applications of iPSC-based technology are extensive, including the establishment of disease models, regenerative cell therapy, drug discovery, and predictive safety pharmacology[78-83,97,98]

In general, many studies on applying stem cells in AA treatment are based on animal models and are mainly preclinical mechanistic studies. Further research is urgently needed to overcome these limitations and promote the research progress in the prediction and treatment of AA using stem cells.

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## Conflict of interest

The authors declare they have no competing interests.

## Author contributions

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ACCSCIENCE PUBLISHING

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REVIEW ARTICLE

# Inflammatory and anti-inflammatory responses in human T-lymphotropic virus Type 1 infection

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## Abstract

Human T-lymphotropic virus Type 1 (HTLV-1) is a viral infectious agent that may cause chronic infection of T lymphocytes. HTLV-1 infection is related to multiple human diseases, including adult T-cell leukemia, which is a neoplastic growth of HTLV-1-infected T cells, and neoplastic inflammatory conditions such as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), Sjögren's syndrome, polymyositis uveitis, and bronchoalveolitis. T regulatory cells (Tregs), also known as regulatory T cells, and T helper 17 (Th17) cells, a distinct subset of cluster differentiation T cells with interleukin-17 as their major cytokine, orchestrate the pathogenesis of anti-inflammatory and inflammatory responses in HTLV-1-mediated diseases. In this review, we aim to evaluate the immune responses of Tregs as anti-inflammatory cells and Th17 cells as inflammatory cells in HTLV-1 infection.

***Keywords:*** Inflammatory responses; Anti-inflammatory responses; Human T-lymphotropic virus type 1; HTLV-1-associated myelopathy/tropical spastic paraparesis

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## 1. Introduction

Human T-lymphotropic virus Type 1 (HTLV-1) is anoncogenic virus that may cause chronic infection of human T cells. It is endemic in several countries, such as Southern Japan, the Caribbean region, areas in South America and tropical Africa, and some foci in the Middle East, Australia, and Melanesia[1-3]. The majority of patients who are infected with HTLV-1 are without certain symptoms, including fever, cough, shortness of breath, nausea, and diarrhea[4,5]. HTLV-1 infection is associated with several disorders, of which the main conditions are adult T-cell leukemia (ATL), which is a neoplastic growth of HTLV-1-infected T cells, and neoplastic inflammatory conditions, such as HTLV-1-associated myelopathy/tropical spastic paraparesis(HAM/TSP), Sjögren's syndrome, polymyositis uveitis, and bronchoalveolitis[2,5,6]. Inflammation may have a major role in the pathogenesis of HTLV-1, as shown in Figure 1.

Neuropilin-1, glucose transporter 1 (GLUT1), and heparin sulfate proteoglycans (HSPGs) are all HTLV-1 receptors[7,8]. Since both GLUT1 and neuropilin-1 are found on various cell surfaces, it is possible that HTLV-1 infects various hematopoietic cells and hematopoietic stem cells (HSCs),such as T cells[7,8]. These receptors facilitate the binding of HTLV-1 with hematopoietic cells. The entry of retroviruses into target cells involves

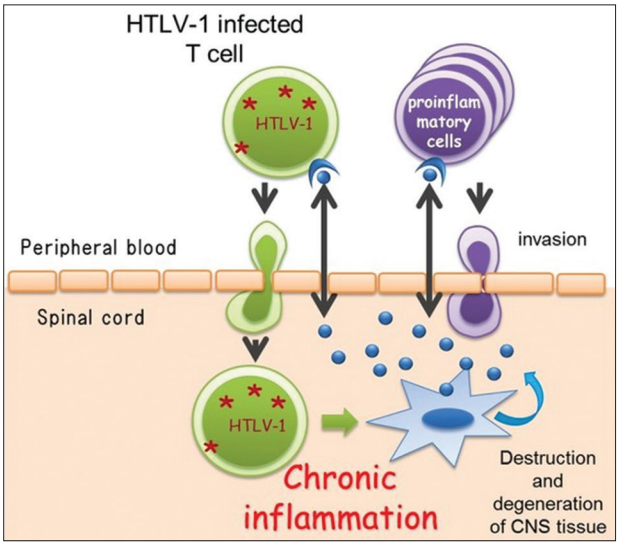


**Figure 1.** Cellular mechanisms underlying the pathogenesis of human T-lymphotropic virus type 1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). HTLV-1-specific immune responses and secondary inflammations inflated in the CNS may lead to subsequent CNS damage. CNS, central nervous system; HTLV-1, human T-lymphotropic virus Type 1; *HTLV-1.

interactions between viral envelope (Env) glycoproteins, a surface glycoprotein (SU) and a transmembrane glycoprotein (TM), with specific cell surface molecules, referred to as receptors. SU is involved in receptor recognition, while TM triggers the fusion of viral and cellular membranes, allowing viral particles to enter target cells[9,10]. GLUT1 has been shown to specifically bind to a truncated soluble form of HTLV-1 and HTLV-2 SU proteins, and the level of GLUT1 in target cells has been found to be correlated with the titer of HTLV-2 Env-pseudotyped virus[10,11]. HSPGs might play a role in HTLV-1 entry. T-helper 17 (Th17) cells and regulatory T cells (Tregs) are two subsets of $CD4^+$ T cells[12].

## 2. Regulatory T cells

Tregs are a subpopulation of $CD4^+$T cells[13]. Tregs have multiple markers, including CD25, glucocorticoid-induced tumor necrosis factor receptor family-related protein(GITR), CD45R, CD62L, CD127, CD103, cytotoxic Tlymphocyte antigen-4 (CTLA-4), and programmed cell death 1 (PD-1) [14]. Forkhead box P3 (FOXP3) is a master transcription factor of Tregs that plays a key role in Tregs activity[15].

Tregs have shown to be useful for immunotherapy in autoimmune and inflammatory conditions as they contribute to establishing and maintaining immune homeostasis[12,16]. Tregs can regulate innate immune cells and adaptive immune cells as well as suppress adverse inflammatory responses of both T and B cells in adaptive immunity. The functions of Tregs include cell contact and production of potent tolerogenic cytokines, such as interleukin (IL)-10, IL-35, and transforming growth factor beta (TGF-β)[17]. Therefore, Tregs can modulate the adverse immune responses that may be involved in the pathogenesis of HTLV-1 infection[18,19]. TGF-β plays a regulatory role in establishing and maintaining peripheral and tissue tolerance[20,21]. IL-10, as a multifunctional cytokine, can effectively limit adverse inflammatory response[20,21]. In addition, IL-10 is also essential for immune homeostasis[20,21]. IL-35 is an inhibitory cytokine composed of Epstein-Barr virus-induced gene 3 and p35 subunits released by Tregs[22]. IL-35 appears to play a critical role in infectious tolerance not only by suppressing the proliferation of effector T cells, but also by inducing the production of IL-35 by non-FOXP3 conventional T cells (Tconvs), which areknown as iTr35 cells[23]. IL-35 has also been associated with the suppression of various autoimmune diseases and atherosclerosis[23,24].

## 3. T-helper 17 cells

Th17 cells are the subpopulation of $CD4^+$ T cells, with a pro-inflammatory cytokine profile, including IL-21, IL-22, IL-17A, IL-17 F, and tumor necrosis factor alpha (TNFα)[25-28]. Retinoicacidreceptor-related orphan nuclear receptor gammat (ROR-γt) is the most important transcription factor involved in Th17 cell differentiation and function[29]. The N terminus of RORγt has a transcription modulation domain (TMD) comprising deoxyribonucleic acid(DNA)-binding amino acid residues and isotype-specific sequences that may play key roles in the functional specificity of RORγt[30]. RORγt, induced at the early stage of Th17 cell differentiation following IL-6 and TGF-β stimulation, has a pivotal role inTh17 cell lineage commitment[31]. RORγt specifically binds to and regulates Th17-associated genes, such as*IL17A*, *IL17F*, and*IL23R*, by activating their transcription in coordination with other transcription factors[32]. Therefore, targeting RORγt by small molecules either genetically or pharmacologically is effective in ameliorating Th17-related inflammatory disorders, including experimental autoimmune encephalomyelitis (EAE), psoriasis, arthritis, colitis, and glomerulonephritis, especially in preventative disease models[33].

IL-17A is responsible for inducing cell types to produce other pro-inflammatory cytokines, chemokines, and metalloproteinases, thereby recruiting neutrophils to the tissue and contributing to the inflammation process[34]. IL-22 is a key cytokine produced by Th17 cells, and it plays an important role in maintaining homeostasis and remodeling epithelial tissues. The importance of IL-22 has been highlighted in the pathogenesis of psoriasis[35,36]. In a study, *IL-22* mRNA expression was found to be

upregulated in psoriatic skin as compared to normal skin, whereas the expression of *IL-22* mRNA in peripheral blood mononuclear cells of both psoriatic patients and normal controls were similar[36]. IL-17F, which is very similar to IL-17, is considered an inflammatory cytokine since it induces many pro-inflammatory cytokines and chemokines[37]. *IL-17F* mRNA has also been reported to be associated with activated monocytes, basophils, and mast cells[37].

IL-6 is a pro-inflammatory cytokine that is a marker of both acute and chronic inflammation[38]. IL-6 is involved in immune responses, inflammation, hematopoiesis, bone metabolism, and embryonic development. IL-6 plays various roles in chronic inflammation (closely related to chronic inflammatory diseases, autoimmune diseases, and cancer) and even in the cytokine storm of the coronavirus disease (COVID-19)[39].

## 4. Role of Treg/Th17 axis in human T-lymphotropic virus Type 1infection

The adverse inflammatory reactions resulting in the central nervous system (CNS) inflammation and tissue damage followingHTLV-1 infection may be caused by the inappropriate function of Th17 cells[40]. The imbalance of the Treg/Th17 axis is a probable factor in the pathogenesis of HTLV-1 infection because Tregs regulate the function of Th17 cells. IL-6 contributes toTh17 cell differentiation by suppressing *FOXP3* and *TGF-β*gene expressions in Tregs[38]. Through direct cell interaction and the release of anti-inflammatory cytokines, both natural and induced (i) Tregs regulate the proliferation and activities of innate immune cells (dendritic cells and macrophages) and suppress self-reactive lymphocytes, such as Th17cells[41].

MT-2 is a human HTLV-1-infected cell line obtained from the leukemic cells of ATL patients[42]. This cell line can be used to determine the molecular and cellular factors that are involved in the pathogenesis of HTLV-1 infection[43]. The majority of MT-2 are regulatory T cells ($CD4^+CD25^+FOXP^+$), which imply that HTLV-1 transforms infected $CD4^+$ T cells into Tregs and causes clonal proliferation[42]. Similarly, Tregs have been suggested to be the cells most infected with HTLV-1 in patients with ATL and HAM/TSP[4]. The proliferation of Tregs increases in response to HTLV-1 infection, but these cells have been shown to be functionally impaired *in vivo* and *in vitro*, which might be one of the mechanisms behind the triggering inflammatory responses[4,44]. Th17-mediated pro-inflammatory responses can enhance viral replication. However, the contributions of Tregs and Th17 cells in HTLV-1-associated illnesses[4] vary depending on the stage of infection and the host's immunological state[4,40,45-47]. The previous research has shown that increased FOXP3 expression in patients with ATL contributes to enhanced Tregs activity, which subsequently leads to increased TGF-β and IL-10secretion, thus activating the immunosuppression phenotype observed in HAM/TSP patients[13]. As mentioned, Th17 cells, as pro-inflammatory cells, express ROR-γt, as a transcription factor, and secrete IL-17A[48,49]. ROR-γt expression has been shown to be elevated in ATL patients' skin and other tissues, which can be attributed to inflammatory reactions[40]. Since Th17 cell actions are regulated by Tregs, it has been hypothesized that Tregs impairment may contribute toTh17 cell overreaction, leading to uncontrolled inflammation and consequently the exacerbation of inflammation in viral infection[40]. However, it has been indicated that Th17 cells may be important for viral transmission suppression in some cases. A study has shown significantly lower levels of Th17 cells in HAM/TSP patients compared to uninfected subjects and a trend toward reduced number of IL-17-secreting cells compared to HTLV-1 asymptomatic carriers. Th17 cell functions vary depending on the stage of infection and the host immune status in viral infection, similar to Tregs[4,40]. Therefore, we conclude that the role of Tregs in the pathogenesis of HTLV-1 infection varies depending on the stage of infection and the host immune status[4,40,45,46].

In the pathogenesis of HTLV-1, the immunosuppressive microenvironment mediated by Tregs may have two opposing roles. On the one hand, the regulatory function of Tregs in suppressing immune responses may have enabled HTLV-1 to escape host immunity, resulting in viral infection progression[50]. In this scenario, Tregs could worsen the HAM/TSP pathogenic process[51]. On the other hand, HTLV-1 functionally inhibits Tregs by increasing its proliferation, which leads to potential impairment of

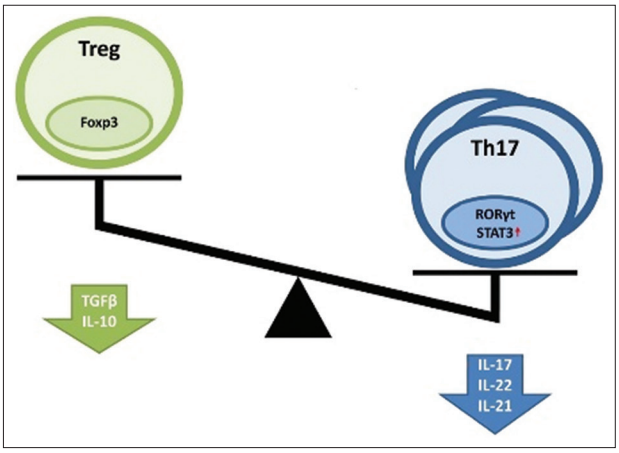


**Figure 2.** Treg/Th17 imbalance in human T-lymphotropic virus Type 1 infection.

**Table 1. Characteristics and results of selected studies**

| Author | Year of publication | Study design | Number of participants | Neurological disease (HAM/TSP) | Hematologic disease (ATL) | Results |
|---|---|---|---|---|---|---|
| Champs *et al.*[47] | 1986 | Experimental (*in vitro*) | – | – | Yes | Production of 1,25($OH_2$) D by lymphoma cells may contribute to the pathogenesis of hypercalcemia in ATL. |
| Satou *et al.*[53] | 1986 | Case series | 5 | – | Yes | Five patients with ATL had 1,25($OH_2$) D levels within or below the normal range, and it was not associated with the usual cause of hypercalcemia in such patients. |
| Nakao *et al.*[62] | 1987 | Experimental (*in vitro*) | – | – | Yes | 1,25($OH_2$) D inhibited the proliferation and *de novo* DNA synthesis of certain HTLV-positive T cell lines. |
| Nakao *et al.*[62] | 1987 | Case series | 18 | – | Yes | Two ATL patients with hypercalcemia had low 1,25($OH_2$) D levels. Hypercalcemia in ATL patients may be a result of the production of factor (s) that stimulate osteoclastic bone resorption by tumor cells. |
| Reichel *et al.*[63] | 1987 | Experimental (*in vitro*) | – | – | Yes | HTLV-1-transformed lymphocytes can produce 1,25($OH_2$) D but the excess production of 1,25($OH_2$) D was unlikely to be associated with the pathogenesis of ATL-associated hypercalcemia. |
| Koizumi *et al.*[64] | 1989 | Experimental (*in vitro*) | – | – | Yes | 1,25($OH_2$) D and glucocorticoid-inhibited cellular proliferation and *c-myc* mRNA expression in HTLV-1-infected T-cell line, KH-2. |
| Inoue *et al.*[65] | 1993 | Experimental (*in vitro*) | – | – | Yes | 22-oxa-1,25($OH_2$)$D_3$ (non-calcemic analog) and 1,25(OH) $_2$D suppressed cell proliferation and *PTHrP* gene expression by binding to overexpressed Vitamin D receptor in HTLV-1-infected T cells. |
| Elstner *et al.*[66] | 1994 | Experimental (*in vitro*) | - | – | Yes | 1,25($OH_2$)-20-epi-$D_3$, the potent 1,25($OH_2$) D analog, was identified with anti-proliferative and differentiating effects on leukemic cells. |
| Peter *et al.*[67] | 1995 | Case report | 2 | – | Yes | Hypercalcemia with normal Vitamin D levels. PTHrP seemed to be the supposed factor for hypercalcemia associated with ATL. |
| Masutani *et al.*[68] | 2005 | Review | – | – | Yes | The expression of TBP-2/VDUP1, a growth suppressor, was suppressed in HTLV-1-transformed cells. |

1,25($OH_2$) D: 1,25-dihydroxyvitamin D; HAM/TSP: HTLV-1-associated myelopathy/tropical spastic paraparesis; HTLV-1: Human T-lymphotropic virus type 1; TBP-2: Thioredoxin-binding protein-2; VDUP1: Vitamin $D_3$ upregulated protein 1

the suppressive activity of Tregs and the up regulation of inflammation in support of virus survival[52-55]. However, the effects of anti-inflammatory and pro-inflammatory responses on viral infection are assumed to be dependent on the stage of infection phase and host immune status[4,7].

Taken together, HTLV-1 infection disrupts immune homeostasis, by altering Treg/Th17 balance (Figure 2), and their associated cytokines, such as IL-17A, IL-10, and TGF-β, which may result in an imbalance of inflammatory and anti-inflammatory responses, tolerance failure, and an exacerbation of inflammation[13,56-60].

Tregs can regulate Th17 cells through direct cell interaction and indirectly by producing potent anti-inflammatory cytokines, such as TGF-β and IL-10. This leads to the inhibition of excessive immune responses that may contribute to the pathogenesis of viral infections, such as HTLV-1 infection. Treg/Th17 axis imbalance is the key contributor to the pathogenesis of HTLV-1 infection.

## 5. Conclusion

A growing number of evidence has indicated that both Tregs impairment and Th17 cell differentiation are

enhanced in HTLV-1 infection, and the inflammatory state in HTL-V1 infection may contribute to the pathogenesis of HTLV-1-related diseases, such as ATL and HAM/TSP. Therefore, immunomodulator agents such as Vitamin D3 (VitD3) may be effective in the prevention and treatment of HTLV-1 infection[61].

Recently, it has been suggested that1,25-dihydroxyvitamin D3 (1,25-VitD3) may act as both an immunoregulatory agent (improving Tregs functions) and an immunosuppressive agent (attenuatingTh17 cell functions), delineating the role of VitD3 as an immunomodulator in HTLV-1 infection. This highlights the importance of sufficient Vitamin D levels and also taking VitD3 supplementation in HTLV-1 infection[61] (Table 1). With regard to its potential, clinical trials in the field investigating the benefits of using VitD3 as a complementary therapy in patients with HTLV-1 infection are recommended. According to the Table 1, the use of VitD3 as a complementary therapy in patients with HTLV-1 infection is recommended.

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## Author contributions

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ACCSCIENCE PUBLISHING

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REVIEW ARTICLE

# Adenine base editing as a promising therapy for cardiovascular diseases

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## Abstract

Cardiovascular diseases (CVDs) are the leading causes of human death worldwide. Genetic variants serve as the major risk factor for CVDs, with limited therapeutic interventions in clinical practice. The recent surge of genome editing technologies offers the hope to correct genetic variants and to cure genetic diseases. Among the diverse genome editing tools, adenine base editors (ABEs) exhibit high efficiency, high specificity, and low off-target effects, successfully entering a clinical trial and demonstrating the tremendous potential to transform modern cardiovascular therapy. In this review, we summarize the basic knowledge about ABE, showcase three hallmark studies using ABE to ameliorate or treat CVDs in experimental animals, and lastly discuss about the key technical concerns that should be addressed to achieve the full potential of ABEs in the future.

***Keywords:*** Adenine base editor; Cardiovascular disease; Gene therapy

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## 1. Introduction

Cardiovascular diseases (CVDs) are the leading causes of morbidity and mortality worldwide. Genomic variants, often in the form of single nucleotide variants (SNVs), are one of the major causes of CVDs[1-3]. In the past decade, many CVD-associated SNVs were discovered, thanks to the advancement in high-throughput sequencing technologies[4]. However, effective therapies for these diseases remain absent.

The recent emergence of the genome editing technology has provided an unprecedented opportunity to treat CVDs. This technology was derived from the clustered regularly interspaced short palindromic repeats (CRISPRs) system in prokaryotes[5,6]. The CRISPR repeats in the prokaryotic genome encode an array of small non-coding RNA called the CRISPR RNA (crRNA). crRNA together with trans-activating crRNA (tracrRNA) was later engineered to form a single guide RNA (sgRNA)[7], which can direct the CRISPR-associated (Cas) nucleases, such as Cas9, to bind to a specific DNA sequence that is base-paired by the crRNA. Next, the nuclease locally digests the DNA and creates a DNA

double-stranded break (DDB), which can be repaired through either non-homologous end jointing (NHEJ) or homology-directed recombination (HDR)[6].

HDR-based genome editing can precisely write DNA sequences at the will of the scientists by providing a template DNA donor. However, the application of HDR is limited by its low editing efficiency, which is further complicated by the small nucleotide insertions and deletions (Indels) that are simultaneously created by NHEJ reaction[5]. Indels usually outnumber the HDR products among the genome-edited cell population, depositing unwanted frame-shifting mutations, so conventional CRISPR/Cas9 genome editing is usually more useful in gene silencing applications, while more efficient and precise genome editing tools, such as the base editors (BEs), are necessary for the correction of disease-causing genetic variants.

BEs are initially derived from the Cas9 nickase (nCas9)[8], which is a Cas9 mutant that only cuts one DNA strand, greatly reducing DDB formation and the introduction of indels at the edited loci. BEs are constructed by fusing nCas9 with an engineered deaminase that preferentially catalyzes nucleotide conversions on DNA[8,9]. The two most widely used BEs are the cytosine base editors (CBEs)[8] and the adenine base editors (ABEs)[9]. In CBEs, nCas9 is fused to the cytidine deaminase APOBEC1 to catalyze the nucleotide conversion of cytidine (C) to uracil (U), which is next modified as a thymidine (T) by the endogenous DNA repair system. In ABEs, the tRNA adenosine deaminase (TadA) is engineered to enable adenosine (A) deamination into inosine (I), which is next converted to guanidine (G) (Figure 1).

Among the SNVs that are known to associate with human diseases, nearly half of them are mutated from the C·G pair to the T·A pair[9,10], falling into the situation in principle reversible by ABEs. ABEs also demonstrate simpler domain structures and lower off-target effects as compared to CBEs[11,12] (Figure 1). Thus, ABEs have become the most popular BE tools that are promising in translational medicine.

In this review, we navigate our focus on the key technical features of ABEs and introduce the recent landmark gene therapy studies for CVDs. We also discuss about the technical concerns on the road toward successful clinical applications.

## 2. Basic parameters for ABE therapy

### 2.1. Editing efficiency

The effort to fuse TadA to nCas9 to edit adenosine initially failed with no detectable edits[9], because natural adenosine deaminases usually function on RNA but not DNA[9]. Dr. David Liu's team solved this problem through a directed evolution experiment on TadA, uncovering mutations at or near the TadA D108 residue as the key modifications to enable ABE activity on DNA substrates[9]. Additional molecular evolutions and trials-and-errors in testing mutation combinations in TadA eventually lead to the TadA7.10 mutant as well as the corresponding ABE prototype called ABE7.10[9].

Because TadA and Cas9 were derived from prokaryotes, ABE7.10 requires additional modifications to adapt to the applications in mammalian cells. First, the codon usage in the ABE7.10 gene was modified by introducing silent mutations so that ABE7.10 was better translated into proteins by tRNAs preferentially expressed in mammals. The second adaption involved the addition of nuclear localization signals (NLSs), so ABE7.10 could be transported into the cell nucleus for genome editing in eukaryotes. After the initial codon optimization and NLS incorporation efforts in ABE7.10[13], these parameters were, further, optimized in following studies, eventually leading to a more robust ABE variant called ABEmax[13,14].

The wildtype TadA protein acts as a dimer. Unlike in bacteria cells, where the TadA in ABEs can pair with the endogenous TadA to facilitate genome editing, mammalian cells do not express TadA. To solve this problem, in ABE7.10 and ABEmax, an evolved TadA was fused in tandem with a wildtype TadA to allow intramolecular dimerization[9]. However, in following studies, additional molecular evolution led to the discovery of ABE8e and ABE8.20, in which the TadA mutant can function as a monomer and exhibit even higher editing activity[15,16] (Table 1).

### 2.2. The scope of the editable adenines

Several factors determine if a specific adenosine is suitable for ABE. First, this nucleotide must be positioned within an "editing window" defined by the position of the sgRNA. In the CRISPR/Cas system, sgRNAs must be placed next to a protospacer adjacent motif (PAM), which is determined by the intrinsic property of the Cas protein. For example, the PAM sequence of a wildtype SpCas9 protein is NGG (N means any nucleotide)[17,18], while natural SaCas9 uses NNGRRT (R means A or G) as the PAM sequence[19]. The PAM sequence determines the location, where the CRISPR/Cas9 system unwinds the DNA to form R-loop[20], which exposes the single-strand DNA in the editing window to TadA for the deamination reaction (Figure 1).

In addition to PAM, the editing window of ABEs is also determined by their enzyme activity and their structural features. Usually, the editing windows of ABEs are 4-5nt wide. With the use of more robust TadA variants, the editing windows of ABEs can be broadened[15,16]. However,

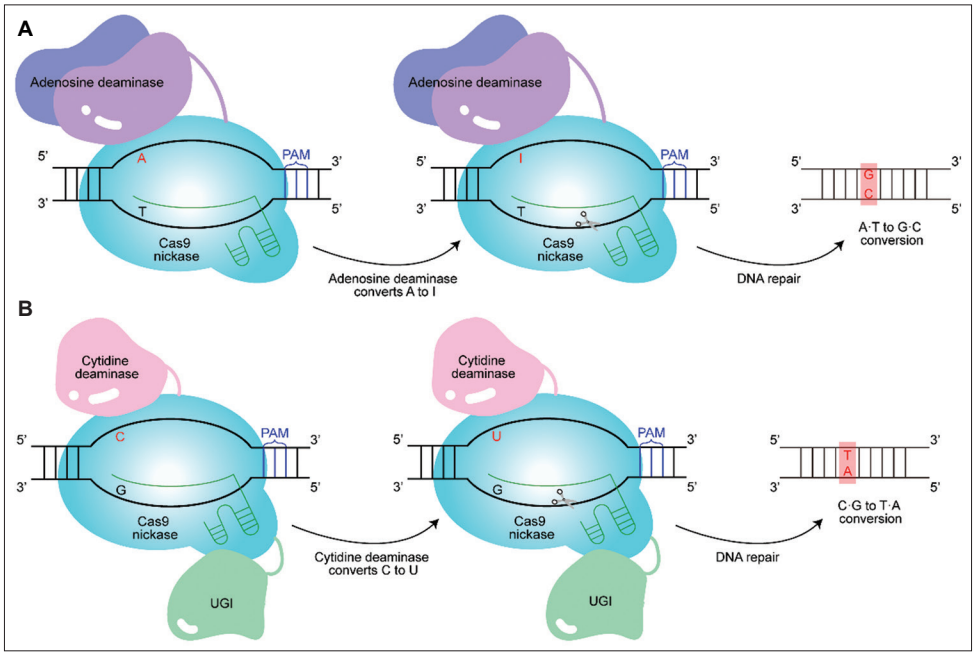



**Figure 1.** The working model of adenine base editing and cytosine base editing. (A) The adenine base editor (ABE) consists of adenosine deaminase TadA and Cas9 nickase (nCas9). SgRNA guides TadA-nCas9 to target the genomic DNA sequence by complementary base pairing. nCas9 unwinds DNA and exposes adenine on a single DNA strand for TadA-based editing. nCas9 also cleaves the non-edited DNA strand to facilitate DNA repair. Adenosine deaminase converts adenosine (A) to inosine (I), which is recognized as guanosine (G) in DNA repairing. Consequently, the ABEs mediate DNA base editing to convert A·T to G·C. (B) The cytosine base editors (CBEs) consist of cytidine deaminase APOBEC1, Cas9 nickase (nCas9), and uracil DNA glycosylase inhibitor (UGI). SgRNA guides APOBEC1-nCas9-UGI to target the genomic DNA by complementary base pairing. nCas9 unwinds DNA to generate an R loop and expose cytidine for APOBEC1-based editing. nCas9 cleaves the non-edited strand to facilitate DNA repair. Cytidine deaminase converts cytosine (C) to uracil (U), which is recognized as thymine (T) in DNA repairing. UGI inhibits uracil N-glycosylase (UNG) to prevent the reversal of U·G mismatch back to C·G base pair. Consequently, the CBEs mediate DNA base editing to convert C·G to T·A.

while a broader editing window means the increased likelihood of editing the target adenosine, it will also increase the bystander effect by introducing unintended editing of other nucleotides, particularly other adenosines, within the same window.

A major strategy to broaden the scope of editable adenosines is to fuse TadA with a Cas effector protein that uses different or less restrictive PAM sequences. These Cas orthologs can be discovered from the wild microbiome. Good examples include the wide variety of Cas9 and Cas12 family members[21,22]. Cas proteins can also be engineered to alter their PAM sequences. Successful examples include SpCas9 variants SpCas9-VRQR[23], which recognizes NGA as the PAM. Other commonly used Cas9 variants include SpCas9-NG[24] and SpG[25], which both use NGN as the PAM. Strikingly, the recently developed SpRY mutant uses the NRN (R means A or G) or NYN (Y means C or T) PAM and almost completely circumvents the PAM restraints[25].

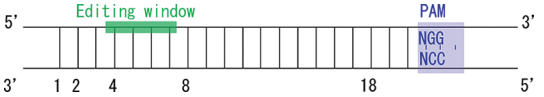
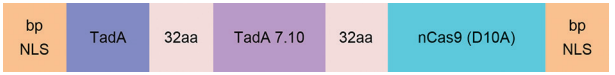
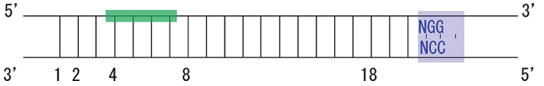
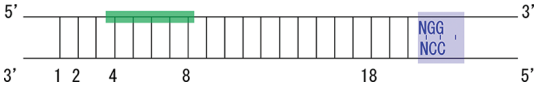
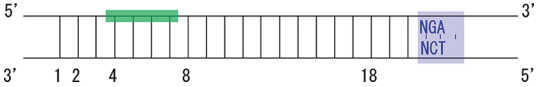
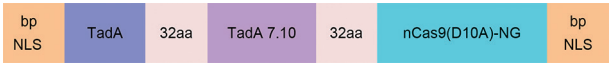
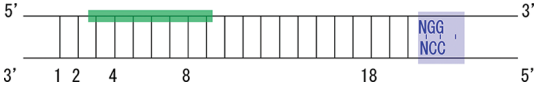
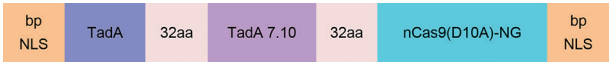
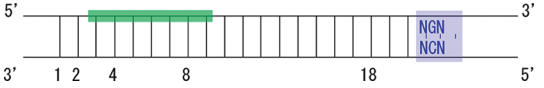
### 2.3. The gene delivery vector

To treat CVDs, the genome editing tools need to be effectively delivered to the cells that play a primary role in the disease. At present, the most successful and popular gene delivery vectors of ABEs include recombinant adeno-associated virus (rAAV) vectors and lipid nanoparticle (LNP) vectors.

rAAVs are viral particles that were engineered from the adeno-associated virus of the dependovirus genus of the parvoviruses[26,27]. An rAAV particle is composed of a protein capsid and an enclosed single-strand DNA of less than ~5kbp. As a non-pathogenic virus, rAAV can effectively transduce a number of organs, including the heart, with relatively low immunogenicity and toxicity. As of January 1, 2023, six rAAV-based gene therapy drugs have been federally approved for the treatment of different diseases, building an excellent safety, and effectiveness record for this new drug format[28]. The trademark names of these drugs are Glybera, Luxturna, Zolgensma, Upstaza, Roctavian, and Hemgenix.

The coding sequence of ABE7.10 is about 5.4 kbp in length, beyond the packaging capacity of rAAV vectors[9]. The mainstream solution of this problem harnesses the split intein system to allow two parts of the proteins to trans-splice into a full-length protein[29]. Therefore, ABE can be split into two halves, each being delivered by two separate

**Table 1. Representative ABE variants**

| ABE variants | Base editor architecture | Editing window and PAM | References |
|---|---|---|---|
| ABE7.10 |  |  | [9] |
| ABEmax |  |  | [12] |
| ABE8e |  |  | [14] |
| ABEmax -VRQR |  |  | [57] |
| ABE8.8 |  |  | [15] |
| ABEmax -NG |  |  | [13] |

NLS: Nuclear localization signal, asa: Amino acid, bpNLS: bipartite NLS. In all panels, the ABEs editing windows are shown in green and PAM sequences in blue

rAAV vectors[30]. Alternatively, the recent engineering of smaller Cas proteins and the more compact designs of the rAAV vector has enabled ABE delivery using a single rAAV particle[31].

Another well-established tool for *in vivo* ABE delivery is the LNPs, which are nanoscale semi-solid particles that are assembled by four types of lipids, namely, cholesterol, phospholipids, ionizable lipids, and PEGylated (PEG means polyethylene glycol) lipids. The ionizable lipid can undergo a pH-dependent charge conversion and allow mRNA encapsulation into LNP[32]. On intravenous administration, conventional LNPs deliver nucleic acids primarily to the liver. The recent development of novel lipid formulas allows LNPs to target the lungs, the spleen, and some other organs[33].

Since the first U.S. Food and Drug Administration (FDA) approval of LNP drugs in 2018[34], LNPs have drawn tremendous attentions from both biotechnological and pharmaceutical researchers. LNPs have demonstrated a great safety record largely, because the lipid components can be quickly metabolized and cleared from the body. As the major vector for COVID-19 mRNA vaccine, the LNP technology and industry are both rapidly growing and maturing[32]. LNPs have recently carried the first ABE drug for CVDs into a clinical trial (NCT05398029), holding the great promise to facilitate ABE-based treatment of more human diseases.

## 3. Landmark studies of ABE therapy for CVDs

### 3.1. Hutchinson-gilford progeria syndrome

One of the first evidence demonstrating the effectiveness of ABE in gene therapy involves the Hutchinson-Gilford Progeria Syndrome (HGPS)[30]. HGPS is a rare disease with whole-body premature aging phenotypes. Among these phenotypes, vascular malformation and dysfunction are most critical as these patients usually die of atherosclerosis and heart attacks in their teens[35]. Therefore, here we treated HGPS as a special type of CVD.

HGPS is commonly caused by a heterozygous *LMNA* c.1824 C>T/p.G608G mutation. This mutation activates a cryptic splicing site in the gene and aberrantly produces a splicing variant protein called progerin[36-38]. Despite much effort to reduce the toxic effects of progerin, particularly with the development of the FDA-approved farnesyltransferase inhibitor drugs[39], the patients can only survive for another 2–3 years.

The *LMNA* c.1824 C>T mutation falls into the SNV category that is editable by ABE. To test this idea, Dr. David Liu's team firstly used a lentiviral vector to deliver the ABEmax-VRQR base editors to treat fibroblasts that are derived from HGPS patients. After a puromycin selection for virus-transduced cells, the authors observed ~84% correction of the pathogenic mutation, substantially reduced progerin expression, and the ameliorated nuclear shape phenotypes[30].

Next, the same team designed a dual-AAV system to deliver ABEs to a transgenic HGPS mouse model that constitutively expresses the human progerin (Figure 2A). A single intravenous injection of these AAVs resulted in variable editing efficiencies (10 – 60%) among the heart, the quad, the liver, the aorta, and the bones. Strikingly, this single-dose treatment was sufficient to reduce the loss of vascular smooth muscle cells and the periadventitial thickening of the aorta, which are key pathological features of HGPS. This ABE treatment also increased the median lifespan of the mouse model from 215 to 510 days, approaching the old age of healthy mice[30]. Therefore, AAV-mediated ABE treatment might potentially be a permanent cure for HGPS in the future.

### 3.2. Inherited hypertrophic cardiomyopathy

In addition to HGPS, hypertrophic cardiomyopathy (HCM) is another CVD that would potentially benefit from ABE treatment. Unlike HGPS, which is a very rare disease with a prevalence of 1 in 20 million people, HCM is the leading cause of cardiac sudden death in people younger than 35-years-old[40]. HCM is featured by the excessive thickening of myocardium and the hypercontractile phenotype of cardiomyocytes[41]. SNVs in genes coding sarcomere proteins, particularly *MYH7* and *MYBPC3*[42], are the major causes of HCMs. Despite the recent development of cardiac myosin inhibitors[43-45] as breakthrough drugs for HCM, their application is usually limited to a subgroup of HCM patients, and their therapeutic effects are far from being satisfactory. Therefore, it is critical to develop a new approach to treat this disease.

Dr. Feng Lan's group performed the first proof-of-concept study to test the ability of ABE to treat HCM in mice[46]. They created a clinically relevant mouse model carrying the HCM pathogenic MYH6-R404Q/+ mutation (*Myh6* c.1211C>T) and validated its pathogenic role in HCM. Then, they microinjected ABEmax-NG mRNA and sgRNA into the mutant zygotes, allowing the embryo to develop to birth, and then genotyped the animals to evaluate the effect of the ABE (Figure 2B). Their results demonstrated that the overall editing efficiency is 91% on the *Myh6* c.1211C>T loci among the mutant embryos. The genetically corrected mice showed normal heart weight, less fibrosis, orderly arranged myofilaments, and normal left ventricular wall thickness, effectively preventing the HCM phenotypes in the R404Q/+ mice.

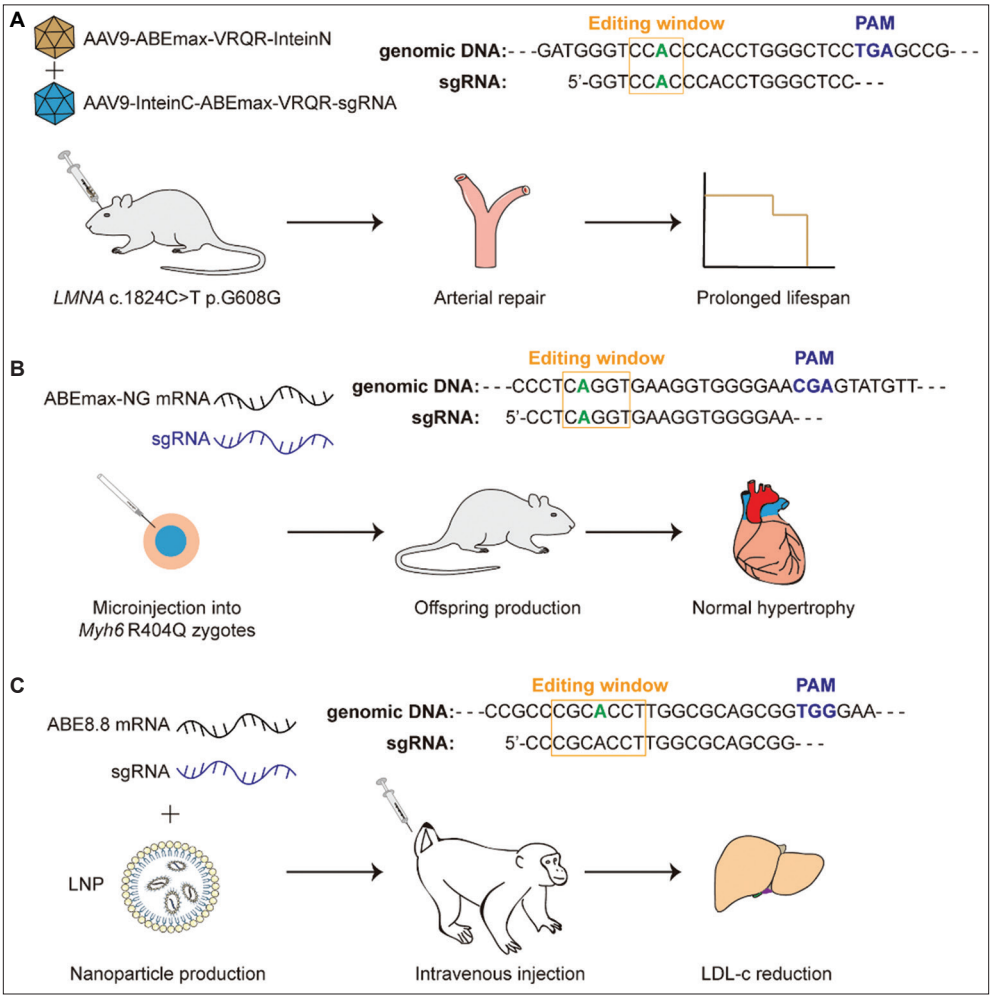



**Figure 2.** Three hallmark studies of ABE-based therapy for CVDs. (A) ABE gene therapy for HGPS in mice. Dual AAV9 expressing ABEmax-VRQR and sgRNA were retro-orbitally injected into HGPS mouse models, which resulted in the prevention of arterial damages and the increase of lifespan. (B) ABE gene therapy for HCM in mice. ABEmax-NG mRNA and sgRNA were co-microinjected into the Myh6-R404Q/+ zygotes, which prevented the development of HCM. (C) ABE gene therapy for hypercholesterolemia and atherosclerotic CVDs. The LNPs that carried ABE8.8 mRNA and sgRNA were delivered into cynomolgus monkey by intravenous injection. The LDL-C was reduced significantly. In all panels, the orange box depicts the editing window with the PAM sequences in blue.

Although zygotic genome editing provides a powerful technique for the proof of concept, germline gene therapy is apparently difficult in clinical practices and would raise serious ethical issues particularly when genome editing is performed[47]. As a test for somatic gene therapy, Dr Lan's group also established a dual-AAV system to deliver ABE into embryonic day-16 mutant fetuses. In contrast to zygotic editing, the AAV system only corrected 25.3% of the pathogenic mutation. This editing efficiency was further reduced if AAV was injected at a later time point, suggesting that the performance of ABE heavily depends on the developmental stage of the heart[46]. The authors argued that the success of base editing required active DNA replication or cell cycle. Thus, mature cardiomyocytes, as a terminally differentiated cell type[48], might be difficult to edit postnatally. This problem might be the major bottleneck in the efforts to treat inherited cardiomyopathy by ABE.

### 3.3. Hypercholesterolemia and atherosclerotic CVDs

Both HGPS and HCM are diseases with clear pathogenic mutations. Such SNVs are often rare, and it is not practical to develop a different ABE drug for each individual SNV. In addition to the correction of missense mutations, ABE can also be harnessed for gene silencing, which greatly expands the application of ABE in gene therapy. Below is an example that has pushed ABE to a clinical trial (NCT05398029), providing an exciting opportunity to treat hypercholesterolemia and to prevent atherosclerotic CVDs, the leading cause of death worldwide[49].

Atherosclerotic CVDs are caused by the narrowing and hardening of the artery walls and the formation of plaques[50]. The elevation of low-density lipoprotein cholesterol (LDL-C) in blood is a major cause of atherosclerosis[51]; thus, reducing LDL-C is a well-accepted strategy to treat atherosclerotic CVDs. PCSK9 is a plasma protein that accelerates the removal of LDL receptors from the surface of hepatocytes, preventing the uptake and clearance of blood LDL-C[52]. Most importantly, non-sense mutations of *PCSK9* in the human population are associated with lower LDL-C levels and a significantly reduced risk of coronary heart disease[53,54], without causing other serious abnormalities. Thus, *PCSK9* has served as a classic therapeutic target to reduce LDL-C for many years.

*PCSK9* is mainly expressed and secreted by the liver; thus, liver-targeted gene delivery vectors, such as LNPs, provide ideal tools to genetically manipulate *PCSK9 in vivo*. In 2021, Musunuru *et al.* designed an elegant ABE system to edit the 5' splice donor sequence in *PCSK9* intron 1, disrupting pre-mRNA splicing, and depositing a premature stop codon that ablates *PCSK9* expression[55]. They used LNPs to deliver ABE8.8 mRNA and the sgRNA targeting *PCSK9* into cynomolgus monkeys (Figure 2C) and detected about 70% editing rate specifically in the liver. This one-time treatment results in almost complete elimination of blood *PCSK9*, as well as over 50% reduction of LDL-C for up to 8 months, suggesting the capacity to lower LDL-C with one shot for life[55]. In July 2022, this investigational drug has been dosed in the first human in a phase 1b clinical trial, as a potential treatment for heterozygous familial hypercholesterolemia[56]. If successful, this game-changing therapy is expected to be repurposed for other forms of atherosclerotic CVDs in the future.

## 4. Future challenges

### 4.1. Identifying and expanding the editable loci

The first step in designing an ABE study is to determine if the target adenosine is editable. At present, this task is challenged by several factors including the availability of a PAM sequence at the appropriate position, the presence of other adenosines in the editing window that might result in unwanted bystander effects, as well as the sequence and chromatin neighborhood that might undermine the editing efficiency.

To overcome these limitations, huge progress has been made to introduce new Cas proteins with distinct or less restrictive PAM sequences[17,24,25,57] in to ABEs. For example, a recent study equipped TadA8e with multiple distinct Cas9 variants and collectively offered editability to about 82% adenosines in the human genome[31]. TadA was further engineered to change the editing windows[15,16,58,59] or to enhance the position precision of ABE so that on-target editing can be achieved while reducing the bystander effects. In addition, machine-learning approaches have also been exploited to predict the outcome of a given ABE reaction *in silico*[60-62], greatly reducing the costs and efforts in the experimental exploration of a good ABE design for a specific application.

### 4.2. Increasing editing efficiency

The editing efficiency of ABE is firstly determined by the design of the ABE machinery. Codon optimization and the proper installation of nuclear localization sequences (NLS) have been shown to enhance the performance of ABE[13]. The modulation of the linker amino acids between TadA and the Cas9 nickase and the coupling of ABE with an uracil glycosylase inhibitor (UGI) could also improve editing efficiency[63]. Most importantly, the directed evolution of TadA has been shown as a powerful approach that continuously increases the editing efficiency of ABEs[9,15,16].

In addition to the intrinsic properties of ABE itself, its editing efficiency is also influenced by the availability and expression levels of ABE components in the cells, which is determined by the gene delivery methods. For example, LNP has be validated as a robust vector to deliver ABEs to the liver to achieve high editing efficiency[55]. With the recent development of new formula, LNPs can also target the lungs, the spleen, and a couple of other organs[33], but whether these new tools will lead to robust editing in these organs remains to be examined.

As compared to LNPs, AAV vectors have been demonstrated to permit base editing in more organs, but the editing efficiency is relatively low[30]. The small payload of AAV vectors has been the major limiting factor, so in the dual-AAV systems, cells can be edited only when both AAV vectors transduce the same cell. With the development of more compact ABE tools and the careful design of AAV vectors, recent studies have started to report all-in-one AAV-ABE vectors[31], which indeed increased editing efficiency as compared to the dual-AAV systems. However, these vectors have pushed the AAV payload to the extreme, leaving little space for further modifications of these vectors.

### 4.3. Reducing the undesired editing

The precision of ABE reaction determines the safety of the relevant therapies. Thus, undesired editing by ABEs needs to be carefully monitored when developing new drugs. These unwanted editing can be grouped into two types, namely, the ones on the targeted site and the ones on the

off-target site (Figure 3). An important form of unwanted editing on the target site is the indels. Because Cas9 nickases are used in ABE, its likelihood to introduce indels is very low. Among the key studies of ABE therapy for CVDs, the indel rate has be reported as 0.2% or lower[30,46,55].

As mentioned previously, the more problematic form of unwanted editing on the target sequence involves the bystander effect (Figure 3A). The three hallmark studies using ABE for CVD therapy all cleverly chose the target sites with only one adenosine in the editing window, circumventing this problem[30,46,55]. However, for most other diseases, it will be inevitable to edit an adenosine near other adenosines. Fortunately, a recent study reported a new version of ABE called ABE9, which exhibited a narrow editing window of only 1-2 nucleotides[59]. By carefully choosing Cas9 variants with less PAM restriction and designing sgRNAs to put only the target adenosine in the editing window, it is promising to drastically reduce the likelihood of bystander effects in the future.

The undesired edits of ABEs on nucleic acid sites distinct from the targeted site are often called the off-target effect (Figure 3B-D). Because TadA was originally an RNA deaminase, a major ABE off-target effect was found on RNA transcripts (Figure 3B), which was seemingly independent from Cas9 and sgRNA[64]. In addition, ABE can deposit unwanted edits on sites with 1-2nt mismatches to sgRNA (Figure 3C), which is known to be tolerated by Cas9[18,65,66]. Interestingly, ABEs can also exert the genomic off–target effect in a Cas9/sgRNA-independent manner (Figure 3D)[15,67]. This type of editing most likely happens in genomic loci that naturally unwind and expose single-strand DNA to the freely available TadA in the nuclei, such as during DNA replication and gene transcription.

Because off-target effects are induced by complicated mechanisms, the prediction and identification of off-target sites by a given ABE reaction are challenging (Table 2). The most convenient and fast methods to nominate off-target sites are through computational prediction. Although these methods usually work fine in predicting sgRNA-dependent off-target sites basing on sgRNA similarity[68,69], or when sufficient prior data are available for machine-learning based prediction[70,71], additional experimental validation is still necessary to avoid false-positive nominations. Off-target effects can also be assessed experimentally by either using purified genomic DNA[72-75] or through cell culture[76,77]. However, these methods still cannot fully characterize the off–target effects in animals or human bodies where ABE is used as a therapy. Whole genome sequencing (WGS) was a universal approach to detect the off-target effect both in cells and animal tissues[78]. The recent development of DISCOVER-seq (discovery of in situ Cas off-targets and verification by sequencing) and GUIDE-tag (GUIDE means genome-wide, unbiased identification of DSB) techniques provides promising tools to directly assess the off-target effects for *in vivo* genome editing[79,80]. However, whether these methods are sensitive

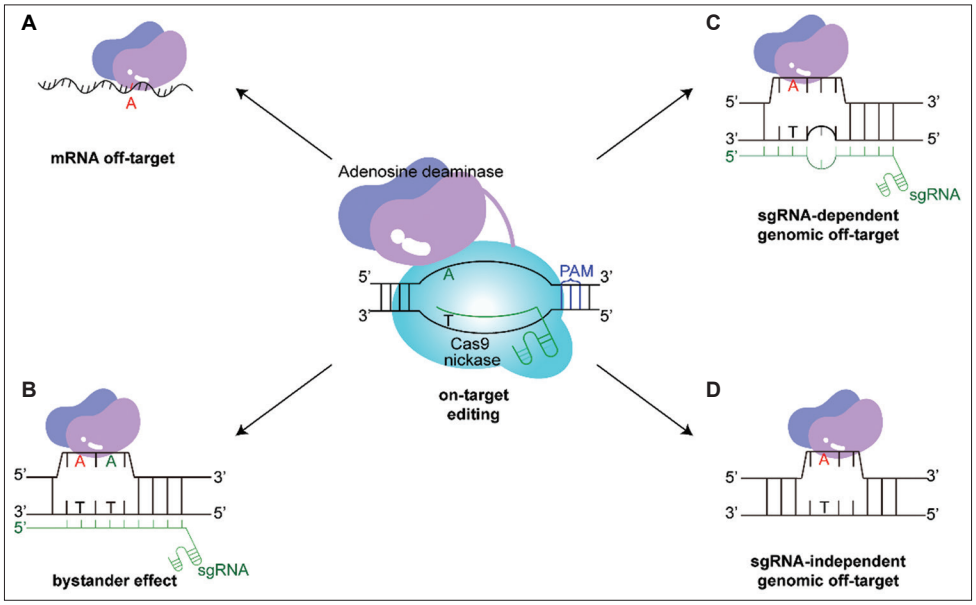



**Figure 3.** The types of undesired ABE editing. (A) Bystander effect. TadA edits other untargeted adenine in the editing window of the on-target site. (B) The off-target effect on mRNA. TadA modified adenine in mRNA independent of Cas9/sgRNA. (C) sgRNA-dependent genomic off-target effect. The sgRNA tolerates 1–2nt mismatches and guides ABE to modify an off-target site. (D) sgRNA-independent genomic off-target effect. TadA converts A to G in some genomic DNA sites independent of sgRNA or Cas9. In all panels, the undesired adenine edits are in red, while the target adenine in green.

**Table 2. Representative methods to determine genomic off-target effects**

| Classification | Name | Advantage | Disadvantage |
|---|---|---|---|
| Computational prediction | Cas-OFFinder[67] | Simple, cheap and expedient | Biased toward sgRNA- dependent off-target sites. Still require further experimental validation. |
| | CasOT[68] | | |
| | DeepCRISPR[69] | | |
| | Elevation[70] | | |
| *In vitro* detection on purified genomic DNA | Digenome-seq[73] | Unbiased and sensitive | Only applicable *in vitro*. With false positive and false negative hits due to the lack of intracellular factors. |
| | CIRCLE-seq[74] | | |
| | SITE-seq[72] | | |
| | EndoV-seq[71] | Specifically designed for ABE | |
| Label and capture in cell culture | IDLV capture[76] | Unbiased, sensitive and in living cells | Only applicable in cell culture. With false positive and false negative hits due to the lack of physiological relevance. |
| | GUIDE-seq[75] | | |
| Direct detection in animal tissues | WGS[77] | Unbiased and suitable for clinical samples | Expensive and relatively low sensitivity |
| | DISCOVER-seq[78] | Unbiased and suitable for clinical samples | Relatively low sensitivity |
| | GUIDE-tag[79] | Unbiased and in animal tissues | Technically complicated and not suitable for clinical samples |

Digenome-seq: *In vitro* Cas9-digested whole-genome sequencing, CIRCLE-seq: Circularization for reporting of cleavage effects by sequencing, SITE-seq: Selective enrichment and identification of adapter-tagged DNA ends by sequencing, IDLV: Integrase-deficient lentivirus, GUIDE-seq: Genome-wide, unbiased identification of DSBs enabled by sequencing, WGS: Whole genome sequencing. DISCOVER-seq: Discovery of *in situ* Cas off-targets and verification by sequencing

and accurate enough to evaluate ABE-induced off-target effects in gene therapy remain to be determined.

So far, two major approaches have been applied to reduce the off-target effects by ABE. The first method harnesses protein structure information or directed evolution technology to engineer more accurate ABE mutants. For example, arginine 153 (R153) within TadA was reported to mediate its RNA editing activity; thus, R153 deletion was implemented in ABEs to minimize its RNA off-target effects[81]. Importantly, the recently established ABE9 also drastically reduced both RNA off-target effects and Cas9-independent DNA off-target effects, in addition to the aforementioned impact on bystander effects[59].

Another plausible method to reduce the off-target effect works by controlling the duration of ABE expression. Because RNA exhibits a high turnover rate, RNA off-target effects will gradually taper off once the ABE stops expressing. Similarly, once the on-target DNA editing is accomplished, ABE activities should be terminated to avoid further accumulation of off-target edits in the genome. Based on this rationale, LNP vectors are more suitable than AAV vectors in delivering ABEs to the liver, as LNP-mediated ABE expression only persists for days, while AAV-mediated gene expression can last for years.

## 5. Conclusions

CRISPR/Cas9-based genome editing has revolutionized biomedical research, including cardiovascular research, in the past decade. With the emergence of more advanced genome editing tools such as ABE, therapeutic genome editing in human bodies has entered clinical trials and will likely become a reality in near future. In this review, we showcase the power of ABE in CVD therapy and recommend more cardiovascular researchers to embrace ABE as a new weapon to tackle CVDs.

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## Conflict of interest

No potential conflicts of interest were disclosed.

## Author contributions

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## Ethics approval and consent to participate

Not applicable.

## Consent for publication

Not applicable.

## Availability of data

Not applicable.

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ACCSCIENCE PUBLISHING

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PERSPECTIVE ARTICLE

# Chronic positive mass balance is the actual etiology of obesity: A living review

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## Abstract

The fundamental cause of obesity is widely assumed to be an energy imbalance between calories consumed and calories expended (i.e., the energy balance theory). However, this century-old obesity paradigm is fallacious. According to known laws of physics, the actual etiology of obesity is chronic positive mass balance, not positive energy balance. Furthermore, the relevant physical law in body mass regulation is the Law of Conservation of Mass, not the Law of Conservation of Energy. It is important to understand that energy balance and mass balance are separate balances in the human body. Since calories simply represent the heat released on food oxidation, they have no impact on body mass. Body mass can only change as a result of net mass flow; thus, the only food property that can augment body mass is its nutrient mass, not its energy content. The recently proposed mass balance model describes the temporal evolution of body weight and body composition under a wide variety of feeding experiments, and it seems to provide a highly accurate description of the very best experimental human feeding data. By shifting to a mass balance paradigm of obesity, a deeper understanding of this condition may follow in the near future. The purpose of this living review is to present the core issues of the upcoming paradigm shift and some practical applications related to the subject.

***Keywords:*** Obesity; Body weight regulation; Macronutrients; Energy balance theory; Mass balance model; Paradigm shift; Living review

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## 1. Introduction

In the *Twilight of the Idols, or, How to Philosophize with a Hammer*, Friedrich Nietzsche emphasized the significance of "sounding out idols," which entails disclosing the inconsistencies in established metaphysical principles, and "philosophizing with a hammer," a metaphor for the process of critical thinking and questioning to destroy illusions, false beliefs, and prejudices that have become ingrained in people's minds.

Reasoning by analogy refers to building knowledge based on prior assumptions and widely accepted paradigms approved by the majority of people, while reasoning from first principles refers to the practice of actively questioning every assumption one thinks he/she "knows" about a given topic and then creating completely new knowledge from scratch. In other words, reasoning from first principles is the act of boiling a process down to the fundamental parts that one believes are true and building up from there. This approach has been used by many great thinkers, including the ancient philosopher Aristotle. With regard to the present time, no one has utilized reasoning from first

principles as effectively as the world's most successful entrepreneur, Elon Musk.

If we start applying this approach to the regulation of body mass, the first question is what body mass consists of. Almost all of the mass of the human body is made up of four elements: Oxygen (65%), carbon (18.5%), hydrogen (10%), and nitrogen (3.2%). It is essential to note that there are no calories or joules listed. Naturally, the next question would be why obesity researchers pay attention only to energy balance given that energy has no mass at all. Even this short reasoning from first principles reveals that mass balance is more relevant than energy balance.

Recently, Arencibia-Albite has published an exceptionally clever article titled "The energy balance theory (EBT) is an inconsistent paradigm" in the *Journal of Theoretical Biology*[1]. This present article deals with the exact same topic but focuses on practical applications. The purpose is to explain in plain language what this far-reaching paradigm shift will mean on a practical level. Therefore, there will be no complex equations or formulas in this article; instead, the core issues are presented in such a way that every university-educated healthcare professional or scientist can understand them.

Due to the growing interest of the scientific community in the impending paradigm shift, this article has taken up a different approach[2]. As a living review, this article will be updated when new and important information appears.

## 2. The EBT is a flawed paradigm

It is widely assumed that the fundamental cause of obesity is an energy imbalance between calories consumed and calories expended (i.e., the EBT: "Calories In, Calories Out"). However, according to known laws of physics, this century-old obesity paradigm must be fallacious. The relevant physical law in body mass regulation is the Law of Conservation of Mass, not the Law of Conservation of Energy (*i.e.*, the First Law of Thermodynamics).

This is not a matter of opinion; rather, it is based on exact natural sciences. If matter (mass) can be exchanged between system and surroundings, then the system is an open one. Therefore, all living organisms are open systems, and such systems can be at mass balance even while the system experiences a persistent energy imbalance. That is to say that while energy balance may be positive ($\Delta E > 0$) or negative ($\Delta E < 0$), the Law of Conservation of Energy does not require the mass change that may occur during energy flux to match the energy balance direction[1]. In practice, this means that an energy imbalance does not always lead to a change in body mass. The latter only occurs when one is in a mass imbalance. Body mass decreases in negative mass balance and increases in positive mass balance[1,3].

Since there are widespread misconceptions about thermodynamics and body mass regulation, a brief recap follows.

An open system is a type of thermodynamic system where energy and mass can be exchanged with its surroundings. Consequently, the mass of the system will vary with time. An example is the human body. A closed system, on the other hand, is a type of thermodynamic system where only energy can be exchanged with its surroundings. Consequently, the mass of the system is constant. An example is the refrigerator.

The EBT falsely assumes that there is no difference between the two thermodynamic systems. Contrary to what is almost universally claimed, the EBT is not a consequence of the First Law of Thermodynamics. The claim that the EBT should be valid because of this law is simply not true. Since this is an extremely stubborn misconception, in even plainer English, this type of textbook argument is fallacious:

> *Due to the first law of thermodynamics, a person gains weight when his/her energy intake is greater than his/her energy expenditure.*

Perhaps, it would be more reasonable to say that the statement is partially correct because energy and mass are closely related. Textbooks, however, should read as follows:

> *Due to the Law of Conservation of Mass, a person gains weight when his/her mass intake is greater than his/her mass expenditure.*

## 3. Law of Conservation of Mass

The Law of Conservation of Mass states that mass can neither be created nor destroyed by chemical or physical changes. In other words, total mass is always conserved. This law dates from Antoine Lavoisier's 1789 discovery par excellence that mass is neither created nor destroyed in any chemical reaction[4]. A clever Frenchman heated mercuric oxide (HgO) and demonstrated that the amount the chemical's mass decreased was equal to the mass of oxygen gas released in the chemical reaction. Lavoisier has proven that mass is conserved in chemical reactions, meaning that the total amount of mass on each side of a chemical equation is always the same. This indicates that the total number of atoms in the reactants must equal the amount in the products regardless of the nature of the chemical change. This forms the basis of stoichiometry, that is, the accounting process by which chemical reactions and equations are mathematically balanced in terms of both mass and number of atoms on each side.

As an example, the oxidation of one generic triglyceride molecule is as follows:

$C_{55}H_{104}O + 78O_2 \rightarrow 55CO_2 + 52H_2O$ + HEAT 8,084 kcal

| Reactants | | Products | |
|---|---|---|---|
| $C_{55}H_{104}O$ | 860 g | $55CO_2$ | 2,420 g |
| $+78O_2$ | 2,496 g | $+ 52H_2O$ | 936 g |
| | 3,356 g | | 3,356 g |

It is important to note that there is mass only in reactants and products but not in energy (calories).

## 4. Mass-energy equivalence principle

The mass-energy equivalence principle implies that when energy is lost in chemical reactions, the system will also lose a corresponding amount of mass. However, as far as the regulation of body mass is concerned, this equivalence principle has been misunderstood. This global misconception requires detailed clarification.

How is energy intake and expenditure not the governing factors that determine if the body stores the food we eat as fat or not? How could one change that? How can the mass of food change that? If the eventual weight loss is from water, urea, or something else, it is still determined by whether or not the body replaces it or even stores more than was used. Where is the gap where energy expenditure is not representative of the substrate (*i.e.*, mass) being used?

To understand why nutrient mass, not nutritional energy, is the entity that determines body mass fluctuations, one must think in terms of arithmetic and analytical chemistry as shown in the next subsections. The caloric values of macronutrients are rounded.

### 4.1. Weight gain is the result of mass accumulation, not energy accumulation

Consider two individuals that gained 1 kg of non-water body mass, as they accumulate 1,000 g of absorbed macronutrients within their body cells. The macronutrient distribution of the first subject is shown below.

(i) 200 g of protein = 200 g × 4 kcal/g = 800 kcal
(ii) 300 g of carbohydrate = 300 g × 4 kcal/g = 1,200 kcal
(iii) 500 g of fat = 500 g × 9 kcal/g = 4,500 kcal

The total stored nutritional energy is 800 kcal + 1,200 kcal + 4,500 kcal = 6,500 kcal.

The macronutrient distribution of the second subject is shown below.

(i) 400 g of protein = 400 g × 4 kcal/g = 1,600 kcal
(ii) 400 g of carbohydrate = 400 g × 4 kcal/g = 1,600 kcal
(iii) 200 g of fat = 200g × 9 kcal/g = 1,800 kcal

The total stored nutritional energy is 1,600 kcal + 1,600 kcal + 1,800 kcal = 5,000 kcal.

This example illustrates, therefore, that the property of food that is related to mass gain is its mass, not energy. The first subject, in effect, has accumulated substantially more nutritional energy than the second subject, yet both have experienced the same degree of weight gain.

### 4.2. Weight loss is the result of mass elimination, not energy expenditure

Consider the oxidation of 100 g of glucose:

$C_6H_{12}O_6 + 6O_2 \rightarrow 6H_2O + 6CO_2$ + HEAT (720 kcal/mol of $C_6H_{12}O_6$)

This requires the uptake of 107 g of oxygen ($O_2$) as 100 g glucose ($C_6H_{12}O_6$) × (192 g $O_2$/180 g $C_6H_{12}O_6$) ≈ 107 g $O_2$. The Law of Conservation of Mass implies that the mass of the products = mass of the reactants. The amount of water ($H_2O$) and carbon dioxide ($CO_2$) formed is 207 g as the mass of the products = mass of the reactants = 100 g $C_6H_{12}O_6$ + 107 g $O_2$ = 207 g.

Now, assume that all the produced water and carbon dioxide are used in the following way:

(i) Water becomes intracellular water in newborn cells;
(ii) Hydrolysis reaction (*i.e.*, the cleavage of a chemical bond by adding a water molecule that becomes part of the reaction products); for example, the release of thyroid hormones thyroxine (T4) and triiodothyronine (T3) requires hydrolysis;
(iii) Carboxylation reaction (*i.e.*, the addition of carbon dioxide to a molecule); for example, carboxylation of acetyl-CoA during fatty acid synthesis.

Notice that in the aforementioned situation, 400 kcal have been expended by oxidizing 100 g of glucose, yet body mass will not decrease when heat is dissipated but rather when the 207 g of reaction products are eliminated, which in the described case did not since, as illustrated, oxidation products become part of the body mass.

The important message in this section is that that energy balance and mass balance are separate balances in the human body[1,3]. This fact should be kept in mind when reading the sections below.

## 5. Energy balance cannot occur at body mass stability

According to the Law of Conservation of Mass, body mass stability (*i.e.*, mass balance) can occur only when the mean absorbed mass of each macronutrient is the same as its respective mean oxidized mass. Otherwise, body mass will

increase (*i.e.*, absorbed mass > oxidized mass) or decrease (*i.e.*, absorbed mass < oxidized mass).

More specifically, energy balance can occur at body mass stability only if the following three conditions are simultaneously satisfied:

(i) Average absorbed fat mass = Average oxidized fat mass;
(ii) Average absorbed carbohydrate mass = Average oxidized carbohydrate mass;
(iii) Average absorbed protein mass = Average oxidized protein mass.

Clearly, this can never happen. If, for example, all the absorbed protein mass (amino acids) is oxidized, where would the body get building blocks? Therefore, energy balance is unattainable at body mass stability[1,3]. This fact refutes the core idea of the EBT, that is, body mass remains constant in energy balance.

To explain clearly, this section highlights that the following textbook argument is fallacious:

> *When a person is in energy balance, his/her weight stays the same.*

On the contrary, textbooks should read the following:

> *When a person is in energy balance, his/her weight does not stay the same. Since this concept can be difficult to understand, more attention should be paid to the matter. However, the energy balance theory should be also understood since it is important to know what has been known before so that one can quickly grasp new information.*

## 6. Regulation of body mass

At this point, it should be clear that the regulation of body mass is entirely about detailed mass balances ("Mass In, Mass Out") rather than energy conservation ("Calories In, Calories Out"). The Law of Conservation of Mass guarantees that (i) the $O_2$ mass that enters cellular respiration plus (ii) the mass of macronutrients that served as energy fuel must be equal to the mass of the excreted oxidation products. This is not a matter of opinion. Daily weight loss must, therefore, be the result of the daily elimination of oxidation products ($CO_2$, water, urea, sulfur trioxide [$SO_3$; "Mass Out") rather than a consequence of the heat release upon nutrient combustion (*i.e.*, daily energy expenditure)[5]. It is macronutrient mass intake ("Mass In") that augments body mass; as dictated by the Law of Conservation of Mass, the absorption of 1 g of glucose, protein, or fat increases body mass by exactly 1 g, independent of the substrate's calories. The absorbed nutrient mass cannot be destroyed, and thus will contribute to the total body mass as long as it remains within the body. Such a contribution ends, however, when the nutrients are eliminated from the body either as products of metabolic oxidation or in other forms (*e.g.*, shedding of dead skin cells).

Animals, including humans, ingest food to obtain energy and mass. While energy refers to the capacity to do work, mass is used to build all bodily structures. Not a single gram of body mass is added through energy intake. Since calories represent the heat release upon food oxidation, they have no impact on body mass[1,3]. The term "calorie" comes from the Latin term *calor* (heat). Calorie is broadly defined as the amount of energy needed to increase the temperature of 1 g of water by 1°C. Heat certainly does not produce mass.

The protons and neutrons (*i.e.*, nucleons) that make up an atom account for nearly all of its mass; nuclei contain >99.9% of the mass of an atom. Countless chemical reactions constantly take place in the human body, and energy is bound or released in them. In all these reactions, only electron clouds undergo changes. Atomic nuclei, therefore, always remain intact; changes to the nuclei only occur during nuclear reactions. Body mass can only change as a result of net mass flow[1,3,6]; thus, the only food property that can augment body mass is its nutrient mass, not its energy content (*i.e.*, calories). Like gravity, this is by no means a matter of opinion.

It inevitably follows that any anti-obesity intervention must decrease the intake of energy-providing mass, increase the elimination of oxidation products, or both.

(i) Decrease intake of energy-providing mass (EPM) ("Mass In"), that is, satiating effect. EPM refers to the daily intake of carbohydrate, fat, protein, soluble fiber, and alcohol.
(ii) Increase elimination of oxidation products ("Mass Out"). Each day, we experience weight loss (*i.e.*, body mass loss) given by the weight of energy expenditure-dependent mass loss (EEDML) plus the weight of energy expenditure-independent mass loss (EEIML)[3]. EEDML refers to the daily excretion of EPM oxidation byproducts ($CO_2$, water, urea, and $SO_3$), whereas EEIML represents the daily weight loss resulting from (a) the daily elimination of non-metabolically produced water; (b) minerals lost in sweat and urine; (c) fecal matter elimination; and (d) the mass loss from renewal of skin, hair, and nails[3].

A recently proposed mass balance model (MBM)[3] describes the temporal evolution of body weight and body composition under a wide variety of feeding experiments, and it seems to provide a highly accurate description of the very best experimental human feeding data[1,3,7,8]. For example, head-to-head comparisons of the predictions by

the MBM with the EBT-based model of Hall *et al.*[9] have demonstrated that the MBM seems to be superior to the EBT-based model[7]. The difference in prediction accuracy is especially clear when the distribution of macronutrient intake changes drastically, for example, a low-carbohydrate diet versus an isocaloric high-carbohydrate diet. The ranking of such models is determined by their predictive accuracy, which is also the reason for such models to be developed. Emphasizing that the MBM predicts not only the change in total body mass, but also the change in fat mass is essential.

Figure 1, which is adapted from Arencibia-Albite's and Manninen's study[7], presents a comparative simulation between the EBT-based model[9] and the MBM[3]. All data can be found in the original source[7]; therefore, there is no reason to repeat them here. However, it is difficult to understand what these simulation results mean. Hence, the results are presented here in a simple manner (Figure 1). In the simulations, the free-living feeding trial data of Kong *et al.* were used[10]. Twenty young female subjects followed a "normal diet" (ND; carbohydrate 44.0 ± 7.6%, protein 15.4 ± 3.3%, and fat 39.6 ± 5.8%) for 4 weeks as a baseline and then switched to a very-low-carbohydrate/high-fat diet (KD; carbohydrate 9.2 ± 4.8%, protein 21.9 ± 3.4%, and fat 69.0 ± 5.4%) for another 4 weeks. The study showed that the 4-week KD intervention led to marked reductions in body mass (−2.9 kg) and body fat percentage (−2.0%). The results of the MBM-based simulations closely resemble those of the feeding trial, whereas the predictions of the EBT-based simulations are clearly incorrect. It is necessary to pay close attention to how the incorrect formula (Daily Fat Loss) = (Daily Fat Intake) – (Daily Net Fat Oxidation) affects the prediction results of the EBT-based model. The aforementioned formula would be valid only if the net fat oxidation is independent of the diet´s macronutrient distribution. If the net fat oxidation increases as dietary fat intake increases (and *vice versa*), fat loss may be similar among isocaloric diets that vary greatly in fat content. Hall *et al.* respiratory quotient (RQ) data demonstrate that this is indeed the case, as shown in Figure 2C, which indicates that the oxidation of fatty acids increases as the proportion

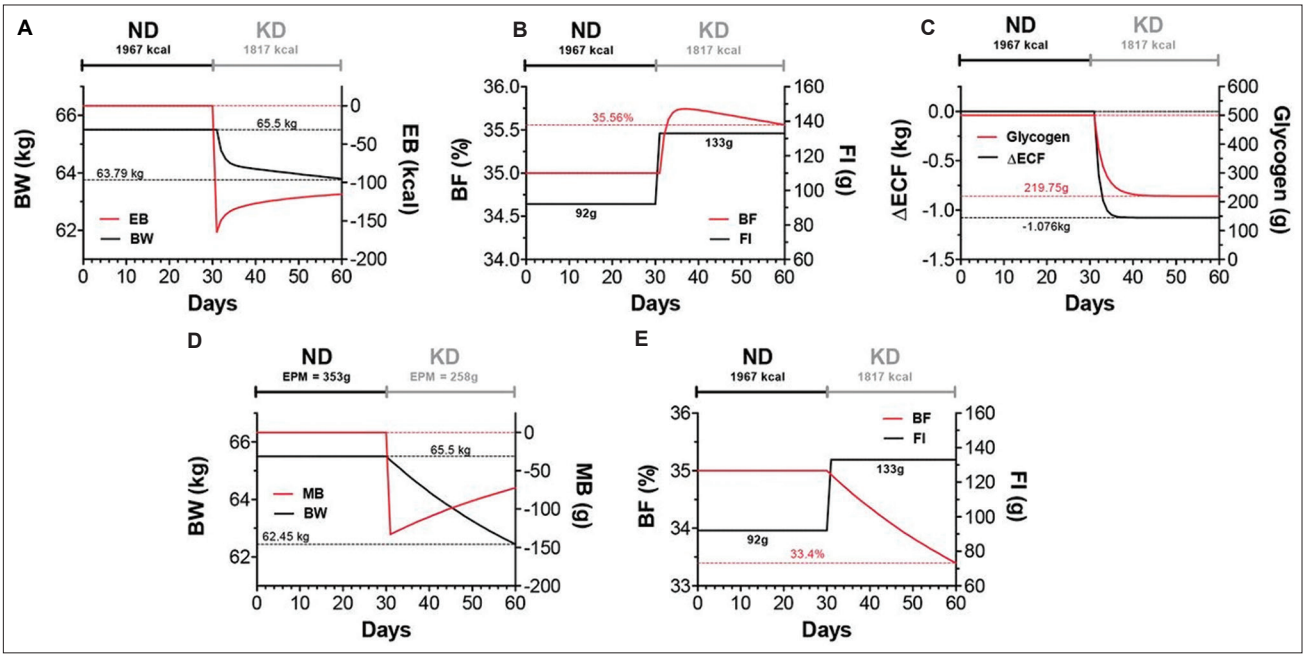


**Figure 1.** Simulations of Kong *et al.* feeding trial (EBT vs. MBM). (A) During the "normal diet" (ND; days 0–30), the energy balance (EB; red curve) was zero. After day 30, EB became negative following the very-low-carbohydrate/high-fat diet (KD), resulting in a 1.71 kg BW loss at day 60. (B) The EBT-based model by Hall *et al.*[9] predicts that BF percentage increases during the KD although BW decreases. This incorrect prediction was due to the 41 g increase in fat intake (FI) during the KD; the EBT incorrectly assumes that Daily Fat Loss = Daily Fat Intake – Daily Net Fat Oxidation[12]. (C) During the ND, the amount of glycogen stored was 500 g, but during the KD, its amount decreased to 280.25 g; extracellular fluid (ECF) also decreased by 1.076 kg; thus, glycogen + ECF = 1.35625 kg. Of the 1.71 kg weight loss in A, 0.35375 kg (1.71 kg – 1.35625 kg) were from other mass sources: 0.2436 kg fat mass + 0.11015 kg fat-free mass. According to the EBT, this indicates that the total weight loss was distributed as 0.2436 kg of fat mass plus 1.4664 kg of fat-free mass. As the decline in fat-free mass is much larger than that of fat mass, the EBT falsely predicts that the BF percentage will increase as illustrated in B. (D) During the ND period (days 0 – 30), the mass balance (MB; red curve) was zero. After day 30, the MB became negative following the KD, resulting in a 3.05 kg weight loss at day 60. (E) According to the MBM, of the 3.05 kg of weight loss, 2.07 kg came from fat mass and 0.98 kg from fat-free mass. Although the FI increased, the decline in fat-free mass was much smaller than that of fat mass, and thus the BF decreased[7].
BF: Body fat; BW: body weight; EBT: Energy balance theory; MBM: Mass balance model.

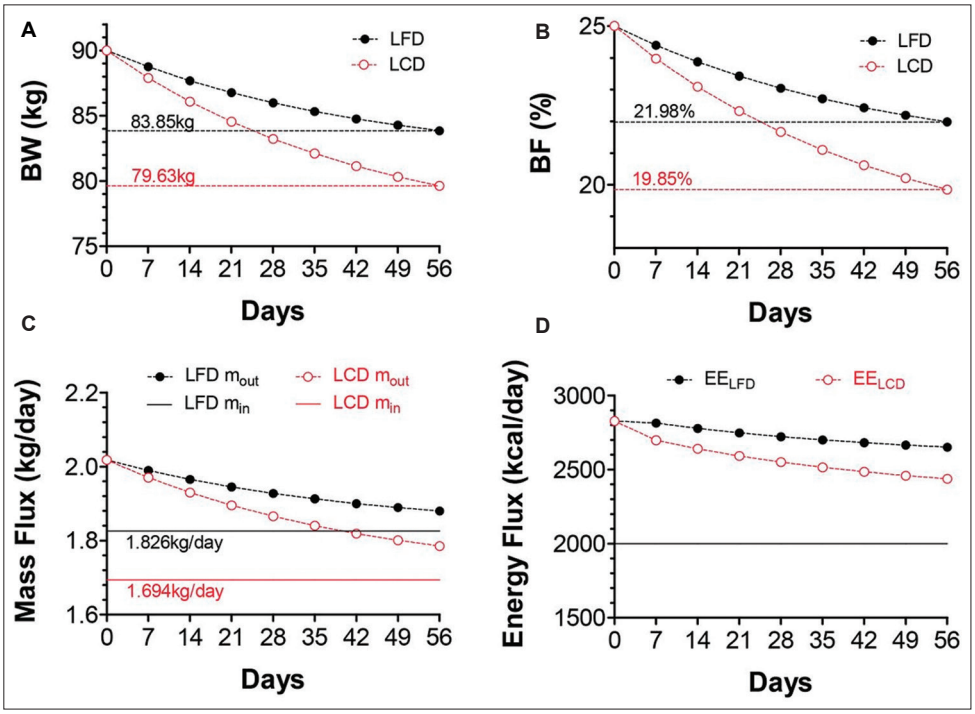


**Figure 2.** (A-D) Mass balance model (MBM)-based simulation of two hypothetical overweight (90 kg) individuals whose body composition and total energy intake were identical but macronutrient distribution was clearly different. In the initial situation, the nutrient intake was as follows: 2,750 kcal/day; 35% fat (F), 50% carbohydrate (C), and 15% protein (P). These individuals began following either a 2,000 kcal high-carbohydrate/low-fat diet (LFD) or a 2000 kcal low-carbohydrate/high-fat diet (LCD), whose macronutrient distribution was as follows: LFD = 20% F, 65% C, and 15% P; LCD = 70% F, 15% C, and 15% P. According to the energy balance theory, these diets should lead to almost identical effects in terms of body mass and fat mass. However, the MBM predicts that the LCD results in a greater body mass and fat mass loss compared with the LFD. As demonstrated, the nutrient mass intake ($m_{in}$) was smaller compared with the eliminated mass ($m_{out}$); thus, the net daily mass loss was larger (*i.e.*, $m_{in} - m_{out}$).
BF: Body fat; BW: body weight.

of fat in the diet increases[11]. For further details, refer to the figure legend.

Although free-living feeding trials are always a mixture of effectiveness and compliance, there is every reason to believe that Kong *et al.* study was a well-controlled study. Several requirements were made to ensure the subjects' adherence to the KD.

(i) All subjects were required to measure their daily urine ketones (early morning or after dinner) and record a 3-day food diary (2 weekdays and 1 week end day) during the experimental period.
(ii) The 3-day food diaries were kept by all subjects for 8 weeks.
(iii) All subjects were given "thorough instructions" on how to estimate portion sizes and record food/beverages intake on food composition tables in advance.
(iv) All subjects were required to report to the laboratory every week to assess changes in body weight and hand-in their logbook with dietary records.
(v) Their energy intake and macronutrient distribution were calculated by the same dietician using the nutrition analysis and management system.
(vi) Their diet compliance was evaluated based on the food diaries and urine ketone results; all subjects received individual follow-up dietary advice and counseling from the dietician.

On the whole, the feeding data produced by Kong *et al.* provide reliable information on the effects of macronutrient distribution on body mass and composition; thus, the simulation comparison would seem to show "beyond reasonable doubt"[13] that the MBM-based simulation provides more accurate predictions than the EBT-based simulation.

As mentioned earlier, Hall *et al.* EBT-based model assumes that body fat fluctuations are the consequence of an imbalance between dietary fat consumption and net fat oxidation. This formula would be valid if the distribution of macronutrients in the diet does not affect the oxidation of fatty acids, but this assumption does not correspond to reality. Hall *et al.* indirectly estimate the reduction in body fat based on the following equations (in g):

Daily Fat Loss = Daily Fat Intake – Daily Net Fat Oxidation (1)

Where Daily Net Fat Oxidation is another estimate calculated by

$$\text{Daily Net Fat Oxidation} = 1.63VO_2 - 1.64VCO_2 - 1.84N \quad (2)$$

Where $VO_2$ and $VCO_2$ are the volume (in L) of consumed $O_2$ and produced $CO_2$, respectively, while N is the urinary nitrogen excretion per 24 h. In these equations, the only precise numeric input is Daily Fat Intake, whereas Daily Net Fat Oxidation is an estimate obtained from estimates that inevitably increase the inaccuracy of measurement. As recently discussed by Arencibia-Albite[1], the aforementioned equations overlook, among other things, the fact that body fat can also decrease through the excretion of fatty acid derivatives.

## 7. A low-carbohydrate diet provides less nutrient mass than an isocaloric high-carbohydrate diet

A low-carbohydrate/high-fat diet leads to a greater body mass and fat mass loss than an isocaloric high-carbohydrate/low-fat diet (LFD) because it provides less nutrient mass[1,3,7,8]. When the energy fraction from dietary fat increases, while energy intake is clamped (*i.e.*, fixed), mass intake decreases due to the significantly higher energy density of fat compared with other energy substrates. Such a difference in mass intake translates into greater body mass and fat loss in a low-carbohydrate diet versus an isocaloric high-carbohydrate diet. If such a feeding response is not observed, then it is simply not a well-controlled study, as alternative results would indicate a violation of the Law of Conversation of Mass.

If two persons eliminate body mass at the same daily rate, then the one ingesting less nutrient mass will express a greater daily body mass and fat loss. For instance, the daily energy intake of 2,500 kcal distributed as 30% fat (9.4 kcal/g), 55% carbohydrate (4.2 kcal/g), and 15% protein (4.7 kcal/g) corresponds to a mass intake of ~487 g, whereas the same energy intake distributed as 60% fat, 30% carbohydrate, and 10% protein reduces mass ingestion by ~96 g. This is not a small difference in the long run.

It has been suggested that a low-carbohydrate diet is more effective than an isocaloric high-carbohydrate diet in losing body mass and fat mass because the former lowers insulin levels[14]. However, it is worth noting that insulin or any other hormones cannot create any kind of mass out of thin air. Only ingested nutrient mass can result in increased body mass. Insulin just ensures that this mass can be stored. Similarly, lowered insulin levels cannot magically destroy any mass. Although insulin levels decrease with a low-carbohydrate diet, it is not a causal factor of body mass and fat mass loss. It just happens simultaneously with decreased nutrient mass intake. What about *de novo* lipogenesis (DNL), that is, the process of synthesizing fatty acids from acetyl-CoA subunits? Hyperinsulinemia, caused by the consumption of a large amount of carbohydrates, can increase DNL, but it is only relevant in extreme overfeeding situations. DNL seems to play a central role in the pathogenesis of non-alcoholic fatty liver disease[15] but this topic is beyond the scope of this article.

However, insulin levels can be important in terms of where body fat is reduced. Since high insulin levels favor fat synthesis and inhibit lipolysis, it seems reasonable that the reduction of body fat occurs primarily through a reduction in dietary fat intake during a high-carbohydrate diet. In contrast, a low-carbohydrate diet lowers insulin levels, thus reducing fat synthesis and stimulating lipolysis, which nullify the effects of high dietary fat intake. These factors may explain why low-carbohydrate diets tend to work well for visceral fat reduction[16-18].

Goss *et al.* have recently reported that the very-low-carbohydrate/high-fat diet group in their 8-week study experienced a 3-fold greater loss of visceral adipose tissue (VAT) and intermuscular adipose tissue (IMAT) when compared to the high-carbohydrate/LFD group[17]. Following a very-low-carbohydrate/high-fat diet, there was a significantly greater decrease in fasting insulin level compared to the high-carbohydrate/LFD ($13.7 \pm 5.6 \rightarrow 9.4 \pm 4.0$ vs. $15.6 \pm 6.5 \rightarrow 16.0 \pm 8.2$) μU/mL. It should be noted, however, that the very-low-carbohydrate/high-fat group consumed significantly lesser total calories, and thus nutrient mass, per day than the high-carbohydrate/low-fat group. Nevertheless, it seems that the very-low-carbohydrate/high-fat diet works particularly well on these metabolically harmful fat depots.

Figure 2, which is adapted from Arencibia-Albite's and Manninen's study[7], presents two hypothetical overweight individuals whose body composition and total energy intake were identical but macronutrient distribution was clearly different.

## 8. A highly controlled metabolic ward feeding trial supporting the mass balance approach

When it comes to proving causality in this matter, metabolic ward studies are considered the gold standard. Unfortunately, the cost of implementing such trials makes it unlikely that any new ones will be conducted in the near future[19]. As of now, the best controlled experiment performed in a metabolic ward is that of Hall *et al.*[11]. The results of the study are in full agreement with the mass

balance approach, and two separate articles by Arencibia-Albite *et al.*[7] and Manninen[12], respectively, have been published on the subject; hence, it will not be discussed further in this present article.

## 9. Recent meta-analyses of randomized and controlled free-living feeding trials supporting the mass balance approach

Feeding trials performed on free-living subjects are always a mixture of effectiveness and compliance. When subjects are randomized to a certain diet, many are often not very committed to following the prescribed diet. Usually, the macronutrient distribution of the compared diets begins to converge at the latest after 1 year. Nevertheless, a couple of recent meta-analyses on the topic will be reviewed in this paper.

In a meta-analysis by Choi *et al.* eight feeding trials reporting changes in weight-related parameters were included in the study[20]. The results indicated that very-low-carbohydrate/high-fat diets were significantly more effective in reducing body mass than higher carbohydrate control diets; these results are in accordance with the mass balance approach. As aforementioned, when the energy fraction from dietary fat increases, while energy intake is fixed, mass intake decreases due to the significantly higher energy density of fat compared with other energy substrates.

A recent meta-analysis by Zaki *et al.* has compared the effectiveness between very-low-carbohydrate diets and low-carbohydrate diets[21]. In this comparison, the difference in nutrient mass intake was not as significant as in the aforementioned meta-analysis. As can be assumed based on the mass balance approach, those who were on very-low-carbohydrate diets lost slightly more body mass than those on low-carbohydrate diets. There were some variations in the results, which must be attributed to poor compliance.

## 10. An "old" well-controlled feeding experiment supporting the mass balance approach

In the early seventies, Young *et al.* compared three diets that contained the same amount of calories (1,800 kcal/day) and protein (115 g/day) but different carbohydrate contents[22]. After 9 weeks on the 30-g, 60-g, and 104-g carbohydrate diet, the weight loss was 16.2 kg, 12.8 kg, and 11.9 kg, respectively, and fat accounted for 95%, 84%, and 75% of the weight loss. The authors thus concluded, "Weight loss, fat loss, and percent of weight loss as fat appeared to be inversely related to the level of carbohydrate in the isocaloric, isoprotein diets." This is consistent with the mass balance approach. Since these diligent researchers have followed the EBT paradigm, "no adequate explanation can be given for weight loss differences." Young *et al.* study has not received any substantial criticism. It is worth noting that they utilized underwater weighing (*i.e.*, hydrodensitometry) to determine body composition. This method is more accurate than other widely available methods of body composition testing. When performed properly, underwater weighing may be accurate up to 1.8%–2.8% in comparison with the state-of-the-art methods (*e.g.*, magnetic resonance imaging [MRI] and computed tomography [CT]).

## 11. Epidemiological data supporting the mass balance approach

As recently pointed out by Mozaffarian[23], the data from the National Health and Nutrition Examination Survey (NHANES) have shown no increase in energy consumption or availability over ≥ 20 years, a time period during which obesity has steadily increased (see Figure 1 in Mozaffarian[23]). In fact, the data from NHANES have suggested a small but statistically significant decline in energy intake over this period[23]. What about the other side of the coin, *i.e.*, energy expenditure? Although there are no similar epidemiological data available on the matter, high-quality studies utilizing the doubly labeled water (DLW) method have indicated that the total energy expenditure (TEE) of Hadza hunter-gatherers was similar to Westerners and other populations in market economies despite their high physical activity level[24]. Therefore, it is clear that the main factor causing the obesity epidemic is increased food intake rather than declined expenditure.

If there has been no change in energy intake and energy consumption, what on earth is causing the obesity epidemic? If we follow the EBT paradigm, this seems paradoxical; however, from the perspective of the mass balance approach, there is nothing surprising about it. According to nutritional recommendations, people should increase their intake of carbohydrates at the expense of fat. If such recommendations are followed, the intake of nutrient mass will increase, while the calorie intake remains the same. The data from NHANES have indicated that the percentage of calories from carbohydrates increased between 1971–1974 and 1999–2000 from 42.4% to 49.0% for men and from 45.4% to 51.6% for women, whereas the percentage of calories from fats decreased from 36.9% to 32.8% for men and from 36.1% to 32.8% for women[25]. Although self-reported dietary intake is subjected to recall bias, there is every reason to assume that strongly marketed nutritional recommendations have produced results in line with the goals at the population level.

In the Women's Health Initiative Observational Study (WHI/OS), the relationship between weight gain and four common diet patterns (a LFD, a reduced-carbohydrate diet, a Mediterranean-style diet, and a diet consistent with the United States Department of Agriculture's [USDA] Dietary Guidelines for Americans [DGA]) was examined[26]. The WHI/OS was a longitudinal study of postmenopausal women aged 49–81 years ($n$ = 93,676) who were enrolled between 1994 and 1998 and followed for up to 8 years. The conclusion made by the researchers states all that is needed: "Our findings therefore challenge prevailing dietary recommendations, suggesting instead that a low-fat (diet) may promote rather than prevent weight gain after menopause." The results were the opposite of what could have been assumed based on the EBT.

## 12. Extremely obese individuals have very high TEE

Based on the EBT, it has been suggested that low TEE is a risk factor for obesity[27], but the evidence does not support this assumption. The results from the investigation of Das *et al.* on the hypothesis stating that both TEE and resting energy expenditure (REE) are low in extremely obese individuals showed the opposite, wherein the TEE of extremely obese subjects was very high[28]. If the EBT were a valid paradigm, a high TEE should protect against obesity. Similarly, Rimbach *et al.* have concluded, "TEE is not a risk factor for, and high TEE is not protective against, weight or body fat gain over the time intervals tested"[29]. These findings cause more gray hairs for the proponents of the EBT.

## 13. The timing of nutrient mass ingestion cannot modify the Law of Conservation of Mass

The timing of nutrient mass ingestion will never be able to modify the Law of Conservation of Mass. The timing of nutrient mass ingestion may have an effect on body mass and fat mass only if it affects mass expenditure. A well-controlled feeding trial by Ruddick-Collins *et al.* demonstrates this fact in an excellent manner[30]. They performed a 4-week crossover isocaloric and eucaloric feeding trial, comparing "morning loaded" (45%:35%:20% calories at breakfast: lunch:dinner) versus "evening loaded" (20%:35%:45% calories at breakfast: lunch:dinner) calorie intake. This was a free-living study, but all food and beverages were provided, making it "the most rigorously controlled study to assess timing of eating in humans to date"[30]. As can be assumed based on the mass balance approach, the results indicated no differences in body mass loss, total daily energy expenditure, and resting metabolic rate in relation to the timing of calorie distribution.

## 14. The results of dietary treatments in the pre-insulin era are in line with the mass balance approach

Some critics of the EBT have rightly pointed out that the results of dietary treatments in the pre-insulin era[31] are not in agreement with this century-old paradigm. In those days, type I diabetics were treated with a diet that was very low in carbohydrate and protein, *that is*, a diet containing mostly fat[31]. The rationality of this was to minimize the excretion of glucose mass in the urine (glucosuria) so that the patient would not starve to death. Protein intake was also kept at low levels since gluconeogenic amino acids can raise blood sugar levels.

The observations of the pre-insulin era in relation to macronutrient distribution and body mass have been simulated. Figure 3, which is adapted from Arencibia-Albite's and Manninen's study[7], shows the results of these simulations.

## 15. A quick look at a recent article that still tries to defend the EBT

In their recent paper, Hall *et al.*[32] attempted to defend the EBT. They stated, "If we are particularly interested in the storage of energy as body fat, then the energy balance equation $E_{in} - E_{out} = E_{storage}$ can be specified as $E_{in} - E_{out} = E_{fat} + E_{protein} + E_{carbohydrate}$," where E = energy.

With all due respect, these authors do not understand that energy balance and mass balance are separate balances, as has already been discussed. The body stores fat as mass, not as energy, because energy has no mass at all. Moreover, changes in both total body mass and fat mass are due to changes in mass balance.

For the above equation to be valid, the mean absorbed mass of each macronutrient must be equal to its respective mean oxidized mass, which is simply not possible (refer to 5. Energy balance cannot occur at body mass stability).

## 16. Diet writers' favorite hormones

### 16.1. Leptin

Leptin, a peptide hormone that is predominantly made by adipose cells, helps to regulate energy balance by inhibiting hunger[33]. According to the EBT, the macronutrient distribution of diet should only have a minimal effect on leptin levels since it has a minimal effect on body mass and fat mass. Hormonal responses have been determined in the aforementioned feeding experiment by Kong *et al.* and their data (Table 1 in Kong *et al.*[10]) have shown the complete opposite of what could be assumed based on the EBT, indicating that switching from a "normal diet"

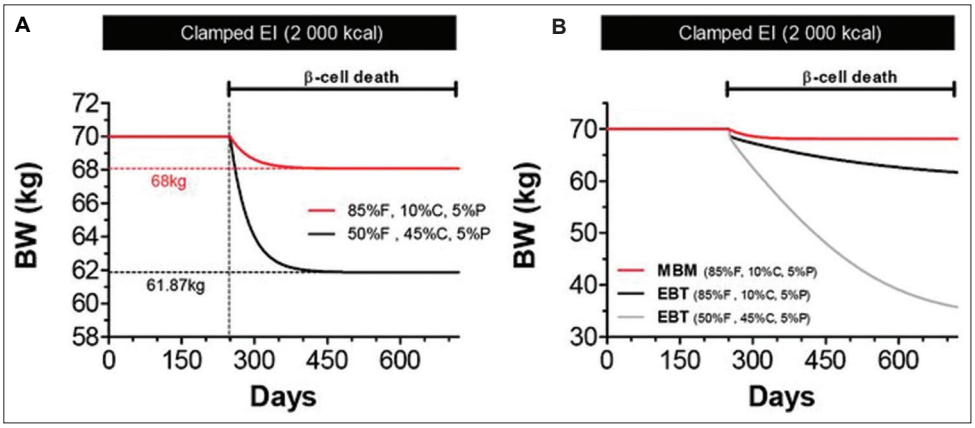


**Figure 3.** Dietary management of type 1 diabetes: MBM versus EBT simulation. (A) MBM predicts that the reduction in body mass caused by beta-cell death (*i.e.*, onset of diabetes) is highly dependent on the macronutrient distribution of diet, a result that is consistent with the results of dietary treatments in the pre-insulin era[51]. From the source[3], we can see how the model parameters have been modified in these diabetes simulations (*i.e.*, $xC = 0$). (B) The EBT-based model by Hall *et al.*[22] predicts that macronutrient distribution has a significantly lesser effect on body mass reduction.
C: Carbohydrate; BW: Body weight; EBT: Energy balance theory; F: Fat; MBM: Mass balance model; P: Protein.

(carbohydrate 44.0 ± 7.6%, protein 15.4 ± 3.3%, and fat 39.6 ± 5.8%) to a very-low-carbohydrate/high-fat diet (carbohydrate 9.2 ± 4.8%, protein 21.9 ± 3.4%, and fat 69.0 ± 5.4%) can significantly reduce leptin levels. This is consistent with the mass balance approach. The lower the body fat level, the lower the leptin level.

### 16.2. Insulin (with special reference to the carbohydrate-insulin model)

Insulin, a peptide hormone that is produced by pancreatic beta cells, is considered the main anabolic hormone of the body. This is probably the reason why this hormone has become a favorite of diet writers. The carbohydrate-insulin model (CIM), an obesity-related model, proposes a reversal of causal direction[14]. According to proponents of the CIM, "increasing fat deposition in the body – resulting from the hormonal responses to a high-glycemic-load diet – drives positive energy balance"[14].

It is worth emphasizing that the CIM operates within the EBT; that is, it assumes that a positive energy balance is the cause of obesity. It is, therefore, not a competing paradigm. The direction of causality in this matter does not affect the laws of physics in any way. The physical basis of both the EBT and the CIM operating within it is flawed, as has already been demonstrated.

One of the central claims of the CIM is that high insulin levels promote weight gain, but as already mentioned, insulin cannot create mass from nothing. Although insulin levels decrease with low-carbohydrate diets, it is not a causal factor in body mass and fat mass loss. It just happens simultaneously with decreased nutrient mass intake. Proponents of the CIM suggest that high insulin levels slow down metabolism, thus promoting weight gain[14]; it is worth mentioning that the universal assumption of metabolism being synonymous with energy expenditure is misleading, as metabolism is actually mass expenditure. Ludwig *et al.* have also claimed that their meta-analysis[34] supports this assumption, but the conclusions do not stand up to critical scrutiny[35].

Although, at a cursory glance, it might appear that the CIM is in agreement with the insulin response data, in reality, it is a free passenger traveling on the wing of mass change. The decrease in insulin level and the intake of nutrient mass occurs simultaneously, but only mass can be a causal factor in mass change. In the MBM, it is assumed that the changes in body mass and composition are independent of the physiological effects of a diet. Rather, the differences are due to different amounts of nutrient mass intake. MBM-based simulations provide very convincing evidence for this assumption[1,3,7,8].

As already discussed, however, insulin levels are considered important in terms of where body fat is reduced. In addition, there are other reasons for maintaining low insulin levels. For instance, individuals with hyperinsulinemia are at higher risk of developing obesity, type 2 diabetes, cardiovascular disease, cancer, and premature mortality[36]. It may seem paradoxical at first glance that the statement "insulin cannot create mass" is made, and then an article[36] that lists hyperinsulinemia as a risk factor for obesity is referred to. To clarify the seemingly contradictory matter, it is important to understand that hyperinsulinemia is often caused by the regular consumption of sugar and other refined carbohydrates, while the individual also gains weight. In this situation, insulin does not cause obesity; rather, obesity is caused by a chronic positive mass balance. Here, it is referring to

the etiology, that is, the root cause. According to *Oxford Learner's Dictionaries*, "etiology" refers to "the cause of a disease or medical condition." Although obesity has only one etiology, that is, chronic positive mass balance, it has numerous risk factors, each of which increases, with a certain probability, the occurrence of a positive mass balance; however, none of these risk factors are capable of creating any kind of mass. It is still a matter of balance between intake and expenditure, but in this case, mass balance.

## 17. Dietary ketosis *per se* does not affect body mass or fat mass

When the rate of mobilization of fatty acids from adipose tissue is accelerated, such as during a very-low-carbohydrate/high-fat ketogenic diet, the liver produces ketone bodies: acetoacetate, beta-hydroxybutyrate, and acetone (a spontaneous breakdown product of acetoacetate). Since the liver cannot utilize ketone bodies, they flow from the liver to extrahepatic tissues (*e.g.*, brain and muscle) for use as fuel.

Although it has been suggested that dietary ketosis could accelerate the reduction of body mass and fat mass, dietary ketosis *per* se does not affect body mass or fat mass if we disregard the scant urine ketone excretion in the initial phase. Mass does not mysteriously disappear. Looking at equation (13) in relation to MBM in Arencibia-Albite[3], it is not necessary to include an extra term for the energy expenditure fraction derived from ketone bodies, as the oxidation of ketone bodies is essentially fat oxidation. For example, the β-oxidation of one palmitic acid molecule yields eight acetyl-coenzyme A molecules, in which two of these react to generate four acetoacetate molecules. Hence, the oxidation of four acetoacetate molecules is equivalent to that of one palmitic acid molecule. For further details, refer to *2.2.1. At energy balance the oxidized macronutrient distribution that results in the* $EE_{avg}$ *is equal to macronutrient distribution in the* $EIa_{vg}$ in Arencibia-Albite[3].

It is impossible to study the matter precisely in a feeding trial setting, as there would be a small difference in the nutrient mass intake between the comparison groups, that is, a very-low-carbohydrate diet group versus an isocaloric low-carbohydrate diet group. More specifically, the nutrient mass intake of the first group is inevitably slightly lower than that of the latter. Remember that when the energy fraction from dietary fat increases, while energy intake is fixed, nutrient mass intake decreases due to the significantly higher energy density of fat compared with other energy substrates. Vidić *et al*. have also investigated the matter and came to a conclusion that a ketogenic diet with sustained hyperketonemia above 1 mol/L has pretty much the same impact on body composition as a low carbohydrate non-ketogenic diet[37].

Dietary ketosis can reduce the feeling of hunger[38], and thereby reduce the intake of nutrient mass; however, this does not undermine the Law of Conservation of Mass.

## 18. Nutrition facts label

The nutrition facts label on packaged food was updated in 2016 to reflect updated scientific information, including information about the association between diet and chronic diseases, such as obesity and heart disease[39]. One of the most prominent updates of the new food labeling regulations released by the Food and Drug Administration (FDA) is found on the calorie line; the font for calories has been significantly enlarged and emboldened for first-glance reference. This well-intentioned update was based on the rationale that caloric values may be easily comprehended without having to examine the food label in detail. Humans need energy (*i.e.*, the capacity to do work), but calories have no impact on body mass; thus, the calorie line should be replaced or complemented with the mass line (*e.g.*, "Nutrient Mass" or "Mass").

It is also worth noting that the concept of "light product" is misleading. In reality, these products are often "heavy products." When the energy fraction from dietary fat increases, while the energy content remains the same, mass intake decreases due to the significantly higher energy density of fat compared with other energy substrates. A high-carbohydrate "light product" containing 200 kcal provides more mass than a high-fat product containing 200 kcal. This fact should have a significant impact on the prevailing legislation and the operation of the food industry.

In short, dietary fats are therefore not problematic in relation to weight management, as they provide a lot of energy relative to their mass. Dietary fats are only a problem when they are used to make high-carbohydrate food highly palatable. The "light product" concept should be removed from legislation as soon as possible because it misleads consumers in many situations.

## 19. A flawed paradigm leads to misinterpretation of research data

In research literature, there is plenty of feeding trial reports that seem to support the EBT. A flawed paradigm, however, almost always leads to incorrect interpretations and conclusions. There are many research reports claiming that the more effective weight loss effect of low-carbohydrate diets compared to isocaloric high-carbohydrate diets is attributed to a methodological error (*e.g.*, under-reporting of food consumption and low sensitivity of research

equipment). The assumption is that such results would violate the Law of Conservation of Energy. As has been discussed before, this is not the case (see 2. The energy balance theory is a flawed paradigm). In studies where low-carbohydrate diets have not been more effective in terms of fat loss, EBT-based calculation formulas have been used, which yielded incorrect results (*e.g.*, Hall *et al.*[11] and a reanalysis in Manninen[12]). Therefore, it is important to consider these points when reading such reports.

The real "acid test" of every theory is how good results can be obtained in practice based on the theory. How effective have EBT-based obesity treatment interventions been? As of 2021, 19 US states and territories have at least 35% of their residents living with obesity, which is more than double the number in 2018[40]. Therefore, it is clearly impossible to even talk about effective interventions. Anyone would think that this upsurge would finally raise concerns. However, in my opinion, wasting time and money on research conducted within the EBT paradigm should be stopped immediately.

## 20. Other applications of the mass balance approach

Although this review focuses on the macronutrient distribution of diet, the mass balance approach has many other practical applications. For example, anti-obesity drugs or dietary supplements that affect satiety should reduce the consumption of carbohydrates instead of fats. Other applications will be covered in the updates to this article.

## 21. The mass balance approach has nothing to do with the metabolic advantage hypothesis

Contrary to what some have thought, the mass balance approach has nothing to do with the metabolic advantage hypothesis[41]. Proponents of this hypothesis postulate that low-carbohydrate diets lead to faster body mass and fat mass loss than isocaloric high-carbohydrate diets because "energetic inefficiency, substrate cycling, and demands of gluconeogenesis support observed advantages for weight [and fat] loss"[42]. Unlike energy, mass has no efficiency. The amount of mass that is taken in always comes out in exactly the same amount. It is worth noting that the metabolic advantage hypothesis operates within the EBT.

With all due respect, it would seem that the proponents of the metabolic advantage hypothesis have not taken notice that efficiency is not related to incomplete substrate oxidation but to how the body uses the available fuel resources. Consider the following example: a man starts to run 5 km every day and soon discovers that he can complete the same distance within the same amount of time (*e.g.*, 0.5 h) but with a lower heart rate. His body has adapted to this cardiovascular stress physiologically, biochemically, and even anatomically in the long run, thus becoming a more efficient fuel utilizer; his body is able to achieve the same running average speed (5 km/0.5 h = 10 km/h), while utilizing less fuel. However, every time his body oxidizes 1 g of glucose while running, the heat released by his body continues to be ~4 kcals, which will not change as he ages or as a function of his genome, epigenome, or proteome.

## 22. The Coca-Cola Company and their insidious marketing arms

The Global Energy Balance Network (GEBN) was a US-based marketing arm of The Coca-Cola Company (hereinafter referred to as Coke), claiming to fund research on the causes of obesity[43]. GEBN's core message was that the primary cause of obesity is physical inactivity, not a poor diet[44-46]. In plain language, their message was that people can continue to enjoy high-carbohydrate junk food, including Coke's drinks, as long as they exercise enough to remain in "energy balance."

With around 1.4 billion inhabitants, China is the world's most populous country, so Coke has naturally taken interest in China's market. In Greenhalgh's article, she covered this topic[46], and her findings were as follows:

(i) Coke's main vehicle for influencing obesity science in China was the International Life Sciences Institute (ILSI), a Coke-sponsored marketing organization that masqueraded as a scientific organization and whose founding president Alex Malaspina was concurrently the vice president of Coke; a few years ago, the ILSI and Coke were practically synonymous;
(ii) ILSI's website emphatically spotlights their long commitment to scientific integrity, but as pointed by Greenhalgh, "[a] close examination of how the organization [ILSI] works in practice reveals a different picture"[46];
(iii) Coke, working through ILSI-Global and ILSI-China, succeeded in redirecting China's obesity science and policy to emphasize physical activity over dietary modifications.

In addition to GEBN and ILSI, Coke leveraged the authority of countless health organizations to spread this message. In Spain, for example, at least 74 health organizations were sponsored by Coke from 2010 to 2016, with a total investment above 6 million euros[47]. The main message was the same as with GEBN: "The most prevalent strategy was to focus on physical activity and sedentary behaviors as key obesogenic risk factors"[47]. Rey-López

*et al*. article[47] is an excellent discussion of Coke's main marketing strategies.

Coke has also utilized US-based governmental organizations in spreading its propaganda. For example, the Centers for Disease Control and Prevention (CDC) has had longstanding ties to Coke[46]. Barbara Bowman, the previous director of the CDC's Division for Heart Disease and Stroke Prevention, resigned after her emails with a former Coke executive were disclosed[48]. These emails proved that Bowman had advised the former Coke and industry association executive on how to influence the Director-General of the World Health Organization (WHO) to stop promoting taxes on sugar. Perhaps prompted by this incident, the CDC website currently states the following: "Frequently drinking sugar-sweetened beverages is associated with weight gain, obesity, type 2 diabetes, heart disease, kidney diseases, non-alcoholic liver disease, tooth decay and cavities, and gout, a type of arthritis. Limiting sugary drink intake can help individuals maintain a healthy weight and have healthy dietary patterns"[49]. However, with regard to individual cases, there is absolutely no reason to assume systematic abuses on the part of the CDC. In my opinion, the CDC website and other bulletins currently have reliable information on the subject.

Although not everyone would agree, the idea that some authoritative nutrition and obesity journals are mainly marketing channels for Coke and other companies that "live" on sugar may hold merit on the basis of the best available evidence collected in 2020, 2011, and 2022. However, this does not, in any way, mean that all the researchers who have had published in these journals are dishonest. Without truthful articles, these misguided journals would not be able to operate any longer. Nevertheless, there is sufficient evidence that these misguided journals have had a considerable detrimental effect on humanity, and thus require very careful legal scrutiny. When the lives and health of hundreds of millions of people are threatened with misinformation, it is a very serious crime. Since the subject, to some extent, falls outside the scope of this article, there is no need to discuss the matter further; however, more detailed information about the activities of these corrupt journals will be presented in the upcoming article.

That having been said, Noakes' recent work *Real Food on Trial: How the diet dictators tried to destroy a top scientist* is a recommended read[50]. This book presents the large-scale corruption in nutrition-related publications in an excellent way. In addition, Lustig's recent *work Metabolical: The Lure and the Lies of Processed Food, Nutrition, and Modern Medicine*[51] is also worth reading. The nutrition industry is clearly tainted from top to bottom. Many researchers are unaware that they are being misled by large corporations like Coke because of the clever marketing strategies they employ.

In essence, contrary to what sugar industry-sponsored "researchers" claim, "you cannot outrun a bad diet"[52].

## 23. Conclusion

In this paper, a new paradigm that paints a more accurate picture of the evolution of body weight is proposed: Chronic positive mass balance is the actual etiology of obesity, not positive energy balance. This opens up a completely new era in obesity research. By shifting to a mass balance paradigm of obesity, a deeper understanding of this disease may follow in the near future. The immediate result of such a shift is that feeding studies will be more accurate, as mass measurements do not suffer from various uncertainty factors, like energy measurements (*e.g*., DLW)[53].

The paradigm shift must begin. The importance of treating and preventing obesity must take precedence over the honor of researchers. At times, fundamental knowledge structures may turn out to be incorrect, and thus must be abandoned. "That is what fundamental novelties of fact and theory do. Produced inadvertently by a game played under one set of rules, their assimilation requires the elaboration of another set," as Kuhn stated in his classic work *The Structure of Scientific Revolutions*[54].

The rational mind's ability for biases, exaggeration, and cover up is the crux[55]. Rationality *per se* does not cause problems, but the rational mind is attracted to the greatest of sins, namely the tendency to think of the things one knows as absolute, "ultimate truths." Researchers celebrate their own theories, claiming that better ones, or ones outside of theirs, do not even have to exist. They begin from the basic assumption that all the most fundamental facts have already been proven beyond doubt, that is, nothing important has been left unexplored. This is sometimes the case, but from time to time, even "ultimate truths" change.

A paradigm shift is an intellectual revolution, accompanied by the chaos and fear inherent in revolution. Sacrifice is always required to correct a mistake, and if the mistake is significant, so must the sacrifice. When the new truth is denied over a long period of time, a significant amount of sacrificial debt may have already accrued. Some leading researchers in the field seem to have already accrued enough debt that they treat the mass balance approach like a plague. They mirror those "unfit to become devotees of knowledge" who refused to be any part of the "new truths" that contradict prevalent opinions, as portrayed in Nietzsche's statement in his classic work *The Gay Science*.

Nietzsche described above the phenomenon that later came to be called cognitive dissonance. The theory of cognitive dissonance proposes that people are averse to inconsistencies within their own minds[56]. More specifically, cognitive dissonance refers to the perception of contradictory information. If a person has been teaching things related to energy balance for decades, the magnitude of dissociation can be at a massive level. Despite this, it is worth considering the fact that if the etiology of obesity is misunderstood, it will have a detrimental effect on practically any type of obesity and weight management research.

Occam's razor (*novacula Occami*) also supports the mass balance approach. This principle states that entities should not be multiplied unnecessarily. By definition, all assumptions introduce possibilities for error. If an assumption does not improve the accuracy of a theory or model, its only effect is to increase the probability that the overall theory or model is wrong. What does a person measure when he/she stands on a bathroom scale? Is it his/her body mass or energy? It is obviously the amount of mass in his/her body. Why then do people continue to study body mass changes in the context of energy changes? Such a perspective only offers a rough second-hand estimate at best. The right starting point is to measure mass directly. As aforementioned, energy balance and mass balance are separate balances in the human body.

The key publications in the reference list and other references that can be found in them may be helpful for further information about the subject. A recent paper by Arencibia-Albite[1] is a recommended read, which should, at least in my humble opinion, provide new insights into this subject.

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Not applicable.

## Availability of data

All data generated or analyzed during this study can be found in the sources cited in this article.

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ORIGINAL RESEARCH ARTICLE

# Leukocyte telomere length and mitochondrial DNA copy number association with colorectal cancer risk in an aging population

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## Abstract

In this study, we evaluated the association of blood leukocyte telomere length (LTL) and mitochondrial DNA copy number (mtDNA-CN) with the risk of incident colorectal cancer (CRC). We studied and followed-up a cohort of Russian men and women (aged 45 – 69 years, $n$ = 9360, 54% female) from the HAPIEE study for 15 years. Using the nested case-control design, we selected cases with incident CRC among those free from any baseline cancer ($n$ = 146) and sex- and age-stratified controls among those free from baseline cancer and cardiovascular disease and alive at the end of the follow-up ($n$ = 799). We employed multivariable-adjusted logistic regression to estimate the odds ratios (ORs) of CRC per 1 decile of LTL or mtDNA-CN. We observed an inverse association of LTL and mtDNA-CN baseline values with the 15-year risk of incident CRC. Carriers of shorter telomeres had an increased 15-year risk of incident CRC with adjusted OR 3.2 (95% CI: 2.56 – 3.87, $P < 0.001$) per 1 decile decrease in LTL, independent of baseline age, sex, smoking, body mass index, blood pressure, lipid levels, and education. Similarly, lower mtDNA-CN was associated with the higher risk of incident CRC with adjusted OR 1.7 (95% CI: 1.12 – 1.89, $P < 0.001$) per 1 decile decrease in mtDNA-CN, independent of the aforementioned factors. Using the modified values of LTL and mtDNA-CN adjusted for multiple factors and their interactions with a case–control status, the ORs of CRC were 2.53 and 1.52 per 1 decile decrease in adjusted baseline LTL and mtDNA-CN, respectively. In conclusion, LTL and mtDNA-CN were independent inverse predictors of the 15-year risk of CRC in the Russian cohort. These findings highlight the relevance for subsequent research to exploit the mechanisms through which LTL and mtDNA-CN may reflect human health.

***Keywords:*** Leukocyte telomere length; Mitochondrial DNA copy number; Aging; Cancer; Case–control; Population

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## 1. Background

The world population is expected to reach 8.6 billion people by 2030, with about 1.4 billion being over 60 years old[1]. An increase in life expectancy goes hand-in-hand with the aging of population and the aggregation of age-related diseases.

Cancer primarily represents an age-related group of pathologies and is among the top 20 causes of death[2]. Colorectal cancer (CRC) is the third most common type of cancer in men and the second in women worldwide, with a mortality rate of 11.0 and 7.2/100,000, respectively[3].

Environmental factors contribute significantly to the risk of CRC development; however, there are also non-modifiable risk factors, such as older age, family history of cancer[4], and molecular genetic contributors[5].

Considering the multifaceted process of aging accompanied by health decline, the molecular markers of "biological age" may reflect the signals of the pace of aging. Among the potential markers of biological age, telomere length and mitochondrial DNA copy number (mtDNA-CN) have been extensively studied. Telomeres are nucleoprotein complexes that are located at chromosome ends and support chromosomal stability by protecting against DNA degradation[6]. Shortened telomeres eventually lead to cellular senescence, and telomere length is regarded as a likely biomarker of a history of replication and cumulative oxidative stress[6]. When telomeres become critically short, the life cycle of cells stops[7]. A shorter leukocyte telomere length (LTL) has been found to be associated with age[8,9], male sex[10], age-related risk factors for cardiovascular disease (CVD) and non-communicable disease[11,12], and all-cause mortality[9,13-15]. These associations are independent of chronological age, which points at an extra value of telomere length as a sign of cellular or biological aging.

In multiple cellular processes such as lipid metabolism, apoptosis, and cell differentiation, mitochondria are engaged in energy production (oxidative phosphorylation) and also participate in the generation of reactive oxygen species (ROS), which is the crux of the free-radical theory of aging[16]. According to experimental results, ROS could act as "mediators" of the stress response to the damage induced by aging[17]. MtDNA-CN is a marker of mitochondrial replication and cellular energy reserve[18]. Although mtDNA-CN is an indirect indicator of mtDNA damage, it is associated with mitochondrial enzyme activity and adenosine triphosphate production[19].

The evaluation of the possible association between LTL and the risk of cancer[14,20-23] and that between alternations in mtDNA-CN and cancer[24-27] has shown inconsistent findings.

We aimed to study the association of LTL and mtDNA-CN with the risk of incident CRC during a 15-year follow-up period.

## 2. Methods

### 2.1. Study participants

A random population sample of men and women aged 45 – 69 years old was examined at baseline in 2003/05 ($n$ = 9360, mainly Caucasoid) and re-examined in 2006/08 and 2015/18 in Novosibirsk (Russia) in the frame of the HAPIEE study (Health, Alcohol and Psychosocial Factors in Eastern Europe; http://www.ucl.ac.uk/easteurope/hapiee-cohort.htm). The baseline cohort was followed-up for cancer, CVD, and all-cause mortality until December 31, 2019, for an average of 15.6 years (standard deviation, SD 0.70; median 15.6; range 14.5 – 17.0), calculated across those alive until censoring date.

In the present analysis, we focused on the most common cancer: CRC. Data on CRC (ICD-10: C18 – C20) in the cohort were collected, including fatal and non-fatal events. For cancer events ascertainment, we used the Cancer Register of Novosibirsk city. In addition, the following sources were used to collect information on all-cause and cause-specific mortality: The Bureau of Population Registration (ZAGS), the State Statistical Bureau of the Novosibirsk Region, and the data obtained from additional sources at two re-examinations of the cohort (including the address bureau and proxy-information on deceased respondents of the study).

### 2.2. Ethical approval and consent

The study was conducted in accordance with the guidelines of the Declaration of Helsinki. The ethical approval for the study was from the Ethics Committee of IIPM – Branch of IC&G SB RAS (Institute of Internal and Preventive Medicine – Branch of Federal State Budgeted Research Institution, "Federal Research Center, Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences") (protocol #1, March 14, 2002, and protocol #12, December 8, 2020). All the study participants signed an informed consent.

### 2.3. Sample selection

Among 9360 people, 160 events of CRC were ascertained during a 15-year follow-up period, including repeated events in several participants. The present study was designed as a nested case-control. We selected incident CRC cases among those free from baseline cancer of any site and with available DNA samples ($n$ = 154) for analysis. The universal control group for the present study was formed based on certain criteria; we excluded those with baseline cancer or CVD, or those who died within the follow-up period. These exclusion criteria were applied to generate universal controls suitable for several outcomes.

We randomly selected controls whose age and sex frequency matched the CRC cases ($n$ = 806). The average follow-up of the studied sample was 15.9 years (SD = 0.74; median = 15.9; range 14.5 – 17.0). After excluding samples with inadequate DNA quality or inappropriate genotyping of LTL and mtDNA-CN, our dataset consisted of 146 CRC cases and 799/785 controls for LTL/mtDNA-CN. The characteristics of the groups are shown in Table 1.

### 2.4. Data collection

In the frame of the HAPIEE study, baseline data were collected using standardized structured interview, physical examination, and the collection of blood and DNA samples. Trained and certified stuff conducted the interview and assessed the participants' medical history in relation to hypertension, diabetes mellitus, CVD, and other chronic diseases; health characteristics; as well as behavioral and socio-economic factors. Briefly, the physical examination included measurements of blood pressure (BP), anthropometry, physical performance, and other optional ones. The protocol details were previously reported[28].

We classified smoking status into three categories (current smoker, former smoker, and never smoked); education level into four categories (primary or less than primary, vocational, middle, and high); and marital status into two categories (married/cohabiting and single/divorced or widow/widower).

We measured BP in sitting position after a 5-min rest over the right arm three times (Omron M-5, OMRON Healthcare) about 2 min apart. For current analysis, we used the average of three BP readings. Height and weight were measured to the nearest 1 mm and 100 g, respectively; body mass index (BMI) was defined as weight/squared height, kg/m$^2$.

Venous blood samples were taken from the cubital vein using the Vacuette blood collection system after an overnight fast (minimum 8 h). After centrifugation (3,000 rate/min, 20 min), the blood cells were separated and the aliquots of serum, plasma, and blood cells were stored in a deep freezer (–80°C). The levels of lipids and glucose were measured within a month from the time blood was drawn. Serum total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), and glucose levels were measured on Konelab 300i Autoanalyzer (Thermo Fisher Scientific Inc., USA) with an enzymatic approach using kits from Thermo Fisher Scientific. Friedewald formula was applied to obtain the low-density lipoprotein cholesterol (LDL-C) values.

DNA samples were isolated from blood cells by phenol-chloroform extraction[29] within 1 month from the time

**Table 1. Distribution of basic parameters of incident CRC cases and controls (HAPIEE study, Russian cohort, men and women, baseline survey 2003 – 2005, 45 – 69 years old)**

| Covariates | Cases (incident CRC) | Controls | *P* |
|---|---|---|---|
| | Mean (SD)/n(%) | | |
| Observed, *n* | 146 | 799 | |
| Baseline age, years (mean, SD) | 60.8 (6.69) | 55.7 (6.51) | <0.001 |
| Women, *n* (%) | 70 (47.9) | 476 (59.6) | 0.009 |
| Systolic blood pressure, mmHg (mean, SD) | 141.8 (20.86) | 137.6 (22.58) | 0.035 |
| Diastolic blood pressure, mmHg (mean, SD) | 89.1 (10.94) | 88.2 (12.86) | 0.422 |
| Heart rate, per min | 70.5 (11.27) | 70.1 (10.37) | 0.680 |
| Body mass index, kg/sqm (mean, SD) | 28.7 (5.12) | 27.9 (4.84) | 0.101 |
| Waist/hip ratio, unit (mean, SD) | 0.91 (0.09) | 0.88 (0.08) | <0.001 |
| Total cholesterol mmol/L (mean, SD) | 6.38 (1.23) | 6.31 (1.21) | 0.512 |
| LDL cholesterol, mmol/L (mean, SD) | 4.13 (1.07) | 4.08 (1.08) | 0.642 |
| HDL cholesterol, mmol/L (mean, SD) | 1.534 (0.40) | 1.55 (0.34) | 0.565 |
| TG, mmol/L (mean, SD) | 1.57 (0.84) | 1.47 (0.74) | 0.149 |
| Glucose, plasma, mmol/L (mean, SD) | 6.03 (1.17) | 5.79 (1.07) | 0.017 |
| Hypertension (HT), *n* (%) | 103 (70.5) | 436 (54.6) | <0.001 |
| HT treatment (among HT), *n* (%) | 56 (54.4) | 206 (47.2) | 0.193 |
| Type 2 diabetes mellitus (T2DM), *n* (%) | 21 (14.4) | 46 (5.8) | <0.001 |
| T2DM treatment (among T2DM), *n* (%) | 4 (19.0) | 10 (21.7) | 0.802 |
| Menopause (women), *n* (%) | 65 (92.9) | 363 (76.3) | 0.005 |
| Smoking, *n* (%) | | | 0.600 |
| Present smoker | 30 (20.5) | 184 (23.0) | |
| Former smoker | 25 (17.1) | 114 (14.3) | |
| Never smoked | 91 (62.3) | 501 (62.7) | |
| Frequency of drinking, *n* (%) | | | 0.664 |
| 5+/week | 2 (1.4) | 16 (2.0) | |
| 1 – 4/week | 39 (26.7) | 185 (23.2) | |
| 1 – 3/month | 32 (21.9) | 216 (27.0) | |
| <1/month | 58 (39.7) | 310 (38.8) | |
| Non-drinker | 15 (10.3) | 72 (9.0) | |
| Education, *n* (%) | | | 0.006 |
| Primary | 11 (7.5) | 20 (2.5) | |
| Vocational | 43 (28.8) | 192 (24.0) | |
| Middle | 47 (32.2) | 297 (37.2) | |
| High | 46 (31.5) | 290 (36.3) | |

(*Cont'd...*)

**Table 1.** (*Continued*)

| Covariates | Cases (incident CRC) | Controls | *P* |
|---|---|---|---|
| | Mean (SD)/n(%) | | |
| Marital status, *n* (%) | | | 0.605 |
| Single | 38 (26.0) | 152 (24.0) | |
| Married | 108 (74.0) | 607 (76.0) | |
| LTL, unit | 0.61 (0.31) | 1.39 (0.39) | <0.001 |
| mtDNA-CN, unit | 0.87 (0.29) | 1.30 (0.49) | <0.001 |

*P* value, Fisher's analysis of variance (ANOVA) or Pearson Chi-square test. CRC: Colorectal cancer, CVD: Cardiovascular disease, HDL: High-density lipoprotein, LDL: Low-density lipoprotein, LTL: Leukocyte telomere length, mtDNA-CN: Mitochondrial DNA copy number, SD: Standard deviation, TG: Triglyceride.

blood was drawn. After isolation from blood cells, genomic DNA samples were stored at −70°C.

## 2.5. Measurement of biomarkers

The assessment of LTL and mtDNA-CN was performed by batches within 6 months from the time blood was drawn with quantitative real-time polymerase chain reaction (qPCR) using StepOnePlus™ System (Applied Biosystems, Thermo Fisher Scientific Inc., USA). The details of these measurement techniques are reported elsewhere[30,31].

### *2.5.1. LTL measurement*

LTL analysis was conducted with the qPCR-based method[32,33]. We performed the quantitative reactions for telomeres and β hemoglobin gene in separate pairs of 96-well plates. The plates included a series of DNA dilutions (0.5, 1, 2, 5, 10, 20, and 30 ng); based on these serial data, a standard calibration curve and the computation of each sample were carried out. Each reaction was done with 10 ng of DNA. The composition of the reaction mixture for telomere analysis included 270 nM tel1b primer (5'-CGGTTT(GTTTGG)5GTT-3'), 900 nM tel2b primer (5'-GGCTTG(CCTTAC)5CCT- 3'), 0.2X SYBR Green I, 5 mM dithiothreitol (DTT), 1% dimethyl sulfoxide (DMSO), 0.2 mM of each deoxynucleoside triphosphate (dNTP), 1.5 mM magnesium chloride ($MgCl_2$), and 1.25 U DNA polymerase. The reaction mixture for β hemoglobin gene contained 300 nm Hbg1 primer (5'-GCTTCTGACACAACTGTGTTCACTAGC-3'), Hgb2 primer (5'-CACCAACTTCATCCACGTTCACC-3'), 0.2X SYBR Green I, 5 mM DTT, 1% DMSO, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, and 1.25 U DNA polymerase. The standard amplifier software was used for calculations. The ratios (T: S) based on single-copy gene were calculated to determine the relative LTL value. We rejected the sample if the SD in three replications was above 0.5. Each plate contained a sample with short LTL and three control samples. We checked the relative signal rates from the controls to ensure the comparability of the plates.

### *2.5.2. mtDNA-CN measurement*

The analysis of mtDNA-CN value was performed by using the qPCR technique based on the modified method of Ajaz *et al.*[34]. Beta-2-microglobulin (B2M) served as a single-copy reference gene (refDNA). We set quantitative reactions separately for mtDNA-CN and B2M in duplicate 96-well plates at identical positions. The plates included a series of DNA dilutions (1.25, 6.25, 25, and 100 ng); these serial data were used to construct a calibration curve and quantitatively process each sample. In each reaction, 10 ng of DNA was taken. The reaction mixture for mtDNA-CN analysis contained the following reagents: 270 nM hMitoF3 primer (5'-CTAAATAGCCCACACGTTCCC-3'), 900 nM hMitoR3 primer (5'-AGAGCTCCCGTGAGTGGTTA-3'), 0.2 Ч SYBR Green I, 5 mM DTT, 1% DMSO, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, and 1.25 U DNA polymerase in a final volume of 15 μL of PCR buffer. The reaction mixture for B2M analysis contained the following reagents: 300 nM hB2MF1 primer (5'-GCTGGGTAGCTCTAAACAATGTATTCA-3'), hB2MR1 primer (5'-CCATGTACTAACAAATGTCTAA AATGGT-3'), 0.2 Ч SYBR Green I, 5 mM DTT, 1% DMSO, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, and 1.25 U DNA polymerase in a final volume of 15 μL buffer.

We used the standard amplifier software for computation. The quality control and calculation of mtDNA/refDNA ratio were performed. We excluded the sample from further analysis if the amplification curves of a sample in three replicates had SD > 0.5. A universal control DNA sample was used for all plates; for comparability of plates, we tested the relative rates of the signal from a control. mtDNA-CN was estimated by the parameter of the threshold cycle (Ct; which represents the intersection point of the DNA accumulation schedule and the threshold line). This procedure allows the estimation of the initial mtDNA-CN and comparison between samples.

## 2.6. Statistical analysis

SPSS (v19.0) was used for statistical analysis. The dataset included 146 CRC cases and 799/785 controls (for LTL and mtDNA-CN, respectively).

In the first step, we conducted a descriptive analysis comparing the general characteristics of the studied groups (using ANOVA for continuous variables, and cross-tabulation techniques for categorical variables). Second, logistic regression was used to evaluate the odds

ratios (ORs) of CRC per 1 decile decrease in LTL and mtDNA-CN as continuous variables. Incident CRC case status was the dependent variable. Model 1 was adjusted for baseline age and sex; Model 2 included additional adjustments for smoking, BMI, systolic blood pressure (SBP), and TC; Model 3 was additionally controlled by the level of education.

Third, we conducted several sensitivity analyses. Analysis stratified by sex was repeated based on the same models, and we conducted analyses separately for CRC at different sites (colon and rectal). To eliminate the potential reverse effect of an early cancer stage on the reduction of biomarkers' values, we excluded cancer cases with onset (i) within the first 2 years from the baseline and (ii) within the first 8 years from the baseline (i.e., below the median period value), and repeated two variants of logistic regression analysis using the same covariates and models as above.

The adjusted LTL and mtDNA-CN values were then generated to incorporate the possible non-linear joint influence of the covariates and case–control status. This was done by adding extra interaction terms between case–control and every covariate into the linear regression analyses of LTL or mtDNA-CN (dependent variable); for example, case-control status, baseline age, and the interaction term between case-control and age. This was repeated for every covariate, including baseline age, sex, smoking, BMI, SBP, TC, education, waist-hip ratio (WHR), and glucose level. The adjusted LTL and mtDNA-CN values were generated (*i.e.*, adjusted for baseline age and sex; controlled for age, sex, smoking, BMI, SBP, TC, education; and, additionally adjusted for WHR and glucose level). Subsequently, we applied logistic regression to evaluate the ORs of CRC risk per 1 decile decrease in adjusted LTL and mtDNA-CN as continuous variables.

## 3. Results

### 3.1. Basic phenotype characteristics of studied groups

**Table 1** presents the basic characteristics of the studied case and control.

Participants with incident CRC were somewhat older; they had higher SBP, serum glucose level, and waist/hip ratio but similar BMI; they were also more likely to have hypertension and diabetes mellitus type 2; in addition, their level of education was lower compared with the control group; and women with CRC were more likely to be in menopause.

In a structure of incident CRC, the proportion of colon cancer was 66%, rectal cancer was 32%, and the combination of both sites was 2%. The mean (SD; median) onset age of cancer as registered was 68.5 (8.01; 69.7); the period between the time blood was drawn and the identification of incident CRC was 7.74 years (4.53; 7.92).

The mean (SD; median) of baseline LTL and mtDNA-CN was 1.27 (0.48; 1.26) and 1.23 (0.49; 1.12), respectively. The baseline LTL and mtDNA-CN values were lower among cases compared to controls: 0.61 (0.31) versus 1.39 (0.39), $P < 0.001$ for LTL; and 0.87 (0.29) versus 1.30 (0.49), $P < 0.001$ for mtDNA-CN (Figure 1). Both biomarkers were correlated well between themselves and negatively correlated with baseline age. The correlation coefficient between LTL and baseline age was −0.211, $P < 0.001$; between mtDNA-CN and age was −0.086,

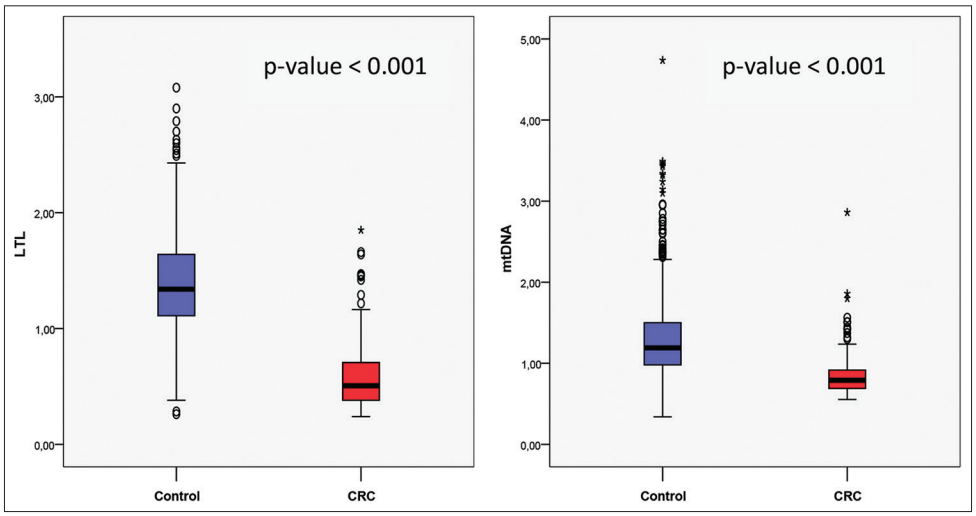


**Figure 1.** Boxplot of leukocyte telomere length (LTL) and mitochondrial DNA copy number (mtDNA-CN) values in cases of colorectal cancer and control groups (cases/controls: $n$ = 146/799 for LTL and $n$ = 146/785 for mtDNA-CN).

$P$ = 0.009; and between LTL and mtDNA-CN was 0.437, $P$ < 0.001. Scatterplots of LTL and mtDNA-CN versus age are presented in Figure 2.

### 3.2. Association between LTL and risk of CRC

Table 2 shows the ORs of incident CRC per 1 decile decrease in baseline LTL. In the model adjusted for age and sex, the OR of CRC per 1 decile decrease in LTL was 3.10 (95% CI: 2.54 – 3.79). The association was independent of smoking, biological covariates, and education; it remained significant in multivariable analyses (fully adjusted Model 3), in which the OR was 3.15 (95% CI: 2.56 – 3.87).

Table 2 (bottom) presents the results separated into men and women. The relationship between LTL and CRC had the same directionality compared to the pooled results; however, the risk of CRC in relation to shorter telomeres was slightly higher in women compared to men. In fully adjusted Model 3, the OR was 3.39 (95% CI: 2.51 – 4.58) in female and 2.96 (95% CI: 2.23 – 3.95) in male.

### 3.3. Association between mtDNA-CN and risk of CRC

Table 3 shows the ORs of incident CRC per 1 decile decrease in baseline mtDNA-CN. In the model adjusted for age and sex, the OR of CRC per 1 decile decrease in mtDNA-CN was 1.68 (95% CI: 1.55 – 1.86). Similarly, in multivariable analyses, the association remained significant, and the OR was 1.71 (95% CI: 1.54 – 1.89) in fully adjusted Model 3 (Table 3).

Table 3 (bottom) presents the results separated into men and women. The relationship between mtDNA-CN and CRC had again the same directionality, with very close ORs in men and women compared to the pooled dataset results.

For sensitivity analyses, we assessed the association of CRC with LTL and mtDNA-CN measures in a cohort excluding early cancer cases that occurred during the first 2 years after baseline examination (Table 4). The results were similar but somewhat weaker, with OR of CRC 3.02 (95% CI: 2.46 – 3.70) per 1 decile decrease in baseline LTL. The relationship between CRC and baseline mtDNA-CN and OR remained practically the same as in the full sample. We also repeated this analysis in a cohort excluding cancer cases that occurred during the first 8 years after baseline examination (below the median of the time between the baseline and onset of CRC). The results were slightly attenuated with OR of CRC 2.90 (95% CI: 2.25 – 3.74) per 1 decile decrease in baseline LTL; for mtDNA-CN, the OR remained close to the results in the full sample (1.74 [95%CI: 1.51 – 2.00]) (Supplementary File, Table S1).

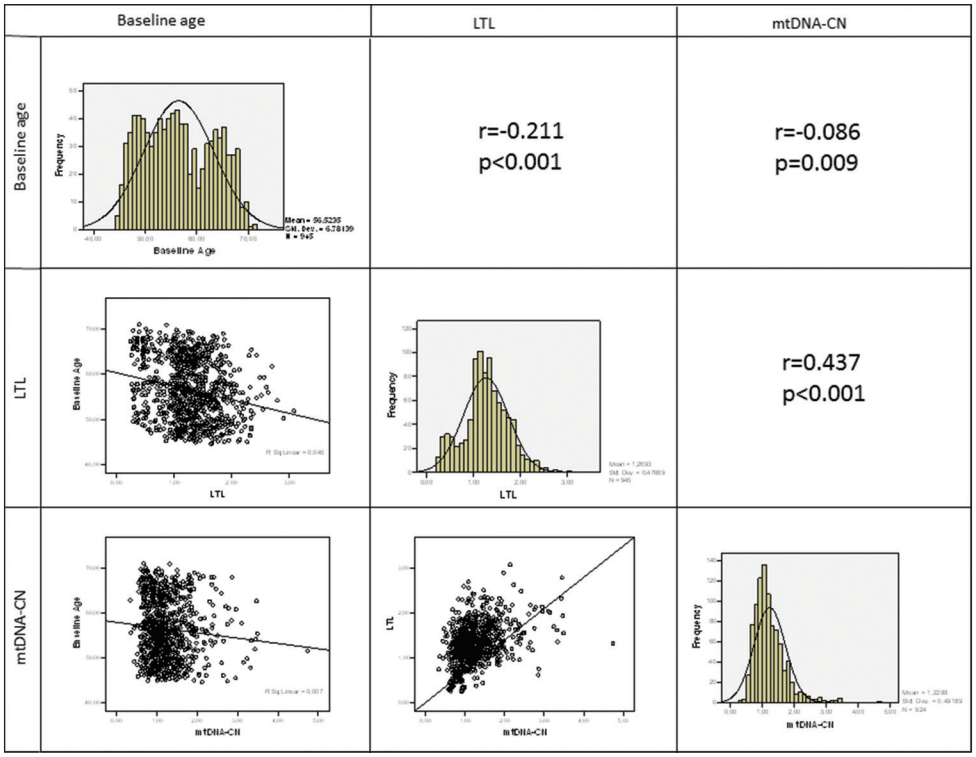


**Figure 2.** A correlation chart for baseline age, leukocyte telomere length, and mitochondrial DNA copy number values, showing distributions of each variable (on the main diagonal); scatterplots with fitted lines for each pair of the variables (below the diagonal); Pearson correlation coefficients (r) and significance level ($P$-value) for each pair of the parameters (above the diagonal).

**Table 2. Relationship between CRC and LTL, per 1 decile decrease in LTL (cases, *n*=146; controls, *n*=799; men and women, 15-year follow-up)**

| Biomarker | Cases/control, *n* | Model 1 | Model 2 | Model 3 |
|---|---|---|---|---|
| | | OR (95% CI), *P* | OR (95% CI), *P* | OR (95% CI), *P* |
| LTL, unit per 1 decile | 146/799 | 3.10 (2.54 – 3.79) | 3.17 (2.58 – 3.89) | 3.15 (2.56 – 3.87) |
| *P* value for trends | | <0.001 | <0.001 | <0.001 |
| Men* | | | | |
| LTL, unit per 1 decile | 76/323 | 2.90 (2.20 – 3.82) | 2.99 (2.25 – 4.00) | 2.96 (2.23 – 3.95) |
| *P* value for trends | | <0.001 | <0.001 | <0.001 |
| Women* | | | | |
| LTL, unit per 1 decile | 70/476 | 3.34 (2.49 – 4.48) | 3.40 (2.51 – 4.59) | 3.39 (2.51 – 4.58) |
| *P* value for trends | | <0.001 | <0.001 | <0.001 |

Model 1: Adjusted for age and sex; Model 2: Adjusted for age, sex, smoking, body mass index (BMI), systolic blood pressure (SBP), and total cholesterol (TC); Model 3: Adjusted for age, sex, smoking, BMI, SBP, TC, and education; *Models 1, 2, and 3 stratified by sex (sex excluded from covariates). LTL: Leukocyte telomere length, OR: Odds ratio, CRC: Colorectal cancer

**Table 3. Relationship between CRC and mtDNA-CN, per 1 decile decrease in mtDNA-CN (cases, *n*=146; controls, *n*=785; men and women, 15-year follow-up)**

| Biomarker | Cases/controls, n | Model 1 | Model 2 | Model 3 |
|---|---|---|---|---|
| | | OR (95% CI), *P* | OR (95% CI), *P* | OR (95% CI), *P* |
| mtDNA-CN, unit per 1 decile | 146/785 | 1.68 (1.55 – 1.86) | 1.70 (1.53 – 1.88) | 1.71 (1.54 – 1.89) |
| *P* value for trends | | <0.001 | <0.001 | <0.001 |
| Men* | | | | |
| mtDNA-CN, unit per 1 decile | 76/311 | 1.72 (1.48 – 2.00) | 1.72 (1.48 – 2.01) | 1.76 (1.50 – 2.06) |
| *P* value for trends | | <0.001 | <0.001 | <0.001 |
| Women* | | | | |
| mtDNA-CN, unit per 1 decile | 70/474 | 1.67 (1.46 – 1.91) | 1.70 (1.48 – 1.95) | 1.71 (1.49 – 1.97) |
| *P* value for trends | | <0.001 | <0.001 | <0.001 |

Model 1: adjusted for age and sex; Model 2: adjusted for age, sex, smoking, body mass index (BMI), systolic blood pressure (SBP), and total cholesterol (TC); Model 3: adjusted for age, sex, smoking, BMI, SBP, TC, and education; *Models 1, 2, and 3 stratified by sex (sex excluded from covariates). Abbreviations: mtDNA-CN, mitochondrial DNA copy number, OR: Odds ratio

**Table 4. Relationship between CRC and LTL and mtDNA-CN, per 1 decile decrease in each biomarker in a cohort, excluding early cancer cases within 2 years from baseline (cases, *n*=131; controls, *n*=799 for LTL/785 for mtDNA-CN; men and women, 15-year follow-up)**

| Biomarker | Cases/controls, n | Model 1 | Model 2 | Model 3 |
|---|---|---|---|---|
| | | OR (95% CI), *P* | OR (95% CI), *P* | OR (95% CI), *P* |
| LTL, unit per 1 decile | 131/799 | 3.00 (2.45 – 3.66) | 3.04 (2.48 – 3.73) | 3.02 (2.46 – 3.70) |
| *P* value for trends | | <0.001 | <0.001 | <0.001 |
| mtDNA-CN, unit per 1 decile | 131/785 | 1. 71 (1.54 – 1.89) | 1.71 (1.54 – 1.91) | 1.72 (1.54 – 1.92) |
| *P* value for trends | | <0.001 | <0.001 | <0.001 |

Model 1: Adjusted for age and sex; Model 2: Adjusted for age, sex, smoking, body mass index (BMI), systolic blood pressure (SBP), and total cholesterol (TC); Model 3: Adjusted for age, sex, smoking, BMI, SBP, TC, and education. LTL: Leukocyte telomere length, mtDNA-CN: Mitochondrial DNA copy number, OR: Odds ratio

In addition, we estimated the association of LTL and mtDNA-CN with cancer of the colon and rectal sites separately. The associations were very close to the pooled dataset results with somewhat higher ORs for the risk of rectal cancer compared to colon cancer, both for LTL and mtDNA-CN (Supplementary File, Table S2).

Finally, we assessed the association of CRC with generated LTL and mtDNA-CN values, preliminary adjusted for multiple covariates, and their interaction with case–control status. The results were weaker with OR of CRC 2.53 (95% CI: 2.16 – 2.97) per 1 decile decrease in fully adjusted baseline LTL (Supplementary File, Table S3) and with OR of CRC 1.52 (1.39 – 1.65) per 1 decile decrease in fully adjusted baseline mtDNA-CN (Supplementary File, Table S4).

## 4. Discussion

The present nested case-control study included individuals with incident CRC (cases) and age and sex frequency-matched controls from a Novosibirsk population cohort (Russia). The carriers of shorter telomeres had an increased 15-year risk of incident CRC with adjusted OR 3.2 per 1 decile decrease in LTL. Lower mtDNA-CN was associated with an increased risk of incident CRC with adjusted OR 1.7 per 1 decile decrease in mtDNA-CN. The risk coefficients of CRC were attenuated to OR 2.53 per 1 decile decrease in adjusted baseline LTL and OR 1.52 per 1 decile decrease in adjusted baseline mtDNA-CN.

A number of epidemiological studies, with varying samples and designs, have explored the associations between LTL and the risk of cancer, but the results are inconsistent[14,20-23]. Specifically for CRC incidence and mortality, both negative and positive relationships, U-shaped, and absence of association with LTL have been reported.

Our findings of an inverse relationship between baseline LTL and CRC incidence are consistent with the prospective Bruneck study[35], a case–control study in China[36], and are in line with the CRC mortality findings in two meta-analyses[37,38]. In the Bruneck study with a 10-year follow-up, the adjusted hazard ratio was 1.56 (95% CI: 1.32 – 1.85) for CRC incidence and 1.88 (95% CI: 1.48 – 2.40) for CRC mortality per 1 SD shorter baseline LTL[35]. In a case-control study of a Chinese population, shorter LTL was modestly associated with higher risk of CRC (OR per LTL tertile 1.13; 95% CI: 1.00 – 1.28)[36]. In a study conducted by Kroupa *et al*., shorter telomeres were found in CRC tumor tissue than in adjacent mucosa[39]. This finding is supported by evidence from recent studies[40]. Zhang *et al*., in a meta-analysis based on 45 prospective studies of incident cancer (2 for CRC), have shown that short telomeres are related to increased CRC mortality, with an RR of 2.54 (95% CI: 1.73 – 3.72) for short telomere length compared to long telomere length as dichotomized variable[37]. In another meta-analysis of prospective and retrospective studies, short LTL was associated with poorer survival for CRC (HR = 2.01; 95% CI: 1.46 – 2.77)[38]. Wentzensen *et al*., in their meta-analysis, have indicated an inverse association between LTL and CRC risk in a retrospective case–control study[41].

On the other hand, our findings are opposite to the data obtained from a prospective study in Singapore ($n$ = 26.000, 12-year follow-up)[42], which has reported a positive association between LTL and CRC risk, with a HR of 1.32 (95% CI: 1.08 – 1.62) for the top quartile of telomere length versus bottom quartile. In a meta-analysis of 28 prospective studies (2 on CRC), no associations were reported between LTL and CRC risk[21]. Similarly, the pooled results of eight prospective studies have revealed that LTL is associated with neither a better or poorer prognosis of CRC patients[43]. Interestingly, in a Mendelian randomization study (MRS) of the UK Biobank data (7.5-year follow-up, $n$ = 261,837), genetically determined longer telomeres were found to be associated with a modestly elevated risk of pooled cancer. However, the risk of CRC was found to be below 1 among those with longer telomeres, while remaining statistically insignificant[22]. A recent MRS using genetic risk score for LTL has found that short LTL score is related to a reduced risk of nine types of cancer (not including CRC) in the UK Biobank dataset, but there has been an observation that is suggestive of an association between short LTL score and a high death hazard of rectum adenocarcinoma in The Cancer Genome Atlas (TCGA) dataset[23], of which the latter is in line with our findings.

Concerning the alternations of mtDNA in relation to cancer, different results have been reported[24]. For a number of cancer types, an inverse, multidirectional, or nonlinear relationship with mtDNA has been reported (colorectal, breast, kidney, and lung)[25,27]; the absence of distinction between tumor tissue and adjacent unaffected tissue has also been reported (colorectal, kidneys, pancreas and thyroid glands, prostate, stomach, and uterus)[26].

Our results of an inverse association between baseline mtDNA-CN and CRC incidence are in line with the Shanghai Women's Health Study[44] and, partly, with a recent prospective Swedish study[45]. In a case–control design for CRC established on a population sample (n= 444/1,423 women, >9 years follow-up) under the Shanghai Women's Health Study, the researchers found an inverse association between baseline mtDNA-CN and incident CRC with adjusted OR = 1.26 (95% CI: 0.93 – 1.70) and 1.44 (95% CI: 1.06 – 1.94) for the middle and bottom tertiles of mtDNA-CN values, respectively[44]. In a prospective Swedish study (women, 15.2 years follow-up), baseline LTL and mtDNA-CN were not associated with the prevalence and incidence of a digestive cancer as a cumulative category (including CRC). However, mtDNA-CN (but not

LTL) was found to be inversely associated with mortality from digestive cancer with OR = 1.53 (95% CI: 1.02 – 2.28) per 1 SD decrease in mtDNA-CN[45]. Van Osch *et al*., in combined CRC cases from hospital series and Netherlands Cohort study, discovered lower mtDNA-CN in primary CRC tissue compared to a resected one as well as an inverse U-shaped relationship between CRC survival and mtDNA-CN[46].

On the other hand, several studies have shown an association between higher mtDNA-CN and risk of incident CRC[47] or CRC progression[48]. In a meta-analysis, Mi *et al*. failed to find any association between mtDNA-CN and several digestive system cancers (including CRC)[49].

The mechanisms of the relationship between LTL and mtDNA-CN and CRC have not been fully understood. A growing body of evidence has suggested the dual role of telomeres (or telomere paradox) in carcinogenesis[39,50]. Tumor cells with increased proliferation undergo faster attrition of telomeres than non-affected cells. Telomere shortening works as a tumor-suppressing mechanism. On the other hand, for cell clones that escape the crisis, critically short telomeres contribute to the mechanisms related to telomere maintenance, including the reactivation of telomerase, which stabilizes short telomeres[50], or rarer alternative telomere lengthening[45,51], thus preventing cell death and promoting tumor invasion. Critically short telomeres also affect genomic aberrations and chromosome instability, which, along with microsatellite instability[40], represent important pathways in the genesis of CRC[39]. The recently discovered telomere-driven chromothripsis (complex chromosomal rearrangement) is a widespread mutational phenomenon found in diverse tumor types[51].

MtDNA-CN, as a proxy for mitochondria function, differs between cancer tissue and non-affected tissue for a number of cancer types[45]. The impact of mitochondria metabolism on tumor onset and progression is heterogeneous by cancer type, and it has been shown to be related to CRC initiation[52,53]. Existing data have suggested that mtDNA-CN changes depend on nuclear or mtDNA mutations and serve as an adaptive response toward these mutations for certain cancer types[52,54]. A recent data have supported the role of mtDNA mutations in metabolic alterations (oxidative phosphorylation defects), facilitating colon tumor[55], and suggested that somatic mutations in mtDNA control region may be shaped by tumor-specific selective pressure and involved in tumorigenesis[56]. Telomere attrition is involved in the regulation of mitochondrial genesis and function in the aging process, leading to their dysfunction and ROS formation; in particular, the role of telomere-p53-mitochondrial axis in cancer has been demonstrated[45].

The heterogeneity of the results for studied biomarkers might be due to the variability across cancer sites, study designs (retrospective or prospective studies with early follow-up may reflect reverse causation), age and other covariates impact, accuracy of LTL or mtDNA-CN measurement, and risk assessment for incident events or cancer progression and mortality[21].

## 5. Limitations and strengths of the study

Our findings should be interpreted within the context of their potential limitations. First, we have to mention the modest sample size. Although our nested case-control analysis involved the complete set of incident CRC cases that developed in a large-scale cohort (9,360) within a long-term follow-up period of 15 years, we ruled out only a few CRC cases (because of the absence of DNA material or insufficient quality of LTL and mtDNA-CN genotyping). The controls satisfied strict exclusion criteria and were frequency-matched to cases by age and sex. We believe that this design likely covers a representative sample of incident CRC in this population.

In addition, to ensure the completeness of registration, we checked multiple sources of information for case ascertainment (including the Cancer Register, Mortality Register, and repeated examinations of the cohort).

Another potential limitation might be related to the effect of cancer per se or cancer treatment on studied biomarkers[57]; thus, retrospective design or early follow-up may reflect reverse causation. To avoid this shortage, we used a prospective design and included only incident cancer cases. Besides that, we fulfilled two sensitivity analyses with the exclusion of CRC cases that occurred within the first 2 years or within the first 8 years after the baseline blood was drawn, which did not substantially alter the results.

Another concern is the sex-dependent variance in LTL and mtDNA-CN (due to the known higher values of both biomarkers in women compared to men). To overcome this potential limitation, we kept a similar sex distribution among cases and controls (nearly 50%–60%), and the estimates were adjusted for sex. In addition, we obtained ORs that were comparable to the combined dataset results after splitting the analysis by sex.

Taking into account the reported difference in the relationship between LTL and subtypes of CRC[42,58], we analyzed the risk of colon cancer and rectal cancer separately. This additional stratification did not materially affect the results.

Furthermore, our results are based on the investigation of a Caucasoid population in Novosibirsk (West Siberia),

and thus have limited generalizability and may not be applicable to another populations. The geographical and ethnic variations as well as the difference in CRC rate, related risk factors, and lifestyle profile between populations might contribute to the population-specific predictive role of studied molecular markers for CRC risk.

Aside from limitations, this study has its strengths. For the first time, this prospective case–control study investigated the links between LTL and mtDNA-CN as well as the risk of incident CRC in the Russian population and altogether the Eastern European population.

It is noteworthy that we used two molecular markers with established association with mortality and several age-related diseases[9,14,15,18,52]. We showed a close correlation between these markers themselves and with age.

Finally, our findings from a Russian cohort offer the first evidence of the value of the association of LTL and mtDNA-CN with CRC in the studied population. Our findings may be useful for further integration with data from different populations for large-sample integrative analysis of the association of LTL and mtDNA-CN with CRC.

## 6. Conclusion

In our nested case–control study, LTL and mtDNA-CN were independent inverse predictors of the 15-year risk of CRC in the middle-aged and elderly (Caucasoid) population cohort in Novosibirsk, West Siberia. These molecular markers may become useful predictors for CRC risk and may have potential implications in cancer prevention. At the same time, our findings highlight the relevance for further research to uncover the mechanisms behind the association of LTL and mtDNA-CN with human health.

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## Conflict of interest

The authors declare no conflict of interest.

## Author contributions

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All authors have contributed substantially to this work and consented to submit the paper to *Global Translational Medicine*, and to the best of the authors' knowledge, the entire research and paper writing process were carried out in adherence to the highest academic conduct standards and publishing ethics code.

## Ethics approval and consent to participate

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of IIPM – Branch of IC&G SB RAS (Institute of Internal and Preventive Medicine – Branch of Federal State Budgeted Research Institution, "Federal Research Center, Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences"), Protocol #1 from March 14, 2002 and Protocol #12 from December 8, 2020. The study was observational and did not involve experiments on humans, animals, or cell lines. Informed consent was obtained from all subjects involved in the study.

## Consent for publication

Informed consent was obtained from all subjects (for study participation, anonymized data analysis and publication).

## Availability of data

The data presented in this study are available in tabulated form on request. The data are not publicly available due to ethical restrictions and project regulations.

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Global Translational Medicine

ORIGINAL RESEARCH ARTICLE

# The vasculoprotective effects of resveratrol are mediated via Kruppel-like factor 2 dependent protection of endothelial barrier function

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## Abstract

Resveratrol is a naturally occurring polyphenolic compound that is thought to have vasculoprotective properties. Its observed effects are proposed, in part, to be mediated through the induction of endothelial Krüppel-like factor 2 (KLF2) expression. KLF2 is a nuclear transcription factor that is highly expressed within the vascular endothelium. Studies from our laboratory and others have shown that this protein mediates vascular function through its transactivation domain, and its targeted expression promotes vascular health, notably by acting as an important positive regulator of endothelial barrier function. In this study, we demonstrate that resveratrol possesses endothelial barrier protective effects dependent on the presence of KLF2, with several key endothelial tight junction proteins expressed in a KLF2-dependent manner. Collectively, our findings identify KLF2 as essential for resveratrol-mediated endothelial barrier protection, thus further implicating KLF2 as a critical vasculoprotective factor.

***Keywords:*** Resveratrol; Krüppel-like factor 2; Barrier function

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## 1. Introduction

Resveratrol (3,5,4′ -trihydroxy-trans-stilbene), a polyphenol that is notably present at high concentrations in grapes and red wine, is occasionally used as a dietary supplement, although its beneficial effects remain unproven. It has generated a significant amount of clinical interest for its many beneficial effects on multiple pathologic processes, including hypertension, diabetic cardiomyopathy, atherosclerosis, heart failure, and several types of cancer[1,2]. Studies performed in rodent models have added to a growing body of evidence that suggests the presence of resveratrol which is vasculoprotective. Resveratrol has been shown to improve endothelial dysfunction and decrease vascular inflammation, resulting in reduced ischemic damage in vital organs[2-4]. Recently, resveratrol has been identified as having protective effects on endothelial barrier function in the vasculature of the brain and retina[5,6]. However, the detailed molecular mechanisms by which

resveratrol exerts its barrier protective effects remain largely unknown.

Krüppel-like factors (KLFs) are a family of zinc finger-containing transcription factors that have been shown to have diverse regulatory roles in biological processes, such as cell proliferation, differentiation, and survival, organ development, and metabolism[7]. Several published reports by us and others have demonstrated that KLF2 serves as a nodal regulator of endothelial biology. It promotes a healthy vascular phenotype by affecting key aspects of vascular function and disease[8,9]. Importantly, *in vivo* studies from our laboratory have identified KLF2's ability to induce endothelial tight junction factors (e.g., occludin)[10,11] and protect the integrity of the vascular barrier of the brain, thereby also serving as a stroke protective factor[11]. These studies have firmly established the role of KLF2 as a critical positive regulator of vascular endothelial barrier function.

Interestingly, *in vitro* studies have demonstrated that resveratrol induces KLF2 expression in endothelial cells (ECs), implicating KLF2 as a potential regulator of its beneficial vascular effects[12]. However, the physiologic importance of this regulation *in vivo* and its specific role in mediating vascular endothelial barrier function has not been investigated thus far. In this study, we examine resveratrol's dependence on KLF2 for its protective effects on vascular endothelial barrier function.

## 2. Materials and methods

### 2.1. Animals

Postnatal KLF2 knockout mice were generated as previously described[11]. KLF2-floxed mice were crossed with CAG-CreERT$^2$ strain (Jackson Laboratory) to generate KLF2$^{fl/fl}$-CAG-CreERT$^2$ mice. Postnatal deletion of KLF2 was induced through intraperitoneal (IP) injection of tamoxifen (0.1 mL at 20 mg/mL, dissolved in sunflower seed oil; T5648, Sigma-Aldrich) to 8 week-old male KLF2$^{fl/fl}$-CAG-CreERT$^2$ mice. Age-matched male CAG-CreERT$^2$ mice that were subjected to the same tamoxifen regimen were used as controls. All animals were maintained in a clean animal facility. All mouse studies were approved by an Institutional Animal Care and Use Committee (IACUC) at Case Western Reserve University and were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

### 2.2. Resveratrol pretreatment

Male 17–20-week-old KLF2$^{-/-}$ and control mice were treated with 75 mg/kg of resveratrol (Sigma) in a saline solution of 20% hydroxypropyl-β-cyclodextrin (American Maize-Products Company) through daily gastric gavage for 10 days.

### 2.3. Stereotactic intracerebral injection of tumor necrosis factor (TNF)-α

Male (15 – 20-week-old) control (tamoxifen-treated CAG-CreERT$^2$ mice) and postnatal KLF2 knockout mice were stereotactically injected with TNF-α (1 μg/kg in 4 μL 1% bovine serum albumin/phosphate-buffered saline (PBS) over 20 – 30 min) into the striatum using the following coordinates from bregma: anteroposterior, 0.0 mm; lateral, 1.5 mm; and ventral, 2.1 mm.

### 2.4. *In vitro* cell culture studies and western blotting

Primary human brain microvascular ECs were purchased from cell systems and cultured in endothelial basal medium-2 (EBM-2) that was supplemented with growth factors. Resveratrol was obtained from Sigma. Polyclonal rabbit anti-KLF2 antibody was provided as a gift by Huck-Hui Ng (Singapore). Mouse anti-β-actin antibody was obtained from Santa Cruz Biotechnology. All small interfering RNA (siRNA) oligonucleotides were obtained from Dharmacon. Transfection of siRNA into endothelial cells was carried out as described[10,11]. Protein isolation and western blot analysis using the indicated antibodies were performed as previously described[10].

### 2.5. Quantitative real-time polymerase chain reaction (RT-PCR)

TRIzol reagent (Invitrogen) was used to extract the total RNA from primary human brain microvascular endothelial cells following the manufacturer's instructions. Two micrograms of total RNA were used for reverse transcription to generate complementary deoxyribonucleic acid (cDNA) using iScript Reverse Transcription kit (Bio-Rad). RT-PCR was performed with Universal SYBR Green PCR Master Mix on Applied Biosystems Step One Real-Time PCR system. Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) using the ΔΔCt method.

### 2.6. *In vitro* endothelial permeability assay

*In vitro* endothelial permeability analysis was performed using a transwell assay as described in our prior publication[11]. To achieve oxygen-glucose deprivation (OGD), cultured endothelial cells were exposed to deoxygenated PBS in a modular incubator chamber flushed with 1% oxygen ($O_2$), 5% carbon dioxide ($CO_2$), and 94% nitrogen ($N_2$), sealed, and placed at 37°C for 30 min, followed by a 2-h exposure to normal culture media under normoxic conditions (21% $O_2$, 5% $CO_2$) at 37°C. Immediately on re-exposure to normal media and

normoxia, fluorescein isothiocyanate (FITC)-dextran was added to the transwell insert. Aliquots of medium collected from the bottom chamber and the fluorescence density of samples were analyzed on a microplate fluorometer.

### 2.7. *In vivo* permeability assay

Following stereotactic injection of TNF-α, tail vein injection of 2% Evans blue dye (EBD; 4 mL/kg) was performed. 2 h later, the mice were euthanized and subjected to saline perfusion, followed by brain tissue removal, weighing, and homogenizing in 50% trichloroacetic acid (TCA). Following EBD extraction, the concentration (ng EBD/mg brain) was determined by fluorescence intensity (excitation 620 nm and emission 680 nm) based on an EBD standard curve.

### 2.8. Statistical analysis

Data were expressed as mean ± standard error of the mean. One-way analysis of variance (ANOVA) was used to compare the differences across 3 or more levels within 1 variable. When comparing a single variable in multiple groups, one-way ANOVA (followed by Dunnett's multiple comparisons test) was performed. Two-way ANOVA (followed by Dunnett's *post hoc* test) was used for two-factor analysis. Statistical analyses were performed using Prism 9.0 software. $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Resveratrol induces KLF2 in microvascular endothelial cells

Previous data have established that resveratrol is able to induce KLF2 expression in human umbilical vein ECs in both time- and dose-dependent manners[12]. We demonstrate similar findings in human primary brain microvascular ECs. The peak induction of *KLF2* mRNA was observed at 8 h after resveratrol treatment (Figure 1A), while all times tested showed significant increase over baseline conditions (0 h). Peak *KLF2* mRNA expression was observed at a concentration of 100 μM, with lower doses also showing upregulation (Figure 1B). Complementary to our mRNA data, resveratrol also induced KLF protein expression in a dose-dependent manner as confirmed by western blot analysis (Figure 1C). Taken together, these data show that resveratrol is a potent activator of KLF2 expression within microvascular ECs.

### 3.2. Resveratrol's induction of key endothelial tight junction factors is KLF2 dependent

We have recently identified KLF2 to be an important regulator of vascular endothelial barrier function through the upregulation of several key endothelial tight junction factors, including occludin, claudin 12, junctional adhesion molecule 1 (JAM-1), and AF-6/afadin[11]. To determine if resveratrol can induce some or all of these factors and whether the induction is KLF2 dependent, the ability of resveratrol to induce a panel of tight junction factors was assessed in the presence and absence of KLF2. Human primary brain microvascular ECs were transfected with *KLF2* siRNA (siKLF2) or control siRNA (non-specific, NS), followed by resveratrol treatment. Resveratrol specifically induced the expression of occludin (Figure 2B), AF-6 (Figure 2C), and JAM-1 (Figure 2D). No significant changes in the mRNA levels of claudin 3, claudin 5, and zona occludens protein 1 (ZO-1) were observed (data not shown). Following siRNA-mediated KLF2 knockdown, resveratrol's induction of KLF2 expression was significantly inhibited (Figure 2A), and the induction of occludin, AF-6, and JAM-1 was abrogated (Figure 2B–D). These findings show that KLF2 is necessary for resveratrol's induction of several key tight junction factors and suggests that an additional mechanism by which resveratrol exerts its vasculoprotective effects may be through the regulation of endothelial barrier function.

### 3.3. Resveratrol's protection of endothelial barrier function is KLF2 dependent

To examine the functional impact of resveratrol on endothelial barrier *in vitro*, transwell assays were performed in human primary brain microvascular ECs. The cells were plated onto a transwell, treated with 100 μM of resveratrol for 16 h, and subjected to OGD. The permeability of FITC-dextran was, then, measured to assess barrier integrity. Under these conditions, resveratrol protects against OGD-mediated endothelial barrier disruption with a significant decrease observed in fluorescence intensity when compared to control (Figure 3A). To determine if resveratrol's endothelial barrier protective effects are KLF2 dependent, transwell experiments with OGD were conducted in ECs following siRNA-mediated KLF2 knockdown. Indeed, the protective effects of resveratrol were significantly abrogated in the absence of KLF2 (Figure 3B). When the control siRNA was transfected into microvascular ECs, resveratrol treatment showed decreased FITC-dextran fluorescence under OGD conditions (comparing siControl + vehicle [OGD] versus siControl + resveratrol [OGD]) (Figure 3B). When siRNA specific for KLF was introduced, this effect was eliminated (comparing siKLF2 + vehicle [OGD] versus siKLF2 + resveratrol [OGD]). To determine the physiologic relevance of our findings *in vivo*, EBD incorporation assays were performed on both control and KLF2-deficient mice. Inflammatory cytokine TNFα was stereotactically injected into the striatum of both control and KLF2 knockout mice,

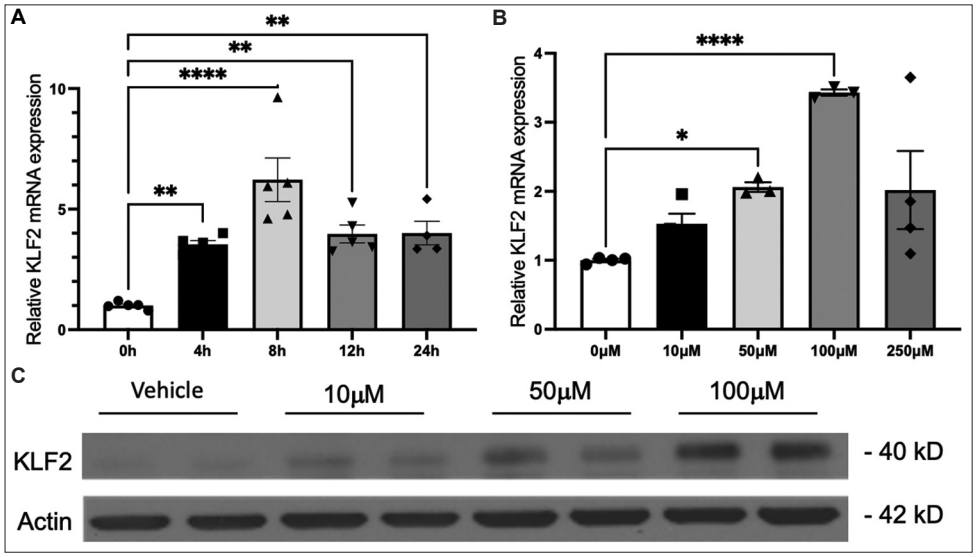


**Figure 1.** Resveratrol induces Krüppel-like factor 2 (KLF2) expression in human primary brain microvascular endothelial cells (ECs). KLF2 mRNA expression in human primary brain microvascular ECs treated with resveratrol (A) (100 μM dose) at different time intervals (n = 3–4), and (B) (8 h) at different concentrations (n = 3–4), *$P < 0.05$, **$P < 0.005$, ****$P < 0.0001$. (C) Elevation of KLF2 protein in human primary brain microvascular ECs treated with resveratrol (12 h) at different concentrations.

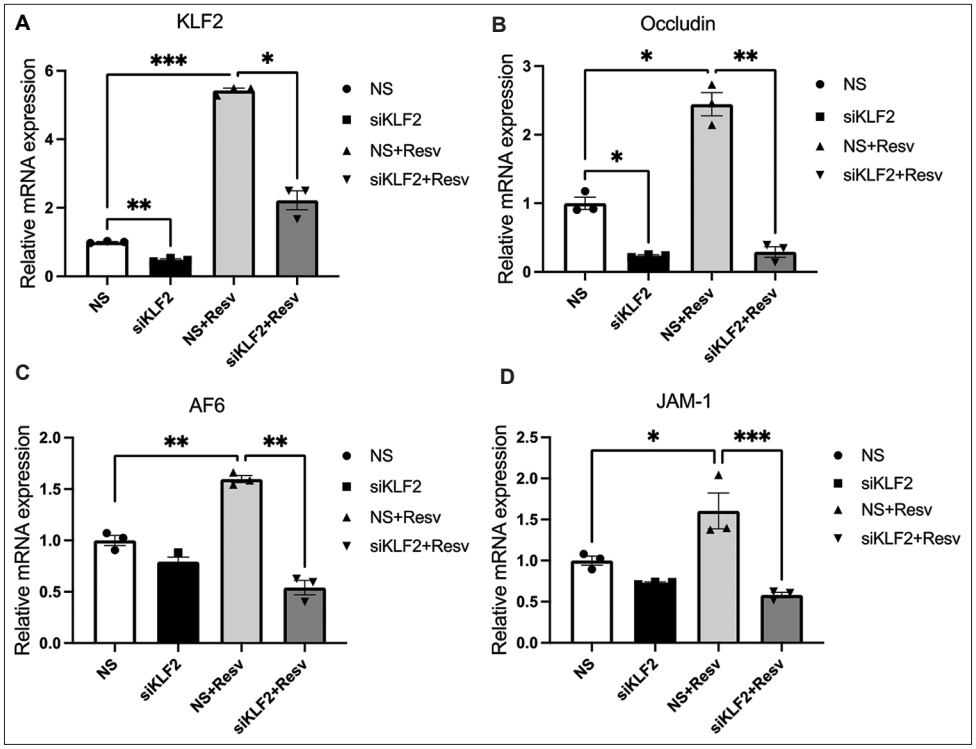


**Figure 2.** Resveratrol-mediated induction of several key tight junction factors is Krüppel-like factor 2 (KLF2) dependent. Quantitative real-time polymerase chain reaction (RT-PCR) analysis of (A) KLF2, (B) occludin, (C) afadin (AF6), and (D) junctional adhesion molecule 1 (JAM-1) in human primary brain microvascular ECs transfected with small interfering ribonucleic acid (siRNA, siControl, or siKLF2) and treated with resveratrol (100 μM for 8 h). NS: Non-specific siRNA, Resv: Resveratrol. *$P < 0.05$; **$P < 0.005$; ***$P < 0.001$.

followed by tail vein injection of EBD. Brain endothelial barrier function was assessed by the quantification of EBD extracted from the harvested brains. The diminished incorporation of EBD into the brain tissue from control mice verifies the endothelial barrier protective effects of resveratrol *in vivo*. In KLF2-deficient mice, there was a

higher rate of EBD incorporation into the tissue, suggesting an overall increase in barrier dysfunction with resveratrol's protective effect being completely abolished (Figure 4). These findings clearly identify KLF2 as a necessary downstream mediator of resveratrol and an important regulator of its vasculoprotective effects.

## 4. Discussion

The blood–brain barrier (BBB) is a critical structural and biochemical barrier composed of the endothelium and the surrounding extracellular matrix. The properties of the BBB are primarily due to junctional complexes within the endothelium. These complexes are categorized as either tight junctions, which seal the endothelium and limit paracellular diffusion, or adherens junctions, which regulate EC cell-cell contacts, cytoskeletal association, and intracellular signaling[13]. To maintain cerebral homeostasis and prevent blood-borne molecules from entering the brain, it is essential to maintain a strict separation between the blood and extravascular compartments[14]. Proteins that contribute to the endothelial barrier function (i.e., claudins, occludin, and JAMs) are critical in the maintenance of BBB integrity. Hence, the disruption of these proteins contributes to a broad spectrum of disease

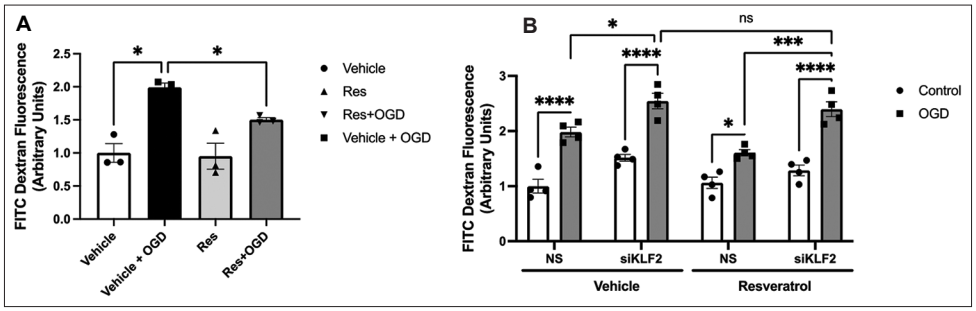


**Figure 3.** Resveratrol has endothelial barrier protective effects, with this barrier protective function being Krüppel-like factor 2 dependent. (A) *In vitro* permeability analysis quantifying passage of fluorescein isothiocyanate (FITC)-dextran across primary human brain microvascular ECs pre-treated with resveratrol (100 μM for 16 h) or vehicle at baseline (Control) and after 30 min oxygen-glucose deprivation, followed by 2 h of normal media ($n$ = 3/group). (B) Permeability assessment as in (A) using cells transfected with small interfering ribonucleic acid (siRNA, si-NS or siKLF2) ($n$ = 4 per group). NS: Non-specific siRNA. $^{*}P < 0.05$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

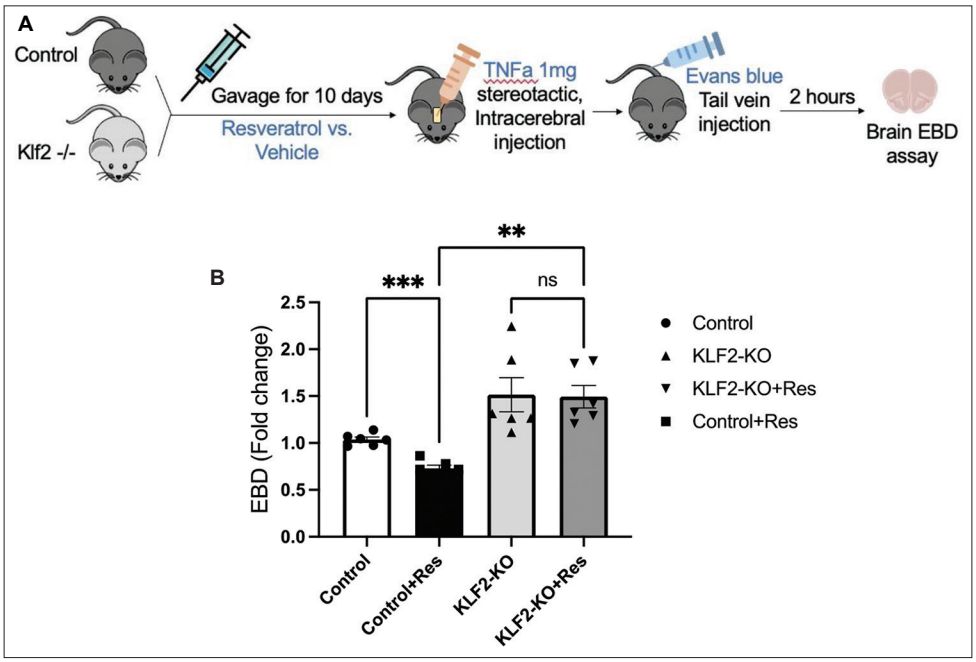


**Figure 4.** Blood–brain barrier (BBB) protection effect of resveratrol is Krüppel-like factor 2 (KLF2) dependent. (A) Schematic diagram for *in vivo* BBB studies. (B) Quantification of Evans blue dye permeability of brains harvested from control and KLF2 knockout mice after pretreatment with vehicle or resveratrol (75 mg/kg) through gastric gavage for 10 days ($n$ = 6/group). Control: CAG-CreERT$^2$, KLF2-/-: postnatal KLF2 knockout, Res: Resveratrol. $^{**}P < 0.005$; $^{***}P < 0.001$.

states[15,16]. Therefore, the discovery of compounds that can help maintain or improve the endothelial barrier function is of significant interest and may be helpful in the treatment of diseases such as Alzheimer's or Parkinson's, where BBB impairment can contribute to worsening neurodegenerative states.

Resveratrol is a naturally occurring vasculoprotective compound. Recently, studies have identified additional beneficial aspects and begun to outline the molecular mechanisms of how resveratrol impacts endothelial function[10]. In the present study, we are the first to identify KLF2 as an obligatory factor for resveratrol's induction of key tight junction factors and subsequent endothelial barrier protective effects. KLF2 is a highly expressed transcription factor within ECs. The previous studies have demonstrated that KLF2 critically regulates key aspects of vascular function and disease, including vascular permeability, EC thrombotic function, and angiogenesis. Importantly, in ECs, KLF2 has also been demonstrated to be critical for resveratrol's induction of endothelial nitric oxide synthase (eNOS) and thrombomodulin (TM), both of which are known to play critical roles in inflammation, vasoreactivity, and thrombosis[12]. Taken together, these findings implicate KLF2 as a central regulator of resveratrol's beneficial effects in ECs.

In addition to promoting vascular health through EC signaling, resveratrol's mechanisms of action have been shown to involve other cell types[1]. One limitation of our studies is that they have been performed using global KLF2-deficient mice; therefore, we cannot rule out the contributing factors from non-EC sources. For that reason, since KLF2 is also expressed in cells of the myeloid lineage, future studies in mice with EC-specific KLF2 deficiency are warranted to further pinpoint the importance of resveratrol's regulation of KLF2 in ECs.

Finally, resveratrol has been proposed to be beneficial in vascular diseases, such as atherosclerosis and ischemic stroke[1-3,5]. Similarly, we have demonstrated KLF2 to be a protective factor against atherosclerosis and stroke[11,17]. Studies treating KLF2-deficient mice with resveratrol in these disease models will, further, define its mechanistic pathway of action and how it, or other similar compounds, can be used as an inexpensive and natural way to help treat and/or alleviate symptoms associated with vascular, cerebral, or neurodegenerative disorders.

## Acknowledgments

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## Funding

None.

## Conflict of interest

The authors have declared that no conflicts of interest exist.

## Author contributions

*Conceptualization:* Hong Shi
*Formal analysis:* Xianming Zhou, Lily Lin, Hong Shi
*Investigation:* Xianming Zhou, Hong Shi
*Writing – original draft:* Xianming Zhou, Lily Lin, Hong Shi
*Writing – review & editing:* Lily Lin, Hong Shi

## Ethics approval and consent to participate

All mouse studies were approved by an Institutional Animal Care and Use Committee (IACUC) at Case Western Reserve University (2011-0136) and were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

## Consent for publication

Not applicable.

## Availability of data

Data will be obtained from the corresponding author on reasonable request.

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ACCSCIENCE PUBLISHING

Global Translational Medicine

ORIGINAL RESEARCH ARTICLE

# Antisense oligonucleotides targeting hepatic angiotensinogen reduce atherosclerosis and liver steatosis in hypercholesterolemic mice

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## Abstract

Hepatocyte-derived angiotensinogen (AGT) is the precursor of angiotensin II (AngII). We determined the effects of hepatocyte-specific (*N*-acetylgalactosamine-conjugated) antisense oligonucleotides targeting AGT (GalNAc AGT ASO) on AngII-mediated blood pressure (BP) regulation and atherosclerosis and compared its effects with losartan, an AngII type 1 (AT1) receptor blocker, in hypercholesterolemic mice. Eight-week-old male low-density lipoprotein (LDL) receptor deficient mice were administered vehicle or GalNAc AGT ASO (1, 2.5, or 5 mg/kg) subcutaneously beginning 2 weeks before the initiation of Western diet feeding. All mice were fed Western diet for 12 weeks. Their systolic BP was monitored by the tail-cuff technique, and the atherosclerotic lesion area was measured by an *en face* method. Although the effects of all 3 doses of GalNAc AGT ASO on plasma AGT concentrations were similar, GalNAc AGT ASO reduced BP and atherosclerotic lesion size in a dose-dependent manner. Subsequently, we compared the effects of GalNAc AGT ASO (5 mg/kg) with losartan (15 mg/kg/day). Compared to losartan, GalNAc AGT ASO led to more profound increases in plasma renin and reduction in BP but had similar effects on atherosclerosis. Remarkably, GalNAc AGT ASO also reduced liver steatosis, which was not observed in losartan-treated mice. In conclusion, the BP increase and atherosclerosis development in hypercholesterolemic mice are dependent on AngII generated from hepatic AGT. Deleting hepatic AGT improves diet-induced liver steatosis, and this occurs in an AT1 receptor-independent manner.

***Keywords:*** Angiotensinogen; Antisense oligonucleotides; Blood pressure; Liver steatosis; Atherosclerosis

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## 1. Introduction

Angiotensinogen (AGT) is cleaved by renin to generate angiotensin (Ang) I and des(AngI)AGT[1,2]. Subsequently, AngI is cleaved by angiotensin-converting enzyme (ACE) to release AngII. AngII, through binding to AngII type 1 (AT1) receptor, contributes to blood pressure (BP) regulation and atherosclerosis[3-5]. In previous studies, the pharmacological inhibition of renin, ACE, or AT1 receptor reduced BP and atherosclerosis in low-density lipoprotein (LDL) receptor$^{-/-}$ mice fed a Western diet[3-7]. Whole-body reduction of AGT, hepatocyte-specific AGT deficiency (hepAGT$^{-/-}$), and global AGT antisense oligonucleotides (ASO) exerted similar effects on BP and atherosclerosis in LDL receptor$^{-/-}$ mice[8].

While the AngII-dependent functions of AGT have been well-documented[1,9], the function of the des(AngI) AGT portion of AGT has not been extensively studied. Recently, the biological function of des(AngI)AGT has been investigated by infecting hepAGT$^{-/-}$ mice with an adeno-associated virus (AAV) encoding des(AngI)AGT. The expression of des(AngI)AGT in hepAGT$^{-/-}$ mice increased Western diet-induced body weight gain and liver steatosis but had no effects on BP and atherosclerosis[8]. These data support the notion that the effects of AGT deletion on BP and atherosclerosis are AngII-dependent, whereas des(AngI)AGT has effects on metabolic disorders in an AngII-independent manner.

Although hepatocytes are the major source of plasma AGT, its synthesis has also been reported at non-hepatic sites[10-13]. The degree to which non-hepatic sources of AGT contribute to the above effects remains unknown. Studies have shown that adipocyte- or macrophage-derived AGT deficiency has marginal or no effect on BP and atherosclerosis[8,10,11]. To verify the importance of hepatic AGT from a pharmacological approach, we targeted hepatocyte AGT mRNA, making use of *N*-acetylgalactosamine (GalNAc)-conjugated AGT ASO[14], in LDL receptor$^{-/-}$ mice fed a Western diet for 12 weeks. A comparison was made versus losartan, a classic AT1 receptor blocker, to distinguish AngII-dependent and independent effects.

## 2. Materials and methods

### 2.1. Animals

Male LDL receptor deficient mice (LDL receptor$^{-/-}$, strain # 002207) were purchased from The Jackson Laboratory (Table S1). Eight-week-old male LDL receptor$^{-/-}$ mice (Table S2) were subcutaneously injected with phosphate-buffered saline (PBS; vehicle) or GalNAc AGT ASO (1, 2.5, or 5 mg/kg) beginning 2 weeks before Western diet feeding (diet # TD.88137, Envigo). Week -2 represents the start of subcutaneous injection of either PBS or GalNAc AGT ASO, while week 0 represents the 1$^{st}$ week of Western diet feeding (Figure 1A). On week -2, the mice were injected with vehicle or any of the 3 doses of GalNAc AGT ASO on days 1, 3, and 5, and then the 1$^{st}$ day of week -1. Two weeks after the initiation of subcutaneous injections (week 0), the mice were fed Western diet for 12 weeks, while vehicle or GalNAc AGT ASO was injected once every week. GalNAc AGT ASO was provided by Ionis Pharmaceuticals Inc. (Carlsbad, CA, USA).

We then tested the rapidity of GalNAc AGT ASO reduction in plasma AGT concentrations. Male C57BL/6J mice (~8 weeks old) were injected with GalNAc AGT ASO 10 mg/kg once (Table S3), and their plasma samples were collected sequentially for 10 days. Subsequently, GalNAc AGT ASO and losartan were compared. Eight-week-old male LDL receptor$^{-/-}$ mice were randomly assigned to three groups (Table S4). The mice in Group 1 (vehicle) were subcutaneously injected with PBS (on days 1 and 3, then once every week) and implanted a pump to deliver water subcutaneously; mice in Group 2 (GalNAc AGT ASO) were subcutaneously injected with GalNAc AGT ASO (5 mg/kg on days 1 and 3, then once every week) and implanted a pump to deliver water subcutaneously; mice in Group 3 (losartan) were subcutaneously injected with PBS (on days 1 and 3, then once every week) and implanted a pump to deliver losartan (15 mg/kg/day; Cat # 61188-100 mg, Millipore Sigma). The mini osmotic pump ALZET Model 2006 (Durect Corp.) was used to deliver water or losartan subcutaneously. The first mini osmotic pumps were replaced by second ones after 6 weeks of infusion. Week -1 represents the start of subcutaneous injection, while week 0 represents the first week of Western diet feeding. On week -1, the mice were injected with PBS or GalNAc AGT ASO on days 1 and 3, and then once each week. Mini osmotic pumps were implanted on day 5 of week -1 to deliver either water or losartan. One week after the initiation of subcutaneous injections (week 0), the mice were fed Western diet for 12 weeks.

All animal experiments (Table S5 and S6) reported in this article were performed with the approval of the University of Kentucky Institutional Animal Care and Use Committee (IACUC protocol number 2018-2968 or 2015-2050).

### 2.2. Systolic blood pressure

Systolic BP was measured in conscious mice using a non-invasive tail-cuff system (BP-2000, Visitech Systems) following our standard protocol[15] and was calculated based on 20 measurements per mouse per day for 3 consecutive days. The mean systolic BP of each mouse from the 3-day measurements was used for data analysis.

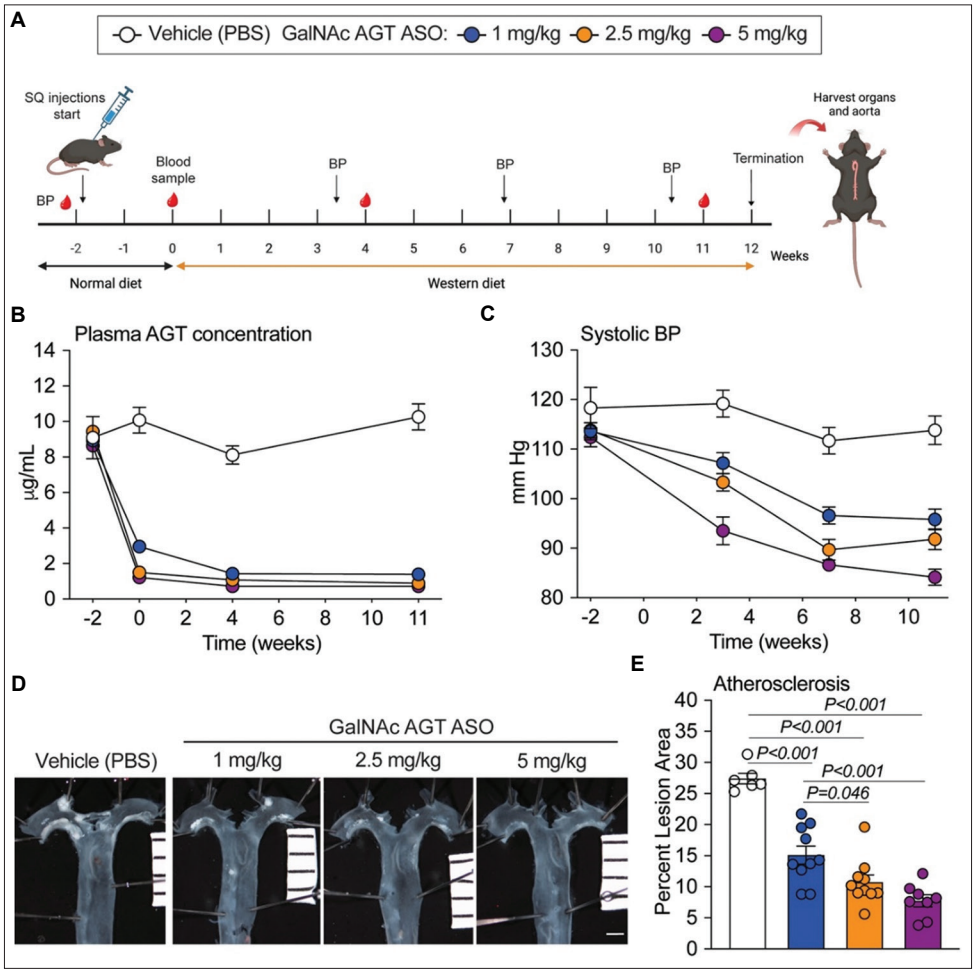


**Figure 1.** Antisense oligonucleotides (ASO) targeting hepatic angiotensinogen (AGT) reduced plasma AGT concentrations, systolic blood pressure, and atherosclerotic lesion area in the thoracic aorta. (A) Experimental protocol. (B) Plasma AGT concentrations on weeks -2, 0, 4, and 11 during ASO administration. A piecewise mixed-effect model was fitted to examine slopes for distinct time points (-2 to 0 and 0 to 11 weeks). Doses 1, 2.5, and 5 mg/kg led to profound reductions in plasma AGT concentrations compared to week -2; $P < 0.001$. (C) Mouse systolic blood pressure (BP) on weeks -2, 3, 7, and 11. A mixed-effect model was used to compare slopes among the four groups. Doses 1, 2.5, and 5 mg/kg led to significant reductions in systolic BP compared to week -2; $P < 0.001$. (D) *En face* aorta images, scale bar = 1 mm. (E) Atherosclerotic lesion area of the thoracic aorta normalized by total intimal surface area; N = 6–10 per group.
GalNAc AGT ASO: N-acetylgalactosamine-conjugated antisense oligonucleotides targeting angiotensinogen; PBS: Phosphate-buffered saline; SQ: Subcutaneous.

### 2.3. Plasma profiles

Mouse blood was collected in the presence of ethylenediaminetetraacetic acid (EDTA; final concentration: 1.8 mg/mL) through submandibular bleeding during the study. At termination, blood was collected through cardiac bleeding through the right ventricle. Plasma AGT concentrations were measured using a mouse AGT enzyme-linked immunosorbent assay (ELISA) kit (Cat # 245718, Abcam), which detects both intact AGT and des(AngI)AGT, thereby measuring total AGT. Plasma renin concentrations in mice were measured by an enzyme-kinetic assay using an AngI ELISA kit (Cat #: IB59131, IBL-America) after being incubated with exogenous recombinant mouse AGT for 1 h.

Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured on the Olympus AU400 clinical analyzer (Olympus, Center Valley, PA) using L-Type ALT.J2 and L-Type AST.J2 reagents and calibrators (Fujifilm Healthcare) in Ionis in a blinded manner.

### 2.4. Quantification of atherosclerosis

After euthanasia, mouse aortas were dissected and fixed in 10% neutrally buffered formalin overnight. Subsequently,

adventitial tissues were removed, and the intimal surface was exposed by a longitudinal cut and pinned on a black rubber surface. Images of *en face* aortas were taken using a digital camera (Nikon Digital Sight DS-Ri1), with a ruler for calibration.

An *en face* method was used to measure atherosclerotic lesions on the intimal surface of the aorta in accord with the American Heart Association (AHA) statement and as detailed in our standard protocol[16,17]. Atherosclerotic lesions were traced manually from the ascending aorta to the proximal part of the descending thoracic aorta (1 mm distal from the orifice of the left subclavian artery) using Nikon NIS-Elements software (NIS-Elements AR 5.11.00.) under a dissecting microscope.

### 2.5. Histology

At termination, a piece of each liver sample was fixed in paraformaldehyde (4% wt/vol) overnight and then embedded in paraffin. Five-micron sections were used for hematoxylin and eosin (H&E) staining. In addition, a piece of liver (fresh frozen) was embedded in optimal cutting temperature compound (OCT; Cat # 14-373-65, Fisher Scientific), sectioned using a cryostat (Leica CM 1850, Leica) at 10 µm/section, and stained with Oil Red O to visualize neutral lipid accumulation.

### 2.6. Quantification of liver steatosis

Liver weights were recorded at termination. A small piece of liver was snap-frozen in liquid nitrogen. Liver lipids were extracted, solubilized with Triton X-100, and quantified using enzymatic assays kits for total cholesterol (Cat # 23-66-201, Pointe Scientific Cholesterol reagent), and triglycerides (Regents 1 and 2: Cat # 994-02891 and Cat # 990-0299, Fujifilm Healthcare)[18].

### 2.7. RNA isolation and quantitative polymerase chain reaction (PCR)

Total RNA was extracted from liver and kidney samples using a commercial kit (Cat # AS1280, Promega) and the automated Maxwell® RSC 48 Instrument (Promega). To quantify mRNA abundance, total RNA was reversely transcribed with iScript™ cDNA Synthesis kit (Cat # 170-8891, Bio-Rad), and quantitative PCR (qPCR) was performed using the TaqMan™ Fast Advances Master Mixes kit (Cat # A44359, Thermo Fisher Scientific) on a Bio-Rad CFX96 cycler. TaqMan assay primers: *Agt* (ID: Mm00599662_m1), *Ren1* (ID: Mm02342889_g1), *Gapdh* (ID: Mm99999915_g1), *Actb* (ID: Mm01205647_g1), and *Ppia* (ID: Mm02342429_g1). Data were analyzed using the ΔΔCt method and normalized with the geometric mean of the three reference genes: *Gapdh, Actb,* and *Ppia*.

### 2.8. Statistical analysis

There were two types of data in this study: non-repeated measures after termination, and repeated measures during the study. Prism v9 (GraphPad Software Inc., La Jolla) was used for non-repeated measures, while R version 4.2.1 was used for repeated measures. Before analyzing non-repeated measures, normality and homogeneous variance assumptions were tested with Shapiro–Wilk and Brown-Forsythe tests, respectively. Since these assumptions were satisfied, all non-repeated data were analyzed using one-way analysis of variance (ANOVA) to compare means among three or more groups, followed by the Sidak *post hoc* test. For repeated measures, mixed-effect models with inverse-variance weights were used with random intercept and slope for time. A piecewise model was fitted to estimate separate slopes for distinct time points (-2 to 0 and 0 to 11 weeks) in plasma AGT concentrations. The mixed-effect models were run using the lme function in the nlme R package. Data of non-repeated measures were represented as individual data points and mean ± standard error of the mean (SEM). $P < 0.05$ or Bonferroni-corrected $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. *N*-acetylgalactosamine-conjugated antisense oligonucleotides targeting angiotensinogen reduced blood pressure, atherosclerosis, and Western diet-induced liver steatosis

In the vehicle group, plasma AGT remained unaltered (Figure 1B) in mice fed either normal laboratory diet (from week -2 to week 0) or Western diet (from week 0 through week 12). GalNAc AGT ASO significantly reduced plasma AGT concentrations in mice when they were fed normal laboratory diet; Western diet did not alter this outcome. All three doses of GalNAc AGT ASO yielded the same degree of AGT lowering, maximally reducing plasma AGT by ~90%. GalNAc AGT ASO reduced systolic BP in a dose-dependent manner versus vehicle (Figure 1C), with the effect at 5 mg/kg being significantly larger than that at 1 mg/kg ($P < 0.001$). Similarly, a dose-dependent reduction in atherosclerotic lesion size was observed (Figure 1D and E).

LDL receptor$^{-/-}$ mice fed a Western diet developed liver steatosis with increased liver weight and liver cholesterol and triglyceride content[8,19-22]. The results obtained in this study (Figure 2A–C) confirmed this outcome. GalNAc AGT ASO administration reduced liver weight and liver total cholesterol and triglyceride content (Figures 2A–C)

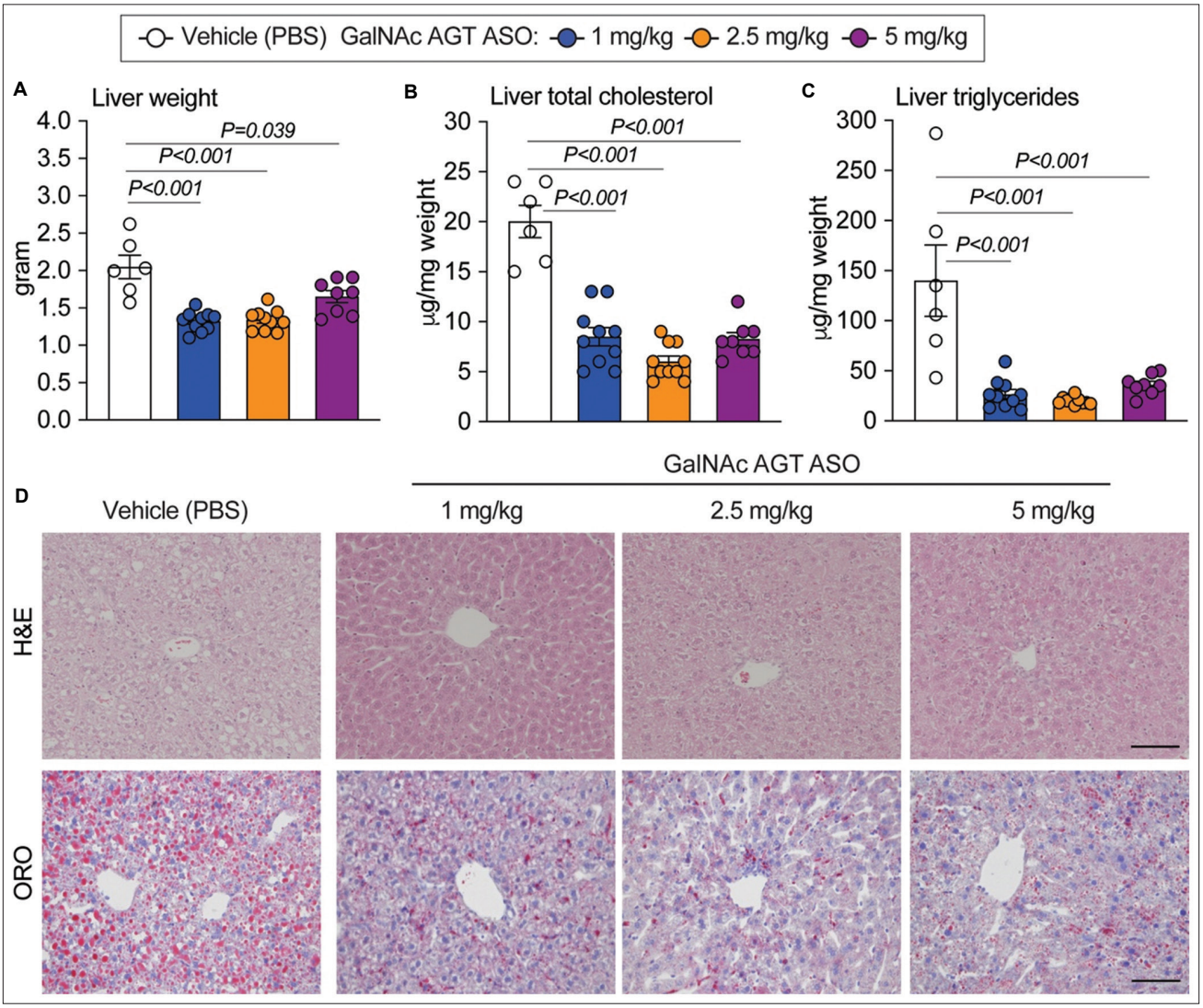


**Figure 2.** Antisense oligonucleotides targeting hepatic angiotensinogen attenuated liver steatosis. (A) Liver weight at termination. (B) Liver total cholesterol concentration (normalized by liver weight). (C) Liver triglycerides concentration (normalized by liver weight). (D) Hematoxylin and eosin (H and E) staining (paraffin-embedded sections) and Oil Red O (ORO) staining (fresh-frozen sections) of liver sections; scale bar = 200 µm. N = 6 – 10 per group. GalNAc AGT ASO: N-acetylgalactosamine-conjugated antisense oligonucleotides targeting angiotensinogen; PBS: Phosphate-buffered saline.

in a dose-independent manner. H&E staining and Oil Red O staining revealed diminished neutral lipid accumulation in mice administered GalNAc AGT ASO at all doses compared to the vehicle group (Figure 2D).

### 3.2. Comparisons between *N*-acetylgalactosamine-conjugated antisense oligonucleotides targeting angiotensinogen and losartan

A single dose of GalNAc AGT ASO reduced plasma AGT maximally within 3 days after the injection (Figure 3A). Therefore, when comparing GalNAc AGT ASO (5 mg/kg) and losartan, we began feeding the Western diet 1 week after the initiation of GalNAc AGT ASO (Figure 3B). GalNAc AGT ASO significantly reduced plasma AGT concentrations, while losartan had no effect (Figure 3C). A similar pattern was observed for hepatic AGT mRNA abundance (Figure 3D). As expected, both drugs increased plasma renin concentrations and renal renin mRNA abundance (Figure 4A and B), but the effects of GalNAc AGT ASO was greater than that of losartan. This coincided with the effect on systolic BP (Figure 4C), which was lowered more significantly by GalNAc AGT ASO. In

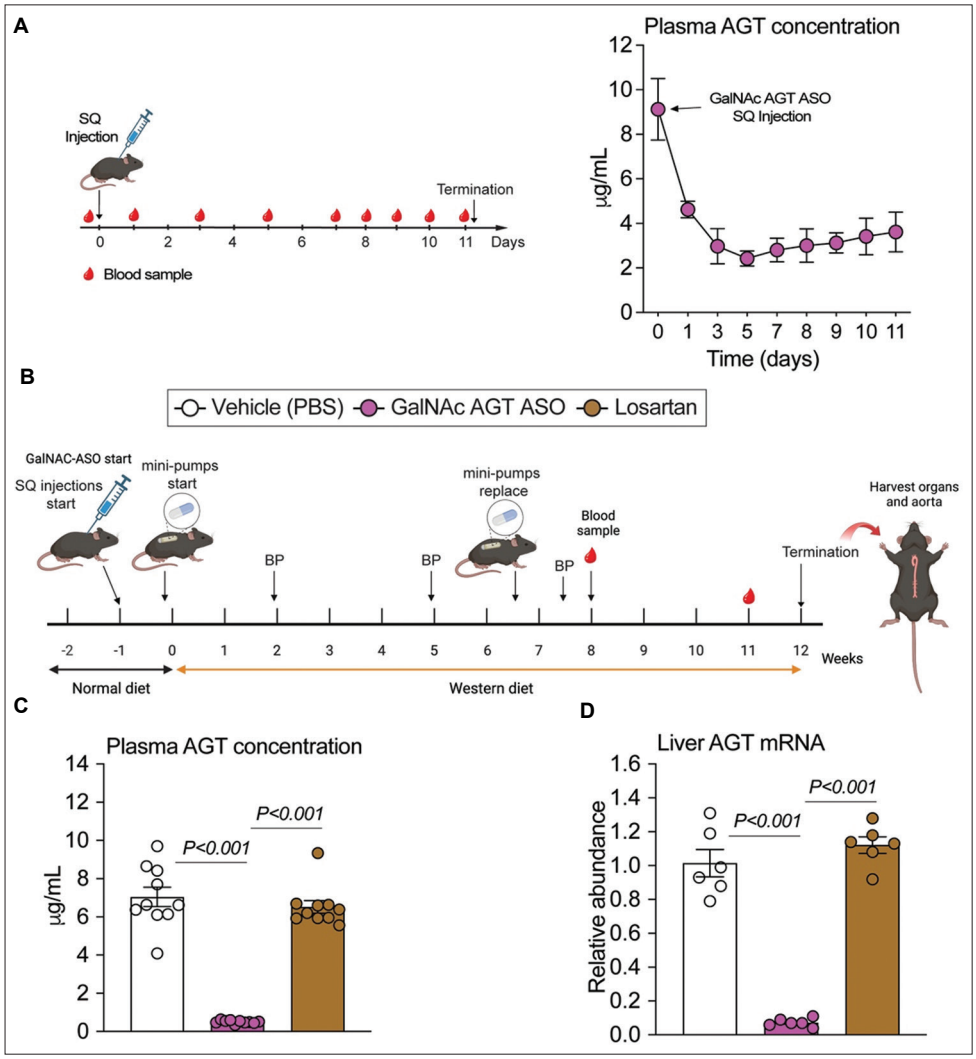


**Figure 3.** Effects of hepatocyte-specific antisense oligonucleotides of angiotensinogen (AGT) versus losartan on AGT protein and mRNA. (A) Experimental protocol for a single dose of GalNAc AGT ASO injection in male C57BL/6J mice. Plasma AGT concentrations were measured using an enzyme-linked immunosorbent assay (ELISA) kit; N = 5. (B) Experimental protocol of GalNAc AGT ASO and losartan comparisons. (C) Plasma AGT concentrations; N = 10 per group. (D) Quantitative polymerase chain reaction of liver AGT mRNA; N = 6 per group.
GalNAc AGT ASO: N-acetylgalactosamine-conjugated antisense oligonucleotides targeting angiotensinogen; PBS: Phosphate-buffered saline; SQ: Subcutaneous.

contrast, both drugs equivalently reduced atherosclerotic lesion areas (Figure 4D and E).

Neither drug increased plasma ALT or AST concentrations (Supplementary File, Figure S1), indicating that GalNAc AGT ASO and losartan do not cause liver damage. Consistent with observations in the first study (Figure 2), GalNAc AGT ASO reduced liver weight and lipid accumulation in the liver (Figure 5). Compared to the vehicle group, losartan did not affect liver weight, nor reduced the total cholesterol, triglycerides, or the severity of neutral lipid accumulation in the liver (Figure 5).

## 4. Discussion

This study demonstrated that GalNAc AGT ASO reduced systolic BP and atherosclerosis in LDL receptor$^{-/-}$ mice fed a Western diet. The effects of GalNAc AGT ASO 5 mg/kg were more pronounced in reducing systolic BP than losartan (15 mg/kg/day). This is likely because this ASO dose induced a greater degree of renin-angiotensin blockade, reflected by the higher renin increase observed after administering GalNAc AGT ASO compared to losartan. Of note, the effects of both drugs on atherosclerosis were not different. Although high BP is an independent

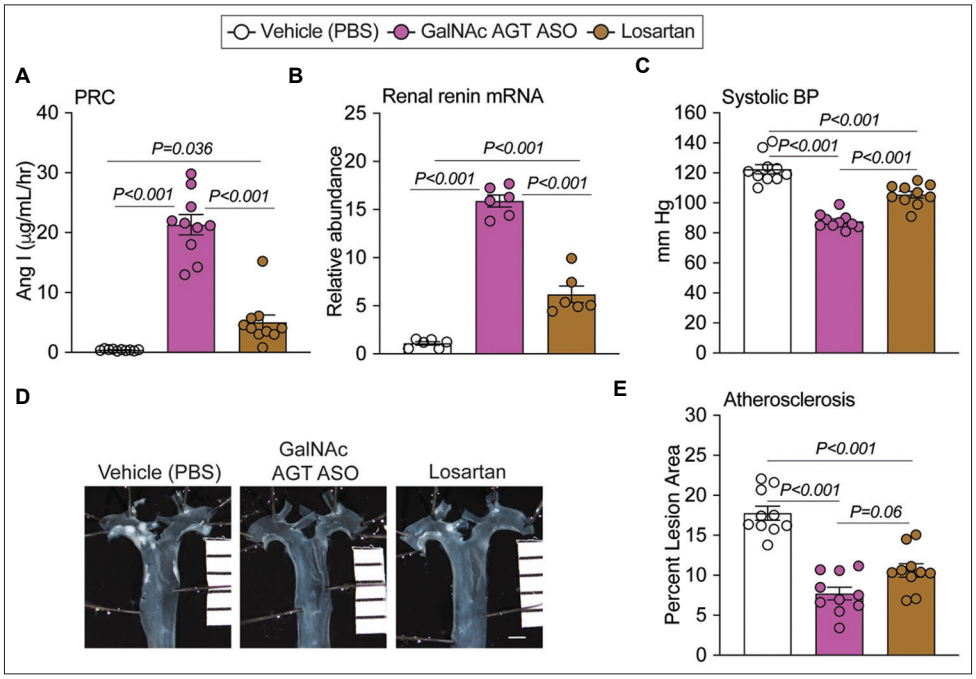


**Figure 4.** Effects of hepatocyte-specific antisense oligonucleotides of angiotensinogen versus losartan on renin, systolic blood pressure, and atherosclerotic lesion size. (A) Plasma renin concentration (PRC); N = 10 per group. (B) Renal renin mRNA abundance by quantitative polymerase chain reaction; N = 6 per group. (C) Systolic blood pressure (BP) at week 7. (D) *En face* aortic images; scale bar = 1 mm. (E) Atherosclerotic lesion area in the thoracic aorta; N = 10 per group.

AngI: Angiotensin I; GalNAc AGT ASO: N-acetylgalactosamine-conjugated antisense oligonucleotides targeting angiotensinogen; PBS: Phosphate-buffered saline.

risk factor for atherosclerosis, there is a growing body of evidence suggesting that BP *per se* does not contribute to atherosclerosis[23]. The different magnitudes of suppressing AGT versus blocking AT1 receptor on BP and atherosclerosis in this study support this notion. Overall, this study unequivocally shows that BP regulation and atherosclerosis in hypercholesterolemic LDL receptor$^{-/-}$ mice are dependent on AngII generated from AGT of hepatic origin, thereby agreeing with the observations made in mice displaying genetic hepatocyte-specific AGT deficiency[8,24,25].

Remarkably, GalNAc AGT ASO, but not losartan, reduced Western diet-induced liver steatosis, which was manifested as increased cholesterol and triglyceride content in the liver. This finding is consistent with our previous studies in hepAGT$^{-/-}$ mice[8,19]. These effects occurred in a dose-independent manner and likely represent the direct consequence of lowering AGT, which occurred following GalNAc AGT ASO administration. Indeed, the expression of des(AngI)AGT induced liver steatosis in hepatocyte-specific AGT deficient mice fed a Western diet[8]. Given the liver-specific activity of GalNAc AGT ASO[14], we would expect non-hepatic AGT to have no effect on liver steatosis.

It is important to note that mice, unlike humans, display a very high turnover of AGT, reflected by lower plasma AGT concentrations than in humans. Hence, the des(AngI)AGT/intact AGT ratio is higher in mice than in humans[26]. Although administration of losartan did not affect AGT and liver steatosis in the present study, earlier studies have reported suppression of liver steatosis in mice administered with RAS inhibitors[27-29]. This may reflect a different and larger degree of renin-angiotensin blockade, potentially lowering AGT[30] and/or affecting the des(AngI)AGT/intact AGT ratio. The molecular mechanism underlying the direct effect of des(AngI) AGT is unknown. It may involve interference with the protein kinase B (Akt)/mammalian target of rapamycin (mTOR)/sterol responsive element-binding protein 1c (SREBP-1c) pathway[19], which is associated with liver steatosis and suppressed in mice with hepatocyte-specific deletion of AGT. It is also unknown whether intact AGT exerts similar effects. Studying this would require the AAV-induced expression of intact AGT in hepAGT$^{-/-}$ mice under complete renin-angiotensin blockade.

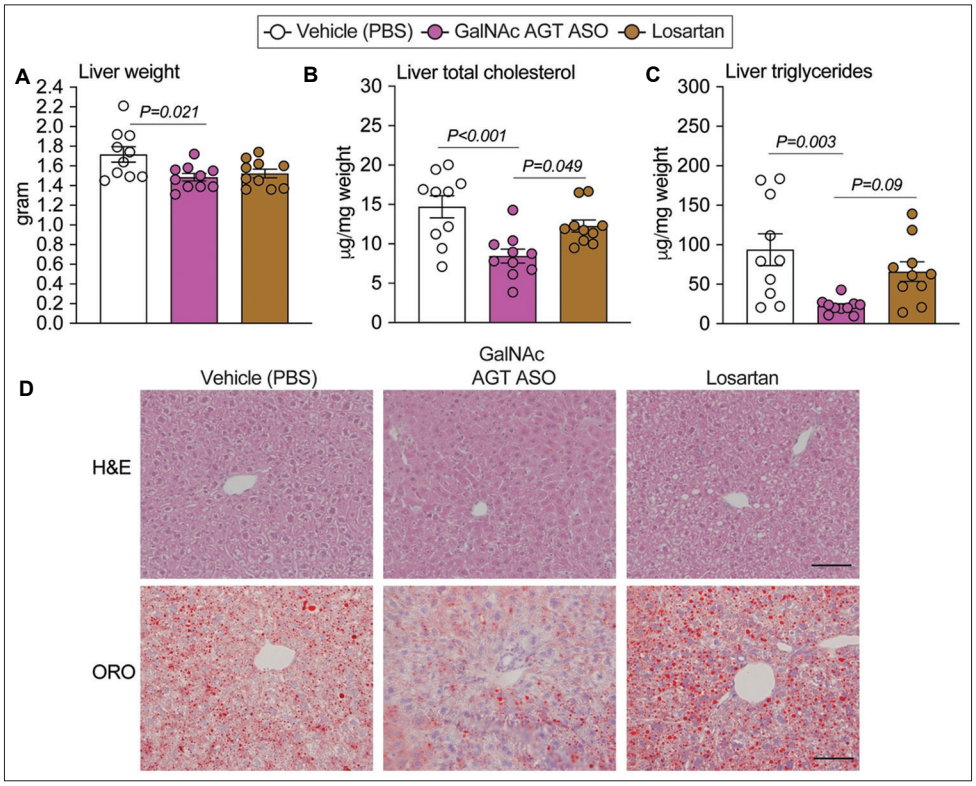


**Figure 5.** Effects of hepatocyte-specific antisense oligonucleotides of angiotensinogen versus losartan on liver steatosis. (A) Liver weight at termination. (B) Liver total cholesterol concentration (normalized by liver weight). (C) Liver triglycerides concentration (normalized by liver weight). (D) Hematoxylin and eosin (H and E) staining and Oil Red O (ORO) staining of liver sections; scale bar = 200 μm. N = 10 per group.
GalNAc AGT ASO: N-acetylgalactosamine-conjugated antisense oligonucleotides targeting angiotensinogen; PBS: Phosphate-buffered saline.

## 5. Conclusions

GalNAc AGT ASO not only reduces BP and atherosclerosis, but also improves diet-induced liver steatosis in mice. Future studies should investigate whether AGT suppression induces similar effects in humans and non-human primates.

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## Conflict of interest

Alan Daugherty and Hong S. Lu have submitted a patent application for use of antisense oligonucleotides targeting angiotensinogen in thoracic aortic aneurysms. Adam E. Mullick is an employee of Ionis Pharmaceuticals, who provided the mouse GalNAc AGT ASO. IONIS-AGT-Lrx and ION904 are both AGT ASO Ionis programs in clinical development. Ionis Pharmaceuticals did not provide any funding for this study. The other authors report no conflicts.

## Author contributions

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*Validation:* Dien Ye, Congqing Wu, Yuriko Katsumata, Hong S. Lu
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## Ethics approval and consent to participate

All animal experiments reported in this article were performed with the approval of the University of Kentucky Institutional Animal Care and Use Committee (IACUC protocol number 2018-2968 or 2015-2050).

## Consent for publication

Not applicable.

## Availability of data

All raw data and analytical methods are available from the corresponding author on appropriate request.

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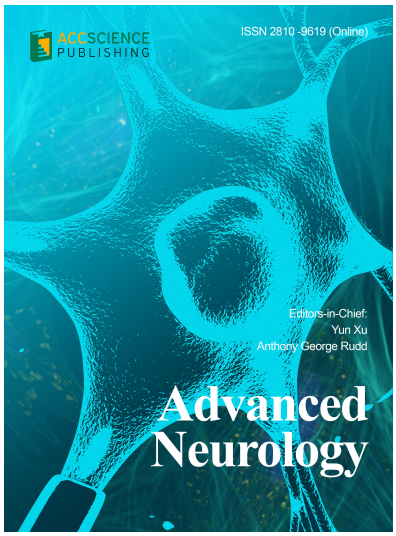


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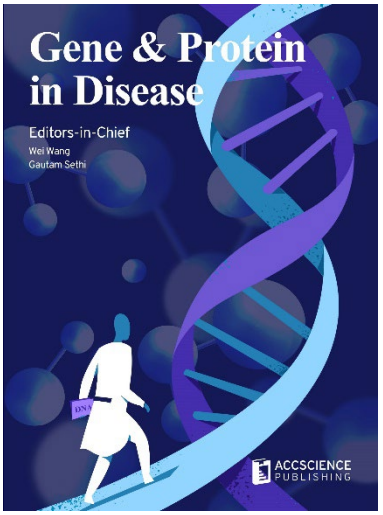


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