ARTERIAL AGING AND AGE-ASSOCIATED ARTERIAL DISEASES

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ARTERIAL AGING AND AGE-ASSOCIATED ARTERIAL DISEASES

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You are only as old as your arteries.
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The underlying mechanisms behind aging has long been a great scientific mystery that the biomedical research community has long studied. In developed nations, as the population grows older, increases in life expectancy is increasingly limited by our relatively poor control of chronic aging-associated diseases such as arterial aging. Among age-related conditions that negatively influence longevity. Growing evidence demonstrates that arterial aging is fundamentally caused by a proinflammatory process that begins at birth and results in detrimental effects that affects every organ system. Vascular aging is the leading risk factor for quintessential cardiovascular diseases such as hypertension and atherosclerosis. With aging, vascular walls develop a pro-inflammatory and stressed microenvironment characterized by both an upregulation of pro-inflammatory chemokines and cytokines as well as a down-regulation anti-inflammatory counterparts. This chronic imbalance in inflammatory

regulatory networks is the key driver of changes at the cellular and molecular level that result in hypertensive and atherosclerotic arterial. Thus, an intervention of proinflammation may be a viable therapeutic intervention to reduce morbidity and mortality from cardiovascular diseases. In this eBook, we aim to supply an updated overview of the physiological and biochemical mechanisms that affect inflammation and vascular aging. We believe that this eBook will contribute to the understanding of the pathogenesis of many chronic or age-related diseases, as well as to their treatment with both current and new therapeutic approaches.

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Editorial: Arterial Aging and Age-Associated Arterial Diseases

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Keywords: aging, arterial remodeling, proinflammation, hypertension, atherosclerosis

Editorial on the Research Topic

Arterial Aging and Age-Associated Arterial Diseases

INTRODUCTION

With advancing age, cellular functions such as the DNA repair, epigenetic modifications, telomere activity, protein folding, mitochondrial respiration, stem cell regeneration, and cellular communication are deviated from their physiological condition, leading to systemic proinflammation. The inflammatory regulatory cascades are the key signaling that drives the cellular changes involved in the adverse arterial wall remodeling thus facilitating the exponential increase in mortality and morbidity related to hypertension and atherosclerosis. In this research topic, titled "arterial aging and age-related arterial diseases," the views of the physiological and biochemical mechanisms that affect vascular proinflammation and inflammatory diseases with aging have been updated. Additional original research articles on molecular and cellular mechanisms, diagnosis, and treatment of arterial adverse remodeling associated with aging have also been included.

Metabolism Disorders and Aging

With advancing age, the glucose metabolism in arterial wall disorders due to insulin resistance, declined growth hormone, and cellular senescence (Strazhesko et al.). Aging increases the deposits of the advanced glycation end-products (AGEs), the long-lived molecules of advanced glycation of extracellular matrix in the basement membrane and interstitial space of cells (Senatus and Schmidt). The basement is thus glycated and as result becomes stiffen and fractured (Senatus and Schmidt). The disrupted and modified basement membrane promotes endothelial proinflammation and dysfunction as well as the synthetic phenotypic shift of vascular smooth muscle cells (VSMCs) (Senatus and Schmidt). The glycated interstitial AGEs interact with their receptor RAGE of vascular cells initiating a cascade of events leading to proinflammation and stiffening of the vasculature (Senatus and Schmidt). Importantly, aging increases the prevalence of the metabolic syndrome and modifies blood coagulation factors increasing vascular inflammation and the risk of thrombosis and atherosclerosis (Lagrange et al.). In addition, apolipoprotein E (APOE) gene exerts important roles in the regulation of lipoprotein metabolism, and its mutation is closely associated with an increased risk of coronary artery disease in individuals with metabolic disorder (Luo et al.).

Inflammation, Calcification, and Arterial Stiffening

Arterial stiffness is a determinant of arterial biological aging rather than arterial chronological aging due in large to genetic and epigenetic factors leading to inflammation and calcification. Aging increases Angiotensin II (Ang II) pro-inflammatory signaling in the arterial wall.

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Ang II modifies the levels of several micro RNA (miR) including miR 21, 22, and 29 (Duggirala et al.; Nanoudis et al.). miR-21 increases arterial fibroblasts survival and extracellular matrix (ECM) deposition (Cheng et al., 2016). miRs-22 promotes VSMC phenotypic shifts from contractile to synthetic phenotype. miR-29 affects the synthesis of elastin and different types of collagen. Thus, Ang II signaling alters the arterial proinflammatory niche modified by miRs, leading to arterial ECM remodeling (Nanoudis et al.).

Arterial calcification is a sequelae of calcium metabolism disorder and inflammation of vascular wall (Nanoudis et al.). Trans-differentiation of VSMCs to a chondrocyte or osteoblast-like phenotype is the main cellular way by which arterial calcification can lead to arterial stiffening (Nanoudis et al.). The medial calcification precedes the atherosclerotic plaque development (Nanoudis et al.). Notably, miRs affect arterial calcification through miR-29-elicited matrix metalloproteinase type II (MMP-2) activation (Nanoudis et al.) and calcium deposition and osteoblast differentiation (Nanoudis et al.).

Arterial stiffening or the extracellular-associated stiffening markedly affects the function of endothelial cells with aging (Kohn et al.). Changes in endothelial cells or the surrounding ECM directly impact endothelial health, such as nitric oxide (NO) production and barrier integrity against leukocyte transmigration (Kohn et al.). VSMCs are durotactic and preferentially migrates toward increased stiffness substrates and ECM chemical cues and proliferates on stiffer matrixes (collagen) (Kohn et al.).

Arterial Stiffening and Hypertension

The age-associated increase in arterial stiffness is well correlated with the incidence of hypertension. In this issue, Kohn et al. comprehensively reviewed the methods of measurement of arterial stiffness at both the macro-and microscale, including the pulse wave velocity macroscale measurement in clinic and microscale atomic force microscopic measurements in the research laboratory (Kohn et al.). As a unique advantage for large epidemiological studies, this issue analyzes the mechanical defects due to arterial aging based on the measured aortic pressure and an assumed triangular flow, paving the way to consider the clinical application of the simplified method for estimating the arterial stiffness (Chang et al.). Arterial intima stiffening measurements have highlighted that hypertension is preceded by arterial stiffening, and elevated arterial stiffening preceded increases in blood pressure in subjects with rheumatoid arthritis (Woodman et al.). This effect is abolished by methotrexate treatment (Woodman et al.).

Aging Atherosclerosis

Atherosclerosis is driven by aging (Head et al.). The mummies studies suggest that atherosclerosis is not a lifestyle dependent disease, rather than, an aging driven disease (Head et al.). Atherosclerosis is a chronic inflammatory, progressive disease with manifestation starting at a young age in children under the age of 10 years and even <1 year old (Head et al.). Aging promotes the dynamic changes of monocytes/macrophages and disorders of lipid. Elderly macrophages assist the living of atheroma and promote the formation of plaques (Head et al.).

Aging Stroke

The incidence of stroke is an early pro-thrombotic phenotype of atherothrombotic events with aging. Such an aspect is reinforced by the pro-coagulant properties of de-differentiated and pro-inflammatory VSMCs due to their increased fibrinogenesis and impaired fibrinolysis and increased saturated fatty acids (Lagrange et al.). Atherothrombotic events are also associated with the metabolic syndrome (Lagrange et al.).

Aging Arterial Dissection/Aneurysms

The dissection and aneurysm are the deadly complications of hypertension and atherosclerosis in the elderly population. Non-coding RNAs, including micro-RNAs and long non-coding RNAs, are recently emerging as important modifiers of gene transcription and cell function (Duggirala et al.). Non-coding RNAs such as miR-21, 29, and 34 are alerted in the arterial wall and involved in the development of arterial aneurysm (Duggirala et al.). Milk fat globule EGF-VIII fragment medin-amyloid, a characterized age-associated arterial amyloid, plays a role in the process of hypertension, atherosclerosis, and aneurysm and dissection (Wang et al.).

Aging Peripheral Arterial Disease

Aging is the major risk factor for the incidence of peripheral arterial disease accompanied by an increase in pulse pressure. Mao et al. performed a prospective cohort observation and investigated the association of pulse pressure with peripheral arterial disease incidence in an elderly general population and they found that the hazard ratio for people with pulse pressure more than 60 mmHg was 2.20 compared with those whose pulse pressure was <40 mmHg (Mao et al.).

Aging Hypertension

Chronic hypertension is one of the most common aging-related disorders. The impairment of endothelial cells function along with the alteration in VSMCs calcium homeostasis plays a fundament role in the development of hypertension. In their review, Eid et al. analyze and discuss the role of different type of Inositol 1,4,5-Trisphosphate Receptors (IP_3R) in the onset and progression of hypertension via their effect on VSMCs physiological functions, such as vascular tone regulation and VSMCs phenotypic switch to proliferative phenotype in age-associated vascular remodeling (Eid et al.).

Anti-aging Molecules

Hydrogen sulfide (H_2S), like a nitric oxygen (NO), may be an anti-inflammatory vascular gaseous mediator. Endogenous H_2S is produced during the cysteine metabolism, which is regulated by the pyridoxal phosphate-dependent enzymes cystathionine β-synthase (CBS) and cystathioine y-lyase (CSE). CSE KO in mice reduces the production of H_2S and predispose the animals to the development of early atherosclerotic lesions, however, supplement of H_2S effectively alleviates the initiation and progression of atherosclerosis (Lin et al.). A further study show that H_2S supplementation in to $ApoE^{-/-}$ mice promotes

the expression of Ang II converting enzyme 2 (ACE2), resulting in a decrease of Ang II in endothelial cells, and eventually diminishing the prevalence and severity of atherosclerosis (Lin et al.).

Apelin is synthesized as 77-amino acid preproapelin and rapidly cleaved into active isoforms, including apelin-13, which is predominantly enriched in the cardiovascular system (Zhou et al.). Apelin-13 is a negative regulator of aging-mediated and Ang II-mediated adverse cardiovascular remodeling and dysfunction via an activation of the apelin receptor (Zhou et al.). Apelin 13 receptor signaling mediates the endothelial-dependent vasodilation (Zhou et al.).

Anti-aging Lifestyles

The regular aerobic exercise effectively alleviates the age-related symptoms and signs. In this issue, Chen et al. report that the habitual exercise improves the aortic endothelial mitochondria function via the activation of an adenosine monophosphate-activated protein kinase α2 (AMPK α2) (Chen et al.). In addition, exercise also improves the trunk flexibility, known as an index of the body stiffness. The flexibility is a predictor of arterial stiffness (Gando et al.). The improvement of the body flexibility reduces the progression of arterial stiffening with aging (Gando et al.).

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REFERENCES

Cheng, M., Wu, G., Song, Y., Wang, L., Tu, L., Zhang, L., et al. (2016). Celastrol-induced suppression of the MiR-21/ERK signalling pathway attenuates cardiac fibrosis and dysfunction. *Cell. Physiol. Biochem.* 38, 1928–1938. doi: 10.1159/000445554

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Growth Hormone, Insulin-Like Growth Factor-1, Insulin Resistance, and Leukocyte Telomere Length as Determinants of Arterial Aging in Subjects Free of Cardiovascular Diseases

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Background: Increased arterial stiffness (AS), intima-media thickness (IMT), and the presence of atherosclerotic plaques (PP) have been considered as important aspects of vascular aging. It is well documented that the cardiovascular system is an important target organ for growth hormone (GH) and insulin-like growth factor (IGF)-1 in humans, and GH /IGF-1 deficiency significantly increases the risk for cardiovascular diseases (CVD). The telomere length of peripheral blood leukocytes (LTL) is a biomarker of cellular senescence and that has been proposed as an independent predictor of (CVD). The aim of this study is to determine the role of GH/IGF-1, LTL and their interaction cardiovascular risk factors (CVRF) in the vascular aging.

Methods: The study group included 303 ambulatory participants free of known CVD (104 males and 199 females) with a mean age of 51.8 ± 13.3 years. All subjects had one or more CVRF [age, smoking, arterial hypertension, obesity, dyslipidemia, fasting

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hyperglycemia, insulin resistance—HOMA (homeostatic model assessment) >2.5, or high glycated hemoglobin]. The study sample was divided into the two groups according to age as "younger" ($m \leq 45$ years, $f \leq 55$ years) and "older" ($m > 45$ years, $f > 55$ years). IMT and PP were determined by ultrasonography, AS was determined by measuring the carotid-femoral pulse wave velocity (c-f PWV) using the SphygmoCor system (AtCor Medical). LTL was determined by PCR. Serum IGF-1 and GH concentrations we measured by immunochemiluminescence analysis.

Results: Multiple linear regression analysis with adjustment for CVRF indicated that HOMA, GH, IGF-1, and LTL had an independent relationship with all the arterial wall parameters investigated in the younger group. In the model with c-f PWV as a dependent variable, $p < 0.001$ for HOMA, $p = 0.03$ for GH, and $p = 0.004$ for LTL. In the model with IMT as a dependent variable, $p = 0.0001$ for HOMA, $p = 0.044$ for GH, and $p = 0.004$ for IGF-1. In the model with the number of plaques as a dependent variable, $p = 0.0001$ for HOMA, and $p = 0.045$ for IGF-1. In the older group, there were no independent significant associations between GH/IGF-1, LTL, HOMA, and arterial wall characteristics.

Conclusions: GH/IGF-1, IR, HOMA, and LTL were the important parameters of arterial aging in younger healthy participants.

Keywords: arterial aging, growth hormone, insulin-like growth factor-1, insulin resistance, leukocytes telomeres length

INTRODUCTION

Cardiovascular disease (CVD) remains a leading cause of death worldwide (Fuster et al., 2011). Steady aging of the population is leading to a significant increase in the number of heart attacks, strokes, and heart failures. More evidence has been obtained on the important prognostic implications of aging-related changes in the structural and functional parameters of the artery wall for the development of cardiovascular events. These changes create a metabolically and enzymatically active environment that promotes the onset and progression of disease.

Age-associated changes in the arteries include increased stiffness of large elastic arteries, for which the "gold standard" measurement method involves the carotid-femoral pulse wave velocity (c-f PWV) (Vlachopoulos et al., 2010). Other changes include an increase in the intima-media thickness (IMT) (Tziomalos et al., 2010) and the presence of atherosclerotic plaques (PP) (Green et al., 2011). Slowing these changes should reduce the risk of clinical CVD. The scientific basis for determining cardiovascular risk factors (CVRF) has increased significantly in recent years, but there is little information about the relationship between conventional risk factors and signs of arterial wall aging in individuals without clinical CVD. There is even less information on the association between signs of arterial wall aging and factors that determine the aging processes in general and in their interaction with CVRF. Factors that influence the aging process include insulin resistance (IR), growth hormone (GH) activity, insulin-like growth factor-1 (IGF-1), and leukocyte telomere length (LTL).

The levels of GH and IGF-1 decrease with age in both laboratory animals and humans (Carter et al., 2002). These hormones are important anabolics that stimulate cell growth, proliferation, and tissue repair. The age-associated decreases of GH and IGF-1 are believed to contribute to the development of many signs of aging, including cardiovascular dysfunction. Cardiomyocytes, smooth muscle cells, and endotheliocytes abundantly express IGF-1 receptors, which makes them highly sensitive to IGF-1 (Chisalita et al., 2009). People with GH deficiency and low circulating IGF-1 levels been documented to have an increased risk of developing CVD and cerebrovascular diseases (Laughlin et al., 2004).

There is a relationship between IR and arterial stiffness in both patients with diabetes and healthy young individuals (Giltay et al., 1999). The role of advanced glycation end-products and the effect of insulin could potentially be important in this context. IR also contributes to the progression of atherothrombosis through the development of dyslipidemia, hyperfibrinogenemia, and increased activity of tissue plasminogen activator inhibitor-1 (Choi et al., 2007).

Several studies have suggested that LTL may be an indicator of the biological age of vessels (Nilsson et al., 2013). Telomeres are the TTAGGG tandem repeats at the ends of chromosomes and progressively shorten with each replication of cultured human somatic cells. LTL reflects both an individual's telomere length at birth and the telomere attrition rates during the course of life, demonstrating the replicative history and cumulative oxidative burden (Aviv, 2004). Individuals with short telomeres in white blood cells are more likely to show accelerated vascular aging (Benetos et al., 2003), atherosclerosis (Samani et al., 2002), coronary heart disease (Fyhrquist et al., 2013) and type 2 diabetes mellitus (T2DM) (Murillo-Ortiz et al., 2012).

One of the least understood issues is the association between risk factors and arterial aging in different age groups. The predictive role of most CVRFs has been proven in studies

involving mostly middle-aged people, and some of these factors are less significant in elderly people (Howard et al., 1997).

The aim of this study is to determine the role of GH/IGF-1, IR, and LTL in the vascular aging process and their interaction with CVRFs in subjects who are free of CVD.

MATERIALS AND METHODS

The study included patients who had passed a preventive outpatient examination at the National Research Center for Preventive Medicine, Moscow, Russia, from 2012 to 2013. To determine eligibility, subjects completed a health screening which included their medical history, physical examination, and a blood sampling for laboratory analyses. We excluded 147 subjects with a previous history of drug medication for diabetes, hypertension, or hyperlipidemia; a history of stroke, coronary heart disease, peripheral arterial disease, arrhythmia, congestive heart failure, or valvular heart disease; hepatic or kidney failure; and cancer. Ultimately, 303 subjects were included in the study. All patients signed a legal informed consent form to participate in the study. The local ethics committee of the National Research Center for Preventive Medicine approved the study protocol.

Patients visited the clinic to undergo the study protocol examinations from 08:00 to 09:00 a.m. after a 12-h period of fasting. Blood pressure (BP) was measured using a brachial cuff (HEM-7200 M3, Omron Healthcare, Kyoto, Japan) on the right hand in a sitting position. The measurements were taken three times for 2-min intervals after a 10-min rest, and the average was used for analysis. Hypertension was diagnosed when BP was >140/90 mmHg.

During the cross-sectional study, anthropometric measurements were carried out with the calculation of the body mass index (BMI) in kg/m^2. Obesity was diagnosed for BMI > 30.0 kg/m^2. Abdominal obesity was indicated by a waist circumference (WC) >80 cm in women and >94 cm in men. The levels of total cholesterol (TC) and triglycerides (TG) were determined by a SAPPHIRE-400 biochemical analyzer (Niigata Mechatronics, Japan) with enzyme kits. The concentration of high-density lipoprotein cholesterol (HDL-C) was determined by the same analyzer in the supernatant after precipitation of serum ApoB-containing lipoproteins. The level of low-density lipoprotein cholesterol (LDL-C) was calculated by the Friedewald equation (when the TG level was no higher than 4.5 mmol/L). Hypercholesterolemia was diagnosed with TC > 5.0 mmol/L. HDL-C was considered to be decreased with a level of <1.2 mmol/L in women and <1.0 mmol/L in men. A level of TG > 1.7 mmol/l was considered to be elevated.

Serum fasting glucose (FG) was determined using routine laboratory methods with the biochemical analyzer. Fasting hyperglycemia was diagnosed when FG was ≥6.1 and <7.0 mmol/l. Serum insulin, GH and IGF-1 were quantified using the chemiluminescent microparticles on an immunoassay analyzer (Architect i 2000SR, Abbot, Canada). HOMA (homeostatic model assessment) was calculated as fasting insulin (mU/ml) × FG (mmol/l)/22.5. The level of glycated hemoglobin (HbA1c) was determined by the immunoturbodimetric method using the SAPPHIRE-400 analyzer.

T2DM was diagnosed when fasting glucose was ≥7.0 mmol/l and ≥11.1 mmol/l within 2 h after taking 75 g of glucose or HbA1c ≥ 6.5%. Participants were considered smokers if they had smoked more than 100 cigarettes during their entire life and if they smoked at the time of the study, whether every day or occasionally. Family history of CVD was considered if cases of CVD had been documented in first-degree relatives under the age of 65 years in women and 55 years in men. Age beyond 45 years for men and 55 years for women was considered a CVRF (National Cholesterol Education Program (NCEP) Expert Panel on Detection, 2002).

LTL was determined according to the method described by Cawthon (2002). Genomic deoxyribonucleic acid (DNA) was extracted directly from blood samples by standard procedures ($OD_{260nm/280nm}$ 1.8–1.9). The assay involved comparing the abundance of telomere DNA to the single-copy genomic DNA number for each sample and by further comparison of the normalized values between DNA from different sources. The ratio of the telomere (T) and single-copy *36B4* gene matrices reflects the length of telomeres (the T/S ratio is approximately $[2^C_t\ (\text{telomeres})/2^C{}_t(36B4)]^{-1} = 2^{-\Delta C}{}_t$ [T1]).

A 1.25× stock mix was also prepared [1× mixture: PCR buffer 1× (Fermentas 10× PCR Hotstartbuf + KCl), 2 mM $MgCl_2$, 0.2 mM dNTP, 0.5 μM of each primer, 0.05 units/μl of Taq polymerase Maxima (Fermentas), and Sybr Green I 0.2×]. The primer sequences were the following: Tel1, GGTTTTTGAGG GTGAGGGTGAGGGTGAGGGTGAGGGT, Tel2, TCCCGACT ATCCCTATCCCTATCCCTATCCCTATCCCTA, 36B4u, CAA GTGGGAAGGTGTAATCC, 36B4d, CCCATTCTATCATCAA CGGGTACAA. Sixteen microliters of master mix were added to each sample well, along with 4 μl of the analyzed genomic DNA with a concentration of 10 ng/μl. Samples were mixed, centrifuged, and amplified in a CFX96 thermocycler.

For polymerase chain reaction (PCR) of the telomeres, we heated the samples at 95°C for 5 min and applied 35 cycles of 95°C for 20 s, and 54°C for 2 min, followed by melting. For the control PCR, we heated the samples at 95°C for 5 min and then applied 35 cycles of 95°C for 20 s, and 58°C for 1 min, followed by melting. The amplification of the corresponding telomeric and control mixtures occupied one cell unit. For each sample, we performed three repeated telomeric reactions and three control reactions. We calculated the difference between cycle thresholds of amplification of the telomere and single copy of the gene (ΔC_t) and determined the relative telomere lengths based on the results.

The genomic DNA of the HEK cell line and a control leukocyte sample was used as a reference point. To take into account the differences in PCR mixtures from time to time, we set the leukocyte reference $\Delta C_{t(leu)}$ value at 8. The relative exponential length L was set as $L = \Delta C_t\text{-}(\Delta C_{t(leu)}\text{-}8)$. We did not obtain the absolute value of the lengths of telomeres, so we used the standard deviation as a measure of the spread of values. In our experiment, the standard deviation in almost all cases was in the range of 0.1–0.4 derived from the relative lengths of 8.30–11.39 (logarithmic scale).

Arterial stiffness was assessed according to the c-f PWV values. It was measured using SphygmoCor 8.0 hardware (Atcor, Sydney) with the help of an applanation tonometer and

electrocardiogram (ECG) gating to obtain pulse waves from both proximal sites (carotid artery) and distal sites (femoral artery). The c-f PWV was calculated from the transit time between the two sites relative to the R-wave within the electrocardiogram complex using the "foot-to-foot method" and the intersecting tangent algorithm (Rajzer et al., 2008). In each subject, two sequences of measurements were performed, and their mean was considered for analysis. The repeatability coefficient value was 0.935. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured with a manual sphygmomanometer. The mean of three consequent readings was recorded. PWV values >10 m/s were considered as elevated (Van Bortel et al., 2012).

Ultrasound examination of the carotid arteries was performed in B-mode by a linear high-resolution 17-5 MHz sensor (PHILIPS iU22, Netherlands). The studies were conducted by one operator. The atherosclerotic plaque was defined as thickening of focal vessel wall by more than 50% compared to the surrounding vessel wall areas or as a focal thickening of the intima-media complex by more than 1.3 mm protruding into the vessel lumen (Touboul et al., 2007). All measurements were performed in diastole, which corresponded to the R-wave on the electrocardiogram. PP was evaluated at six sites of the carotid arterial system: both common carotid arteries, both bifurcations, and both internal carotid arteries. The total number of all plaques was also determined. The average IMT was measured in automatic mode on both sides in the longitudinal section in the distal third of the common carotid at a distance of 1 cm proximal to the bifurcation. The posterior-wall IMT was measured as the distance between the vessel lumen and adventitia. The larger of the values on the right and left sides was taken into account. IMT values >0.9 mm were considered to indicate thickened walls (Perk et al., 2012).

Statistical Analysis

Statistical processing of the results was carried out using the statistical software package SAS 9.1 (SAS Institute, Cary, NC, USA). The mean value (M) and standard deviation (SD) were used to describe the quantitative indicators in the case of normal distributions, while the median and upper (UQ) and lower quartiles (LQ) were used in the case of abnormal distributions. To compare two groups with normal sample distribution the paired Student's *t*-test was used, for the abnormal distribution of variables the Mann-Whitney criterion was used. To compare the proportions Fisher's exact test was used. For the statistical description of the relationship between different parameters, Spearman's rank correlation analysis and intergroup comparison were conducted. Linear regression models were created to reveal the independence of the association between the risk factors and the parameters of the arterial wall. The vascular wall parameters were considered as dependent variables. The independent variables were indicators that demonstrated statistically significant associations with the vascular wall parameters in the correlation analysis and intergroup comparison. To assess the associations between the risk factors and the vascular wall parameters the logistic regression analysis was also used. Statistical significance level was set to $p < 0.05$.

RESULTS

We assumed that the nature of the relationship between the studied indicators and the artery wall parameters may differ between younger and older ages, which is why separate analyses were conducted for these groups. The younger group included men of 45 years of age and under and women of 55 years of age and under, while the older subjects formed the older group. The clinical characteristics of patients are presented in **Table 1**. In the older group, the SBP values were higher, the metabolic status indicators (WC, BMI, TC, LDL-C, TG, FG, HOMA) were worse, and levels of GH and IGFR-1 were lower, which are consistent with existing representations.

A comparison of the arterial wall characteristics in both groups is presented in **Table 2**. In the older group, the arterial stiffness and IMT were higher, there were more atherosclerotic plaques, and values of the studied parameters associated with an increased risk of CVD were more common. The results of Spearman's correlation analysis of the association of arterial wall parameters and conventional CVRF, GH, IGF-1, LTL, and HOMA are presented in **Table 3**. In the general group, the arterial wall characteristics are associated with almost all studied parameters.

Taking into account the available data on the decrease of the role of conventional CVRF with age (Howard et al., 1997), we separately analyzed the relationship between the studied parameters and the vascular wall characteristics in the two age groups (see **Table 4**). Notably, almost all parameters demonstrated an association with the arterial wall characteristics in the younger group. However, some of them lost the connection with arterial wall characteristics in the older group. This also applies to GH, IGF-1, and HOMA. The strength of the association, even if it is preserved, is weaker in the older group.

To identify the independent relationships of some of the clinical and biological factors with c-f PWV, IMT, atherosclerotic plaque number (APN), linear regression models were compiled using the arterial wall indicators as a dependent variable. Parameters that demonstrated a statistically significant relationship with the vascular wall in the correlation analysis were used as independent variables. Thus, to create a forecast model for each dependent variable, a set of explanatory variables was formed. Only statistically significant variables were presented in the final regression equation. Considering the previously identified differences between the younger and older groups in listed factors associated with a particular arterial wall parameter, the regression models were performed for not only the general group, but separately for older and younger groups.

In the general group, only c-f PWV as a dependent variable was associated with GH, IGF-1, and LTL (see **Table 5**). A model that evaluated age, $HbA_{1c,}$ LTL, HOMA, GH, and IGF-1 explained 36% of the c-f PWV variability. IMT and the APN did not demonstrate this relationship. In the younger group, HOMA, GH, and IGF-1 demonstrated an independent relationship with all the studied arterial wall parameters, but LTL only had a relationship with c-f PWV. The highest coefficient of $R^2 = 0.45$ was in the IMT variability model. The results of multiple linear regression analysis (with adjustment for CVRF) in the younger

TABLE 1 | Characteristics of the patients included in the study ($n = 303$).

Characteristic	All patients ($n = 303$)	Younger group ($n = 144$)	Older group ($n = 159$)	p
Age (years), $M \pm SD$	51.5 ± 13.3	40.9 ± 8.7	61.1 ± 8.5	< 0.001
Men (%)	34%	26.4	41.5	< 0.001
Smoking (%)	19.1	20.8	17.6	0.478
Arterial hypertension (%)	25.4	17.4	32.0	0.003
Hypercholesteremia (%)	66.3	61.1	71.1	0.068
Fasting hyperglycaemia (%)	22.4	10.4	33.3	< 0.001
T2DM (%)	16.5	5.6	26.4	< 0.001
Obesity (%)	24.4	23.6	25.2	0.754
Family history of CVD (%)	21.0	24.3	18.0	0.182
SBP (mmHg), $M \pm SD$	125.4 ± 16.4	120.6 ± 15.3	129.7 ± 16.2	< 0.001
DBP (mmHg), $M \pm SD$	78.2 ± 10.2	77.5 ± 10.4	78.9 ± 10.1	0.250
WC (cm), $M \pm SD$	89.6 ± 15.3	85.8 ± 15.3	93 ± 14.5	< 0.001
BMI (kg/m2), $M \pm SD$	27.4 ± 5.1	26.7 ± 5.6	27.9 ± 4.6	0.041
TC (mmol/l), $M \pm SD$	5.7 ± 1.2	5.4 ± 1.0	5.9 ± 1.2	< 0.001
LDL-C (mmol/l), $M \pm SD$	3.9 ± 1.1	3.7 ± 0.9	4.0 ± 1.4	0.008
HDL-C (mmol/l), $M \pm SD$	1.2 ± 1.3	1.2 ± 0.3	1.2 ± 0.3	0.208
TG (mmol/l), Med (LQ-UQ)	1.04 (0.76–1.51)	0.92 (0.63–1.37)	1.2 (0.86–1.67)	< 0.001
Fasting glucose (mmol/l)	5.8 ± 1.4	5.3 ± 1.0	6.1 ± 1.6	< 0.001
HOMA, Med (LQ-UQ)	1.83 (1.31–2.93)	1.7 (1.19–2.52)	2.03 (1.41–3.22)	0.01
HbA1c (%), $M \pm SD$	5.48 ± 0.96	5.17 ± 0.71	5.77 ± 1.06	< 0.001
Insulin (mIU/L), $M \pm SD$	9.07 ± 4.71	8.32 ± 4.66	10.06 ± 5.69	0.134
CRP (mg/l), Med (LQ-UQ)	2.3 (1.6–3.9)	2.1 (1.5–3.1)	2.7 (1.7–4.2)	0.257
GH, ng/ml, Med (LQ-UQ)	0.49 (0.12–2.0)	0.67 (0.11–2.84)	0.39 (0.12–1.06)	0.006
IGF-1, ng/ml, $M \pm SD$	150.8 ± 58.6	161.9 ± 63.2	141 ± 52.6	0.004
LTL, $M \pm SD$	9.77 ± 0.50	9.93 ± 0.48	9.63 ± 0.46	< 0.001

BMI, body mass index; CRP, high sensitive C-reactive protein; CVD, cardiovascular diseases; DBP, diastolic blood pressure; FG, fasting glucose; GH, growth hormone; HbA1c, glycated hemoglobin; HDL-C, cholesterol of high-density lipoproteins; HOMA, insulin resistance index; IGF-1, insulin-like growth factor 1; LDL-C, cholesterol of low-density lipoproteins; LTL, leukocytes telomere length; SBP, systolic blood pressure; TC, total cholesterol; TG, triglycerides; T2DM, diabetes mellitus type 2; WC, waist circumference; p, p-value between younger and older groups.

TABLE 2 | The characteristics of arterial wall in both age groups ($n = 303$).

	All patients ($n = 303$)	Younger group ($n = 144$)	Older group ($n = 159$)	p
c-f PWV (m/s), $M \pm SD$	8.8 ± 2.1	7.9 ± 1.7	9.6 ± 2.1	<0.001
c-f PWV > 10 m/s (n, %)	88 (31.2)	20 (14.9)	68 (46)	<0.001
IMT (mm), $M \pm SD$	0.75 ± 0.19	0.64 ± 0.15	0.86 ± 0.17	<0.001
IMT > 0.9 mm (n,%)	67 (23.5)	11 (8.1)	56 (37.3)	<0.001
AP (n), $M \pm m$	1.09 ± 0.09	0.35 ± 0.07	1.77 ± 0.13	<0.001
PP (n, %)	138 (48.3)	30 (22.1)	108 (72)	<0.001

AP, atherosclerotic plaques; IMT, intima-media thickness; c-f PWV, carotid-femoral pulse wave velocity; PP, plaques presence; p, p-value when comparing the younger and older groups.

group are presented in **Table 6**. In contrast, there were no independent significant associations of GH/IGF-1 and arterial wall characteristics obtained through multiple linear regression analysis in the older group.

A logistic regression method was used to evaluate the associations between the studied parameters and vascular wall characteristics. The selection of predictors was carried out based on the linear regression models. The method of stepwise exclusion of predictors was used. Only statistically significant predictors ($p < 0.05$) were retained in the final model. The relative contribution of individual predictors was determined by the value of the Wald chi-squared statistics. Binary indicators based on median hormone levels were used as characteristics of the hormonal status: the median GH was 0.50 ng/ml and the median IGF-1 was 140 ng/ml. The median of individual LTL values was 9.75. Telomeres were considered to be short if their length was less than this value and long if TL ≥ 9.75. Long telomeres were observed in 156 people, while 141 people had short telomeres. Telomeres were considered to be the shortest if LTL was <9.25, which corresponded to the first quartile of distribution (Q1) ($n = 35$). Telomeres were considered the longest with LTL > 10.25 (IV quartile – QIV) ($n = 42$).

Logistic regression analysis in the general group showed that the probability of having stiff arteries (c-f PWV > 10m/s) increased with HOMA > 2.5 and the presence of short telomeres (**Table 7**). In the younger group the results of logistic regression analysis were statistically significant for only the PP. The probability of having atherosclerotic plaques was increased in Q1 TL. IGF-1 greater than the median was associated with a five-fold lower probability of atherosclerotic plaques being present (**Table 8**).

The results indicate that the level of hormones (GH, IGF-1) and HOMA index were significantly related to the vascular wall

parameters in the general and younger groups but not the older group. These data once again confirm the idea of a decrease in the role of certain factors in the older group in comparison with the younger one. Differences between older and younger people can be explained by the fact that people whose risk factors led to subclinical changes at a younger age after reaching old age had clinical manifestations of CVD and could not become participants in our study.

TABLE 3 | Association between the arterial wall parameters and studied indicators in the general group.

	c-f PWV	IMT	AP
Age	0.45 ($p < 0.001$)	0.68 ($p < 0.001$)	0.58 ($p < 0.001$)
SBP	0.35 ($p < 0.001$)	0.39 ($p < 0.001$)	0.36 ($p < 0.001$)
DBP	0.22 ($p < 0.001$)	0.18 ($p = 0.003$)	0.16 ($p = 0.008$)
WC	0.31 ($p < 0.001$)	0.34 ($p < 0.001$)	0.20 ($p < 0.001$)
BMI	0.28 ($p < 0.001$)	0.29 ($p < 0.001$)	0.17 ($p = 0.003$)
TC	0.22 ($p < 0.001$)	0.25 ($p < 0.001$)	0.21 ($p < 0.001$)
LDL-C	0.17 ($p = 0.004$)	0.20 ($p < 0.001$)	0.18 ($p = 0.003$)
HDL-C	−0.13 ($p = 0.028$)	−0.16 ($p = 0.007$)	−0.12 ($p = 0.04$)
TG	0.38 ($p < 0.001$)	0.44 ($p < 0.001$)	0.30 ($p < 0.001$)
FG	0.37 ($p < 0.001$)	0.35 ($p < 0.001$)	0.31 ($p = 0.015$)
HOMA	0.33 ($p < 0.001$)	0.29 ($p < 0.001$)	0.18 ($p = 0.003$)
GH	−0.17 ($p = 0.007$)	−0.13 ($p = 0.034$)	0.006 ($p = 0.929$)
IGF-1	−0.05 ($p = 0.462$)	−0.023 ($p < 0.001$)	−0.22 ($p < 0.001$)
LTL	−0.33 ($p < 0.001$)	−0.30 ($p < 0.001$)	−0.29 ($p < 0.001$)

The results of the Spearman's correlation analysis (n = 303). BMI, body mass index; DBP, diastolic blood pressure; FG, fasting glucose; GH, growth hormone; HDL-C, cholesterol of high-density lipoproteins; HOMA, insulin resistance index; IGF-1, insulin-like growth factor 1; LDL-C, cholesterol of low-density lipoproteins; LTL, leukocytes telomere length; SBP, systolic blood pressure; TC, total cholesterol; TG, triglycerides; WC, waist circumference; p, p-value between younger and older groups.

GH was associated with c-f PWV and IMT, while IGF-1 was associated with APN and IMT. The association of these hormones with IMT was higher than with other parameters of the vascular wall. It is important that the negative relationship testifies to the protective role of GH and IGF-1 for pathological changes in the arteries. The HOMA index was associated with all arterial wall parameters. LTL is associated with both increased arterial stiffness and subclinical atherosclerosis. It can be traced both in the younger and general groups. The obtained results determine potential therapeutic targets to prevent arterial changes.

DISCUSSION

We consider that the first important result of this work is the identification of the significant association of GH/IGF- 1 (independently of CVRF) with the main arterial wall parameters in healthy people without hypopituitarism (HP). The role of IGF-1 in cardiovascular pathology was firstly observed in studies of the causes of death in patients with pituitary diseases. Epidemiological studies have shown that in patients with a long period of IGF-1 deficiency and somatotropic insufficiency, cardiovascular mortality was two times higher than in the general population (Higashi et al., 2012). GH/IGF-1 deficiency leads to physiological age-related changes in the cardiovascular system, such as a decrease in the cardiomyocyte number, fibrosis, collagen accumulation, and decreases in the synthesis of proteins, including contractile actin and myosin (LeRoith et al., 1995).

Rosén and Bengtsson (1990) were the first to demonstrate a reduction in the quality of life in patients with HP. They analyzed data from 333 patients with HP who visited endocrinology clinics between 1956 and 1987. During the follow-up period,

TABLE 4 | Associations between the arterial wall characteristics and studied parameters in different age groups.

	c-f PWV		IMT		AP	
	Younger group	Older group	Younger group	Older group	Younger group	Older group
Age	0.48 ($p < 0.001$)	0.05 ($p = 0.477$)	0.52 ($p < 0.001$)	0.43 ($p < 0.001$)	0.43 ($p < 0.001$)	0.27 ($p < 0.001$)
SBP	0.25 ($p = 0.003$)	0.21 ($p = 0.011$)	0.38 ($p < 0.001$)	0.23 ($p = 0.004$)	0.25 ($p = 0.003$)	0.29 ($p < 0.001$)
DBP	0.31 ($p < 0.001$)	0.07 ($p = 0.371$)	0.35 ($p < 0.001$)	0.04 ($p = 0.645$)	0.20 ($p = 0.020$)	0.15 ($p = 0.074$)
WC	0.29 ($p < 0.001$)	0.12 ($p = 0.131$)	0.47 ($p < 0.001$)	0.02 ($p = 0.767$)	0.21 ($p = 0.016$)	−0.02 ($p = 0.8$)
BMI	0.36 ($p < 0.001$)	0.12 ($p = 0.157$)	0.46 ($p < 0.001$)	0.05 ($p = 0.562$)	0.20 ($p = 0.019$)	0.02 ($p = 0.8$)
TC	0.30 ($p < 0.001$)	0.06 ($p = 0.456$)	0.36 ($p < 0.001$)	0.05 ($p = 0.561$)	0.20 ($p = 0.018$)	0.10 ($p = 0.240$)
LDL-C	0.25 ($p = 0.004$)	0.02 ($p = 0.807$)	0.33 ($p < 0.001$)	−0.02 ($p = 0.849$)	0.18 ($p = 0.033$)	0.06 ($p = 0.437$)
HDL-C	−0.12 ($p = 0.157$)	−0.05 ($p = 0.567$)	−0.27 ($p = 0.001$)	−0.03 ($p = 0.747$)	−0.18 ($p = 0.033$)	−0.04 ($p = 0.665$)
TG	0.43 ($p < 0.001$)	0.22 ($p = 0.006$)	0.60 ($p < 0.001$)	0.22 ($p = 0.009$)	0.31 ($p < 0.001$)	0.12 ($p = 0.158$)
FG	0.33 ($p < 0.001$)	0.23 ($p = 0.004$)	0.21 ($p = 0.015$)	0.20 ($p = 0.015$)	0.18 ($p = 0.038$)	0.18 ($p = 0.025$)
HOMA	0.35 ($p < 0.001$)	0.24 ($p = 0.005$)	0.39 ($p < 0.001$)	0.14 ($p = 0.102$)	0.21 ($p = 0.017$)	0.11 ($p = 0.19$)
GH	−0.23 ($p = 0.014$)	−0.008 ($p = 0.919$)	−0.27 ($p = 0.003$)	0.06 ($p = 0.476$)	−0.001 ($p = 0.989$)	0.06 ($p = 0.505$)
IGF-1	−0.12 ($p = 0.200$)	−0.15 ($p = 0.081$)	−0.34 ($p < 0.001$)	−0.05 ($p = 0.556$)	−0.27 ($p = 0.003$)	−0.10 ($p = 0.209$)
LTL	−0.36 ($p = 0.0001$)	−0.15 ($p = 0.112$)	−0.08 ($p = 0.543$)	−0.06 ($p = 0.434$)	−0.13 ($p = 0.137$)	−0.03 ($p = 0.637$)

The result of the Spearman's correlation analysis.

BMI, body mass index; DBP, diastolic blood pressure; FG, fasting glucose; GH, growth hormone; HDL-C, cholesterol of high-density lipoproteins; HOMA, insulin resistance index; IGF-1, insulin-like growth factor 1; LDL-C, cholesterol of low-density lipoproteins; LTL, leukocytes telomere length; SBP, systolic blood pressure; TC, total cholesterol; TG, triglycerides; WC, waist circumference.

TABLE 5 | Stepwise multiple linear regression analysis of c-f PWV as dependent variable in general group (with adjustment for CVRF).

Predictor	β ± *SE*	Type II SS	*p*	R^2
Intercept	12.569 ± 3.852	48.853	0.001	
Age	0.076 ± 0.013	146.890	0.0001	
HbA1c	0.408 ± 0.167	27.438	0.015	
HOMA	0.182 ± 0.075	26.985	0.016	
GH	−0.153 ± 0.065	25.986	0.018	
IGF-1	−0.005 ± 0.003	29.118	0.013	
LTL	0.896 ± 0.356	17.424	0.053	0.3595

GH, growth hormone; HbA1c, glycated hemoglobin; HOMA, insulin resistance index; IGF-1, insulin-like growth factor 1; LTL, leukocytes telomere length; S.E., Standard error; Type II SS, type II sum of squares.

TABLE 6 | Stepwise multiple linear regression analysis (with adjustment for CVRF) of c-f PWV, IMT, Number of Plaques as dependent variables in the younger group.

Predictor	β ± *SE*	Type II SS	*P*	Model R^2
c-f PWV – DEPENDENT VARIABLE				
Intercept	22.871 ± 4.678	70.130	0.0001	
HOMA	0.371 ± 0.108	34.541	< 0.001	
GH	−0.138 ± 0.062	14.185	0.03	
LTL	−1.370 ± 0.468	25.130	0.004	0.2868
IMT – DEPENDENT VARIABLE				
Intercept	0.582 ± 0.052	1.430	0.0001	
GH	−0.009 ± 0.005	0.044	0.056	
IGF-1	−0.001 ± 0.0001	0.102	0.004	
HOMA	0.041 ± 0.007	0.399	0.0001	0.4528
NUMBER OF PLAQUES – DEPENDENT VARIABLE				
Intercept	0.254 ± 0.198	0.641	0.203	
IGF-1	−0.002 ± 0.001	1.615	0.045	
HOMA	0.192 ± 0.034	12.130	0.0001	0.2920

GH, growth hormone; IMT, intima-media thickness; HbA1c, glycated hemoglobin; HOMA, insulin resistance index; IGF-1, insulin-like growth factor 1; LTL, leukocytes telomere length; c-f PWV, carotid-femoral pulse wave velocity; SE, Standard error; Type II SS, type II sum of squares.

104 patients died, which corresponded to a significantly higher mortality rate compared to the general population. The excess mortality was related to deaths from CVD, and the most common causes were myocardial infarction (MI), coronary artery disease (CAD), congestive heart failure, and cerebrovascular disease. Subsequently, the relationship between the level of GH/IGF-1 and the prognosis of CVD was revealed in non-HP patients.

In a cross-sectional study, Spallarossa et al. showed that a low level of IGF-1 was associated with angiographically confirmed CAD (Spallarossa et al., 1996). A prospective case-control study observed 600 participants for 15 years and showed that the level of IGF-1 below the median increased the risk of CAD (Juul et al., 2002). The level of IGF-1 predicted death from CAD in 1,185 people of both sexes in a study conducted by Laughlin et al. (2004) A low level of IGF-1 was associated with a worse prognosis in the early period of MI (Conti et al., 2001).

The explanation of these relationships may lie in the connection of the hormone levels with the arterial wall, even at subclinical changes. Thus, flow-dependent vasodilatation of peripheral arteries was impaired in patients with GH deficiency (Smith et al., 2002). Galderisi et al. revealed a positive relationship between IGF-1 and coronary reserve (Galderisi et al., 2002). A cross-sectional study on 400 elderly men conducted by van Den Beld et al. revealed an inverse correlation between IGF-1 and IMT (van den Beld et al., 2003). These results become clear when considering that the cardiovascular system is the target of GH and IGF-1. Smooth muscle cells (SMCs) and endotheliocytes abundantly express IGF-1 receptors (Chisalita and Arnqvist, 2004). IGF-1 is a powerful mitogenic, anti-apoptotic, and promigrant factor for both endotheliocytes and SMC (Arnqvist, 2008). That is, IGF-1 can be pro-atherogenic by its ability to stimulate the migration and proliferation of SMC and macrophage migration (Renier et al., 1996) to promote the expression of adhesion molecules (Li et al., 2009). On the other hand, a decrease in the level of IGF-1 can cause plaque destabilization (Libby and Sasiela, 2006).

In our study, IGF-1 apparently played a protective role in plaque formation, arterial wall thickening, and increasing stiffness. These effects can be associated with pronounced anti-inflammatory and antioxidant properties of IGF-1 and its ability to enhance reparative mechanisms (Sukhanov et al., 2007), primarily in the endothelium. Most clinical studies support the concept that normal levels of GH and IGF-1 are necessary to maintain endothelial health. IGF-1 is involved in the synthesis of nitrogen monoxide (NO) in endothelial cells, causing additional vasodilation of the arteries. This leads to a decrease in the concentration of free fatty acids (FFA) and an increase in sensitivity to native insulin. Our studies are consistent with others indicating that patients with GH deficiency and low circulating IGF-1 had an increased risk of CVD (Vasan et al., 2003; Conti et al., 2004; Laughlin et al., 2004).

It is impossible not to recall contradictory results on the role of GH/IGF-1 in life expectancy. In some, not all, studies on rodents, it was shown that a decrease in the activity of GH/IGF-1 leads to an increase in life expectancy (Brown-Borg et al., 1996; Coschigano et al., 2000; Flurkey et al., 2001). The most important question remains the following: "Does the deficiency of these hormones in humans increase the life expectancy like animals?" It should be noted that although there are isolated cases when patients with dwarfism lived longer than their peers, the overall life expectancy of people with dwarfism did not differ from healthy ones, which was shown in patients with Laron's syndrome (a hereditary disease with an autosomal recessive type of inheritance-variety dwarfism caused by a congenital defect in the GHR receptor gene), leading to insensitivity of peripheral tissues to the action of GH (Laron, 2005). A meta-analysis of 12 studies, including 14,906 people, showed that there is a U-shaped curve of the relationship between the level of IGF-1 and overall mortality. Low levels of IGF-1 are associated with higher mortality due to the increase in cardiovascular mortality, high IGF-1 values are associated with higher cancer mortality (Burgers et al., 2011). Thus, the data from clinical studies do not support the concept of the role of GH/ IGF-1 in increasing the life expectancy that was observed in invertebrates and rodents. It can be assumed that the signaling pathway GH/ IGF-1 is

TABLE 7 | Results of logistic regression analysis in general group where c-f PWV > 10 m/s is dependent variable.

Predictor	β ± *SE*	χ^2 Wald statistic	*p*	OR	95% CI
Model 1. Predictors: Old age, male sex, ↑SBP, ↑HOMA, Fasting hyperglycemia, ↑TG, LTL< 9.75					
Old age	1.521 ± 0.330	21.330	0.0001	4.58	2.40–8.73
↑HOMA	0.658 ± 0.319	4.261	0.039	1.93	1.03–3.61
LTL < 9.75	0.893 ± 0.313	8.141	0.004	2.44	1.32–4.51

HOMA, insulin resistance index; LTL, leukocytes telomere length.

TABLE 8 | Results of logistic regression analysis in younger group, where the presence of AP is a dependent variable.

Predictor	β ± *SE*	χ^2 Wald statistic	*p*	OR	95% CI
Model 3. Predictors: male sex, smoking, ↑SBP, ↑HbA1c, ↑HOMA, ↑BMI, ↑CRP, IGF-1>Med, Q1TL					
↑CRP	2.737 ± 0.897	9.304	0.002	15.44	2.66–89.63
Q1LTL	2.832 ± 1.066	7.059	0.008	16.98	2.10–137.01
IGF-1>Med	−1.75 ± 0.731	5.725	0.017	0.174	0.04–0.73

BMI, body mass index; CRP, high sensitive C-reactive protein; HbA1c, glycated hemoglobin; HOMA, insulin resistance index; Med, median; SBP, systolic blood pressure; Q1LTL, LTL was <9.25, which corresponded to the first quartile of distribution.

not an evolutionarily conservative mechanism for regulating life expectancy. At the same time, this signaling pathway plays an important role in the development and prevention of age-associated diseases.

The second important result of this work is the detection of a negative relationship between the HOMA index and arterial wall parameters. This can be explained by the fact that despite the PI3K-Akt signaling pathway blockade in IR, the pathway of mitogen-activating protein kinases (MAPK), which does not depend on the receptor sensitivity to insulin, continues to function. This stimulates the SMC proliferation and migration and causes a prothrombotic state. Compensatory hyperinsulinemia accompanying IR shifts the balance of signaling pathways toward mitogenic action, which promotes an accelerated atherogenesis. The stimulation of insulin by the local renin-angiotensin system of blood vessels causes an increase in NADPH-oxidase activity, a decrease in the bioavailability of NO, and an increase in the production of reactive oxygen species (ROS) (Wang et al., 2007).

In IR angiotensin II, oxidative stress, endothelial dysfunction, pro-inflammatory cytokines, and adhesion molecules activate matrix metalloproteinases (MMPs), which cause fragmentation of elastin molecules and increase collagen stiffness (Jacob, 2003). As the number of collagen molecules increases, collagen binds to glucose molecules with the formation of cross-links represented by advanced glycation end products (AGEs), which significantly increase collagen rigidity and disrupt normal processes of its transformation. Activated MMPs promote basal membrane degradation as well as enhance the migration of SMCs and intimal proliferation (Wang and Lakatta, 2002).

IR is characterized by the development of dyslipidemia, which is an increase in TG and LDL, as well as a decrease in the level of HDL. LDL is mainly represented by the subfraction of highly atherogenic dense particles. Their ability to bind to LDL receptors is reduced, so they circulate for a long time in the bloodstream, become oxidized, and are actively captured by macrophages. Macrophages secreting growth factors and cytokines cause a thickening of the vessel wall and contribute to the plaque development and destabilization (Ford et al., 2002). It was shown that hyperinsulinemia caused hyperfibrinogenemia and an increase in the activity of plasminogen activator inhibitor-1, which led to fibrinolysis failure. Violations in the fibrinolysis system contribute to the progression of atherothrombosis (Choi et al., 2007).

The third important result of this work is the detection of the independent inverse relationship of LTL with both increased arterial stiffness (arteriosclerosis) and atherosclerosis. It is well-known, that the process of vascular aging is characterized, even in apparently healthy subjects, by number of deleterious changes within the vascular wall that are involved both in atherosclerosis and arterial stiffening (Palombo and Kozakova, 2016). Aging is associated with endothelial dysfunction, decreased bioavailability of NO, increased bioavailability of ROS as well as with low-grade inflammation. Age also induces degradation and fragmentation of elastic fibers and a non-enzymatic cross-linkage between collagen fibers. The functional capacity of stem and progenitor cells play key role in these processes. These cells participate in damage repair and tissue differentiation processes, and they play an important role in maintaining tissue homeostasis, including the vessel wall (Sharpless and DePinho, 2007). In clinical practice, the length of telomeres is determined in leukocytes and it reflects the length of telomeres in stem cells and progenitor cells. There is increasing evidence that low telomerase activity and telomere shortening are key components of the reduction in stem cell reserves, age-associated tissue degeneration, and increased vascular stiffness (Sharpless and DePinho, 2007). We have shown that the presence of short telomeres with a length corresponding to the first quartile of the distribution was associated with 17 times higher probability of the presence of AP in the younger group. These data are consistent with the results of the latest meta-analyses, which proved that short telomeres were associated with atherosclerotic CVD (D'Mello et al., 2015). Moreover, it is now widely acknowledged that LTL is not a passive marker but an active determinant of atherosclerosis development, since it determines the replicative and reparative abilities of tissue (Calado and Young, 2009) in response to the influence of risk factors.

Another interesting result is the differences revealed in the relationship of risk factors and arterial wall condition in different age groups. Our study has shown that the value of traditional risk factors for the vascular wall changes was reduced in the older group. Similar results indicating a decrease in the association of conventional risk factors and subclinical changes in arteries in older age were obtained in the Cardiovascular Health Study and the Atherosclerosis Risk in Communities Study (Howard et al., 1997).

The level of lipids and BMI in the elderly was significantly less associated with CVD risk than in the young (Psaty et al., 1999). Obesity and hyperlipidemia in the older group were recognized as unimportant risk factors. In the Italian longitudinal study of

aging the metabolic syndrome was not associated with a risk of MI and stroke in the elderly (Motta et al., 2009) According to other data, an increase in the BMI to 27.0 in the elderly did not result in an increase in the number of cardiovascular events and mortality from all causes (Heiat et al., 2001). It was suggested that elderly people with risk factors but not having clinical CVD are resistant to the influence of CVRF.

CONCLUSION

GH/IGF-1 along with IR and LTL play important roles in the development of arterial aging. The negative relationship between GH/IGF-1 and arterial wall characteristics attests to the protective role of these hormones in arterial wall changes. The arterial walls should be evaluated at a young age, even in the absence of clinical manifestations of CVD and primarily in people with CVRF. To predict changes in the vascular wall, it is advisable to study not only conventional risk factors, but also indicators such as GH/IGF-1, LTL, and HOMA.

AUTHOR CONTRIBUTIONS

IS, OT, NB, NS, OI, DK, VV, IO, MP, DS, SB: Creation of the conception and design of the work, revision the work, final approval of the version to be published, agreement for all aspects of the work. DA, ED, EP, VP, AK, MP, DS: Acquisition, analysis, interpretation of data for the work, revision the work, final approval of the version to be published, agreement for all aspects of the work.

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REFERENCES

Arnqvist, H. J. (2008). The role of IGF-system in vascular insulin resistance. *Horm. Metab. Res.* 40, 588–592. doi: 10.1055/s-0028-1082325

Aviv, A. (2004). Telomeres and human aging: facts and fibs. *Sci. Aging. Knowledge. Environ.* 22:pe43. doi: 10.1126/sageke.2004.51.pe43

Benetos, A., Okuda, K., Lajemi, M., Kimura, M., Thomas, F., Skurnick, J., et al. (2003). Telomere length as an indicator of biological aging: the gender effect and relation with pulse pressure and pulse wave velocity. *Hypertension* 37, 381–385. doi: 10.1161/01.HYP.37.2.381

Brown-Borg, H. M., Borg, K. E., Meliska, C. J., and Bartke, A. (1996). Dwarf mice and the ageing process. *Nature* 384:33. doi: 10.1038/384033a0

Burgers, A. M., Biermasz, N. R., Schoones, J. W., Pereira, A. M., Renehan, A. G., Zwahlen, M., et al. (2011). Meta-analysis and dose-response etaregression: circulating insulin-like growth factor I (IGF-I) and mortality. *J. Clin. Endocrinol. Metab.* 96, 2912–2920. doi: 10.1210/jc.2011-1377

Calado, R. T., and Young, N. S. (2009). Telomere diseases. *N. Engl. J. Med.* 361, 2353–2365. doi: 10.1056/NEJMra0903373

Carter, C. S., Ramsey, M. M., Ingram, R. L., Cashion, A. B., Cefalu, W. T., Wang, Z. Q., et al. (2002). Models of growth hormone and IGF-1 defciency: applications to studies of aging processes and life-span determination. *J. Gerontol. A Biol. Sci. Med. Sci.* 57, B177–B188. doi: 10.1093/gerona/57.5.B177

Cawthon, R. M. (2002). Telomere measurement by quantitative PCR. *Nucleic. Acids. Res.* 30:e47. doi: 10.1093/nar/30.10.e47

Chisalita, S. I., and Arnqvist, H. J. (2004). Insulin-like growth factor I receptors aremore abundant than insulin receptors in human micro- and macrovascular endothelial cells. *Am. J. Physiol. Endocrinol. Metab.* 286, E896–E901. doi: 10.1152/ajpendo.00327.2003

Chisalita, S. I., Johansson, G. S., Liefvendahl, E., Bäck, K., and Arnqvist, H. J. (2009). Human aortic smooth muscle cells are insulin resistant at the receptor level but sensitive to IGF1 and IGF2. *J. Mol. Endocrinol.* 43, 231–239. doi: 10.1677/JME-09-0021

Choi, H. K., Ford, E. S., Li, C., and Curhan, G. (2007). Prevalence of the metabolic syndrome in patients with gout: the third national health and nutrition examination survey. *Arthr. Rheum.* 57, 109–115. doi: 10.1002/art.22466

Conti, E., Andreotti, F., Sciahbasi, A., Riccardi, P., Marra, G., Menini, E., et al. (2001). Markedly reduced insulinlike growth factor-1 in the acute phase of myocardial infarction. *J. Am. Coll. Cardiol.* 38, 26–32. doi: 10.1016/S0735-1097(01)01367-5

Conti, E., Carrozza, C., Capoluongo, E., Volpe, M., Crea, F., Zuppi, C., et al. (2004). Insulin-like growth factor-1 as a vascular protective factor. *Circulation* 110, 2260–2265. doi: 10.1161/01.CIR.0000144309.87183.FB

Coschigano, K. T., Clemmons, D., Bellush, L. L., and Kopchick, J. J. (2000). Assessment of growth parameters and life span of GHR/BP gene-disrupted mice. *Endocrinology* 141, 2608–2613. doi: 10.1210/endo.141.7.7586

D'Mello, M. J., Ross, S. A., Briel, M., Anand, S. S., Gerstein, H., and Paré, G. (2015). Association between shortened leukocyte telomere length and cardiometabolic outcomes: systematic review and meta-analysis. *Circ. Cardiovasc. Genet.* 8, 82–90. doi: 10.1161/CIRCGENETICS.113.000485

Flurkey, K., Papaconstantinou, J., Miller, R. A., and Harrison, D. E. (2001). Lifespan extension and delayed immune and collagen aging in mutant mice with defects in growth hormone production. *Proc. Natl. Acad. Sci. U.S.A.* 98, 6736–6741. doi: 10.1073/pnas.111158898

Ford, E. S., Giles, W. H., and Dietz, W. H. (2002). Prevalence of the metabolic syndrome among US adults: findings from the third National Health and Nutrition Examination Survey. *J. Am. Med. Assoc.* 287, 356–359. doi: 10.1001/jama.287.3.356

Fuster, V., Kelly, B. B., and Vedanthan, R. (2011). Global cardiovascular health: urgent need for an intersectoral approach. *J. Am. Coll. Cardiol.* 58, 1208–1210. doi: 10.1016/j.jacc.2011.05.038

Fyhrquist, F., Saijonmaa, O., and Strandberg, T. (2013). The roles of senescence and telomere shortening in cardiovascular disease. *Nat. Rev. Cardiol.* 10, 274–283. doi: 10.1038/nrcardio.2013.30

Galderisi, M., Caso, P., Cicala, S., De Simone, L., Barbieri, M., Vitale, G., et al. (2002). Positive association between circulating free insulin-like growth factor-1 levels and coronary flow reserve in arterial systemic hypertension. *Am. J. Hypertens.* 15, 766–772. doi: 10.1016/S0895-7061(02)02967-9

Giltay, E. J., Lambert, J., Elbers, J. M., Gooren, L. J., Asscheman, H., and Stehouwer, C. D. (1999). Arterial compliance and distensibility are modulated by body composition in both men and women but by insulin sensitivity only in women. *Diabetologia* 42, 214–221. doi: 10.1007/s001250051141

Green, D. J., Jones, H., Thijssen, D., Cable, N. T., and Atkinson, G. (2011). Flow-mediated dilation and cardiovascular event prediction: does nitric oxide matter? *Hypertension* 57, 363–369. doi: 10.1161/HYPERTENSIONAHA.110.167015

Heiat, A., Vaccarino, V., and Krumholz, H. M. (2001). An evidence-based assessment of federal guidelines for overweight and obesity as they apply to elderly persons. *Arch. Intern. Med.* 161, 1194–1203. doi: 10.1001/archinte.161.9.1194

Higashi, Y., Sukhanov, S., Anwar, A., Shai, S. Y., and Delafontaine, P. (2012). Aging, atherosclerosis, and IGF-1. *J. Gerontol. A Biol. Sci. Med. Sci.* 67A, 626–639. doi: 10.1093/gerona/gls102

Howard, G., Manolio, T. A., Burke, G. L., Wolfson, S. K., and O'Leary, D. H. (1997). Does the association of risk factors and atherosclerosis change with age?

An analysis of the combined ARIC and CHS cohorts. *Stroke* 28, 1693–1701. doi: 10.1161/01.STR.28.9.1693

Jacob, M. P. (2003). Extracellular matrix remodeling and matrix metalloproteinases in the vascular wall during aging and in pathological conditions. *Biomed. Pharmacother.* 57, 195–202. doi: 10.1016/S0753-3322(03)00065-9

Juul, A., Scheike, T., Davidsen, M., Gyllenborg, J., and Jorgensen, T. (2002). Low serum insulin-like growth factor I is associated with increased risk of ischemic heart disease: a population-based case-control study. *Circulation* 106, 939–944. doi: 10.1161/01.CIR.0000027563.44593.CC

Laron, Z. (2005). Do deficiencies in growth hormone and insulin-like growth factor-1 (IGF-1) shorten or prolong longevity? *Mech Ageing Dev.* 126, 305–307. doi: 10.1016/j.mad.2004.08.022

Laughlin, G. A., Barrett-Connor, E., Criqui, M. H., and Kritz-Silverstein, D. (2004). The prospective association of serum insulin-like growth factor I (IGF-I) and IGF-binding protein-1 levels with all cause and cardiovascular disease mortality in older adults: the Rancho Bernardo Study. *J. Clin. Endocrinol. Metab.* 89, 114–120. doi: 10.1210/jc.2003-030967

LeRoith, D., Werner, H., Beitner-Johnson, D., and Roberts, C. T. Jr. (1995). Molecular and cellular aspects of the insulin-like growth factor I receptor. *Endocr. Rev.* 16, 143–163. doi: 10.1210/edrv-16-2-143

Li, G., Barrett, E. J., Ko, S. H., Cao, W., and Liu, Z. (2009). Insulin and insulin-like growth factor-I receptors differentially mediate insulin-stimulated adhesion molecule production by endothelial cells. *Endocrinology* 150, 3475–3482. doi: 10.1210/en.2009-0172

Libby, P., and Sasiela, W. (2006). Plaque stabilization: can we turn theory into evidence? *Am. J. Cardiol.* 98, 26–33. doi: 10.1016/j.amjcard.2006.09.017

Motta, M., Bennati, E., Cardillo, E., Passamonte, M., Ferlito, L., and Malaguarnera, M. (2009). The metabolic syndrome (MS) in the elderly: considerations on the diagnostic criteria of the International Diabetes Federation (IDF) and some proposed modifications. *Arch. Gerontol. Geriatr.* 48, 380–384. doi: 10.1016/j.archger.2008.03.005

Murillo-Ortiz, B., Albarrán-Tamayo, F., Arenas-Aranda, D., Benítez-Bribiesca, L., Malacara-Hernández, J. M., Martínez-Garza, S., et al. (2012). Telomere length and type 2 diabetes in males: a premature aging syndrome. *Aging Male* 15, 54–58. doi: 10.3109/13685538.2011.593658

National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) (2002). Third report of the national cholesterol education program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel III) final report. *Circulation* 106, 3143–3421.

Nilsson, P. M., Tufvesson, H., Leosdottir, M., and Melander, O. (2013). Telomeres and cardiovascular disease risk: an update 2013. *Transl. Res.* 162, 371–380. doi: 10.1016/j.trsl.2013.05.004

Palombo, C., and Kozakova, M. (2016). Arterial stiffness, atherosclerosis and cardiovascular risk: pathophysiologic mechanisms and emerging clinical indications. *Vascul Pharmacol.* 77, 1–7. doi: 10.1016/j.vph.2015.11.083

Perk, J., De Backer, G., Gohlke, H., Graham, I., Reiner, Z., Verschuren, M., et al. (2012). European Guidelines on cardiovascular disease prevention in clinical practice (version 2012). The Fifth Joint Task Force of the European Society of Cardiology and Other Societies on Cardiovascular Disease Prevention in Clinical Practice (constituted by representatives of nine societies and by invited experts). *Eur. Heart J.* 33, 1635–1701. doi: 10.1093/eurheartj/ehs092

Psaty, B. M., Furberg, C. D., Kuller, L. H., Bild, D. E., Rautaharju, P. M., Polak, J. F., et al. (1999). Traditional risk factors and subclinical disease measures as predictors of first myocardial infarction in older adults: the Cardiovascular Health Study. *Arch. Intern. Med.* 159, 1339–1347. doi: 10.1001/archinte.159.12.1339

Rajzer, M. W., Wojciechowska, W., Klocek, M., Palka, I., Brzozowska-Kiszka, M., and Kawecka-Jaszcz, K. (2008). Comparison of aortic pulse wave velocity measured by three techniques: Complior, SphygmoCor and Arteriograph. *J. Hypertens.* 26, 2001–2007. doi: 10.1097/HJH.0b013e32830a4a25

Renier, G., Clément, I., Desfaits, A. C., and Lambert, A. (1996). Direct stimulatory effect of insulin-like growth factor-I on monocyte and macrophage tumor necrosis factor-alpha production. *Endocrinology* 137, 4611–4618. doi: 10.1210/endo.137.11.8895324

Rosén, T., and Bengtsson, B. A. (1990). Premature mortality due to cardiovascular disease in hypopituitarism. *Lancet* 336, 285–288. doi: 10.1016/0140-6736(90)91812-O

Samani, N. J., Boultby, R., Butler, R., Thompson, J. R., and Goodall, A. H. (2002). Telomere shortening in atherosclerosis. *Lancet* 358, 472–473. doi: 10.1016/S0140-6736(01)05633-1

Sharpless, N. E., and DePinho, R. A. (2007). How stem cells age and why this makes us grow old. *Nat. Rev. Mol. Cell. Biol.* 8, 703–713. doi: 10.1038/nrm2241

Smith, J. C., Evans, L. M., Wilkinson, I., Goodfellow, J., Cockcroft, J. R., Scanlon, M. F., et al. (2002). Effects of GH replacement on endothelial function and large-artery stiffness in GH-defcient adults: a randomized, double-blind, placebo-controlled study. *Clin. Endocrinol.* 56, 493–501. doi: 10.1046/j.1365-2265.2002.01514.x

Spallarossa, P., Brunelli, C., Minuto, F., Caruso, D., Battistini, M., Caponnetto, S., et al. (1996). Insulin-like growth factor-I and angiographically documented coronary artery disease. *Am. J. Cardiol.* 77, 200–202. doi: 10.1016/S0002-9149(96)90600-1

Sukhanov, S., Higashi, Y., Shai, S. Y., Vaughn, C., Mohler, J., Li, Y., et al. (2007). IGF-1 reduces inflammatory responses, suppresses oxidative stress, and decreases atherosclerosis progression in ApoE-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* 27, 2684–2690. doi: 10.1161/ATVBAHA.107.156257

Touboul, P. J., Hennerici, M. G., Meairs, S., Adams, H., Amarenco, P., Bornstein, N., et al. (2007). Mannheim carotid intima-media thickness consensus (2004-2006). An update on behalf of the Advisory Board of the 3rd and 4th Watching the Risk Symposium, 13th and 15th European Stroke Conferences, Mannheim, Germany, 2004, and Brussels, Belgium, 2006. *Cerebrovasc. Dis.* 23, 75–80. doi: 10.1159/000097034

Tziomalos, K., Athyros, V. G., Karagiannis, A., and Mikhailidis, D. P. (2010). The role of ankle brachial index and carotid intima-media thickness in vascular risk stratification. *Curr. Opin. Cardiol.* 25, 394–398. doi: 10.1097/HCO.0b013e328338c109

Van Bortel, L. M., Laurent, S., Boutouyrie, P., Chowienczyk, P., Cruickshank, J. K., De Backer, T., et al. (2012). Artery Society; European Society of Hypertension Working Group on Vascular Structure and Function; European Network for Noninvasive Investigation of Large Arteries. Expert consensus document on the measurement of aortic stiffness in daily practice using carotid-femoral pulse wave velocity. *J. Hypertens.* 30, 445–448. doi: 10.1097/HJH.0b013e32834fa8b0

van den Beld, A. W., Bots, M. L., Janssen, J. A., Pols, H. A., Lamberts, S. W., and Grobbee, D. E. (2003). Endogenous hormones and carotid atherosclerosis in elderly men. *Am. J. Epidemiol.* 157, 25–31. doi: 10.1093/aje/kwf160

Vasan, R. S., Sullivan, L. M., D'Agostino, R. B., Roubenoff, R., Harris, T., Sawyer, D. B., et al. (2003). Serum insulin-like growth factor I and risk for heart failure in elderly individuals without a previous myocardial infarction: the Framingham Heart Study. *Ann. Intern. Med.* 139, 642–648. doi: 10.7326/0003-4819-139-8-200310210-00007

Vlachopoulos, C., Aznaouridis, K., and Stefanadis, C. (2010). Prediction of cardiovascular events and all-cause mortality with arterial stiffness: a systematic review and meta-analysis. *J. Am. Coll. Cardiol.* 55, 1318–1327. doi: 10.1016/j.jacc.2009.10.061

Wang, M., and Lakatta, E. G. (2002). Altered regulation of matrix metalloproteinase-2 in aortic remodeling during aging. *Hypertension* 39, 865–873. doi: 10.1161/01.HYP.0000014506.13322.66

Wang, M., Zhang, J., Jiang, L. Q., Spinetti, G., Pintus, G., Monticone, R., et al. (2007). Proinflammatory profile within the grossly normal aged human aortic wall. *Hypertension* 50, 219–227. doi: 10.1161/HYPERTENSIONAHA.107.089409

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REVIEW
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The AGE-RAGE Axis: Implications for Age-Associated Arterial Diseases

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The process of advanced glycation leads to the generation and accumulation of an heterogeneous class of molecules called advanced glycation endproducts, or AGEs. AGEs are produced to accelerated degrees in disorders such as diabetes, renal failure, inflammation, neurodegeneration, and in aging. Further, AGEs are present in foods and in tobacco products. Hence, through both endogenous production and exogenous consumption, AGEs perturb vascular homeostasis by a number of means; in the first case, AGEs can cause cross-linking of long-lived molecules in the basement membranes such as collagens, thereby leading to "vascular stiffening" and processes that lead to hyperpermeability and loss of structural integrity. Second, AGEs interaction with their major cell surface signal transduction receptor for AGE or RAGE sets off a cascade of events leading to modulation of gene expression and loss of vascular and tissue homeostasis, processes that contribute to cardiovascular disease. In addition, it has been shown that an enzyme, which plays key roles in the detoxification of pre-AGE species, glyoxalase 1 (GLO1), is reduced in aged and diabetic tissues. In the diabetic kidney devoid of *Ager* (gene encoding RAGE), higher levels of *Glo1* mRNA and GLO1 protein and activity were observed, suggesting that in conditions of high AGE accumulation, natural defenses may be mitigated, at least in part through RAGE. AGEs are a marker of arterial aging and may be detected by both biochemical means, as well as measurement of "skin autofluorescence." In this review, we will detail the pathobiology of the AGE-RAGE axis and the consequences of its activation in the vasculature and conclude with potential avenues for therapeutic interruption of the AGE-RAGE ligand-RAGE pathways as means to forestall the deleterious consequences of AGE accumulation and signaling via RAGE.

Keywords: RAGE, glycation, arterial diseases, inflammation, aging

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INTRODUCTION

Our population is aging and living longer. Although the average life expectancy of the population is increasing in the United States, there remain significant consequences of the aging process. An average 1 death every 40 s is due to cardiovascular disease (Mozaffarian et al., 2016). The prevalence of heart failure is highest among the adult population 65 years or older (Lakatta and Levy, 2003). As one in four, or 25% of individuals, will be over 65 years of age in the United States by the year 2035, these statistics underscore the fact that significant increases in heart failure are to be expected over this time frame (Lakatta and Sollott, 2002). Clinical and experimental evidence suggests that natural aging imbues inherent risk for the development of cardiovascular complications, such as arterial

stiffness, atherosclerosis, and hypertension, which eventually may lead to myocardial infarction, stroke, and heart failure (Safar et al., 2003; Izzo, 2004; Sethi et al., 2014). Hence, understanding the underlying mechanisms may aid in the discovery of new therapeutic approaches to deter pathological vascular aging.

Changes in the components of large arteries due to advancing age have been described in humans and animals (Spinetti et al., 2004; Pepe and Lakatta, 2005). Age-associated blood vessel remodeling includes such features as dilation of the lumina, intimal and medial thickening, changes in the extracellular matrix (ECM), and augmented stiffness (Gaballa et al., 1998). In addition to these structural changes, other mechanisms contribute to the overall consequences of aging to the arterial wall, including such phenomena as inflammation, endothelial dysfunction, and oxidative stress (Xu et al., 2003). Fibroblasts and smooth muscle cells (SMC) contribute to aging in the vasculature, in part by increasing ECM; macrophages contribute by increasing inflammatory factors that have a wide range of possible consequences, such as vascular hyperpermeability and an increase in the procoagulant state (Sprague and Khalil, 2009; Strait and Lakatta, 2012). These pathobiological events adversely affect the vessel wall and all of its components (Najjar et al., 2005; Greenwald, 2007), potentially contributing to arterial aging.

It has been shown that the aged human arterial wall exhibits a more proinflammatory signature, with increased expression and activity of matrix metalloproteinases (MMPs) and chemokines (Wang et al., 2007). Atop these considerations is the effect of co-morbid conditions in aging, which may augment production of inflammatory mediators and exacerbate the impact of arterial aging, examples of which include diabetes mellitus (types 1 or 2 or the rarer forms of diabetes); chronic renal disease; and chronic immune/inflammatory disorders.

AGEs: PRODUCTION AND FUNCTIONS IN ARTERIAL AGING

Advanced glycation endproducts (AGEs) are a diverse group of macromolecules and at least 20 different specific AGEs have been described to date. Among the major groups of AGEs are carboxymethyl lysine (CML), carboxyethyl lysine (CEL), pentosidine, glucosepane, methylglyoxal lysine dimer, glyoxal lysine dimer, and glycolic acid lysine amide (Henning and Glomb, 2016). AGEs form throughout life via the process of non-enzymatic glycation of proteins and lipids, and this process is accelerated during hyperglycemia, oxidative stress, aging, advanced renal disease, and inflammation (Daffu et al., 2013; Singh et al., 2014; Baig et al., 2017). Humans and animals are also exposed to exogenous sources of AGEs ingested through food-derived AGEs and tobacco products (Luevano-Contreras and Chapman-Novakofski, 2010; Uribarri et al., 2015). It has been shown that restriction in dietary AGE intake may increase the lifespan in animals (Cai et al., 2002; Luevano-Contreras and Chapman-Novakofski, 2010).

AGEs accumulate in aging tissues and on vulnerable plasma proteins. Higher levels of circulating AGEs have been linked to chronic diseases in aging subjects (Semba et al., 2015). The accumulation of AGEs is increased and accelerated in hypertensive subjects (McNulty et al., 2007) and is also associated with diabetes (Soulis et al., 1997; Yan et al., 2003). In fact, aged subjects, even though healthy, may have higher AGE accumulation compared to younger subjects with diabetes and its complications, thus underscoring that AGE production and accumulation accompanies the normal aging process (Hadi and Suwaidi, 2007). Therefore, multiple factors such as the rate of accumulation of AGE ligand, the absolute concentration of the ligand, and individual susceptibility to AGE formation may be important in determining an individual's AGE burden.

AGEs modify collagen and elastin in the vascular wall (Meerwaldt et al., 2004); because of reduced turnover of such proteins, they become more susceptible to glycation during the aging process (Schleicher et al., 1997; Sell and Monnier, 2012). Elevated levels of plasma CML-AGEs are associated with diastolic dysfunction in aging (Campbell et al., 2012). In experiments in diabetic rats, higher AGE crosslinking of collagen was associated with increased stiffness of the aorta (Reddy, 2004). This may change the beneficial functions of several important molecules of the ECM, which can mediate vascular dysfunction. Numerous studies have confirmed the correlation between AGE accumulation and increased artery stiffness (Goldin et al., 2006; Campbell et al., 2011). Arterial stiffness is associated with greater risk for aging-associated cardio- and cerebrovascular diseases and mortality (Laurent et al., 2001; Mattace-Raso et al., 2006; Kaess et al., 2012). AGE accumulation causes upregulation of inflammation and destruction of collagen and elastin, along with other proteins of the ECM (Sims et al., 1996; Greenwald, 2007; Peppa and Raptis, 2008; Baulmann et al., 2010).

MEASURING AGES

AGEs can affect virtually every tissue in the body, either through mediation of cellular damage via protein cross-linking and/or through their binding to cell surface receptors. Since various diseases have been linked to AGEs, it is plausible that AGEs can be utilized as biomarkers, such as for predilection to disease, state of disease activity, and/or response to therapeutic interventions.

Measurment of skin autofluorescence (SAF) estimates the skin tissue AGE content and may predict cardiovascular complications, at least in certain subjects (Lutgers et al., 2009; Noordzij et al., 2012; Tanaka et al., 2012). Some AGEs are fluorescent and can be non-invasively measured in skin by autofluorescence, as a representative marker of the total AGE

Abbreviations: AGEs, advanced glycation endproducts; ALT7-11, Alagebrium; AR, aldose reductase; ARI, aldose reductase inhibitors; CML, carboxymethyl lysine; CEL, carboxyethyl lysine; ctRAGE, cytoplasmic domain of RAGE; DIAPH1, diaphanous-1; ELISA, enzyme-linked immunosorbent assay; esRAGE, endogenous secretory RAGE; ECM, extracellular matrix; FH1, formin homology 1; Glo1, Glyoxalase 1; HMGB1, high mobility group Box-1; HPLC, high performance liquid chromatography; LC-MS, liquid chromatography tandem mass spectrometry; MG, methylglyoxal; ROS, reactive oxygen species; VCAM-1, vascular cell adhesion molecule−1; VEGF, vascular endothelial growth factor; RAGE, receptor for advanced glycation end products; SAF, Skin autofluorescence; SMC, smooth muscle cells; sRAGE, soluble RAGE; SRFs, serum response factors; Tg, transgenic.

modifications of other long-lived tissues in association with vascular disease. In a recent study, SAF was measured using an autofluorescent spectrometer followed by measurement of endothelial function and arterial stiffness. The results indicated that SAF was associated with increased arterial stiffness in the older individuals and that arterial function was blunted by the advancing age of the subject (Sturmer et al., 2015). It is important to note, however, that there are limitations to the use of SAF; first, it only measures fluorescence and not all AGEs are fluorescent; second, skin fluorophores exist that are not related to AGEs, and therefore, such measurement is not reflective of the AGE pool; third, in subjects with darker skin pigmentation, SAF measurements have been found to be less reliable, thereby possibly reducing the applicability of this technique across diverse groups of subjects; and fourth, certain skin creams, such as agents used to "tan" or "brown" the skin may cause direct interference with SAF measurements. Hence, although the technique does not require biopsies or invasive approaches, there are notable limitations that must be considered in its use (Da Moura Semedo et al., 2017).

Traditionally, the precise detection of AGE measurement includes HPLC (High-Performance liquid chromatography) (Ashraf et al., 2015) with fluorescence-based detection. Using HPLC methods, high levels of serum AGE, such as CML-AGE and pentosidine were shown to increase with age and in patients with diabetes (Aso et al., 2000). The use of ELISA (Enzyme-linked immunosorbent assay), an immunological technique for the determination of AGEs, proved to be an alternative for detection of AGEs in samples such as serum and plasma (Munch et al., 1997; Takeuchi et al., 1999). Tissue AGE concentrations using immunohistochemical methods can also be measured using antibodies to detect CML-AGE (Soulis et al., 1997). Using these methods, AGE have been identified in arterial disease and have been localized to early atherosclerotic plaques and cellular constituents, including macrophages and SMCs (Stitt et al., 1997). LC-MS (liquid chromatography- mass spectroscopy) is another technique for the detection of AGEs and the early glycation products. LC-MS allows for AGEs such as pyrraline to be detected in human skin and plasma in very low concentrations (picomolar range) (Pitt, 2009). The possibility of using AGE measurements to gauge the state of AGE-related disease activity and the effectiveness of therapeutic intervention underscores the importance of using reliable methods for the detection of AGEs.

AGES AND THEIR PATHOBIOLOGICAL ACTIONS: INTERACTIONS WITH CELLULAR RECEPTOR RAGE

RAGE (Receptor for Advanced glycation end products) was identified in 1992 from bovine lung as a protein that bound AGEs in a dose-dependent manner (Wautier et al., 1994). RAGE has many ligands that increase in aging, even beyond AGEs, and it is a cell surface macromolecule. RAGE contains extracellular domains composed of one V (variable)-type domain and two C (constant)-type immunoglobulin—like domains (C1 And C2); these are followed by a single transmembrane spanning helix (Koch et al., 2010), and the cytoplasmic domain, which is essential for signal transduction (Xue et al., 2014). RAGE binds a diverse group of ligands, including AGEs, at least certain members of the S100/calgranulins, high mobility group Box-1 (HMGB1), Mac-1, and amyloid- β peptide, particularly its oligomeric forms (Herold et al., 2007).

Mechanisms by which AGEs could alter the vasculature and increase arterial stiffness include generation of inflammation (Chavakis et al., 2004) and oxidative stress (Tan et al., 2007). Further, AGEs binding to endothelial cell surface RAGE can lead to stimulation of NADPH oxidase, thereby increasing the production of reactive oxygen species (ROS) (Wautier et al., 2001). Additional mechanisms such as mitochondrial stress may further increase the production of ROS (Rubattu et al., 2013; Li et al., 2014; Montezano and Touyz, 2014). Previous studies proposed that one of the mechanisms by which AGE/RAGE contributes to endothelial dysfunction is through regulation of the production and expression of tumor necrosis factor (TNF)-α. The transcription factor nuclear factor-κB (NF-κB), triggered by inflammation and by ROS, plays a key role in cytokine and inflammatory mediator expression, thereby exacerbating microvasculopathy and mediating pathological changes in gene expression, at least in part through RAGE ligand-RAGE interactions and activation of cellular signal transduction (Gao et al., 2008; Kay et al., 2016).

Evidence of RAGE-mediated perturbation *in vivo* has also been demonstrated. Diabetic *apolipoprotein E* (*ApoE*) deficient mice that are also devoid of *Ager* (gene encoding RAGE) display reduced atherosclerosis and lower expression of vascular cell adhesion molecule (VCAM)-1 and tissue factor (Kislinger et al., 2001). AGEs also induce vascular endothelial growth factor (VEGF) expression in microvascular endothelial cells (Yamagishi et al., 1997), which may have implications for the diabetic retina, as an example. In addition to the chronic conditions of AGE formation discussed above, such as aging, diabetes, and chronic inflammatory conditions, research has illustrated that AGEs may form rapidly in settings of acute stress as well. For example, endothelial cells subjected to *in vitro*-applied hypoxia release AGEs within minutes of exposure to reduced levels of oxygen (Chang et al., 2008). These considerations indicate that it was important to identify means to block AGE-RAGE interactions in the vasculature.

In animal studies, treatment with soluble RAGE (sRAGE), the soluble extracellular domains of RAGE, which sequester AGEs and RAGE ligands, thereby blocking their interaction with RAGE demonstrated significant reductions in atherosclerotic lesion area (Park et al., 1998), in a manner independent of changes in lipid or glucose levels. In other studies, sRAGE treatment in rodents significantly mitigated diabetic vascular hyperpermeability (Schmidt et al., 1999).

Hallam and colleagues demonstrated that aged 24 month-old Fischer 344 rats displayed higher vascular RAGE expression in the aorta, and higher expression of the polyol pathway enzyme, aldose reductase (AR), which stimulates metabolic pathways

that increase AGE formation. Aging-related vascular dysfunction was evident in these rats on account of impaired endothelial relaxation in response to acetylcholine (Hallam et al., 2010). Treatment of aged Fischer 344 rats with sRAGE improved endothelial dependent relaxation in response to acetylcholine (Hallam et al., 2010). Taken together, these studies illustrate that increased AGE burden and RAGE expression mediate vascular dysfunction and that these perturbations may be suppressed by administration of antagonists of ligand-RAGE interactions *in vivo*.

GLYOXALASE 1 (GLO1) AND AMPLIFICATION OF AGE ACCUMULATION

In addition to increased direct mediators of damage in aging, defense mechanisms may also be attenuated in aging. (GLO1) contributes to the regulation of the levels of the pre-AGE methylglyoxal (MG) and MG-derived AGEs (Giacco et al., 2014). MG, formed mainly inside cells, is a potent glycating agent (Rabbani and Thornalley, 2014).

Published work has suggested a link between RAGE and *Glo1*. Exposure of cultured endothelial cells to high glucose increases expression of RAGE and various RAGE ligands, such as S100B, AGEs, and HMGB1; this was prevented by overexpression of *Glo1* (Brouwers et al., 2011, 2014). Reiniger and colleagues showed that renal accumulation of AGEs promotes kidney dysfunction and that when *Ager* is deleted in OVE26 diabetic mice, reduced pathological, and functional derangements in the kidney ensued, in parallel with reduced MG levels and higher levels of GLO1 in the kidney (Reiniger et al., 2010). These authors showed that in *Ager* null diabetic OVE26 kidney, levels of MG were lower than those of wild-type diabetic OVE26 controls, despite equal levels of high glucose. Reiniger and colleagues traced the mechanism to RAGE-dependent downregulation of *Glo1* mRNA and activity in diabetes (Reiniger et al., 2010). Thus, RAGE activation may perpetuate AGE accumulation and deletion of *Ager* may exert its protection, at least in part by downregulation of *Glo1*.

It is possible that increasing GLO1 expression and/or activity may slow down age-related damage, as acceleration in glycation in aged rats was attenuated by transgenic (Tg) expression of *Glo1* in these animals (Jo-Watanabe et al., 2014). Interestingly, exercise training in aged rats resulted in activation of GLO1, with consequent reduction in the formation of MG and CML, along with lower RAGE expression in the aorta (Gu et al., 2014). Overall, agents that augment GLO1 to block formation of AGEs may serve as therapeutic strategies for averting complications in vascular disorders in which AGEs accumulate.

RAGE/DIAPH1 SIGNAL TRANSDUCTION AXIS: LINK TO VASCULAR DYSFUNCTION

RAGE requires its cytoplasmic domain for signal transduction. Hudson and colleagues demonstrated the interaction of the cytoplasmic domain of RAGE tail with mammalian diaphanous 1 or DIAPH1 (Hudson et al., 2008). The cytoplasmic domain or tail of RAGE (ctRAGE) binds specifically to the formin homology 1 (FH1) domain of DIAPH1 (Hudson et al., 2008). Formins are actin-binding molecules that contribute to Rho GTPase downstream signals (Hudson et al., 2008) in cells such as vascular cells, monocytes/macrophages, and transformed cells. DIAPH1 has also been shown to be an effector of serum response factors (SRFs), which are linked to gene regulation mechanisms, and cellular signaling mechanisms such as AKT and GSK-3beta (Toure et al., 2012).

In SMCs, DIAPH1 was required for RAGE ligand (S100B)-induced c-Src translocation to the plasma membrane, RAC1 activation, generation of ROS and cellular migration. RAC1 modulates the actin cytoskeleton, the arrangement of which governs cell motility and regulates signal transduction pathways (Toure et al., 2012). To verify the RAGE-DIAPH1 interaction, Shekhtman and colleagues used NMR spectroscopy to identify the four key amino acids in the RAGE cytoplasmic domain (Q3, R4, R5, and Q6 corresponding to Q364, R365, R366, and Q367 of the full-length RAGE) that are essential for the interaction of ctRAGE with the FH1 domain of DIAPH1. When R5/Q6 were mutated to alanine residues and expressed in murine SMCs, AKT signaling and cellular migration and proliferation in response to RAGE ligand S100B, but not to non-RAGE ligands, were significantly reduced (Rai et al., 2012).

A role for DIAPH1 in RAGE signaling in macrophages has also been demonstrated. In macrophages devoid of *Diaph1*, hypoxia-mediated upregulation of the transcription factor *Egr1,* which upregulates inflammatory and prothrombotic mediators, was prevented (Xu et al., 2010). To test these points *in vivo* and the role of DIAPH1 in mediating the effects of RAGE ligands, studies are underway in animals of diabetes, aging, and vascular perturbation to probe the potential impact of DIAPH1 in vascular dysfunction.

Taken together, extensive evidence is building to implicate AGEs and RAGE in the pathogenesis of vascular perturbation, which stimulate processes that lead to the development of arterial stiffness, an established harbinger of cardiovascular disease and aging. AGEs, via RAGE stimulate endothelial cells to generate ROS and to activate cellular signaling pathways, at least in part through the cytoplasmic domain of RAGE binding to DIAPH1; processes which lead to activation of seminal transcription factors such as NF-κB (**Figure 1**). In addition to AGE-RAGE activation of endothelial cells and mediation of endothelial dysfunction, AGEs, via RAGE, may also stimulate macrophages and other immune cells, to induce migration and recruitment of inflammatory cells into AGE-laden foci in the tissues. Further, research has shown that a natural anti-AGE mechanism, the enzyme GLO1, which detoxifies pre-AGE species, is downregulated by the actions of RAGE, such as in the diabetic kidney. Hence, AGE-RAGE activation stimulates a feed forward loop, in which AGE-RAGE interaction causes vascular perturbation and, in parallel, a mechanism to perpetuate AGE production and accumulation.

In the section to follow, we consider therapeutic opportunities in halting the detrimental actions of this AGE-RAGE pathway.

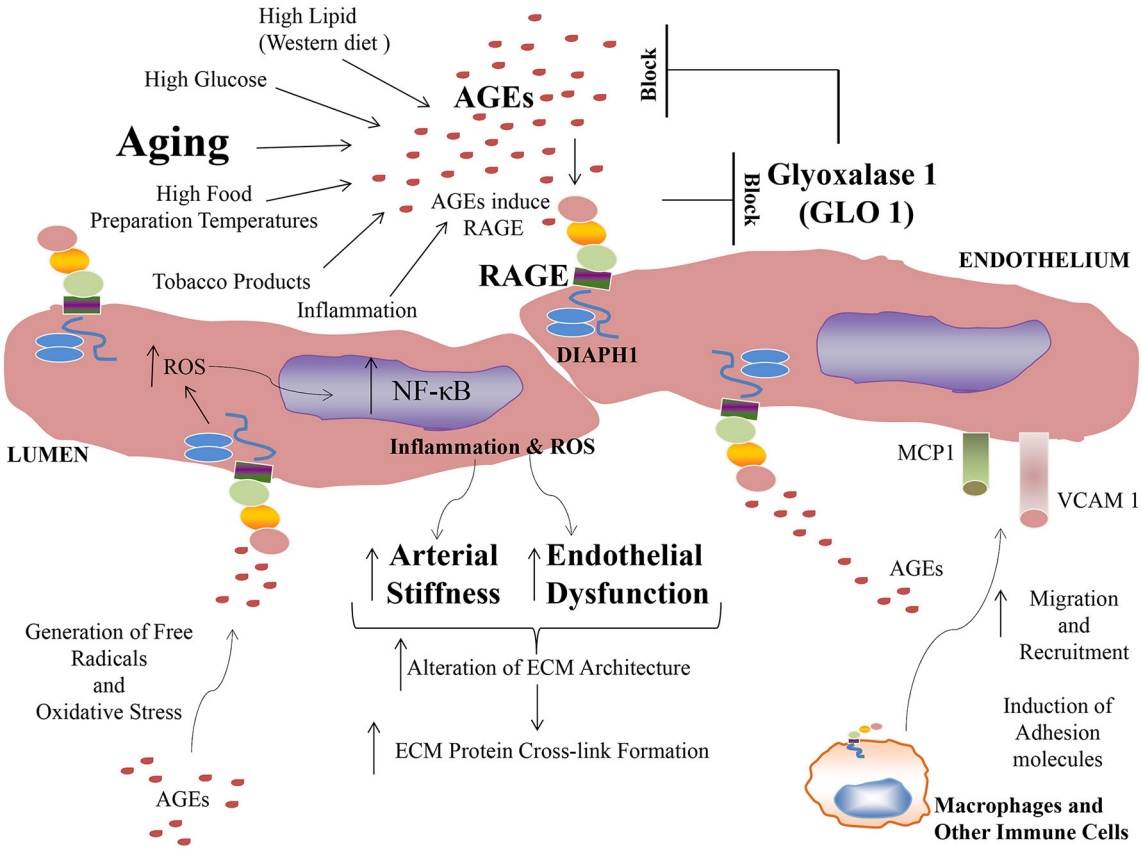

FIGURE 1 | Formation of AGEs and mechanisms of their accumulation and pathobiological actions via the signal transduction receptor RAGE.

THERAPEUTIC STRATEGIES: TARGETING AGE AND RAGE

Propelled by the epidemiological and experimental evidence linking AGE and RAGE to the pathogenesis of arterial stiffness and vascular perturbation, AGEs and RAGE have been identified as targets for therapeutic intervention in these settings. In the sections to follow, we detail examples of some of the strategies to block AGEs and RAGEs for their possible benefits in cardiovascular diseases (**Figure 2**).

Anti-AGE Strategies

Alagebrium, or ALT7-11, is an AGE cross-link breaker. In animals and humans, this agent improved ventricular function and arterial compliance (Kass et al., 2001; Vaitkevicius et al., 2001); reduced expression of RAGE and collagen accumulation in vascular tissues; and in patients with systolic hypertension, it improved endothelial function (Zieman et al., 2007). Although Alagebrium is no longer available, its use served as an important test of the AGE hypothesis in vascular stiffness and function.

Other anti-AGE strategies, such as aminoguanidine, which blocks AGE production, also had beneficial effects in increasing vascular elasticity and in augmenting left ventricular arterial coupling, as well as decreasing vascular permeability in diabetic rats (Wu et al., 2008). Atherosclerosis was attenuated in streptozotocin-induced diabetic *Apoe*-deficient mice treated with aminoguanidine (Forbes et al., 2004). Aminoguanidine also reduced AGE accumulation from food sources (He et al., 1999). Finally, other strategies to reduce AGEs are being investigated, such as aldose reductase inhibitors (ARI). ARI have been shown to suppress AGE accumulation in the atherosclerotic plaques and, in parallel, to reduce atherosclerotic plaque lesions (Vikramadithyan et al., 2005).

In summary, it is noteworthy that despite testing of multiple classes of anti-AGE agents, none have obtained, at least to date, approval for anti-AGE indications. Although there are many possible reasons for this, we propose that one reason is that solely targeting AGEs fails to capture the pathobiological effects of distinct RAGE ligands. Therefore, it is not surprising that attempts are underway to directly target RAGE as a therapeutic strategy.

Anti-RAGE Strategies

Approaches to limit RAGE ligand AGEs have been accompanied by efforts to block RAGE itself and these have been tested *in vitro* and *in vivo*; in addition, human clinical trial testing is also underway. *In vitro,* pre-treatment of AGE-stimulated endothelial cells with anti-RAGE antibodies or anti-oxidants blocked cellular

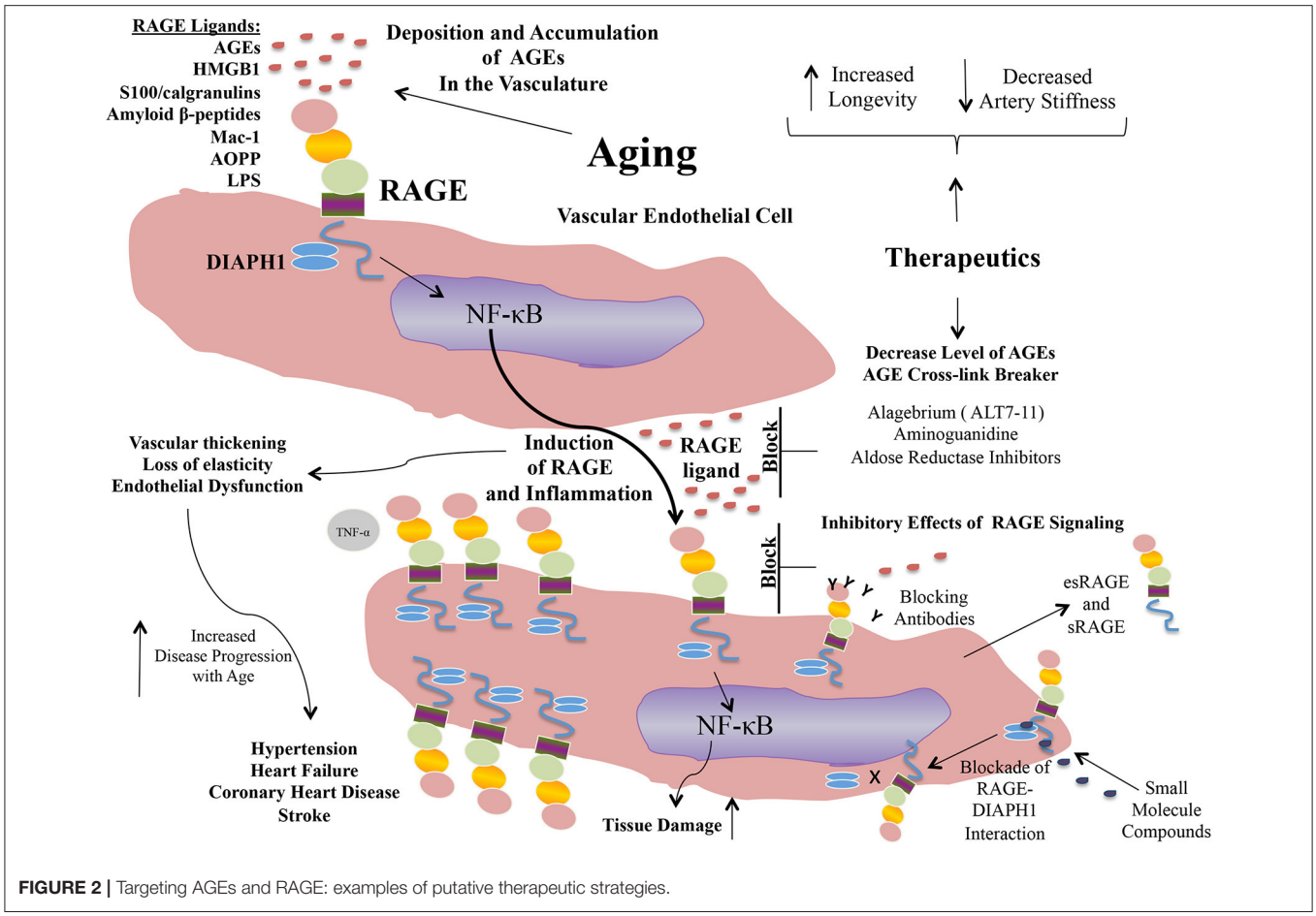

FIGURE 2 | Targeting AGEs and RAGE: examples of putative therapeutic strategies.

perturbation (Yan et al., 1994). Another RAGE blocking agent currently in Phase III clinical trials in Alzheimer's disease is the small molecule Azeliragon, which inhibits the receptor for advanced glycation endproducts through its first extracellular V-type domain, which prevents RAGE ligands from interacting with RAGE. It is orally bioavailable (Sabbagh et al., 2011).

The *AGER* gene may be alternatively spliced to result in the generation of several RAGE isoforms (Yonekura et al., 2003; Kalea et al., 2009). The C- terminally truncated RAGE (known as endogenous secretory RAGE (esRAGE) or RAGEv1, does not contain the transmembrane domain, and is secreted. Other forms of soluble RAGE also exist, as the cell surface RAGE can be proteolytically cleaved by MMPs or ADAM10, thereby resulting in the release of soluble RAGE (sRAGE) (Hanford et al., 2004).

In mouse models, sRAGE treatment suppressed acceleration and blocked the progression of established atherosclerosis in diabetic *Apoe* null mice (Park et al., 1998; Bucciarelli et al., 2002). Various studies in human subjects have sought to determine whether the plasma sRAGE or esRAGE level is associated with cardiometabolic diseases (Choi et al., 2009). Generally, plasma sRAGE/esRAGE levels are lower in subjects with these disorders vs. healthy controls. Thus, recombinant sRAGE might be of benefit in arterial aging and metabolic diseases.

Beyond targeting RAGE and the extracellular domains, distinct therapeutic opportunities have arisen regarding RAGE signaling via blockade of RAGE-DIAPH1 interaction. Manigrasso and colleagues developed a high throughput RAGE tail-DIAPH1 binding assay and screened a library of >58,000 small molecule compounds to identify molecules that blocked this interaction. A series of 13 compounds was identified that exhibited high affinity binding to ctRAGE domain. *In vitro* and *in vivo* studies illustrated that these compounds displayed inhibitory effects on RAGE signal transduction in SMCs *in vitro,* and *in vivo*, on RAGE ligand-stimulated inflammatory gene expression in liver and kidney tissue (Manigrasso et al., 2016). Therefore, the discovery that the cytoplasmic domain of RAGE bound DIAPH1 may aid in the identification of a distinct class of RAGE signaling intracellular antagonists.

CONCLUSIONS AND PERSPECTIVES

Evidence is accruing that exposure to AGEs contributes to detrimental aging-related outcomes and to reduced health and life span. *In vitro* and *in vivo* animal model studies have shown that AGEs affect and disrupt cellular and tissue homeostasis. AGEs can cause alteration of ECM architecture, thereby affecting

TABLE 1 | Key anti-AGE and anti-RAGE therapeutic approaches.

Examples of anti-AGE and anti-RAGE therapeutic strategies	Biochemical target/Mechanisms of action
Alagebrium (ALT7-11)	AGE cross-link breaker
Aldose reductase inhibitors (ARI)	Blockade of Aldose Reductase on glucose metabolism that contributes to AGE formation
Aminoguanidine	Blockade of AGE production
Azeliragon	Small molecule inhibitor of RAGE ligand binding to the RAGE extracellular V-domain
Anti-RAGE antibodies	Blockade of ligand binding to RAGE
Inhibitors of RAGE tail-DIAPH1 binding	Blockade of RAGE signal transduction
Upregulation/Activation of glyoxalase-1	Augmentation of detoxification of AGE precursors
Soluble RAGE (sRAGE) or Endogenous secretory RAGE (esRAGE)	RAGE ligand binding species that sequester RAGE ligands and block their biological effects
Vitamin C, Vitamin E	Anti-oxidants, possible effects on reduction of AGE formation and AGE effects

cellular permeability and signaling; mediate ECM and circulating protein cross-linking; and they can activate cellular signaling and modulate transcription factor activities and subsequent gene expression via receptors such as RAGE. AGE accumulation may result in the increased expression of RAGE in a ligand-enriched environment and exacerbate proinflammatory mechanisms, thereby accelerating aging-associated arterial diseases.

RAGE is expressed on a number of important cell types implicated in arterial aging and vascular pathology. Once AGEs are formed, albeit by diverse intrinsic and environmentally-triggered mechanisms, their interaction with RAGE on endothelial cells, SMCs, and immune cells such as macrophages, results in upregulation of inflammatory and oxidative stress-provoking factors, thereby providing a mechanism to link AGE-RAGE to arterial aging and its consequences, such as stroke, hypertension, atherosclerosis, myocardial infarction, and heart failure. Of note, as hyperglycemia accelerates AGE formation, these AGE-RAGE processes are amplified in diabetes. Epidemiological studies assuredly support the exacerbation of cardiovascular disease in subjects with diabetes.

Certainly, more research is required to understand the entire scope of RAGE signaling and the extent to which blocking AGEs/RAGE/DIAPH1 interaction may intercept the full pathobiology of RAGE activation. Key remaining questions include whether interventions to reduce AGEs and/or to block RAGE extracellular and/or intracellular domains, might provide the greatest protection in attenuation of arterial aging and vascular dysfunction (See **Table 1** for a summary of some of the key anti-AGE and anti-RAGE therapeutic approaches). Clinical studies to address these concepts are required to optimize strategies to protect the vasculature from the adverse effects of AGEing.

MATERIALS AND METHODS

Search Strategies
Arterial Aging:
11,140 refs
https://www.ncbi.nlm.nih.gov/pubmed/?term=arterial+aging
Arterial aging and glycation:
116 refs:
https://www.ncbi.nlm.nih.gov/pubmed/?term=arterial+aging+and+glycation
Arterial aging and advanced glycation end product:
95 refs:
https://www.ncbi.nlm.nih.gov/pubmed/?term=arterial+aging+and+advanced+glycation+end+product
Arterial aging and receptor for advanced glycation end products
22 refs:
https://www.ncbi.nlm.nih.gov/pubmed/?term=arterial+aging+and+receptor+for+advanced+glycation+end+products.

AUTHOR CONTRIBUTIONS

LS and AS: Wrote and edited the manuscript.

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REFERENCES

Ashraf, J. M., Ahmad, S., Choi, I., Ahmad, N., Farhan, M., Tatyana, G., et al. (2015). Recent advances in detection of AGEs: immunochemical, bioanalytical and biochemical approaches. *IUBMB Life* 67, 897–913. doi: 10.1002/iub.1450

Aso, Y., Inukai, T., Tayama, K., and Takemura, Y. (2000). Serum concentrations of advanced glycation endproducts are associated with the development of atherosclerosis as well as diabetic microangiopathy in patients with type 2 diabetes. *Acta Diabetol.* 37, 87–92. doi: 10.1007/s005920070025

Baig, M. H., Jan, A. T., Rabbani, G., Ahmad, K., Ashraf, J. M., Kim, T., et al. (2017). Methylglyoxal and advanced glycation end products: insight of the regulatory machinery affecting the myogenic program and of its modulation by natural compounds. *Sci. Rep.* 7:5916. doi: 10.1038/s41598-017-06067-5

Baulmann, J., Nurnberger, J., Slany, J., Schmieder, R., Schmidt-Trucksass, A., Baumgart, D., et al. (2010). [Arterial stiffness and pulse wave analysis]. *Dtsch. Med. Wochenschr.* 135(Suppl. 1), S4–S14. doi: 10.1055/s-0030-1249183

Brouwers, O., Niessen, P. M., Ferreira, I., Miyata, T., Scheffer, P. G., Teerlink, T., et al. (2011). Overexpression of glyoxalase-I reduces hyperglycemia-induced levels of advanced glycation end products and oxidative stress in diabetic rats. *J. Biol. Chem.* 286, 1374–1380. doi: 10.1074/jbc.M110.144097

Brouwers, O., Niessen, P. M., Miyata, T., Ostergaard, J. A., Flyvbjerg, A., Peutz-Kootstra, C. J., et al. (2014). Glyoxalase-1 overexpression reduces endothelial

dysfunction and attenuates early renal impairment in a rat model of diabetes. *Diabetologia* 57, 224–235. doi: 10.1007/s00125-013-3088-5

Bucciarelli, L. G., Wendt, T., Qu, W., Lu, Y., Lalla, E., Rong, L. L., et al. (2002). RAGE blockade stabilizes established atherosclerosis in diabetic apolipoprotein E-null mice. *Circulation* 106, 2827–2835. doi: 10.1161/01.CIR.0000039325.03698.36

Cai, W., Gao, Q.-D., Zhu, L., Peppa, M., He, C., and Vlassara, H. (2002). Oxidative stress-inducing carbonyl compounds from common foods: novel mediators of cellular dysfunction. *Mol. Med.* 8, 337–346.

Campbell, D. J., Somaratne, J. B., Jenkins, A. J., Prior, D. L., Yii, M., Kenny, J. F., et al. (2011). Impact of type 2 diabetes and the metabolic syndrome on myocardial structure and microvasculature of men with coronary artery disease. *Cardiovasc. Diabetol.* 10, 80–80. doi: 10.1186/1475-2840-10-80

Campbell, D. J., Somaratne, J. B., Jenkins, A. J., Prior, D. L., Yii, M., Kenny, J. F., et al. (2012). Diastolic dysfunction of aging is independent of myocardial structure but associated with plasma advanced glycation end-product levels. *PLoS ONE* 7:e49813. doi: 10.1371/journal.pone.0049813

Chang, J. S., Wendt, T., Qu, W., Kong, L., Zou, Y. S., Schmidt, A. M., et al. (2008). Oxygen deprivation triggers upregulation of early growth response-1 by the receptor for advanced glycation end products. *Circ. Res.* 102, 905–913. doi: 10.1161/CIRCRESAHA.107.165308

Chavakis, T., Bierhaus, A., and Nawroth, P. P. (2004). RAGE (receptor for advanced glycation end products): a central player in the inflammatory response. *Microb. Infect.* 6, 1219–1225. doi: 10.1016/j.micinf.2004.08.004

Choi, K. M., Yoo, H. J., Kim, H. Y., Lee, K. W., Seo, J. A., Kim, S. G., et al. (2009). Association between endogenous secretory RAGE, inflammatory markers and arterial stiffness. *Int. J. Cardiol.* 132, 96–101. doi: 10.1016/j.ijcard.2007.10.047

Daffu, G., del Pozo, C. H., O'Shea, K. M., Ananthakrishnan, R., Ramasamy, R., and Schmidt, A. M. (2013). Radical roles for RAGE in the pathogenesis of oxidative stress in cardiovascular diseases and beyond. *Int. J. Mol. Sci.* 14, 19891–19910. doi: 10.3390/ijms141019891

Da Moura Semedo, C., Webb, M., Waller, H., Khunti, K., and Davies, M. (2017). Skin autofluorescence, a non-invasive marker of advanced glycation end products: clinical relevance and limitations. *Postgrad. Med. J.* 93, 289–294. doi: 10.1136/postgradmedj-2016-134579

Forbes, J. M., Yee, L. T., Thallas, V., Lassila, M., Candido, R., Jandeleit-Dahm, K. A., et al. (2004). Advanced glycation end product interventions reduce diabetes-accelerated atherosclerosis. *Diabetes* 53, 1813–1823. doi: 10.2337/diabetes.53.7.1813

Gaballa, M. A., Jacob, C. T., Raya, T. E., Liu, J., Simon, B., and Goldman, S. (1998). Large artery remodeling during aging: biaxial passive and active stiffness. *Hypertension* 32, 437–443. doi: 10.1161/01.HYP.32.3.437

Gao, X., Zhang, H., Schmidt, A. M., and Zhang, C. (2008). AGE/RAGE produces endothelial dysfunction in coronary arterioles in type 2 diabetic mice. *Am. J. Physiol. Heart Circ. Physiol.* 295, H491–H498. doi: 10.1152/ajpheart.00464.2008

Giacco, F., Du, X., D'Agati, V. D., Milne, R., Sui, G., Geoffrion, M., et al. (2014). Knockdown of glyoxalase 1 mimics diabetic nephropathy in nondiabetic mice. *Diabetes* 63, 291–299. doi: 10.2337/db13-0316

Goldin, A., Beckman, J. A., Schmidt, A. M., and Creager, M. A. (2006). Advanced glycation end products: sparking the development of diabetic vascular injury. *Circulation* 114, 597–605. doi: 10.1161/CIRCULATIONAHA.106.621854

Greenwald, S. E. (2007). Ageing of the conduit arteries. *J. Pathol.* 211, 157–172. doi: 10.1002/path.2101

Gu, Q., Wang, B., Zhang, X. F., Ma, Y. P., Liu, J. D., and Wang, X. Z. (2014). Contribution of receptor for advanced glycation end products to vasculature-protecting effects of exercise training in aged rats. *Eur. J. Pharmacol.* 741, 186–194. doi: 10.1016/j.ejphar.2014.08.017

Hadi, H. A. R., and Suwaidi, J. A. (2007). Endothelial dysfunction in diabetes mellitus. *Vasc. Health Risk Manag.* 3, 853–876.

Hallam, K. M., Li, Q., Ananthakrishnan, R., Kalea, A., Zou, Y. S., Vedantham, S., et al. (2010). Aldose reductase and AGE–RAGE pathways: central roles in the pathogenesis of vascular dysfunction in aging rats. *Aging Cell* 9, 776–784. doi: 10.1111/j.1474-9726.2010.00606.x

Hanford, L. E., Enghild, J. J., Valnickova, Z., Petersen, S. V., Schaefer, L. M., Schaefer, T. M., et al. (2004). Purification and characterization of mouse soluble receptor for advanced glycation end products (sRAGE). *J. Biol. Chem.* 279, 50019–50024. doi: 10.1074/jbc.M409782200

He, C., Sabol, J., Mitsuhashi, T., and Vlassara, H. (1999). Dietary glycotoxins: inhibition of reactive products by aminoguanidine facilitates renal clearance and reduces tissue sequestration. *Diabetes* 48, 1308–1315. doi: 10.2337/diabetes.48.6.1308

Henning, C., and Glomb, M. A. (2016). Pathways of the Maillard reaction under physiological conditions. *Glycoconj. J.* 33, 499–512. doi: 10.1007/s10719-016-9694-y

Herold, K., Moser, B., Chen, Y., Zeng, S., Yan, S. F., Ramasamy, R., et al. (2007). Receptor for advanced glycation end products (RAGE) in a dash to the rescue: inflammatory signals gone awry in the primal response to stress. *J. Leukoc. Biol.* 82, 204–212. doi: 10.1189/jlb.1206751

Hudson, B. I., Kalea, A. Z., Del Mar Arriero, M., Harja, E., Boulanger, E., D'Agati, V., et al. (2008). Interaction of the RAGE cytoplasmic domain with diaphanous-1 is required for ligand-stimulated cellular migration through activation of Rac1 and Cdc42. *J. Biol. Chem.* 283, 34457–34468. doi: 10.1074/jbc.M801465200

Izzo, J. L. Jr. (2004). Arterial stiffness and the systolic hypertension syndrome. *Curr. Opin. Cardiol.* 19, 341–352. doi: 10.1097/01.hco.0000126581.89648.10

Jo-Watanabe, A., Ohse, T., Nishimatsu, H., Takahashi, M., Ikeda, Y., Wada, T., et al. (2014). Glyoxalase I reduces glycative and oxidative stress and prevents age-related endothelial dysfunction through modulation of endothelial nitric oxide synthase phosphorylation. *Aging Cell* 13, 519–528. doi: 10.1111/acel.12204

Kaess, B. M., Rong, J., Larson, M. G., Hamburg, N. M., Vita, J. A., Levy, D., et al. (2012). Aortic stiffness, blood pressure progression, and incident hypertension. *JAMA* 308, 875–881. doi: 10.1001/2012.jama.10503

Kalea, A. Z., Reiniger, N., Yang, H., Arriero, M., Schmidt, A. M., and Hudson, B. I. (2009). Alternative splicing of the murine receptor for advanced glycation end-products (RAGE) gene. *FASEB J.* 23, 1766–1774. doi: 10.1096/fj.08-117739

Kass, D. A., Shapiro, E. P., Kawaguchi, M., Capriotti, A. R., Scuteri, A., deGroof, R. C., et al. (2001). Improved arterial compliance by a novel advanced glycation end-product crosslink breaker. *Circulation* 104, 1464–1470. doi: 10.1161/hc3801.097806

Kay, A. M., Simpson, C. L., and Stewart, J. A. (2016). The role of AGE/RAGE signaling in diabetes-mediated vascular calcification. *J. Diabetes Res.* 2016:6809703. doi: 10.1155/2016/6809703

Kislinger, T., Tanji, N., Wendt, T., Qu, W., Lu, Y., Ferran, L. J. Jr., et al. (2001). Receptor for advanced glycation end products mediates inflammation and enhanced expression of tissue factor in vasculature of diabetic apolipoprotein E-null mice. *Arterioscler. Thromb. Vasc. Biol.* 21, 905–910. doi: 10.1161/01.ATV.21.6.905

Koch, M., Chitayat, S., Dattilo, B. M., Schiefner, A., Diez, J., Chazin, W. J., et al. (2010). Structural basis for ligand recognition and activation of RAGE. *Structure* 18, 1342–1352. doi: 10.1016/j.str.2010.05.017

Lakatta, E. G., and Levy, D. (2003). Arterial and cardiac aging: major shareholders in cardiovascular disease enterprises: part I: aging arteries: a "set up" for vascular disease. *Circulation* 107, 139–146. doi: 10.1161/01.CIR.0000048892.83521.58

Lakatta, E. G., and Sollott, S. J. (2002). Perspectives on mammalian cardiovascular aging: humans to molecules. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* 132, 699–721. doi: 10.1016/S1095-6433(02)00124-1

Laurent, S., Boutouyrie, P., Asmar, R., Gautier, I., Laloux, B., Guize, L., et al. (2001). Aortic stiffness is an independent predictor of all-cause and cardiovascular mortality in hypertensive patients. *Hypertension* 37, 1236–1241. doi: 10.1161/01.HYP.37.5.1236

Li, H., Horke, S., and Förstermann, U. (2014). Vascular oxidative stress, nitric oxide and atherosclerosis. *Atherosclerosis* 237, 208–219. doi: 10.1016/j.atherosclerosis.2014.09.001

Luevano-Contreras, C., and Chapman-Novakofski, K. (2010). Dietary advanced glycation end products and aging. *Nutrients* 2, 1247–1265. doi: 10.3390/nu2121247

Lutgers, H. L., Gerrits, E. G., Graaff, R., Links, T. P., Sluiter, W. J., Gans, R. O., et al. (2009). Skin autofluorescence provides additional information to the UK Prospective Diabetes Study (UKPDS) risk score for the estimation of cardiovascular prognosis in type 2 diabetes mellitus. *Diabetologia* 52, 789–797. doi: 10.1007/s00125-009-1308-9

Manigrasso, M. B., Pan, J., Rai, V., Zhang, J., Reverdatto, S., Quadri, N., et al. (2016). Small molecule inhibition of ligand-stimulated RAGE-DIAPH1 signal transduction. *Sci. Rep.* 6:22450. doi: 10.1038/srep22450

Mattace-Raso, F. U., van der Cammen, T. J., Hofman, A., van Popele, N. M., Bos, M. L., Schalekamp, M. A., et al. (2006). Arterial stiffness and risk of coronary heart disease and stroke: the Rotterdam Study. *Circulation* 113, 657–663. doi: 10.1161/CIRCULATIONAHA.105.555235

McNulty, M., Mahmud, A., and Feely, J. (2007). Advanced glycation end-products and arterial stiffness in hypertension. *Am. J. Hypertens.* 20, 242–247. doi: 10.1016/j.amjhyper.2006.08.009

Meerwaldt, R., Graaff, R., Oomen, P. H. N., Links, T. P., Jager, J. J., Alderson, N. L., et al. (2004). Simple non-invasive assessment of advanced glycation endproduct accumulation. *Diabetologia* 47, 1324–1330. doi: 10.1007/s00125-004-1451-2

Montezano, A. C., and Touyz, R. M. (2014). Reactive oxygen species, vascular noxs, and hypertension: focus on translational and clinical research. *Antioxid. Redox Signal.* 20, 164–182. doi: 10.1089/ars.2013.5302

Mozaffarian, D., Benjamin, E. J., Go, A. S., Arnett, D. K., Blaha, M. J., Cushman, M., et al. (2016). Executive summary: heart disease and stroke statistics—2016 update: a report from the american heart association. *Circulation* 133, 447–454. doi: 10.1161/CIR.0000000000000366

Münch, G., Keis, R., Wessels, A., Riederer, P., Bahner, U., Heidland, A., et al. (1997). Determination of advanced glycation end products in serum by fluorescence spectroscopy and competitive ELISA. *Eur. J. Clin. Chem. Clin. Biochem.* 35, 669–677. doi: 10.1515/cclm.1997.35.9.669

Najjar, S. S., Scuteri, A., and Lakatta, E. G. (2005). Arterial aging: is it an immutable cardiovascular risk factor? *Hypertension* 46, 454–462. doi: 10.1161/01.HYP.0000177474.06749.98

Noordzij, M. J., Lefrandt, J. D., Loeffen, E. A., Saleem, B. R., Meerwaldt, R., Lutgers, H. L., et al. (2012). Skin autofluorescence is increased in patients with carotid artery stenosis and peripheral artery disease. *Int. J. Cardiovasc. Imaging* 28, 431–438. doi: 10.1007/s10554-011-9805-6

Park, L., Raman, K. G., Lee, K. J., Lu, Y., Ferran, L. J. Jr., Chow, W. S., et al. (1998). Suppression of accelerated diabetic atherosclerosis by the soluble receptor for advanced glycation endproducts. *Nat. Med.* 4, 1025–1031. doi: 10.1038/2012

Pepe, S., and Lakatta, E. G. (2005). Aging hearts and vessels: masters of adaptation and survival. *Cardiovasc. Res.* 66, 190–193. doi: 10.1016/j.cardiores.2005.03.004

Peppa, M., and Raptis, S. A. (2008). Advanced glycation end products and cardiovascular disease. *Curr. Diabetes Rev.* 4, 92–100. doi: 10.2174/157339908784220732

Pitt, J. J. (2009). Principles and applications of liquid chromatography-mass spectrometry in clinical biochemistry. *Clin. Biochem. Rev.* 30, 19–34.

Rabbani, N., and Thornalley, P. J. (2014). The critical role of methylglyoxal and glyoxalase 1 in diabetic nephropathy. *Diabetes* 63, 50–52. doi: 10.2337/db13-1606

Rai, V., Maldonado, A. Y., Burz, D. S., Reverdatto, S., Schmidt, A. M., and Shekhtman, A. (2012). Signal transduction in receptor for advanced glycation end products (RAGE): solution structure of c-terminal rage (ctRAGE) and its binding to mDia1. *J. Biol. Chem.* 287, 5133–5144. doi: 10.1074/jbc.M111.277731

Reddy, G. K. (2004). AGE-related cross-linking of collagen is associated with aortic wall matrix stiffness in the pathogenesis of drug-induced diabetes in rats. *Microvasc. Res.* 68, 132–142. doi: 10.1016/j.mvr.2004.04.002

Reiniger, N., Lau, K., McCalla, D., Eby, B., Cheng, B., Lu, Y., et al. (2010). Deletion of the receptor for advanced glycation end products reduces glomerulosclerosis and preserves renal function in the diabetic OVE26 mouse. *Diabetes* 59, 2043–2054. doi: 10.2337/db09-1766

Rubattu, S., Mennuni, S., Testa, M., Mennuni, M., Pierelli, G., Pagliaro, B., et al. (2013). Pathogenesis of chronic cardiorenal syndrome: is there a role for oxidative stress? *Int. J. Mol. Sci.* 14, 23011–23032. doi: 10.3390/ijms141123011

Sabbagh, M. N., Agro, A., Bell, J., Aisen, P. S., Schweizer, E., and Galasko, D. (2011). PF-04494700, an oral inhibitor of receptor for advanced glycation end products (RAGE), in Alzheimer disease. *Alzheimer Dis. Assoc. Disord.* 25, 206–212. doi: 10.1097/WAD.0b013e318204b550

Safar, M. E., Levy, B. I., and Struijker-Boudier, H. (2003). Current perspectives on arterial stiffness and pulse pressure in hypertension and cardiovascular diseases. *Circulation* 107, 2864–2869. doi: 10.1161/01.CIR.0000069826.36125.B4

Schleicher, E. D., Wagner, E., and Nerlich, A. G. (1997). Increased accumulation of the glycoxidation product N(epsilon)-(carboxymethyl)lysine in human tissues in diabetes and aging. *J. Clin. Invest.* 99, 457–468. doi: 10.1172/JCI119180

Schmidt, A. M., Yan, S. D., Wautier, J. L., and Stern, D. (1999). Activation of receptor for advanced glycation end products: a mechanism for chronic vascular dysfunction in diabetic vasculopathy and atherosclerosis. *Circ. Res.* 84, 489–497. doi: 10.1161/01.RES.84.5.489

Sell, D. R., and Monnier, V. M. (2012). Molecular basis of arterial stiffening: role of glycation - a mini-review. *Gerontology* 58, 227–237. doi: 10.1159/000334668

Semba, R. D., Sun, K., Schwartz, A. V., Varadhan, R., Harris, T. B., Satterfield, S., et al. (2015). Serum carboxymethyl-lysine, an advanced glycation end product, is associated with arterial stiffness in older adults. *J. Hypertens.* 33, 797–803; discussion: 803. doi: 10.1097/HJH.0000000000000460

Sethi, S., Rivera, O., Oliveros, R., and Chilton, R. (2014). Aortic stiffness: pathophysiology, clinical implications, and approach to treatment. *Integr. Blood Press. Control* 7, 29–34. doi: 10.2147/IBPC.S59535

Sims, T. J., Rasmussen, L. M., Oxlund, H., and Bailey, A. J. (1996). The role of glycation cross-links in diabetic vascular stiffening. *Diabetologia* 39, 946–951. doi: 10.1007/BF00403914

Singh, V. P., Bali, A., Singh, N., and Jaggi, A. S. (2014). Advanced glycation end products and diabetic complications. *Korean J. Physiol. Pharmacol.* 18, 1–14. doi: 10.4196/kjpp.2014.18.1.1

Soulis, T., Thallas, V., Youssef, S., Gilbert, R. E., McWilliam, B. G., Murray-McIntosh, R. P., et al. (1997). Advanced glycation end products and their receptors co-localise in rat organs susceptible to diabetic microvascular injury. *Diabetologia* 40, 619–628. doi: 10.1007/s001250050725

Spinetti, G., Wang, M., Monticone, R., Zhang, J., Zhao, D., and Lakatta, E. G. (2004). Rat aortic MCP-1 and its receptor CCR2 increase with age and alter vascular smooth muscle cell function. *Arterioscler. Thromb. Vasc. Biol.* 24, 1397–1402. doi: 10.1161/01.ATV.0000134529.65173.08

Sprague, A. H., and Khalil, R. A. (2009). Inflammatory cytokines in vascular dysfunction and vascular disease. *Biochem. Pharmacol.* 78, 539–552. doi: 10.1016/j.bcp.2009.04.029

Stitt, A. W., He, C., Friedman, S., Scher, L., Rossi, P., Ong, L., et al. (1997). Elevated AGE-modified ApoB in sera of euglycemic, normolipidemic patients with atherosclerosis: relationship to tissue AGEs. *Mol. Med.* 3, 617–627.

Strait, J. B., and Lakatta, E. G. (2012). Aging-associated cardiovascular changes and their relationship to heart failure. *Heart Fail. Clin.* 8, 143–164. doi: 10.1016/j.hfc.2011.08.011

Stürmer, M., Šebeková, K., Fazeli, G., Bahner, U., Stäb, F., and Heidland, A. (2015). 25-hydroxyvitamin d and advanced glycation endproducts in healthy and hypertensive subjects: are there interactions? *J. Ren. Nutr.* 25, 209–216. doi: 10.1053/j.jrn.2014.10.027

Takeuchi, M., Makita, Z., Yanagisawa, K., Kameda, Y., and Koike, T. (1999). Detection of noncarboxymethyllysine and carboxymethyllysine advanced glycation end products (AGE) in serum of diabetic patients. *Mol. Med.* 5, 393–405.

Tan, A. L., Forbes, J. M., and Cooper, M. E. (2007). AGE, RAGE, and ROS in diabetic nephropathy. *Semin. Nephrol.* 27, 130–143. doi: 10.1016/j.semnephrol.2007.01.006

Tanaka, K., Tani, Y., Asai, J., Nemoto, F., Kusano, Y., Suzuki, H., et al. (2012). Skin autofluorescence is associated with severity of vascular complications in Japanese patients with type 2 diabetes. *Diabet. Med.* 29, 492–500. doi: 10.1111/j.1464-5491.2011.03448.x

Touré, F., Fritz, G., Li, Q., Rai, V., Daffu, G., Zou, Y. S., et al. (2012). Formin mDia1 mediates vascular remodeling via integration of oxidative and signal transduction pathways. *Circ. Res.* 110, 1279–1293. doi: 10.1161/CIRCRESAHA.111.262519

Uribarri, J., del Castillo, M. D., de la Maza, M. P., Filip, R., Gugliucci, A., Luevano-Contreras, C., et al. (2015). Dietary advanced glycation end products and their role in health and disease. *Adv. Nutr.* 6, 461–473. doi: 10.3945/an.115.008433

Vaitkevicius, P. V., Lane, M., Spurgeon, H., Ingram, D. K., Roth, G. S., Egan, J. J., et al. (2001). A cross-link breaker has sustained effects on arterial and ventricular properties in older rhesus monkeys. *Proc. Natl. Acad. Sci. U.S.A.* 98, 1171–1175. doi: 10.1073/pnas.98.3.1171

Vikramadithyan, R. K., Hu, Y., Noh, H. L., Liang, C. P., Hallam, K., Tall, A. R., et al. (2005). Human aldose reductase expression accelerates diabetic atherosclerosis in transgenic mice. *J. Clin. Invest.* 115, 2434–2443. doi: 10.1172/JCI24819

Wang, M., Zhang, J., Jiang, L. Q., Spinetti, G., Pintus, G., Monticone, R., et al. (2007). Proinflammatory profile within the grossly normal aged human aortic wall. *Hypertension* 50, 219–227. doi: 10.1161/HYPERTENSIONAHA.107.089409

Wautier, J. L., Wautier, M. P., Schmidt, A. M., Anderson, G. M., Hori, O., Zoukourian, C., et al. (1994). Advanced Glycation End Products (AGEs) on the surface of diabetic erythrocytes bind to the vessel wall via a specific receptor inducing oxidant stress in the vasculature: a link between surface-associated AGEs and diabetic complications. *Proc. Natl. Acad. Sci. U.S.A.* 91, 7742–7746. doi: 10.1073/pnas.91.16.7742

Wautier, M. P., Chappey, O., Corda, S., Stern, D. M., Schmidt, A. M., and Wautier, J. L. (2001). Activation of NADPH oxidase by AGE links oxidant stress to altered gene expression via RAGE. *Am. J. Physiol. Endocrinol. Metab.* 280, E685–E694.

Wu, M. S., Liang, J. T., Lin, Y. D., Wu, E. T., Tseng, Y. Z., and Chang, K. C. (2008). Aminoguanidine prevents the impairment of cardiac pumping mechanics in rats with streptozotocin and nicotinamide-induced type 2 diabetes. *Br. J. Pharmacol.* 154, 758–764. doi: 10.1038/bjp.2008.119

Xu, B., Chibber, R., Ruggiero, D., Kohner, E., Ritter, J., and Ferro, A. (2003). Impairment of vascular endothelial nitric oxide synthase activity by advanced glycation end products. *FASEB J.* 17, 1289–1291. doi: 10.1096/fj.02-0490fje

Xu, Y., Toure, F., Qu, W., Lin, L., Song, F., Shen, X., et al. (2010). Advanced glycation end product (AGE)-receptor for AGE (RAGE) signaling and up-regulation of Egr-1 in hypoxic macrophages. *J. Biol. Chem.* 285, 23233–23240. doi: 10.1074/jbc.M110.117457

Xue, J., Ray, R., Singer, D., Böhme, D., Burz, D. S., Rai, V., et al. (2014). The receptor for advanced glycation end products (RAGE) specifically recognizes methylglyoxal-derived AGEs. *Biochemistry* 53, 3327–3335. doi: 10.1021/bi500046t

Yamagishi, S., Yonekura, H., Yamamoto, Y., Katsuno, K., Sato, F., Mita, I., et al. (1997). Advanced glycation end products-driven angiogenesis *in vitro*. Induction of the growth and tube formation of human microvascular endothelial cells through autocrine vascular endothelial growth factor. *J. Biol. Chem.* 272, 8723–8730. doi: 10.1074/jbc.272.13.8723

Yan, S. D., Schmidt, A. M., Anderson, G. M., Zhang, J., Brett, J., Zou, Y. S., et al. (1994). Enhanced cellular oxidant stress by the interaction of advanced glycation end products with their receptors/binding proteins. *J. Biol. Chem.* 269, 9889–9897.

Yan, S. F., Ramasamy, R., Naka, Y., and Schmidt, A. M. (2003). Glycation, inflammation, and RAGE: a scaffold for the macrovascular complications of diabetes and beyond. *Circ. Res.* 93, 1159–1169. doi: 10.1161/01.RES.0000103862.26506.3D

Yonekura, H., Yamamoto, Y., Sakurai, S., Petrova, R. G., Abedin, M. J., Li, H., et al. (2003). Novel splice variants of the receptor for advanced glycation end-products expressed in human vascular endothelial cells and pericytes, and their putative roles in diabetes-induced vascular injury. *Biochem. J.* 370, 1097–1109. doi: 10.1042/bj20021371

Zieman, S. J., Melenovsky, V., Clattenburg, L., Corretti, M. C., Capriotti, A., Gerstenblith, G., et al. (2007). Advanced glycation endproduct crosslink breaker (alagebrium) improves endothelial function in patients with isolated systolic hypertension. *J. Hypertens.* 25, 577–583. doi: 10.1097/HJH.0b013e328013e7dd

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Implication of Free Fatty Acids in Thrombin Generation and Fibrinolysis in Vascular Inflammation in Zucker Rats and Evolution with Aging

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Background: The metabolic syndrome (MetS) and aging are associated with modifications in blood coagulation factors, vascular inflammation, and increased risk of thrombosis.

Objectives: Our aim was to determine concomitant changes in thrombin generation in the blood compartment and at the surface of vascular smooth muscle cells (VSMCs) and its interplay with adipokines, free fatty acids (FFA), and metalloproteinases (MMPs) in obese Zucker rats that share features of the human MetS.

Methods: Obese and age-matched lean Zucker rats were compared at 25 and 80 weeks of age. Thrombin generation was assessed by calibrated automated thrombography (CAT).

Results: Endogenous thrombin potential (ETP) was increased in obese rats independent of platelets and age. Clot half-lysis time was delayed with obesity and age. Interleukin (IL)-1β and IL-13 were increased with obesity and age respectively. Addition of exogenous fibrinogen, leptin, linoleic, or palmitic acid increased thrombin generation in plasma whereas adiponectin had an opposite effect. ETP was increased at the surface of VSMCs from obese rats and addition of exogenous palmitic acid further enhanced ETP values. Gelatinase activity was increased in aorta at both ages in obese rats and MMP-2 activity was increased in VSMCs from obese rats.

Conclusions: Our study demonstrated in MetS an early prothrombotic phenotype of the blood compartment reinforced by procoagulant properties of dedifferentiated and inflammatory VSMCs. Mechanisms involved (1) increased fibrinogen and impaired fibrinolysis and (2) increased saturated fatty acids responsible for additive procoagulant effects. Whether specifically targeting this hypercoagulability using direct thrombin inhibitors would improve outcome in MetS is worth investigating.

Keywords: vascular aging, blood coagulation test, obesity, fatty acids, thrombin generation

INTRODUCTION

Atherothrombotic events and venous thromboembolism are associated with the metabolic syndrome (MetS), a cluster of risk factors for cardiovascular disease including insulin resistance (IR), abdominal adiposity, dyslipidemia, and hypertension (Dandona et al., 2005). Likewise, obesity is causally related to the high prevalence of MetS. Inflammation in MetS results in endothelial dysfunction and increased arterial stiffness (Weiss et al., 2013), probably through the action of matrix metalloproteinases (MMPs; Halcox et al., 2009). Aging is also associated with intimal thickening, breaks in the internal elastic lamina and impaired endothelial function leading to increased arterial stiffness (Wang et al., 1996).

A further cascade of obesity-induced chronic inflammation leads to increased tissue factor (TF; Samad et al., 2001) through the NF-κB pathway (Sonnenberg et al., 2004). Von Willebrand factor (VWF) participates in the prothrombotic state found in MetS (Lim et al., 2004). Total thrombin generation and platelet reactivity are increased in type 2 diabetes and older obese women (Beijers et al., 2010). Furthermore, as far as fibrinolysis is concerned, chronic inflammation, abdominal obesity, and IR all increase plasminogen activator inhibitor-1 (PAI-1) production, so reducing plasminogen conversion and leading to a hypofibrinolytic state (Alessi and Juhan-Vague, 2008; Suehiro et al., 2012).

Adipokine levels (adiponectin, leptin) as well as free fatty acid (FFA) metabolism are changed significantly in MetS (Matsuzawa et al., 2004; Wakil and Abu-Elheiga, 2009). Both are known also to be directly or indirectly implicated in haemostasis and increased thrombosis (Konstantinides et al., 2001; Restituto et al., 2010). Since haemostasis is modified in the MetS and during aging our hypothesis is that MetS, the related adipokines, and FFAs have a major impact on haemostasis changes, increased thrombotic risk and worsen the vascular phenotype. A major challenge is to elucidate the mechanisms leading to increased thrombosis during MetS and in the natural course of aging, and how they are related to the interaction between blood haemostasis and the vascular wall. Rodent models that mimic human MetS are major tools for understanding this pathophysiology (Sloboda et al., 2012).

Obese Zucker rats have a missense point mutation (fa/fa) in the leptin receptor gene that leads to hyperphagia and marked obesity (Phillips et al., 1996). These rats display also many other aspects of the human condition, such as IR, hypertension, and increased plasma lipid levels. We have shown previously that obese Zucker rats exhibited an increased age-dependent arterial stiffening which was greater in obese than lean, as well as endothelial dysfunction with increased systemic oxidative stress (Sloboda et al., 2012).

We have developed therefore a strategy combining "adult" (25-week-old) and "old" (80-week-old) Zucker rats with MetS characteristics and their lean controls and a vascular smooth muscle cell (VSMC) approach to investigate the role of FFAs and vascular inflammation in the prothrombotic properties of MetS. We first explored thrombin generation and its functional consequences on the fibrin network and on fibrinolysis in the blood compartment. To get insights into the underlying mechanisms we then examined thrombin generation at the surface of Zucker rat VSMCs and their MMP activity. We demonstrated that obesity from at least 25 weeks triggers increased thrombin generation in the blood compartment and at the surface of VSMCs via increased FFAs and associated vascular inflammation.

MATERIALS AND METHODS

Animals

Male Zucker rats with the MetS (MSZR, fa/fa; $n = 18$) and their age-matched male lean Zucker rat controls (LZR, FA/-; $n = 18$) were obtained from the breeding colony (animal facility, Faculty of Medicine, University of Lorraine, France). The animals were maintained at a constant temperature of 22–24°C, with a 12 h light-dark cycle (light beginning at 8 a.m.) and given free access to water and standard chow (A04, Scientific Animal Food and Engineering advance, Augy, France). The metabolic status of MSZR and LZR has been published previously (Sloboda et al., 2012).

Eighty weeks of age corresponds to 5 weeks before the mean maximum life span of rats from our local breeding colony.

This study was carried out in accordance with recommendations of the Animal Ethics Committee of the Institut National de la Santé et de la Recherche Médicale and conformed to the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health. The protocols were approved by the Animal Ethics Committee of the Institut National de la Santé et de la Recherche Médicale.

Blood Sampling

Rats were anesthetized with isoflurane and whole blood was collected via a carotid catheter into syringes containing one-tenth the volume of 0.106 M sodium citrate. Platelet count

was determined with an automatic cell counter (Micros 60 ABX model, Montpellier, France). Blood was centrifuged at 190 g for 10 min at room temperature to obtain platelet-rich plasma (PRP) and then at 1,750 g for 10 min to obtain platelet-poor plasma. PRP was adjusted to 200×10^9 platelets/l by addition of autologous platelet-poor plasma and used for platelet aggregation and thrombin generation. Platelet-free plasma (PFP) was obtained by centrifugation of platelet-poor plasma at 13,000 g for 30 min at 4°C, and frozen at −80°C.

Preparation of Arterial Cryo-Sections

Artery cryo-sections were collected in the cross-sectional orientation and used subsequently for *in situ* gelatin zymography. The descending thoracic aorta was embedded in Optimal Cutting Temperature (OCT) medium and frozen using iso-pentane pre-cooled in liquid N_2 and stored at −80°C until cryo-sectioning. Cryo-sections were cut at a thickness of 5 μm and mounted onto glass slides (Leica, Milton Keynes, UK) and stored at −80°C until use.

Cell Culture

The descending thoracic aorta was excised from rats after isoflurane anesthesia (4.5% in 1.5 l/min dioxygen) and exsanguination. VSMCs were isolated as described previously (Ait Aissa et al., 2015). VSMCs were grown in DMEM/F12 supplemented with 10% fetal bovine serum (Lonza, Basel, Switzerland). For thrombin generation assays, VSMCs at passages 3–5 were seeded (7,500 cells/well) in 96-well tissue culture flat-bottom plates (MICROTEST^{TM}96), grown to subconfluence and washed with HBS before use.

Platelet Aggregation

Blood was centrifuged at 190 g for 4 min followed by 70 s at 1,900 g at room temperature to obtain PRP and then platelets were sedimented by centrifugation at 5,000 g for 4 min. Platelets were re-suspended in Tyrode buffer (5 mM Hepes, 137 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM glucose, pH 7.3). Platelet aggregation was measured by turbidimetry at 37°C under stirred conditions. PRP or washed platelets were adjusted to 200×10^9 platelets/l and were stimulated by 5 μg/ml collagen or 5 μM ADP (SD Innovation, Frouard, France). Aggregation was followed for 10 min using a TA-8V aggregometer (SD Innovation).

Thrombin Generation Assay

Calibrated automated thrombinography (CAT) in PRP or PFP was performed in a microtiter plate fluorometer (Fluoroskan Ascent, ThermoLabsystems, Helsinki, Finland) using a dedicated software program (Thrombinoscope BV, Maastricht, The Netherlands) as reported previously (Regnault et al., 2004). All reagents were used at half the ordinary volume as follows: 40 μl PRP or PFP, 10 μl of 5 pM recombinant human tissue factor (TF) (Dade Behring, Marburg, Germany) and phospholipid vesicles (PV) consisted of phosphatidylcholine-serine-ethanolamine (PC/PS/PE) 60/20/20 mole% at a final concentration of 4 μM equivalent PS, 10 μl fluorogenic substrate and calcium. PV were replaced by buffer in PRP and VSMC experiments. Round-bottom 96-well Greiner blue plates were used for PFP and PRP, and MICROTEST^{TM}96 plates for VSMC monolayers. Thrombin generation curves were recorded in triplicate. Thrombin generation was monitored also following supplementing PFP with adiponectin or leptin (BioVision, San Francisco, USA), with fibrinogen (Sigma-Aldrich, St Louis, USA), or with palmitic acid or linoleic acid (Sigma-Aldrich).

Coagulation and Circulating Parameters

Prothrombin and FVIII were measured in PFP samples diluted 1:40–80 in factor diluent (Instrumentation Laboratory, Le Pré Saint Gervais, France). For each assay 50 μl of diluted sample were added to 50 μl of human prothrombin-deficient plasma (Siemens Healthcare Diagnostics SAS, Saint-Denis, France) or FVIII deficient plasma (Dade Behring, Deerfield, USA). After 1 min of incubation at 37°C in a KC10 coagulometer, coagulation was started by addition of 80 μl of Thromborel® S. Calibration curves were generated using the reference plasma Unicalibrator (Diagnostica Stago, Asnières, France). Fibrinogen was measured in PFP samples diluted 1:10–20 in Owren–Koller buffer (Diagnostica Stago, Asnières, France). Unicalibrator was used to generate calibration curves. After 4 min of incubation at 37°C in a KC10 coagulometer, coagulation was started by addition of 100 μl of Fibriquik (Biomérieux-Trinity Biotech, Bray, Ireland). Antithrombin levels were measured with the Coamatic® antithrombin test kit from Chromogenix, and TAT with the Enzygnost® TAT micro (Instumentation Laboratory). TF and TF pathway inhibitor (TFPI) activities were measured in PFP using the Actichrome® tissue factor and Actichrome® TFPI activity assay respectively (American Diagnostica, Stamford, CT). PAI-1 levels were measured with the rat PAI-1 total antigen ELISA kit from Innovative Research, Inc. IL-13 and IL-1β concentrations were measured with the IL-13 and IL-1 beta rat ELISA kits from Invitrogen. MMP-9 levels were measured with the Quantikine rat total MMP-9 immunoassay from R&D Systems. VCAM-1 was assessed with the rat VCAM-1 ELISA kit from Elabscience.

In Vitro Fibrinolytic Test

PFP (20 μl) was diluted by addition of 40 μl buffer containing 5 pM recombinant TF, PV at 4 μM equivalent PS, 5 nM rabbit thrombomodulin (TM) (American Diagnostica, Greenwich, USA) and 4 μg/ml recombinant human tissue Plasminogen Activator (tPA) Actilyse® (Boehringer Ingelheim, Ingelheim am Rhein, Germany). Clot formation was initiated by addition of 10 μl of 100 mM $CaCl_2$. To monitor clot lysis, absorbance was read kinetically at 405 nm using a microplate reader. To standardize the figure, for each sample basal optical density (OD) after lysis was subtracted from each point of the curve. Half lysis time was defined as the time required to reach half-maximal variation in OD.

Microscopy of Fibrin Fiber Ultrastructure

The thrombin generation assay was performed in order to generate fibrin for fixation using the same TF and PV concentrations as in the CAT experiments. This was done using plasma on paper disks and a Rhodamine substrate was used

(Ninivaggi et al., 2012). Immediately after thrombin generation was finished (50 min for each run), the mineral oil was removed from the well and a solution of glutaraldehyde (grade I) in phosphate buffered saline (PBS) (Sorensen's PBS, pH 7.2) was applied. This was put at room temperature for 1 h and then kept at 4°C overnight. The samples were then washed 5 times with PBS and a secondary fixation was performed in OsO_4 (1%) in sodium cacodylate (200 nM, pH 7.4) for 1 h at RT. The samples were then dehydrated with increasing concentrations of ethanol each during 3 min (30, 50, 70, 90, 100%) and the last step (100%) was performed three times. Further dehydration was accomplished by a hexamethyldisilazane (HMDS)/ethanol solution (1:1) for 3 min and HMDS for 10 min. The samples were removed from the wells and left to dry. In order to visualize the samples with a Phenom G2Pro scanning electron microscopy (SEM) (Phenom-World, Eindhoven, the Netherlands), they were put on stubs using carbon tabs and coated with gold.

For each sample, 3–5 pictures were analyzed. Fiber thickness was measured using ImageJ software (version 1.48v). For each picture 100 measurements were performed. The density of the fibers was calculated from the pictures by counting the number of fibers that crossed a line of 26.8 μm (Konings et al., 2011).

Rat Cytokine Antibody Array

The Rat Cytokine Array Panel A (Cat # ARY008) from R&D system (Minneapolis, MN) was used to probe cytokines in PFP from MSZR and LZR by following the procedures recommended by the manufacturer. Bound antibodies were detected by chemiluminescence using the Immobilon™ Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA). This was performed once with a plasma pool from 5 to 6 animals to reduce inter-animal variability in each group.

Phospholipid Procoagulant Activity

The chromogenic assay measuring the phospholipid-related procoagulant activity (PPA) in VSMCs was performed as described previously for plasma (Wagenvoord et al., 1994; Membre et al., 2008). VSMCs cultured in 96 well plates were washed and 50 μl of 50 mM Tris, 175 mM NaCl, pH 7.9 (TBS) containing 2 g/l bovine serum albumin (BSA) were added as well as 50 μl of activated factor X (1.2 nM), activated factor V (2.4 nM), $CaCl_2$ (15 mM) and 50 μl of bovine prothrombin (6 μM) plus Z-Gly-Gly-Arg-AMC substrate (1.25 mM) in 20 mM HEPES pH 7.5 containing 60 g/l BSA. The plate was placed in the Fluoroskan Ascent fluorometer and allowed to warm up to 37°C for 5 min before kinetic readings were taken over 10 min. Phospholipid concentration was estimated from the initial rate of thrombin formation by reference to a standard curve constructed with PV, and expressed as PS equivalents.

Western Blot

Cell extracts were obtained by lysing VSMCs in complete Lysis-M buffer (Roche Diagnostics Corporation, Basel, Switzerland). Detergent-soluble fractions were retained, and protein concentrations in samples were determined using a Bradford protein assay (Bio-Rad, Hercule, USA). Lysates containing 30 μg of protein were electrophoresed on polyacrylamide gels (8% gel), transferred to Hybond-C nitrocellulose membranes (transblot turbo, Bio-Rad, Hercule, USA) and blotted with the following antibodies: α-smooth muscle actin (αSMA), 4/1,000 (Sigma-Aldrich), smooth muscle myosin heavy chain (SM-MHC), 1/1,000 (Abcam; Cambridge, UK); smoothelin, 1/500 (Santa Cruz Biotechnology, USA); integrin α_v, 1/1,000 (Santa Cruz Biotechnology, Dallas Texas); integrin β_3 1/500 (Merck Millipore, Billerica, USA) and tubulin, 2/1,000 (Sigma-Aldrich). After rinsing, incubation with a secondary rabbit antibody 1/1,000 (α_v, β_3, smoothelin, SM-MHC, Sigma-Aldrich) and mouse antibody 1/1000 (αSMA, tubulin, Sigma-Aldrich). Reactions were visualized by the ECL Western Blot Detection Kit (Bio-Rad, Hercule, USA) after incubation with peroxidase conjugates 1/2000 (GE Healthcare, Little Chalfont, UK). Tubulin was used as loading control and the protein expression was normalized to tubulin.

In Situ Gelatin Zymography

In situ gelatin zymography was performed to determine the gelatinase activity across the aortic wall using DQ-gelatin (Life Technologies, Paisley, UK) as described previously (Mook et al., 2003). Fluorescein isothiocyanate (FITC, 1/110), and 4′,6-diamidino-2-phenylindole (DAPI, 1/150) filters were used to visualize the degree of gelatinase activity and the localisation of nuclear tissue by fluorescence microscopy using a x20 optical objective (Keynece, Osaka, Japan). Analysis of average fluorescence was performed for three 20 μm thick profile lines across 3 arterial wall regions for each sample.

Zymography Analysis

VSMCs from LZR or MSZR (passage 4–6) were seeded (50,000 cells/well) in 6-well culture plates in DMEM/F-12 supplemented with 10% fetal bovine serum (life technology Thermo Fisher Scientific, Waltham, USA). Cells were grown to subconfluence and after 16 h in serum-free medium, cells were washed with PBS (Sigma-Aldrich), the medium was changed and cells were incubated for 4, 8, or 20 h at 37°C. Conditioned media were then removed and centrifuged at 500 g for 10 min at room temperature and used for the determination of MMP-2 secretion.

Conditioned media were analyzed for gelatin degradation by electrophoresis under non-reducing conditions on a 10% polyacrylamide-SDS gel containing 0.1% gelatin. Gels were washed for 1 h at room temperature in a 2% triton X-100 solution and incubated overnight at 37°C in 50 mM Tris–HCl/10 mM $CaCl_2$ (pH 7.6) buffer.

Gels were stained in a 0.1% coomassie Blue (G250)/45% methanol/10% acetic acid solution and de-stained in a 10% acetic acid/20% methanol solution. White lysis strips, indicative of gelatinolytic activity, were revealed and scanned (Fujifilm LAS 4000, Life sciences, Branford, USA). Densitometric analysis was made using MultiGauge software (Fuji, Tokyo, Japan). Fetal bovine serum diluted at 1% in serum free medium was used as a positive control.

Statistical Analysis

Results are presented as mean ± standard error of the mean. Data were analyzed by a one-way or two-way ANOVA, followed by a Fisher's test for multiple comparisons to evaluate the influence of age and strain and their interaction on the different variables. In the case of SEM measurements, the differences in fiber thickness were analyzed using the Mann Whitney *U*-test.

RESULTS

Platelet Aggregation, Thrombin Generation, and Fibrinolysis Were All Impaired with the MetS and/or Aging

Platelet count in blood was increased in MSZR at both ages compared to the same aged LZR (**Table 1**). Platelet aggregation using washed platelets and collagen as a strong agonist was not significantly modified as shown by the mean maximum aggregation (**Figure 1A**). For platelet aggregation in PRP using ADP, mean maximum aggregation was increased in 80 week-old MSZR and LZR compared to 25 week-old controls (**Figure 1B**). The F1+2 fragment was analyzed to evaluate the *in vivo* reactivity of the coagulation system. The amount of F1+2 fragment was increased in 25 week-old MSZR compared to the same aged LZR (**Table 1**). Thrombin generation measurement was performed as an integrative *in vitro* phenotype of coagulation. Adult and very old MSZR had a significantly increased endogenous thrombin potential (ETP) compared to same aged LZR. The other thrombin generation parameters (lag time, peak, and velocity) were not changed significantly except for the time to peak which was increased in obese at both ages (**Table 1**; **Figure 1C**). The ratio of thrombin generation in PFP and PRP compared to 25 week-old LZR was made to evaluate the platelet reactivity impact on thrombin generation. Interestingly, thrombin generation was more increased in PRP from MSZR at 25 week of age compared to 80 week-old rats (**Figure 1D**). The coagulation parameters, TF, TFPI, prothrombin and fibrinogen, were all increased in MSZR compared to LZR at both ages. TFPI was decreased and fibrinogen was increased with age in MSZR and prothrombin was increased with age in LZR. FVIII was increased significantly with age and MetS in 80 week-old MSZR. Antithrombin measurements showed no modification in MSZR and LZR rats (**Table 1**). Fibrin clots were characterized by SEM. Computerised analysis of the SEM images showed a decrease of fibrin fiber thickness in MSZR compared to LZR at both ages while fiber density was only increased in 80 week-old LZR (**Figures 1E-G**). Circulating levels of PAI-1 were increased in both 80 week-old LZR and MSZR (**Figure 1H**). In a fibrinolysis test (**Figure 1I**), half-time lysis was increased in MSZR compared to LZR at both ages and aging significantly increased half-time lysis in both groups (**Figure 1J**). Maximal lysis speed was not modified (**Figure 1K**).

Inflammation, Metabolic Factors, and Free Fatty Acids Modified Thrombin Generation

Fibrinogen concentration was correlated highly to ETP ($r = 0.069$) and supplementing plasma with exogenous fibrinogen at concentrations that agreed with the changes between MSZR and LZR gradually increased ETP (**Figures 2A,B**). The 1.2-fold increase in ETP with the 2.5 mg/mL concentration is consistent with the 1.4 increase in plasma fibrinogen in MSZR. We have then tested the effects of addition of exogenous leptin, adiponectin, linoleic acid, and palmitic acid to PFP at concentrations selected to encompass the range previously

TABLE 1 | Blood coagulation parameters and thrombin generation parameters of LZR and MSZR at 25 and 80 weeks of age.

	25 week-old		80 week-old		ANOVA		
	LZR	MSZR	LZR	MSZR	Strain	Age	Interaction
n	9	10	12	9			
Platelets (10^3/mm^3)	574 ± 37	789 ± 34*	633 ± 29	834 ± 63*	≤0.0001	0.009	0.013
F1+2 (pmol/l)	4.1 ± 0.5	7.9 ± 1.0*	5.8 ± 1.2	5.5 ± 0.9	0.009	0.7	0.05
TF (pM)	0.3 ± 0.1	12.2 ± 1.7*	2.0 ± 0.4	9.9 ± 1.5*	≤0.0001	0.8	0.09
TFPI activity (U/ml)	4.9 ± 0.2	11.2 ± 0.2*	5.4 ± 0.2	9.9 ± 0.6*#	≤0.0001	0.3	0.01
FVIII (%)	104 28	190 34	124 28	466 52*#	≤0.0001	0.002	0.001
Prothrombin (%)	94 ± 3	223 ± 19*	155 ± 14#	264 ± 16*	≤0.0001	0.002	0.5
AT (%)	129 ± 2	125 ± 2	127 ± 1	123 ± 3	0.04	0.5	0.9
Fibrinogen (g/l)	2.8 ± 0.1	4.0 ± 0.2*	3.1 ± 0.1	4.9 ± 0.2*#	≤0.0001	0.0003	0.2
n	11	11	10	7			
Lag time (min)	1.5 ± 0.1	1.7 ± 0.1	1.4 ± 0.1	1.5 ± 0.1	0.09	0.4	0.6
Peak (nM)	99 ± 8	121 ± 9	102 ± 10	117 ± 15	0.07	0.98	0.7
Time to peak (min)	4.4 ± 0.1	5.2 ± 0.3*	4.1 ± 0.1	5.3 ± 0.2*	≤0.0001	0.6	0.3
ETP (nM.min)	395 ± 37	549 ± 52*	362 ± 34	553 ± 76*	0.001	0.8	0.8
Velocity (nM/min)	35 ± 3	37 ± 4	40 ± 4	31 ± 4	0.6	0.98	0.2

*Results are mean ± standard error to the mean. *$p < 0.05$, SMZR vs. LZR at the same age; #$p < 0.05$, 80 vs. 25 week-old rats in the same strain. F1+2, fragment 1+2; TF, tissue factor; TFPI, tissue factor pathway inhibitor; AT, antithrombin; ETP, endogenous thrombin potential.*

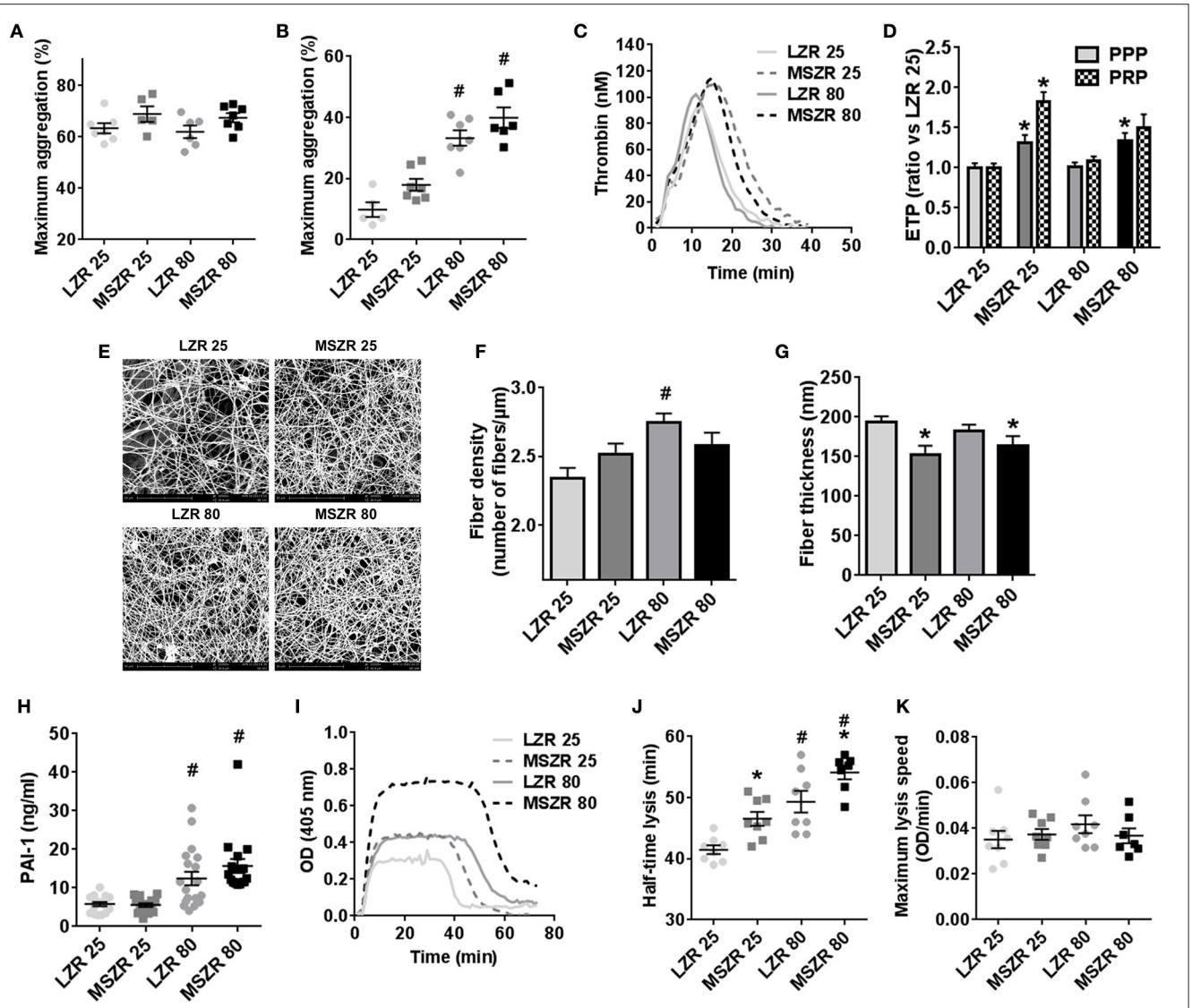

FIGURE 1 | Platelet aggregation, thrombin generation, and fibrinolysis in LZR and MSZR rats**. (A)** Mean maximum aggregation in washed platelets in response to collagen (5 µg/ml) and in **(B)** platelet-rich plasma (PRP) in response to ADP (5 µM), with the platelet count adjusted to 200 × 10^9 platelets/l. **(C)** Calibrated automated thrombinography (CAT) in rat plasma. Mean thrombin generation curves in platelet free plasma (PFP) triggered by 5 pM tissue factor in LZR and MSZR at 25 and 80 weeks of age. **(D)** Endogenous thrombin potential (ETP) in PFP and PRP of 25 and 80 week-old LZR and MSZR, expressed as ratios of values for 25 week-old LZR. **(E)** Ultrastructure of fibrin fibers was visualized by scanning electron microscopy. Pictures were made at 10,000 × magnification. **(F,G)** Fiber thickness and fiber density of fibrin clot in LZR and MSZR. **(H)** ELISA results of PAI-1 measured in PFP (n = 17–19). **(I)** Representative curves of fibrinolytic tests in PFP in LZR and MSZR. **(J,K)** Half-lysis time and maximal lysis speed of fibrinolytic tests in LZR and MSZR. Results are mean ± standard error of the mean (n = 7–11). $^*p < 0.05$ vs. LZR at the same age; $^\#p < 0.05$ vs. 25 week-old rats in the same strain.

reported for each molecule in MSZR (Sloboda et al., 2012; Godin et al., 2013). Addition of leptin or adiponectin elicited similar concentration-dependent changes in ETP whatever the group of rat. The two adipokines had opposite effects on thrombin generation, leptin increased ETP whereas adiponectin decreased it (**Figures 2C,D**). The two lower concentrations of added linoleic acid (0.75 and 1.5 mg/mL) had clear procoagulant effects whereas the higher concentration (3 mg/mL) was less effective in increasing thrombin generation (**Figures 2E,F**). There was a significant increase in thrombin generation for all added concentrations of palmitic acid whatever the group of rat. The results show an additive effect of FFAs on MSZR plasma.

Plasma Cytokines Were Increased Both with MetS and Aging

To explore inflammation in our model we performed a plasma cytokine array of 27 cytokines in order to provide qualitative data that will subsequently be used to quantify cytokines known likely to promote prothrombotic phenotypes (**Figure 3**). Panel

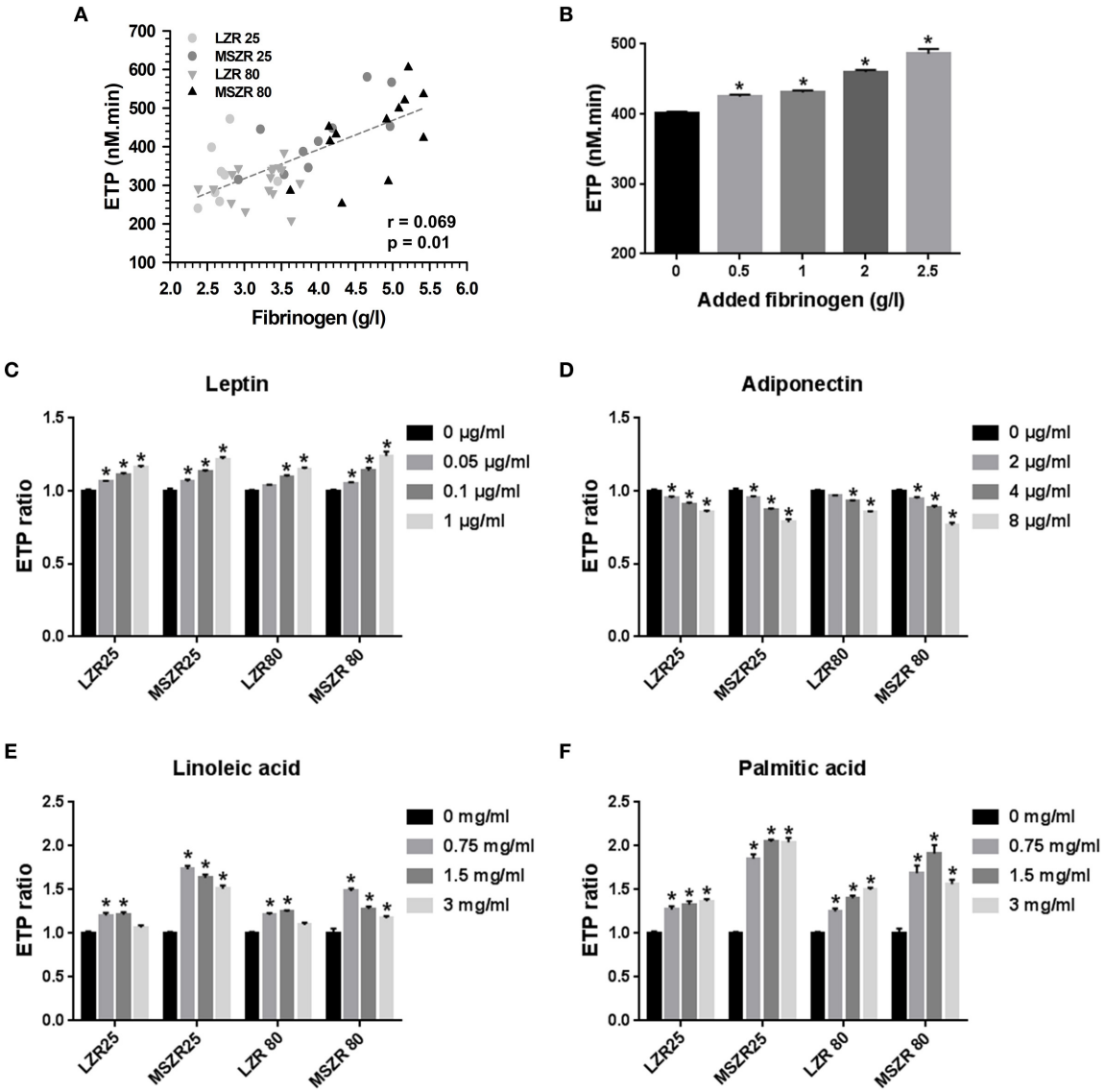

FIGURE 2 | Effect of fibrinogen, adipokines, and free fatty acids on thrombin generation. **(A)** Correlation between ETP and plasma fibrinogen concentration of 25 and 80 week-old LZR and MSZR ($r = 0.069$, $p = 0.01$). **(B)** ETP values in 25 week-old LZR platelet free plasma supplemented with 0.5, 1.0, 2.0, or 2.5 g/l fibrinogen. **(C–F)** ETP values, expressed as ratios of values in presence of adipokines or free fatty acids to those obtained with no addition for each group, in platelet free plasma supplemented with 0.05, 0.1, or 1.0 ng/ml leptin **(C)**, with 2, 4, or 8 µg/ml adiponectin **(D)**, with 0.75, 1.5, or 3 mg/ml of linoleic acid **(E)** or with 0.75 1.5, or 3 mg/ml of palmitic acid **(F)**. Results are mean ± standard error of the mean ($n = 11$–16). *$p < 0.05$ vs. no addition.

A presents pictures of the cytokine array membranes. A 50% variation between two groups was chosen as a threshold to classify cytokines into four groups. The first group of five cytokines showed no modifications (**Figure 3B**), a second group of eight cytokines were increased with MetS (**Figure 3C**), a third group of three cytokines were increased with aging (**Figure 3D**) and a last group of 11 cytokines were increased with both MetS and aging (**Figure 3E**). The highest variation between 25 week-old MSZR and LZR was found for IL-1β (>3,000% variation) and the highest variation between 80 and 25 week-old rats was observed for IL-13 (>400% variation). ELISAs performed with individual rat PFP for IL-1β and IL-13 showed an increase of these cytokine levels in LZR and MSZR with age (**Figures 3F,G**). IL-13 was increased also in 80 week-old MSZR compared to same aged LZR.

MetS and Aging-Induced Inflammation and Haemostasis Impairment Were Related to Alteration of VSMCs

To explore the contribution of VSMCs, thrombin generation was measured at the surface of cultured VSMCs isolated from LZR and MSZR. Thrombin generation with PFP from LZR and MSZR was always increased at the surface of MSZR VSMCs

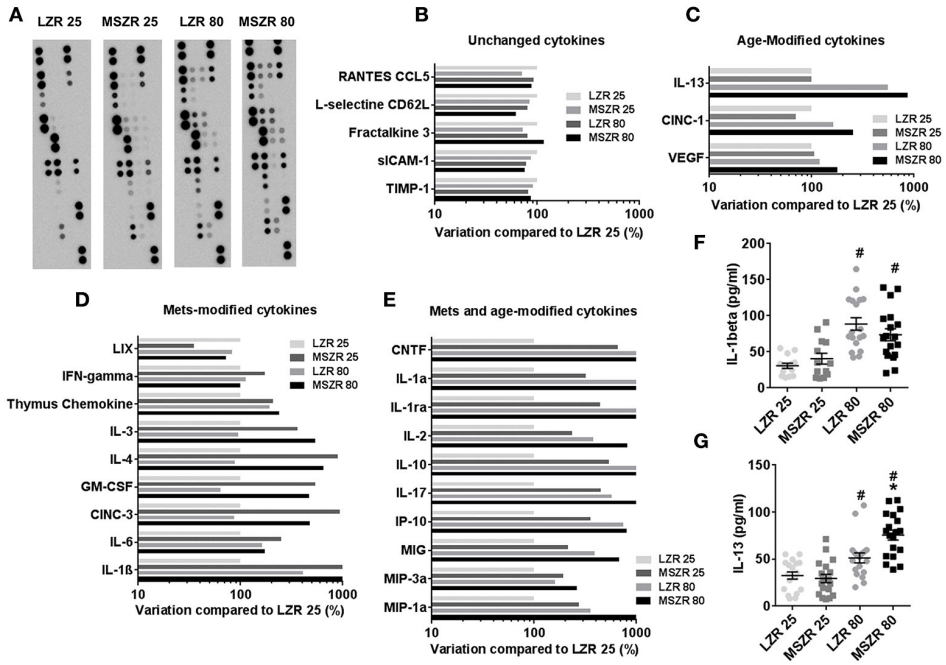

FIGURE 3 | Plasma cytokine array in Zucker rats**. (A)** Cytokine arrays of pooled platelet free plasma from 25 to 80 week-old MSZR and LZR. Relative chemoluminescence compared to 25 week-old LZR was measured. **(B)** Unchanged cytokines, **(C)** cytokines modified with age, **(D)** with MetS, or **(E)** both with MetS and age. ELISAs results for IL-1β **(F)** and IL-13 **(G)** measured in PFP ($n = 14$–18), results are mean ± standard error of the mean, $^{*}p < 0.05$ vs. LZR at the same age; $^{\#}p < 0.05$ vs. 25 week-old rats in the same strain. VEGF, vascular endothelial growth factor; CINC-1, cytokine-induced neutrophil chemoattractant 1; CINC-3, cytokine-induced neutrophil chemoattractant 3; GM-CSF, granulocyte macrophage colony stimulating factor; MIP, Macrophage Inflammatory Protein; MIG, C-X-C motif ligand 9; IP-10, interferon gamma-induced protein 10; CNTF, ciliary neurotrophic factor; INFγ, interferon γ; IL, interleukin.

compared to LZR VSMCs. Remarkably, addition of palmitic acid in LZR VSMCs increased thrombin generation to the level of MSZR independently of the PFP used (**Figure 4A**). MSZR VSMCs displayed increased procoagulant phospholipids at their surface compared to LZR VSMCs (**Figure 4B**). Integrin subunit α_v was increased in MSZR compared to LSZ VSMCs while the β_3 subunit was not modified. VSMC differentiation markers α-SMA, SM-MHC, and smoothelin, interestingly, were all decreased in MSRZ VSMCs compared to LZR VSMCs (**Figures 4C,D**). Thus, *in situ* gelatin zymography was performed to explore MMP activity through gelatinase activity (**Figure 4E**). **Figure 4E** shows representative photographs of *in situ* gelatin zymography in aorta, gelatinase activity is in green. Mean gelatinase activity in the aortic wall was increased in 25 and 80 week-old MSZR compared to age matched LZR aortas (**Figure 4F**). However, age did not modulate gelatinase activity. At the cellular level MSZR VSMCs displayed increased MMP-2 secretion compared to LZR VSMCs (**Figures 4G,H**). Circulating levels of MMP-9 were increased in 80 week-old MSZR whereas VCAM-1 was increased in 25 week-old MSZR compared to same aged LZR and in 80 week-old LZR (**Figures 4I,J**).

DISCUSSION

The aim of the present study was to determine concomitant changes in the haemostasis system and VSMC phenotype and their interplay with FFAs and MMPs during aging in obese rats compared to lean rats of the same age. Our results demonstrated (1) increased thrombin generation in MetS in plasma as early as 25 weeks of age, independently of platelets and at the surface of VSMCs; (2) reinforcement of this hypercoagulability by reduced plasma fibrinolysis; (3) no influence of aging on plasma thrombin generation; (4) an age-related increase in platelet aggregation and clot half lysis time and, (5) contribution of saturated FFAs to the increased thrombin generation both in plasma and at the surface of VSMCs.

Increased thrombotic risk can be attributed to three factors: abnormalities in the vessel wall, in blood flow, and in haemostasis including coagulation and fibrinolysis. We found previously that MSZR presented endothelial dysfunction as shown by increased circulating VWF. This endothelial dysfunction was exacerbated during aging as shown by increases in both VWF and soluble CD146 (Sloboda et al., 2012).

Few studies have used Zucker rats to look at haemostasis and to our knowledge none have been performed in very old Zucker rats. Paul et al. found that 12 week-old diabetic Zucker rats presented unmodified *in vitro* platelet reactivity (Paul et al., 2007). Recently Shang et al. have shown increased thrombosis, increased thrombin generation and decreased fibrinolysis in 7–10 week-old diabetic Zucker rats (Shang et al., 2014). They found also decreased platelet reactivity to collagen and ADP in obese rats in PRP. In PRP, we found increased platelet aggregation using ADP in 80 week-old MSZR and LZR rats compared to

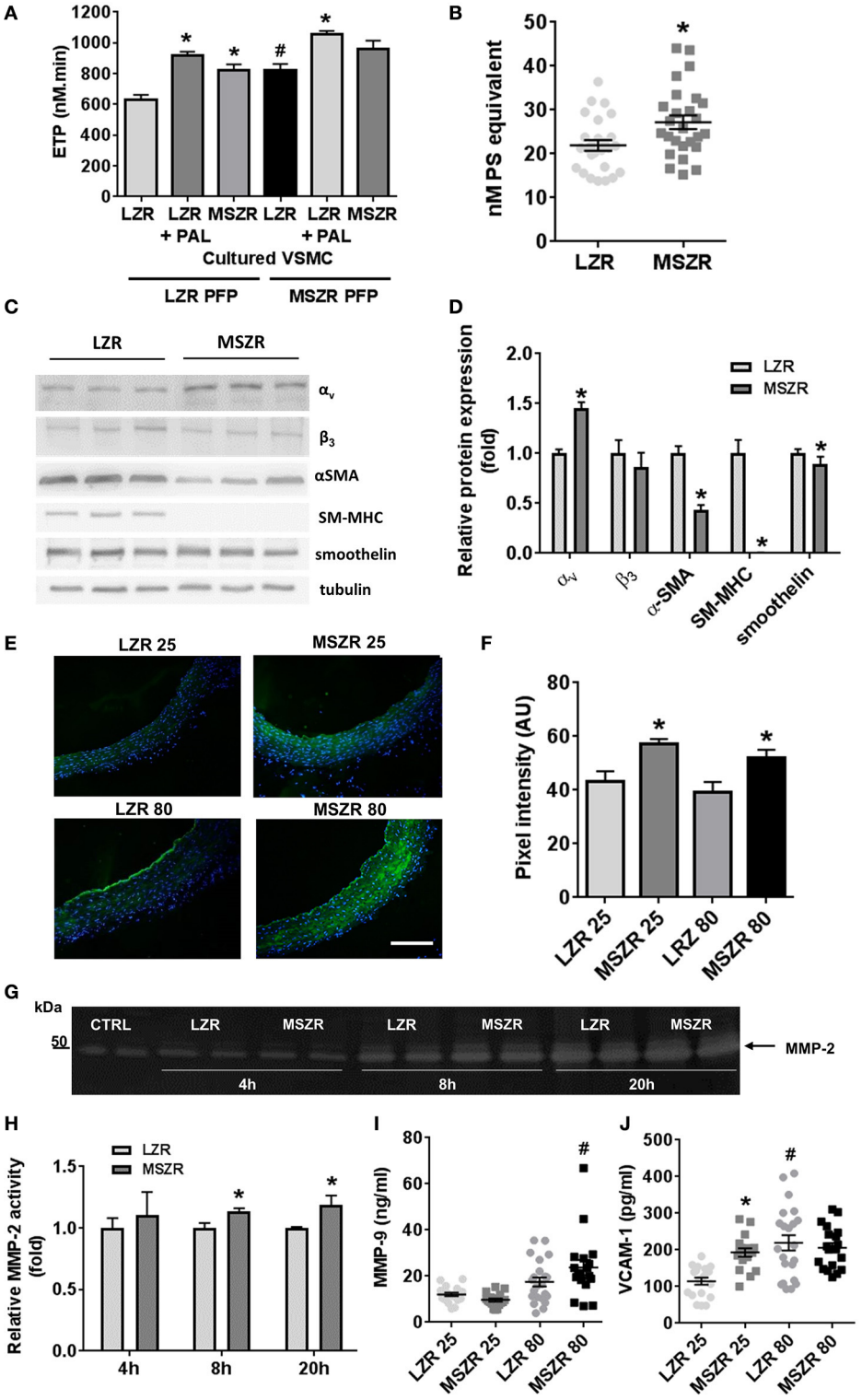

FIGURE 4 | Role of smooth muscle cells in thrombin generation. **(A)** ETP values measured at the surface of vascular smooth muscle cells (VSMCs) from LZR and MSZR, with LZR or MSZR platelet free plasma (PFP), and with or without 1.5 g/l exogenous added palmitic acid (PAL). Results are mean ± standard error of the mean, $n = 3$ with 6 wells per condition per experiment. $^{*}p < 0.05$ vs. LZR VSMC, $^{\#}p < 0.05$ vs. LRZ PFP and LRZ VMSC. **(B)** VSMC associated procoagulant activity reported as phosphatidylserine (PS) equivalent in LRZ and MSZR. Results are mean ± standard error of the mean ($n = 25$). $^{*}p < 0.05$ vs. LZR. **(C)** Typical Western blot and **(D)** quantification analysis of VSMC differentiation markers (αSMA, SM-MHC, and smoothelin) and integrin subunits (α_V and β_3) in cultured VSMCs.

(Continued)

FIGURE 4 | Continued
Results, expressed as fold change vs. VSMCs from LZR, are mean ± standard error of the mean ($n = 6$). *$p < 0.05$, MSZR vs. LZR. **(E)** Representative images of gelatinolytic metalloproteinase activity in the aorta was measured using *in situ* gelatin zymography for each group of Zucker rats. Fluorescence as marker for intra-plaque gelatinolytic activity was quantified. Nuclei were visualized by DAPI staining. **(F)** Average wall fluorescence of the gelatinolytic metalloproteinase activity in the aorta. **(G)** Representative images of zymography gels of LZR and MSZR VSCMCs supernatant at 4, 8, and 20 h. **(H)** Relative MMP-2 activity in LZR and MSZR VSMC supernatant at 4, 8, and 20 h. Results are mean ± standard error of the mean ($n = 5$). *$p < 0.05$, MSZR vs. LZR. ELISAs results of MMP-9 **(I)** and VCAM-1 **(J)** measured in PFP ($n = 17$–22). *$p < 0.05$ vs. LZR at the same age; $^{\#}p < 0.05$ vs. 25 week-old rats in the same strain.

25 week-old controls, but not between rats of the same age. In addition, we were not able to aggregate platelets using collagen. Washed platelets were able to aggregate when triggered with collagen but we did not find any significant changes with obesity or with age. These changes might be related to the metabolic differences existing between rats since they used diabetic Zucker rats while we used obese Zucker rats that only develop diabetes very late with age. Moreover, platelet count was not modified in the diabetic Zucker rats of the Shang et al. study while we found a 25% increased count in MSZR compared to LZR at both ages. Interestingly, platelet-related thrombin generation showed a very important increase in 25 week-old MSZR compared to thrombin generation made with PFP. Altogether, increased platelet aggregation to ADP with age concomitant to increased platelet count in obese Zucker rats is in favor of a prothrombotic state.

To better assess the prothrombotic state in obese and aged rats we investigated *in vivo* thrombin generation by measuring F1+2 fragments, which were increased in MSZR indicating increased *in vivo* formation of thrombin with MetS. As expected, MetS also increased the *in vitro* thrombin generation capacity of plasma, but this ability was not modified with age. This change in the *in vitro* reactivity of the coagulation system points out the role of several components including metabolic factors and the vascular wall. Regarding individual clotting factors it was clear that TF increased in MSZR as well as its inhibitor (TFPI). Increased prothrombin concentration leads to higher thrombin generation and can contribute to the increased ETP in MSZR. Other procoagulant factors such as FVII, FVIII, and VWF are known to be increased with MetS and aging. Metabolic factors such as leptin and adiponectin can participate in haemostasis. Leptin has been suggested previously to represent a link between obesity and atherothrombosis (Petrini et al., 2016). It has been reported that leptin enhanced platelet aggregation while adiponectin reduced it (Konstantinides et al., 2001; Restituto et al., 2010). Adiponectin has been involved also in the endothelium anticoagulation function (Lee et al., 2011) since it increased endothelial TFPI synthesis (Chen et al., 2008). We found in all Zucker rats a strong positive correlation between plasma TPFI and adiponectin concentrations dosed previously (data not shown; Sloboda et al., 2012). Moreover, in our study, we found for the first time that leptin increased ETP and that adiponectin decreased it. Despite it being a modest effect, it argues for a major involvement of adipokines in the regulation of thrombin generation.

Fibrinogen concentration was correlated also to ETP and we confirmed that increased plasma fibrinogen increased ETP (Kumar et al., 1994). Thrombin linked to fibrin can possibly be protected from inhibition by antithrombin, in the same way as it is protected from inhibition when bound to TM (Bourin, 1987). This may participate in explaining the increased time to peak observed in MSZR and increased ETP with no significantly increased peak.

We found that fibrinogen concentration was increased in MSZR and during aging. In favor of the relevance of this result it has been shown that synthesis of fibrinogen is upregulated by inflammatory cytokines such as IL-6 (Morozumi et al., 2009). The consequence of an increased thrombin generation was an increased fibrin network formation in MSZR as shown by thinner fibrin fibers (Wolberg, 2007). The increase in PAI-1 with aging in LZR as well as in MSZR is relevant to human physiology since it is known that during aging PAI-1 is associated with an increased thrombotic risk. In addition, the fibrinogen concentration increased during aging but the mechanisms underlying this association with thrombotic risk are unclear (Cesari et al., 2010). Human fibrinolysis is also impaired in the MetS with a decrease in clot lysis ability linked to increased PAI-1 (Pandolfi et al., 2001). Organization of the fibrin network is likely due to the increased thrombin generation found in MSZR (Wolberg, 2007). Moreover, clots with thinner fibrin fibers are more resistant to lysis than clots with thick fibers (Gabriel et al., 1992). This is supported by the increased half-time lysis found in MSZR and very old Zucker rats. Other factors must be implicated since fiber thickness was unchanged with age in both groups whereas fibrinolysis time increased only during aging indicating the formation of a denser clot. In line with this, adiponectin may act as an anticoagulant molecule. Indeed, full length adiponectin reduces platelet aggregation, inhibits TF and enhances TFPI expression at the surface of endothelial cells (Chen et al., 2008; Restituto et al., 2010). Both adiponectin and IL-13 increase the expression of MMPs which can degrade fibrinogen (Hotary et al., 2002; Wanninger et al., 2011; Firszt et al., 2014). Consistent with this, we found an increase in IL-13 plasmatic concentration with aging and also with the MetS in 80 week-old MSZR which presents the same variations as plasma levels of MMP-9 and FVIII. Whether adiponectin interplays directly with fibrinogen remains an open question. The increase in FVIII with MetS and associated inflammatory stimuli was anticipated in Zucker rats as it is in humans (Begbie et al., 2000; Kotronen et al., 2011).

Inflammation during aging and in the MetS triggers vascular remodeling. Fibrinogen (Lominadze et al., 2010) as well as fibrin and fibrin degradation products have proinflammatory functions that can modify VSMC phenotype (Lu et al., 2011). Cytokines in the plasma, as shown in the array presented here, are increased by the MetS, aging, or both. Our data indicated that the more relevant proinflammatory cytokines such as IL-1α, IL-1β, IL-2, IL-3, and IL-6 were increased early with the MetS while few

anti-inflammatory cytokines were increased with MetS and aging (IL-10, IL-1ra, IL-17). Our cytokine array made with a pool of plasma for each group was checked using ELISA measurements with individual samples for the two main cytokines involved in the regulation of haemostasis (IL-13 and IL-1β). IL-13 changes were confirmed while IL-1β increased only with aging but not with the MetS at 25 weeks of age. This points to a determinant role of age in complex vascular pathologies including several comorbidities. IL-1β has a pleiotropic effect in the development of atherothrombosis through its action on leukocyte adhesion to the vascular wall and induction of procoagulant activity (Libby et al., 1986; Dinarello, 2011). Recently, inhibition of IL-1β and subsequent reduction of inflammation (without modification of lipid levels) in patients with previous episodes of myocardial infarction was found to reduce recurrent cardiovascular events (Ridker et al., 2017). These findings are in line with the increase of circulating IL-1β and increased activity of haemostasis with age we observed in MSZR. Therefore, exploration of haemostasis function in MSZR with inhibition of IL-1β could be of interest.

Other factors related to MetS that can potentiate the modifications we observed in MSZR haemostasis are FFAs. Saturated FFAs such as palmitic acid are known to be associated with ischemic heart disease and increase postprandial concentrations of fibrinogen (Simon et al., 1995; Pacheco et al., 2006). One other mechanism proposed recently to explain the thrombogenic effect of palmitic acid was its ability to induce extracellular release of histones (Shrestha et al., 2013). Histones are known to promote thrombin generation through platelet activation (Semeraro et al., 2011). Additionally, palmitic acid was measured recently in diabetic Zucker rats pointing out a 2.75 times increased concentration in obese rats (0.68 g/l in LZR vs. 1.87 g/l in MSZR) (Godin et al., 2013). A similar increase was observed for a polyunsaturated FFA, linoleic acid. We supplemented 25 week-old LZR PFP with linoleic or palmitic acid to reach MSZR plasma concentrations. We showed for the first time a direct effect of FFAs on thrombin generation confirming the prothrombotic effect of palmitic acid.

All these FFAs, pro-inflammatory cytokines and coagulation factors can have deleterious effects on the vascular wall. We have shown previously the presence of endothelial dysfunction in MSZR (Sloboda et al., 2012). In the present study we studied VSMCs in more detail. Interestingly, thrombin generation measured at the surface of VSMCs from MSZR was increased compared to LZR VSMCs. This increase can be related to the increased procoagulant phospholipids at the surface of MSZR VSMCs. We showed recently that thrombin generation at the surface of VSMC from spontaneously hypertensive rats (SHR) leads to increased ETP and VSMCs were responsible for a prothrombotic phenotype in SHR rats. In the same way as for SHR rats, increased VSMC-supported thrombin generation can be a mechanism implicated in the prothrombotic phenotype we have observed in Zucker rats. In these cellular experiments addition of palmitic acid exacerbated also thrombin generation over MSZR VSMCs.

MMPs are related to FFAs, obesity-related diseases such as type 2 diabetes and overall, inflammation. In our model, mean gelatinase activity, focusing on MMP-2 and-9 activities, was increased in MSZR. These molecules are responsible for the degradation of type IV collagen, elastin, fibronectin, and laminin, among other proteins. It is known that FFAs and insulin lead to hyperactivity of MMP-2 and-9 (Boden et al., 2008). The close relation between MMPs and insulin was demonstrated also in Zucker rats (Zhou et al., 2005). IL-13 was increased in old rats and is known to be an activator of MMPs (Firszt et al., 2014). This increase in aortic MMP activity in the intima with aging has been described in rats and was 2-fold higher in old vs. young non-human primates (Li et al., 1999; Wang et al., 2007). In addition, MMP activity may participate also in age-related vascular remodeling in the aortic media since MMPs accumulate around elastic fibers in the aortic media (Li et al., 1999), which become fragmented with age-associated increases in arterial stiffness which thus increases cardiovascular risk. Interestingly, MMP production can be stimulated through integrin $\alpha_v\beta_3$ (Bendeck et al., 2000). Concerning this pathway, we found an increase of the α_v subunit in MSZR VSMCs and MMP-2 secretion was increased in MSZR compared to LZR. Moreover, we have shown previously that this integrin is responsible for thrombin generation supported by VSMCs and it argues for its role in vascular remodeling (Mao et al., 2012). Very interestingly all VSMC differentiation markers we tested were downregulated in MSZR and even absent concerning SM-MHC. This illustrates a phenotype switch from contractile to secreting VSMCs occurring in vascular diseases such as atherosclerosis (Lacolley et al., 2012).

In conclusion, our study demonstrates in MetS a prothrombotic phenotype of the blood compartment reinforced by procoagulant properties of the vascular wall. Regarding the mechanisms, fibrinogen contributes to this hypercoagulable phenotype in plasma at an early stage of MetS. Leptin and adiponectin exert moderate opposite effects on thrombin generation precluding a major contribution of adipokines. An increase in proinflammatory cytokines likely increased MMP activity inducing a VSMC dedifferentiated phenotype exhibiting procoagulant properties. An increase in FFAs contributes to the increased thrombin generation both in plasma and at the surface of VSMCs. Plasma from MSZR and palmitic acid elicit additive procoagulant effects. The potential benefit of direct thrombin inhibitors should be investigated both on haemostatic balance in blood compartments and on the cellular phenotypic modulation within the vessel wall, and MMP production in MetS and its complications with aging.

AUTHOR CONTRIBUTIONS

JL: performed experiments, analyzed data and wrote the manuscript; AM, MD, and HL: performed experiments and analyzed data; LW, SB, and RA: contributed to the collection, analysis and interpretation of data and writing of the manuscript; BdL, BD, MS, ST, BF, PC, and JKC: contributed to critical writing and revising the intellectual content and final approval of the version of manuscript; PL and VR: designed research and supervised the work, analyzed the data, wrote and reviewed the manuscript.

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REFERENCES

Ait Aissa, K., Lagrange, J., Mohamadi, A., Louis, H., Houppert, B., Challande, P., et al. (2015). Vascular smooth muscle cells are responsible for a prothrombotic phenotype of spontaneously hypertensive rat arteries. *Arterioscler. Thromb. Vasc. Biol.* 35, 930–937. doi: 10.1161/ATVBAHA.115.305377

Alessi, M.-C., and Juhan-Vague, I. (2008). Metabolic syndrome, haemostasis, and thrombosis. *Thromb. Haemost.* 99, 995–1000. doi: 10.1160/TH07-11-0682

Begbie, M., Notley, C., Tinlin, S., Sawyer, L., and Lillicrap, D. (2000). The Factor VIII acute phase response requires the participation of NFκB and C/EBP. *Thromb. Haemost.* 84, 216–222.

Beijers, H. J., Ferreira, I., Spronk, H. M., Bravenboer, B., Dekker, J. M., Nijpels, G., et al. (2010). Body composition as determinant of thrombin generation in plasma: the Hoorn study. *Arterioscler. Thromb. Vasc. Biol.* 30, 2639–2647. doi: 10.1161/ATVBAHA.110.211946

Bendeck, M. P., Irvin, C., Reidy, M., Smith, L., Mulholland, D., Horton, M., et al. (2000). Smooth muscle cell matrix metalloproteinase production is stimulated via $\alpha_v\beta_3$ integrin. *Arterioscler. Thromb. Vasc. Biol.* 20, 1467–1472. doi: 10.1161/01.ATV.20.6.1467

Boden, G., Song, W., Pashko, L., and Kresge, K. (2008). *In vivo* effects of insulin and free fatty acids on matrix metalloproteinases in rat aorta. *Diabetes* 57, 476–483. doi: 10.2337/db07-1261

Bourin, M. C. (1987). [Effect of rabbit thrombomodulin on the inhibition of thrombin by the antithrombin-heparin complex: role of the acid domain of thrombomodulin]. *Comptes Rendus Académie Sci. Sér. III Sci. Vie* 304, 173–176.

Cesari, M., Pahor, M., and Incalzi, R. A. (2010). Plasminogen activator inhibitor-1 (PAI-1): a key factor linking fibrinolysis and age-related subclinical and clinical conditions. *Cardiovasc. Ther.* 28, e72–e91. doi: 10.1111/j.1755-5922.2010.00171.x

Chen, Y.-J., Zhang, L.-Q., Wang, G.-P., Zeng, H., Lü, B., Shen, X.-L., et al. (2008). Adiponectin inhibits tissue factor expression and enhances tissue factor pathway inhibitor expression in human endothelial cells. *Thromb. Haemost.* 100, 291–300. doi: 10.1160/TH08-02-0124

Dandona, P., Aljada, A., Chaudhuri, A., Mohanty, P., and Garg, R. (2005). Metabolic syndrome: a comprehensive perspective based on interactions between obesity, diabetes, and inflammation. *Circulation* 111, 1448–1454. doi: 10.1161/01.CIR.0000158483.13093.9D

Dinarello, C. A. (2011). Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. *Blood* 117, 3720–3732. doi: 10.1182/blood-2010-07-273417

Firszt, R., Francisco, D., Church, T. D., Thomas, J. M., Ingram, J. L., and Kraft, M. (2014). Interleukin-13 induces collagen type-1 expression through matrix metalloproteinase-2 and transforming growth factor-β1 in airway fibroblasts in asthma. *Eur. Respir. J.* 43, 464–473. doi: 10.1183/09031936.00068712

Gabriel, D. A., Muga, K., and Boothroyd, E. M. (1992). The effect of fibrin structure on fibrinolysis. *J. Biol. Chem.* 267, 24259–24263.

Godin, J.-P., Ross, A. B., Cléroux, M., Pouteau, E., Montoliu, I., Moser, M., et al. (2013). Natural carbon isotope abundance of plasma metabolites and liver tissue differs between diabetic and non-diabetic Zucker diabetic fatty rats. *PLoS ONE* 8:e74866. doi: 10.1371/journal.pone.0074866

Halcox, J. P. J., Donald, A. E., Ellins, E., Witte, D. R., Shipley, M. J., Brunner, E. J., et al. (2009). Endothelial function predicts progression of carotid intima-media thickness. *Circulation* 119, 1005–1012. doi: 10.1161/CIRCULATIONAHA.108.765701

Hotary, K. B., Yana, I., Sabeh, F., Li, X.-Y., Holmbeck, K., Birkedal-Hansen, H., et al. (2002). Matrix metalloproteinases (MMPs) regulate fibrin-invasive activity via MT1-MMP-dependent and -independent processes. *J. Exp. Med.* 195, 295–308. doi: 10.1084/jem.20010815

Konings, J., Govers-Riemslag, J. W. P., Philippou, H., Mutch, N. J., Borissoff, J. I., Allan, P., et al. (2011). Factor XIIa regulates the structure of the fibrin clot independently of thrombin generation through direct interaction with fibrin. *Blood* 118, 3942–3951. doi: 10.1182/blood-2011-03-339572

Konstantinides, S., Schäfer, K., Koschnick, S., and Loskutoff, D. J. (2001). Leptin-dependent platelet aggregation and arterial thrombosis suggests a mechanism for atherothrombotic disease in obesity. *J. Clin. Invest.* 108, 1533–1540. doi: 10.1172/JCI13143

Kotronen, A., Joutsi-Korhonen, L., Sevastianova, K., Bergholm, R., Hakkarainen, A., Pietiläinen, K. H., et al. (2011). Increased coagulation factor VIII, IX, XI and XII activities in non-alcoholic fatty liver disease. *Liver Int.* 31, 176–183. doi: 10.1111/j.1478-3231.2010.02375.x

Kumar, R., Béguin, S., and Hemker, H. C. (1994). The influence of fibrinogen and fibrin on thrombin generation–evidence for feedback activation of the clotting system by clot bound thrombin. *Thromb. Haemost.* 72, 713–721.

Lacolley, P., Regnault, V., Nicoletti, A., Li, Z., and Michel, J.-B. (2012). The vascular smooth muscle cell in arterial pathology: a cell that can take on multiple roles. *Cardiovasc. Res.* 95, 194–204. doi: 10.1093/cvr/cvs135

Lee, S., Park, Y., Dellsperger, K. C., and Zhang, C. (2011). Exercise training improves endothelial function via adiponectin-dependent and independent pathways in type 2 diabetic mice. *Am. J. Physiol. Heart Circ. Physiol.* 301, H306–H314. doi: 10.1152/ajpheart.01306.2010

Li, Z., Froehlich, J., Galis, Z. S., and Lakatta, E. G. (1999). Increased expression of matrix metalloproteinase-2 in the thickened intima of aged rats. *Hypertension* 33, 116–123. doi: 10.1161/01.HYP.33.1.116

Libby, P., Ordovas, J. M., Auger, K. R., Robbins, A. H., Birinyi, L. K., and Dinarello, C. A. (1986). Endotoxin and tumor necrosis factor induce interleukin-1 gene expression in adult human vascular endothelial cells. *Am. J. Pathol.* 124, 179–185.

Lim, H. S., Lip, G. Y. H., and Blann, A. D. (2004). Plasma von Willebrand factor and the development of the metabolic syndrome in patients with hypertension. *J. Clin. Endocrinol. Metab.* 89, 5377–5381. doi: 10.1210/jc.2004-0616

Lominadze, D., Dean, W. L., Tyagi, S. C., and Roberts, A. M. (2010). Mechanisms of fibrinogen-induced microvascular dysfunction during cardiovascular disease. *Acta Physiol. Oxf. Engl.* 198, 1–13. doi: 10.1111/j.1748-1716.2009.02037.x

Lu, P., Liu, J., Liu, N., Guo, F., Ji, Y., and Pang, X. (2011). Pro-inflammatory effect of fibrinogen and FDP on vascular smooth muscle cells by IL-6, TNF-α and iNOS. *Life Sci.* 88, 839–845. doi: 10.1016/j.lfs.2011.03.003

Mao, X., Said, R., Louis, H., Max, J.-P., Bourhim, M., Challande, P., et al. (2012). Cyclic stretch-induced thrombin generation by rat vascular smooth muscle cells is mediated by the integrin $\alpha_v\beta_3$ pathway. *Cardiovasc. Res.* 96, 513–523. doi: 10.1093/cvr/cvs274

Matsuzawa, Y., Funahashi, T., Kihara, S., and Shimomura, I. (2004). Adiponectin and metabolic syndrome. *Arterioscler. Thromb. Vasc. Biol.* 24, 29–33. doi: 10.1161/01.ATV.0000099786.99623.EF

Membre, A., Wahl, D., Latger-Cannard, V., Max, J.-P., Lacolley, P., Lecompte, T., et al. (2008). The effect of platelet activation on the hypercoagulability induced by murine monoclonal antiphospholipid antibodies. *Haematologica* 93, 566–573. doi: 10.3324/haematol.12364

Mook, O. R. F., Van Overbeek, C., Ackema, E. G., Van Maldegem, F., and Frederiks, W. M. (2003). *In situ* localization of gelatinolytic activity in the extracellular matrix of metastases of colon cancer in rat liver using quenched fluorogenic DQ-gelatin. *J. Histochem. Cytochem.* 51, 821–829. doi: 10.1177/002215540305100613

Morozumi, T., Sharma, A., and De Nardin, E. (2009). The functional effects of the−455G/A polymorphism on the IL-6-induced expression of the beta-fibrinogen gene may be due to linkage disequilibrium with other functional polymorphisms. *Immunol. Invest.* 38, 311–323. doi: 10.1080/08820130902745153

Ninivaggi, M., Apitz-Castro, R., Dargaud, Y., de Laat, B., Hemker, H. C., and Lindhout, T. (2012). Whole-blood thrombin generation monitored with a calibrated automated thrombogram-based assay. *Clin. Chem.* 58, 1252–1259. doi: 10.1373/clinchem.2012.184077

Pacheco, Y. M., Bermúdez, B., López, S., Abia, R., Villar, J., and Muriana, F. J. G. (2006). Ratio of oleic to palmitic acid is a dietary determinant of thrombogenic and fibrinolytic factors during the postprandial state in men. *Am. J. Clin. Nutr.* 84, 342–349.

Pandolfi, A., Cetrullo, D., Polishuck, R., Alberta, M. M., Calafiore, A., Pellegrini, G., et al. (2001). Plasminogen activator inhibitor type 1 is increased in the arterial wall of type II diabetic subjects. *Arterioscler. Thromb. Vasc. Biol.* 21, 1378–1382. doi: 10.1161/hq0801.093667

Paul, W., Queen, L. R., Page, C. P., and Ferro, A. (2007). Increased platelet aggregation *in vivo* in the Zucker Diabetic Fatty rat: differences from the streptozotocin diabetic rat. *Br. J. Pharmacol.* 150, 105–111. doi: 10.1038/sj.bjp.0706957

Petrini, S., Neri, T., Lombardi, S., Cordazzo, C., Balìa, C., Scalise, V., et al. (2016). Leptin induces the generation of procoagulant, tissue factor bearing microparticles by human peripheral blood mononuclear cells. *Biochim. Biophys. Acta* 1860, 1354–1361. doi: 10.1016/j.bbagen.2016.03.029

Phillips, M. S., Liu, Q., Hammond, H. A., Dugan, V., Hey, P. J., Caskey, C. J., et al. (1996). Leptin receptor missense mutation in the fatty Zucker rat. *Nat. Genet.* 13, 18–19. doi: 10.1038/ng0596-18

Regnault, V., Hemker, H. C., Wahl, D., and Lecompte, T. (2004). Phenotyping the haemostatic system by thrombography–potential for the estimation of thrombotic risk. *Thromb. Res.* 114, 539–545. doi: 10.1016/j.thromres.2004.06.017

Restituto, P., Colina, I., Varo, J. J., and Varo, N. (2010). Adiponectin diminishes platelet aggregation and sCD40L release. Potential role in the metabolic syndrome. *Am. J. Physiol. Endocrinol. Metab.* 298, E1072–E1077. doi: 10.1152/ajpendo.00728.2009

Ridker, P. M., Everett, B. M., Thuren, T., MacFadyen, J. G., Chang, W. H., Ballantyne, C., et al. (2017). Antiinflammatory therapy with canakinumab for atherosclerotic disease. *N. Engl. J. Med.* 377, 1119–1131. doi: 10.1056/NEJMoa1707914

Samad, F., Pandey, M., and Loskutoff, D. J. (2001). Regulation of tissue factor gene expression in obesity. *Blood* 98, 3353–3358. doi: 10.1182/blood.V98.12.3353

Semeraro, F., Ammollo, C. T., Morrissey, J. H., Dale, G. L., Friese, P., Esmon, N. L., et al. (2011). Extracellular histones promote thrombin generation through platelet-dependent mechanisms: involvement of platelet TLR2 and TLR4. *Blood* 118, 1952–1961. doi: 10.1182/blood-2011-03-343061

Shang, J., Chen, Z., Wang, M., Li, Q., Feng, W., Wu, Y., et al. (2014). Zucker diabetic fatty rats exhibit hypercoagulability and accelerated thrombus formation in the Arterio-Venous shunt model of thrombosis. *Thromb. Res.* 134, 433–439. doi: 10.1016/j.thromres.2014.04.008

Shrestha, C., Ito, T., Kawahara, K., Shrestha, B., Yamakuchi, M., Hashiguchi, T., et al. (2013). Saturated fatty acid palmitate induces extracellular release of histone H3: a possible mechanistic basis for high-fat diet-induced inflammation and thrombosis. *Biochem. Biophys. Res. Commun.* 437, 573–578. doi: 10.1016/j.bbrc.2013.06.117

Simon, J. A., Hodgkins, M. L., Browner, W. S., Neuhaus, J. M., Bernert, J. T., and Hulley, S. B. (1995). Serum fatty acids and the risk of coronary heart disease. *Am. J. Epidemiol.* 142, 469–476. doi: 10.1093/oxfordjournals.aje.a117662

Sloboda, N., Fève, B., Thornton, S. N., Nzietchueng, R., Regnault, V., Simon, G., et al. (2012). Fatty acids impair endothelium-dependent vasorelaxation: a link between obesity and arterial stiffness in very old Zucker rats. *J. Gerontol. A Biol. Sci. Med. Sci.* 67, 927–938. doi: 10.1093/gerona/glr236

Sonnenberg, G. E., Krakower, G. R., and Kissebah, A. H. (2004). A novel pathway to the manifestations of metabolic syndrome. *Obes. Res.* 12, 180–186. doi: 10.1038/oby.2004.24

Suehiro, A., Wakabayashi, I., Uchida, K., Yamashita, T., and Yamamoto, J. (2012). Impaired spontaneous thrombolytic activity measured by global thrombosis test in males with metabolic syndrome. *Thromb. Res.* 129, 499–501. doi: 10.1016/j.thromres.2011.06.019

Wagenvoord, R. J., Hendrix, H. H., Kai, H., and Hemker, H. C. (1994). A chromogenic test to determine the procoagulant phospholipids in platelet-rich plasma and whole blood. Thromb. *Haemost.* 72, 582–587.

Wakil, S. J., and Abu-Elheiga, L. A. (2009). Fatty acid metabolism: target for metabolic syndrome. *J. Lipid Res.* 50, S138–S143. doi: 10.1194/jlr.R800079-JLR200

Wang, M. Y., Zhou, Y. T., Newgard, C. B., and Unger, R. H. (1996). A novel leptin receptor isoform in rat. *FEBS Lett.* 392, 87–90. doi: 10.1016/0014-5793(96)00790-9

Wang, M., Zhang, J., Jiang, L.-Q., Spinetti, G., Pintus, G., Monticone, R., et al. (2007). Proinflammatory profile within the grossly normal aged human aortic wall. *Hypertension* 50, 219–227. doi: 10.1161/HYPERTENSIONAHA.107.089409

Wanninger, J., Walter, R., Bauer, S., Eisinger, K., Schäffler, A., Dorn, C., et al. (2011). MMP-9 activity is increased by adiponectin in primary human hepatocytes but even negatively correlates with serum adiponectin in a rodent model of non-alcoholic steatohepatitis. *Exp. Mol. Pathol.* 91, 603–607. doi: 10.1016/j.yexmp.2011.07.001

Weiss, T. W., Arnesen, H., and Seljeflot, I. (2013). Components of the interleukin-6 transsignalling system are associated with the metabolic syndrome, endothelial dysfunction and arterial stiffness. *Metab. Clin. Exp.* 62, 1008–1013. doi: 10.1016/j.metabol.2013.01.019

Wolberg, A. S. (2007). Thrombin generation and fibrin clot structure. *Blood Rev.* 21, 131–142. doi: 10.1016/j.blre.2006.11.001

Zhou, Y.-P., Madjidi, A., Wilson, M. E., Nothhelfer, D. A., Johnson, J. H., Palma, J. F., et al. (2005). Matrix metalloproteinases contribute to insulin insufficiency in Zucker diabetic fatty rats. *Diabetes* 54, 2612–2619. doi: 10.2337/diabetes.54.9.2612

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The Associations between Apolipoprotein E Gene Epsilon2/Epsilon3/Epsilon4 Polymorphisms and the Risk of Coronary Artery Disease in Patients with Type 2 Diabetes Mellitus

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Background and Objective: Apolipoprotein E (APOE) plays important roles in lipoprotein metabolism and cardiovascular disease. Evidence suggests the *APOE* gene epsilon2/epsilon3/epsilon4 (ε2/ε3/ε4) polymorphisms might be associated with the susceptibility of coronary artery disease (CAD) in patients with type 2 diabetes mellitus (T2DM). However, no clear consensus has yet been established. Therefore, the aim of this meta-analysis is to provide a precise conclusion on the potential association between *APOE* ε2/ε3/ε4 polymorphisms and the risk of CAD in patients with T2DM based on case-control studies.

Methods: Pubmed, Embase, Chinese National Knowledge Infrastructure (CNKI), and Wanfang databases were searched for all relevant studies prior to August 2017 in English and Chinese language. The pooled odds ratios (ORs) and their corresponding 95% confidence intervals (CIs) were used to assess the strength of the relationships. The between-study heterogeneity was evaluated by Cochran's Q-test and the I^2 index to adopt fixed- or random- effect models.

Results: A total of 13 studies were eligible for inclusion. There was evidence for significant associations between *APOE* ε4 mutation and the risk of CAD in patients with T2DM (for ε3/ε4 vs. ε3/ε3: OR = 1.69, 95% CI = 1.38–2.08, $P < 0.001$; for ε4/ε4 vs. ε3/ε3: OR = 2.72, 95% CI = 1.61–4.60, $P < 0.001$; for ε4/ε4+ε3/ε4 vs. ε3/ε3: OR = 1.83, 95% CI = 1.52–2.22, $P < 0.001$; for ε4 allele vs. ε3 allele: OR = 1.64, 95% CI = 1.40–1.94, $P < 0.001$). In contrast, no significant associations were found in genetic model of *APOE* ε2 mutation (for ε2/ε2 vs. ε3/ε3: OR = 1.67, 95% CI = 0.90–3.09, $P = 0.104$; for ε2/ε3 vs. ε3/ε3: OR = 1.18, 95% CI = 0.93–1.51, $P = 0.175$; for ε2/ε2+ε2/ε3 vs. ε3/ε3: OR = 1.26, 95% CI = 0.88–1.82, $P = 0.212$; for ε2 allele vs. ε3 allele: OR = 1.34, 95% CI = 0.98–1.84, $P = 0.07$).

Conclusions: The *APOE* gene ε4 mutation is associated with an increased risk of CAD in patients with T2DM, while the ε2 variation has null association with this disease.

Keywords: coronary artery disease, type 2 diabetes mellitus, apolipoprotein E, epsilon2, epsilon3, epsilon4, genetic polymorphism

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a long-term metabolic disease with a high incidence and prevalence in the world. T2DM is often accompanied by various complications such as hypertension, dyslipidemia and coronary artery disease (CAD) (Naito and Miyauchi, 2017). As the disease progresses, patients with T2DM have a 2 to 4-fold increased risk for developing CAD compared with non-diabetic individuals (Mohan et al., 2001; Emerging Risk Factors et al., 2010). In addition, cardiovascular disease including CAD in patients with T2DM is associated with significant mortality (Zhang et al., 2014b; Freitas Lima et al., 2015). Therefore, early prevention and vigorous control of T2DM and its complications are becoming an ever-increasing global health priority. A better understanding of the etiology of CAD in patients with T2DM will result in better clinical management.

Dyslipidemia, hypertension, obesity, and smoking status are well-established risk factors for T2DM (Paneni et al., 2013; Wang et al., 2015a). Additionally, human genetic association studies have revealed that numerous genetic mutations and polymorphisms also play a critical role (Wei et al., 2014; Raj et al., 2015; Sumi et al., 2017). Among the previous studies, apolipoprotein E (APOE) gene has been regarded as one of the most likely candidate genes which may be associated with CAD in T2DM patients.

APOE is a class of plasma apolipoprotein totaling 299 amino acids, and it is involved in lipoprotein metabolism and the development of cardiovascular diseases (Zheng et al., 1998). The *APOE* gene is mapped to chromosome 19q13.2 in a cluster with apolipoprotein C1 and C2 gene, and it consists of three introns and four exons. *APOE* is a polymorphic gene and the most commonly studied alleles/isoforms are: epsilon2 (ε2), epsilon3 (ε3), and epsilon4 (ε4). The differences between the three isoforms are the location of 112 and 158 in the amino acid chain where cysteine or arginine is present. These three *APOE* alleles are determined by the rs7412 and rs429358 single-nucleotide polymorphisms. The three alleles, *APOE*-ε2 (cys112 and cys158), *APOE*-ε3 (cys112 and arg158) and *APOE*-ε4 (arg112 and arg158), yield six different genotypes for the *APOE* gene: ε2/ε2, ε2/ε3, ε2/ε4, ε3/ε3, ε3/ε4, and ε4/ε4. Because the ε3 allele or ε3/ε3 genotype is the most common allele or genotype among the population, they are well accepted as the "wild-type" and used as the "reference" in the genetic models (Zhang et al., 2000; Guo et al., 2007; Izar et al., 2009; Chaudhary et al., 2012; Hong et al., 2017).

The role of *APOE* ε2/ε3/ε4 polymorphisms in the development of CAD in patients with T2DM is widely studied, but the results are still controversial and conflicting. In 1998, Zheng et al. firstly investigated the association between *APOE* gene polymorphism and T2DM complicated with CAD in the Chinese population. The results showed that *APOE*-ε4 allele increased the risk of CAD in T2DM (Zheng et al., 1998). Other studies have also confirmed Zheng's findings (Chaaba et al., 2008; Hong et al., 2017). However, *APOE*-ε2 allele was also found to be associated with the risk of CAD in T2DM (Halim et al., 2012). In addition, no significant association between *APOE* ε2/ε3/ε4 polymorphisms and the risk of CAD in T2DM was reported in some studies (Zhang et al., 2000; Guo et al., 2007; Izar et al., 2009). To demonstrate with certainty the associations between the *APOE* ε2/ε3/ε4 polymorphisms and the risk of CAD in patients with T2DM, we conducted a systematic review and meta-analysis on published case-control studies.

MATERIALS AND METHODS

Literature Search

This study was undertaken according to the methodology of MOOSE (Meta-analysis of Observational Studies in Epidemiology) statement (Stroup et al., 2000). We thoroughly searched all published studies in the Embase, PubMed, China National Knowledge Infrastructure (CNKI) and Wanfang databases up to August 2, 2017. The included articles were limited to Chinese and English language. The following keywords were used for searching: "apolipoprotein E" OR "APOE" AND "polymorphism" OR "single nucleotide polymorphism" OR "SNP" OR "variant" OR "variation" AND "coronary artery disease" OR "coronary heart disease" OR "CAD" OR "CHD" OR "atherosclerosis" OR "myocardial infarction" OR "myocardial infarct" OR "heart attack" OR "MI" AND "type 2 diabetes" OR "non-insulin dependent diabetes mellitus" OR "diabetes mellitus, type 2" OR "diabetes, type 2" OR "diabetes mellitus, non-insulin dependent" The Chinese databases were searched using the equivalent Chinese terms. In addition, hand searches for all related articles were performed. The detailed search strategies are presented in Supplementary Table 1.

Inclusion and Exclusion Criteria

The first two investigators independently accessed the eligibility of the studies by screening the title, abstract and full-text, based on the inclusion and exclusion criteria. The inclusion criteria for all studies were as follows: (1) study on the associations between *APOE* ε2/ε3/ε4 polymorphisms and CAD in patients with T2DM, regardless of sample size. (2) case-control design. (3) detailed data for the *APOE* alleles or genotype distribution in case and control groups to estimate odds ratio (OR) with 95% confidence interval (CI). Exclusion criteria: (1) duplication of previous data; (2) review, comment and editorial; (3) no sufficient

genotype data. Any dispute about the eligibility of an article was resolved by discussion.

Data Extraction

The data was drawn out based on a standard protocol. The following information was carefully extracted from all eligible publications independently by two authors (JQL and HR) using a standardized form: last name of first author, year of publication, study country, sample size in cases and controls, methods of genotyping, number genotypes and alleles. If similar data sets presented in different articles by the same research group, the data would be adopted only once. The collected data were compared, and possible disagreements were discussed by the authors and resolved with consensus.

Quality Score Assessment

The study quality was independently assessed by two reviewers. Quality assessment of genetic associations between *APOE* ε2/ε3/ε4 polymorphisms and CAD in patients with T2DM is described in the Supplementary Table 2. The scores were adjusted according to the criteria developed for meta-analysis of molecular association studies by Thakkinstian et al. (2005). The total scores ranged from 0 to 13, with 13 representing the highest quality.

Statistics Analysis

All the statistical analysis in this study was performed using Stata 12.0 (StataCorp, College Station, TX). Hardy-Weinberg equilibrium was performed in control groups using the chi-square test. The combined OR and 95%CI were calculated to evaluate the strength of the association between the *APOE* ε2/ε3/ε4 polymorphisms and risk of CAD in T2DM subjects. The pooled ORs were, respectively, performed for nine genetic models (ε2/ε2 vs. ε3/ε3; ε2/ε3 vs. ε3/ε3; ε2/ε4 vs. ε3/ε3; ε3/ε4 vs. ε3/ε3; ε4/ε4 vs. ε3/ε3; ε2 allele vs. ε3 allele; ε4 allele vs. ε3 allele; ε2/ε2+ε2/ε3 vs. ε3/ε3; ε4/ε4+ε3/ε4 vs. ε3/ε3). The statistically significant level of the combined OR was determined

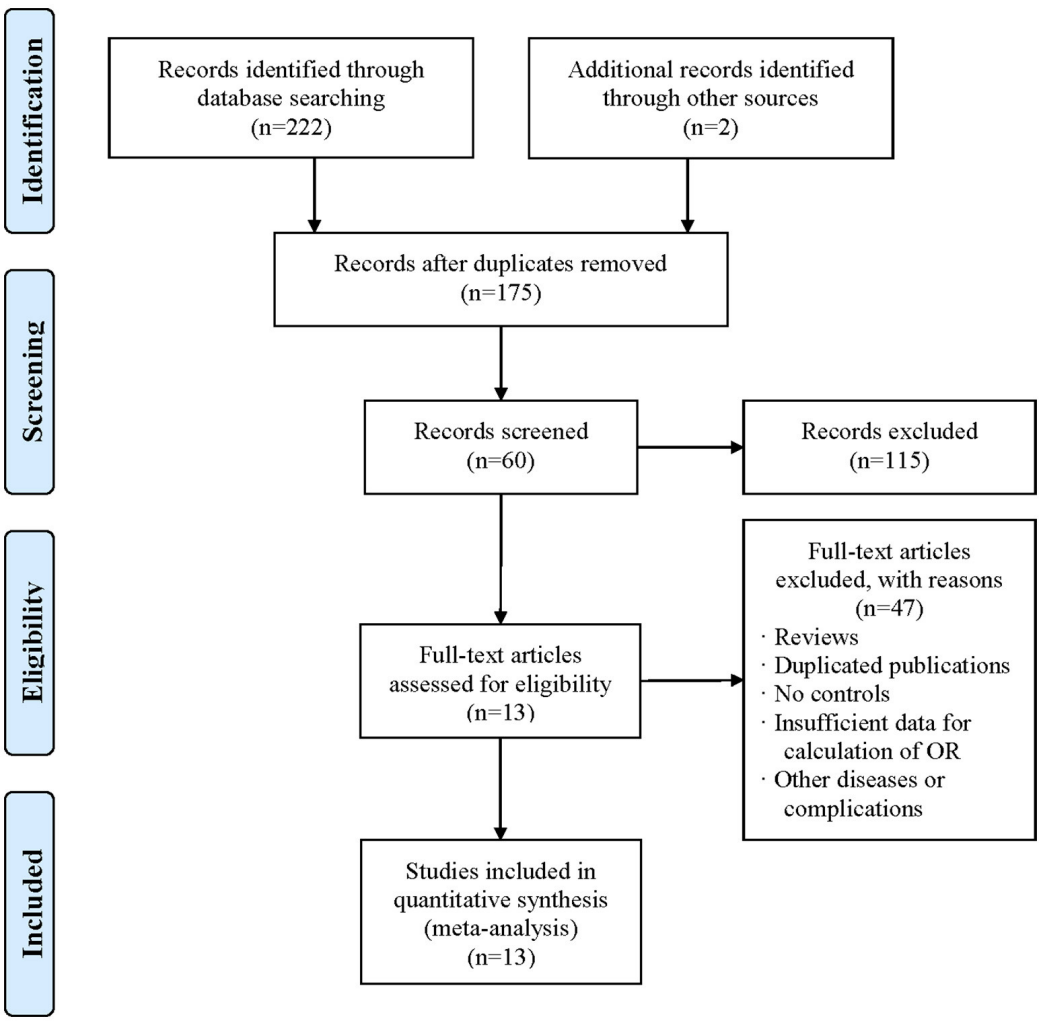

FIGURE 1 | Flow diagram of study selection process. The term "n" in the boxes represens the number of corresponding studies.

TABLE 1 | Characteristics of the included studies for this meta-analysis.

First-author	Year	Country	Genotyping methods[a]	Quality score	Sample size		APOE genotypes distribution (case/control)									
					Case	Control	ε2/ε2	ε2/ε3	ε3/ε3	ε3/ε4	ε4/ε4	ε2/ε4	ε2	ε3	ε4	ε2/ε2+ ε2/ε3	ε3/ε4+ ε4/ε4
Hong	2017	China	RT-PCR	10	114	106	1/1	14/11	61/72	31/20	2/0	5/2	21/15	167/175	40/22	15/12	33/20
Chaudhary	2012	Thailand	PCR-RFLP	12	147	155	1/1	11/2	88/117	46/30	1/4	0/1	13/5	233/266	48/39	12/3	47/34
Halim	2012	Egypt	PCR-RFLP	5	35	35	6/0	5/2	18/31	6/2	0/0	0/0	17/2	47/66	6/2	11/2	6/2
Al-Majed	2011	Kuwaiti	PCR-RFLP	9	41	105	3/7	1/2	21/73	4/6	12/15	0/2	7/18	47/154	28/38	4/9	16/21
Vaisi-Raygani	2010	Iran	PCR-RFLP	12	172	118	4/0	31/26	91/69	31/20	12/3	3/0	42/26	244/184	58/26	35/26	43/23
Shi	2009	China	PCR-RFLP	9	98	110	0/0	4/3	44/71	36/27	2/0	12/9	16/12	128/172	52/36	4/3	38/27
Izar	2009	Brazil	PCR-RFLP	11	386	604	3/7	60/86	241/388	57/81	9/4	14/31	80/131	599/943	89/120	63/93	66/85
Chaaba	2008	Tunisia	PCR-RFLP	9	71	86	0/0	3/9	57/68	NA	NA	0/1	NA	NA	NA	3/9	11/8
Zhang L	2008	China	PCR-RFLP	10	100	100	2/4	12/15	54/67	30/13	2/1	0/0	16/23	150/162	34/15	14/19	32/14
Guo	2007	China	Multi-ARMS-PCR	11	40	40	0/0	2/1	22/29	13/7	1/0	2/3	4/4	59/66	17/10	2/1	14/7
Pan	2002	China	PCR-RFLP	11	24	63	0/1	4/7	12/45	6/8	0/0	2/2	6/11	34/105	8/10	4/8	6/8
Zhang WH	2000	China	PCR-RFLP	9	61	63	1/0	2/7	46/50	10/6	1/0	1/0	5/7	104/113	13/6	3/7	11/6
Zheng	1998	China	PCR-RFLP	8	33	78	NA	NA	22/59	NA	NA	NA	NA	NA	NA	3/15	8/4

[a]*Multi-ARMS-PCR: multiplex amplification refractory mutation system-polymerase chain reaction; PCR-RFLP: polymerase chain reaction restriction fragment length polymorphism; RT-PCR: real-time polymerase chain reaction.*

NA: not available.

by the *Z*-test with $P < 0.05$. Heterogeneity between studies was calculated by using the Cochran's Q-test and Higgins I^2 index. In the absence of between-study heterogeneity ($I^2 < 50\%$), the fixed effect model (Mantel–Haenszel method) was chosen to calculate the pooled estimates. Otherwise, random effect model (DerSimonian and Laird method) would be adopted if the $I^2 > 50\%$ (Higgins et al., 2003). Subgroup analysis was performed according to the source of patients (Chinese and non-Chinese). Galbraith plot analysis and sensitivity analysis were conducted to detect whether there were outliers that could be the potential sources of heterogeneity between studies when heterogeneity was moderately large. Publication bias was evaluated by Begg's funnel plot and Egger's regression test (Begg and Mazumdar, 1994; Egger et al., 1997). If there is evidence of significant publication bias, the trim and fill method was performed to assess the potential influence of publication bias (Duval and Tweedie, 2000).

RESULTS

The Characteristics of the Included Studies

As depicted in **Figure 1**, a total of 222 articles were obtained by online search, and 2 articles were included by manual search. After removing duplicates, 175 articles were included. After screening title and abstract, 115 articles were excluded. As a result, 13 articles (Zheng et al., 1998; Zhang et al., 2000, 2008; Pan et al., 2002; Guo et al., 2007; Chaaba et al., 2008; Izar et al., 2009; Shi et al., 2009; Vaisi-Raygani et al., 2010; Al-Majed et al., 2011; Chaudhary et al., 2012; Halim et al., 2012; Hong et al., 2017) were eligible for the meta-analysis. The characteristics of the included articles are summarized in **Table 1**. The included studies were conducted in several countries including China, Brazil, Thailand, Egypt, Iran, Kuwait, and Tunisia. All studies were performed in a case-control design and the sample sizes varied from 70 to 990. The quality score of the included studies ranged from 5 to 12 (mean: 9.69) out of a maximal score of 13.

Quantitative Synthesis

The main results of this meta-analysis for the association between *APOE* ε2/ε3/ε4 polymorphisms and the risk of CAD in T2DM patients are presented in **Table 2**. There was significant association in three genetic models which demonstrate, ε4 mutation contributed to an increased risk of CAD in patients with T2DM (**Figure 2**). The pooled results for the three genetic models in the overall analysis were as follows: for ε3/ε4 vs. ε3/ε3: OR = 1.69, 95% CI = 1.38–2.08, $P < 0.001$; for ε4/ε4 vs. ε3/ε3: OR = 2.72, 95% CI = 1.61–4.60, $P < 0.001$; for ε4/ε4+ε3/ε4 vs. ε3/ε3: OR = 1.83, 95% CI = 1.52–2.22, $P < 0.001$. In contrast, the ε2 variation had null association with this disease (**Figure 3**). No significant association in the overall analysis was found in genetic model of ε2/ε2 vs. ε3/ε3 (OR = 1.67, 95% CI = 0.90–3.09, $P = 0.104$); ε2/ε3 vs. ε3/ε3 (OR = 1.18, 95% CI = 0.93–1.51, $P = 0.175$); ε2/ε4 vs. ε3/ε3 (OR = 1.20, 95% CI = 0.78–1.84, $P = 0.405$); ε2/ε2+ε2/ε3 vs. ε3/ε3 (OR = 1.26, 95% CI = 0.88–1.82, $P = 0.212$). In addition, the genetic models of allele-based contrasts in the overall analysis also revealed a statistically significant pooled estimates for ε4 allele vs. ε3 allele (OR = 1.64, 95% CI = 1.40–1.94, $P < 0.001$) but not for ε2 allele vs. ε3 allele (OR = 1.34, 95% CI = 0.98–1.84, $P = 0.07$).

In the subgroup analysis according to the source of patients (Chinese and non-Chinese), the pooled ORs of all genetic models except the ε2/ε4 vs. ε3/ε3 model were consistent with the results

TABLE 2 | Meta-analysis results of the associations between *APOE* ε2/ε3/ε4 polymorphisms and risk of coronary artery diseases in type 2 diabetes patients.

Genetic model	Pooled OR (95%CI)	*Z*-value	$P_{meta\text{-}analysis}$	NO. of studies	Model[a]	$P_{heterogeneity}$[b]	I^2%
ε2/ε2 vs. ε3/ε3	1.67(0.90–3.09)	1.62	0.104	9	F	0.532	0.00
Chinese	2.03(0.98–4.21)	0.02	0.984	4	F	0.841	0.00
Non-Chinese	1.01(0.31–3.32)	1.90	0.058	5	F	0.208	32.00
ε2/ε3 vs. ε3/ε3	1.18(0.93–1.51)	1.36	0.175	12	F	0.151	30.10
Chinese	1.21(0.76–1.95)	0.80	0.423	6	F	0.450	0.00
Non-Chinese	1.32(0.72–2.42)	0.89	0.374	6	R	0.053	54.30
ε2/ε4 vs. ε3/ε3	1.20(0.78–1.84)	0.83	0.405	10	F	0.493	0.00
Chinese	2.17(1.10–4.28)	2.22	0.026	5	F	0.852	0.00
Non-Chinese	0.79(0.44–1.41)	0.79	0.428	5	F	0.746	0.00
ε3/ε4 vs. ε3/ε3	1.69(1.38–2.08)	4.99	<0.001	11	F	0.312	13.90
Chinese	2.22(1.59–3.09)	4.71	<0.001	6	F	0.954	0.00
Non-Chinese	1.42(1.09–1.85)	2.57	0.010	5	F	0.186	35.30
ε4/ε4 vs. ε3/ε3	2.72(1.61–4.60)	3.72	<0.001	9	F	0.807	0.00
Chinese	4.26(1.16–15.61)	2.18	0.029	5	F	0.980	0.00
Non-Chinese	2.45(1.37–4.37)	3.03	0.002	4	F	0.291	19.70
ε2/ε2+ε2/ε3 vs. ε3/ε3	1.26(0.88–1.82)	1.25	0.212	13	R	0.071	39.50
Chinese	1.08(0.71–1.65)	0.34	0.734	7	F	0.538	0.00
Non-Chinese	1.52(0.81–2.85)	1.30	0.193	6	R	0.012	66.00
ε4/ε4+ε3/ε4 vs. ε3/ε3	1.83(1.52–2.22)	6.24	<0.001	13	F	0.384	6.20
Chinese	2.44(1.78–3.36)	5.51	<0.001	7	F	0.890	0.00
Non-Chinese	1.55(1.22–1.97)	3.60	<0.001	6	F	0.360	8.80
ε2 allele vs. ε3 allele	1.34(0.98–1.84)	1.81	0.070	11	R	0.054	44.70
Chinese	1.19(0.84–1.69)	0.99	0.324	6	F	0.536	0.00
Non-Chinese	1.67(0.93–3.03)	1.71	0.088	5	R	0.007	71.50
ε4 allele vs. ε3 allele	1.64(1.40–1.94)	5.97	<0.001	11	F	0.284	16.80
Chinese	2.08(1.58–2.74)	5.21	<0.001	6	F	0.987	0.00
Non-Chinese	1.44(1.17–1.77)	3.50	<0.001	5	F	0.138	42.60

OR, odd ratio; CI, confidence interval.

[a] *F: fixed random effect model; R: random effect model.*

[b] $P_{heterogeneity}$ *value for between-study heterogeneity based on Cochran's Q test.*

in the overall population. In the Chinese population, the ε2/ε4 genotype increased the risk of CAD in patients with T2DM (OR = 2.17, 95% CI = 1.10–4.28, $P = 0.026$).

Heterogeneity Analysis

As shown in **Table 2**, there was moderate between-study heterogeneity in the genetic model of ε2 allele vs. ε3 allele ($P_{heterogeneity} = 0.054$, $I^2 = 44.70\%$) and ε2/ε2+ε2/ε3 vs. ε3/ε3 ($P_{heterogeneity} = 0.071$, $I^2 = 39.50\%$) in the overall analysis. However, no significant heterogeneity was found in other genetic models (for ε2/ε2 vs. ε3/ε3: $P_{heterogeneity} = 0.532$, $I^2 = 0\%$; for ε2/ε3 vs. ε3/ε3: $P_{heterogeneity} = 0.151$, $I^2 = 30.10\%$; for ε2/ε4 vs. ε3/ε3: $P_{heterogeneity} = 0.493$, $I^2 = 0\%$; for ε3/ε4 vs. ε3/ε3: $P_{heterogeneity} = 0.312$, $I^2 = 13.90\%$; for ε4/ε4 vs. ε3/ε3: $P_{heterogeneity} = 0.807$, $I^2 = 0\%$; for ε4 allele vs. ε3 allele: $P_{heterogeneity} = 0.284$, $I^2 = 16.80\%$; for ε4/ε4+ε3/ε4 vs. ε3/ε3: $P_{heterogeneity} = 0.384$, $I^2 = 6.20\%$). The heterogeneity analysis results indicated that the pooled results of this meta-analysis in most genetic models were statistically steady and robust. In addition, subgroup analysis indicated that there was no heterogeneity under all nine genetic models in the Chinese population.

Galbraith Plot Analysis and Sensitivity Analysis

There was evidence of moderately large between-study heterogeneity in the genetic model of ε2 allele vs. ε3 allele ($P_{heterogeneity} = 0.054$, $I^2 = 44.70\%$) and ε2/ε2+ε2/ε3 vs. ε3/ε3 ($P_{heterogeneity} = 0.071$, $I^2 = 39.50\%$), so Galbraith plot analysis and sensitivity analysis were performed to detect the possible sources of heterogeneity. Under the genetic model of ε2 allele vs. ε3 allele, the Galbraith plot analysis (**Figure 4A**) showed that the Halim et al. study was the outlier, which is consistent with the results of sensitivity analysis (**Figure 4B**). No heterogeneity existed after this outlier study was omitted ($P_{heterogeneity} = 0.460$, $I^2 = 0\%$). Thus, the study by Halim et al. may be the source of heterogeneity in the meta-analysis for the ε2 allele vs. ε3 genetic model.

Similarly, under the genetic model of ε2/ε2+ε2/ε3 vs. ε3/ε3, the Galbraith plot analysis (**Figure 4C**) and sensitivity analysis (**Figure 4D**) indicated that Halim and Chaudhary's

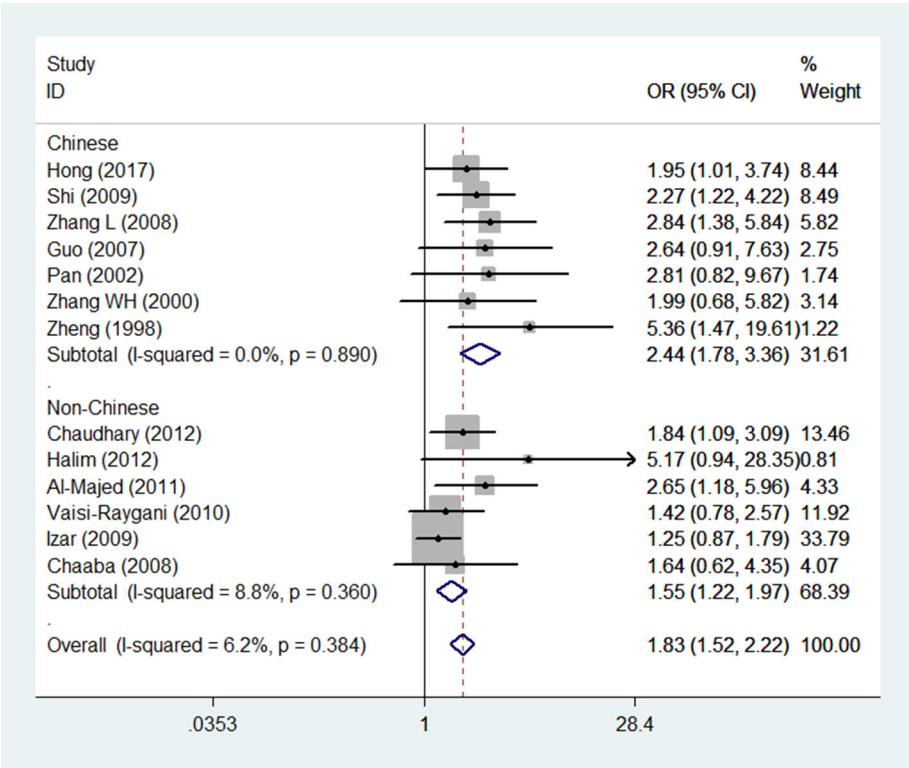

FIGURE 2 | Forest plot for the association between *APOE* gene polymorphism and the risk of coronary artery diseases in type 2 diabetes patients under the genetic model of ε4/ε4+ε3/ε4 vs. ε3/ε3. The center of each square represents the OR, the area of the square is for the weight of studies, and the horizontal line indicates the 95% CI.

study were the outliers. When the two outlier studies were omitted, no heterogeneity existed in the remaining studies ($P_{\text{heterogeneity}} = 0.681$, $I^2 = 0\%$). Therefore, the studies of Halim et al. and Chaudhary et al. may be the main contributors to the source of heterogeneity in the meta-analysis for the ε2/ε2+ε2/ε3 vs. ε3/ε3 genetic model.

Publication Bias

No obvious asymmetry was observed in the shape of the funnel plot for the following genetic models: ε2/ε2 vs. ε3/ε3 (**Figure 5A**); ε2/ε3 vs. ε3/ε3 (**Figure 5B**); ε2/ε4 vs. ε3/ε3 (**Figure 5C**); ε4/ε4 vs. ε3/ε3 (**Figure 5D**); ε2 allele vs. ε3 allele (**Figure 5E**); ε2/ε2+ε2/ε3 vs. ε3/ε3 (**Figure 5F**). In addition, the Begg's test and Egger's test also did not show any evidence of publication bias ($P_{\text{Begg}} = 0.251$ and $P_{\text{Egger}} = 0.08$ for ε2/ε2 vs. ε3/ε3, $P_{\text{Begg}} = 0.373$ and $P_{\text{Egger}} = 0.320$ for ε2/ε3 vs. ε3/ε3, $P_{\text{Begg}} = 0.283$ and $P_{\text{Egger}} = 0.403$ for ε2/ε4 vs. ε3/ε3, $P_{\text{Begg}} = 0.466$ and $P_{\text{Egger}} = 0.988$ for ε4/ε4 vs. ε3/ε3, $P_{\text{Begg}} = 0.119$ and $P_{\text{Egger}} = 0.053$ for ε2 allele vs. ε3 allele, $P_{\text{Begg}} = 0.300$ and $P_{\text{Egger}} = 0.331$ for ε2/ε2+ε2/ε3 vs. ε3/ε3).

The results from the following three genetic models ε3/ε4 vs. ε3/ε3; ε3/ε4+ε4/ε4 vs. ε3/ε3 and ε4 allele vs. ε3 allele performed by Begg's test ($P_{\text{Begg}} = 0.213$, $P_{\text{Begg}} = 0.033$, and $P_{\text{Begg}} = 0.043$, respectively) or Egger's test ($P_{\text{Egger}} = 0.013$; $P_{\text{Egger}} = 0.001$ and $P_{\text{Egger}} = 0.001$, respectively) revealed publication bias. Nevertheless, by using the trim and fill method, the recalculated estimates (OR = 1.50, 95%CI = 1.24–1.82; OR = 1.59, 95%CI = 1.34–1.89 and OR = 1.40, 95%CI = 1.22–1.62, respectively) remained statistically significant, which indicated that our meta-analysis results were steady and not influenced by publication bias. **Figure 6** shows the funnel plot of trim and fill method in the genetic model of ε3/ε4 vs. ε3/ε3 (**Figure 6A**), ε3/ε4+ε4/ε4 vs. ε3/ε3 (**Figure 6B**), ε4 allele vs. ε3 allele (**Figure 6C**).

DISCUSSION

T2DM is a well-established risk factor for the development of CAD. The management of CAD in patients with T2DM poses great challenges to the medical profession (Wei et al., 2015). The identification of susceptibility genes would be very helpful for the management of CAD in patients with T2DM. The link between *APOE* ε2/ε3/ε4 polymorphisms and CAD in diabetic patients has been highlighted in our study. This meta-analysis provides evidence for the significant associations between *APOE* ε4 mutation (ε3/ε4 vs. ε3/ε3; ε4/ε4 vs. ε3/ε3; ε4/ε4+ε3/ε4 vs. ε3/ε3; ε4 allele vs. ε3 allele) and an elevated risk of CAD in patients with T2DM. In contrast, no significant association was found in genetic model of *APOE* ε2 variation (ε2/ε2 vs. ε3/ε3; ε2/ε3 vs. ε3/ε3; ε2/ε2+ε2/ε3 vs. ε3/ε3; ε2 allele vs. ε3

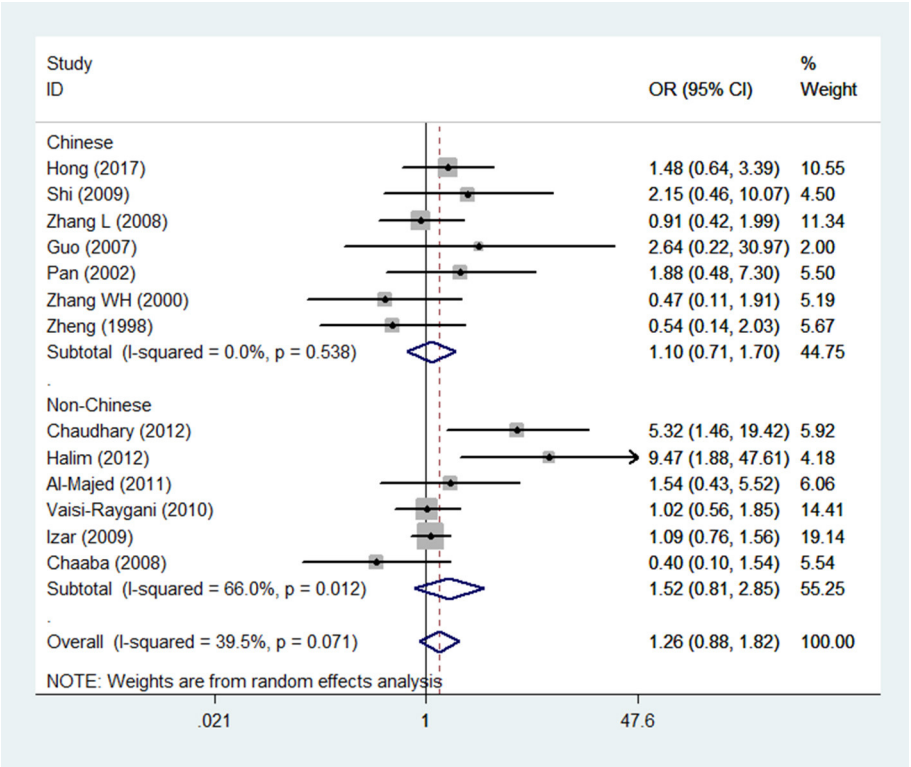

FIGURE 3 | Forest plot for the association between *APOE* gene polymorphism and the risk of coronary artery diseases in type 2 diabetes patients under the genetic model of ε2/ε2+ε2/ε3 vs. ε3/ε3. The center of each square represents the OR, the area of the square is for the weight of studies, and the horizontal line indicates the 95% CI.

allele). However, CAD in patients with T2DM is believed to be multifactorial and involved in many susceptibility genes with small individual effects. Therefore, the integration of information derived from several polymorphisms in multiple susceptibility genes may become clinically useful.

It has been reported that lipoprotein-related mechanisms are associated with the impairment of the cardiovascular system among patients with diabetes (Jenkins et al., 2004). For example, serum low-density lipoprotein cholesterol (LDL-C) level was identified as an independent risk factor for CAD in T2DM patients (Jayashankar et al., 2016). APOE is initially recognized for its important role in plasma lipid metabolism and thus affects the serum lipid profiles in the body. The three *APOE* alleles (ε2, ε3, ε4) differ from each other by only one or two amino acids at positions 112 and 158, but these slight differences alter the structure and function of APOE. In general, the *APOE*-ε4 allele is associated with higher and the *APOE*-ε2 allele with lower total plasma cholesterol and LDL-C concentrations compared with the *APOE*-ε3 allele (Bennet et al., 2007; Larifla et al., 2017). Therefore, abnormalities of lipoprotein metabolism may explain, at least in part, the associations between *APOE* ε2/ε3/ε4 polymorphisms and the risk of CAD in patients with T2DM.

Several meta-analysis studies have been conducted to assess the association between *APOE* ε2/ε3/ε4 polymorphisms and risk of CAD in the general population. In 2004, Song et al firstly found that carriers of the *APOE*-ε4 allele had a 42% increased risk for CAD (OR = 1.42, 95% CI = 1.26–1.61) compared with the ε3/ε3 genotypes (Song et al., 2004). Xu et al. found similar results which showed that the ε4 allele had a 46% higher risk of CAD (OR = 1.46, 95% CI = 1.28–1.66) (Xu et al., 2016). Similar findings were also observed in other meta-analysis (Yin et al., 2013; Xu et al., 2014, 2016; Zhang et al., 2014a, 2015; Wang et al., 2015b). Interestingly, the role of *APOE*-ε2 allele in the risk of CAD may be dependent on the patient ethnicity (Xu et al., 2016). In addition, the association between *APOE* ε2/ε3/ε4 polymorphisms and the risk of T2DM in the general population was also well explored in previous meta-analysis (Anthopoulos et al., 2010; Yin et al., 2014). The results indicated that both *APOE* ε2 and ε4 alleles were associated with an increased risk of T2DM in the general population. In 2015, Wu et al. performed a meta-analysis on the association between *APOE* ε2/ε3/ε4 polymorphisms and T2DM patients with CAD among Chinese Han population. They found that *APOE*-ε4 allele resulted in an increased risk of T2DM patients with CAD in China (Wu et al., 2015). However, only five individual studies were included in their meta-analysis. To our knowledge, our meta-analysis represents the largest study to investigate the association between *APOE* ε2/ε3/ε4 polymorphisms and risk of CAD in the T2DM patients.

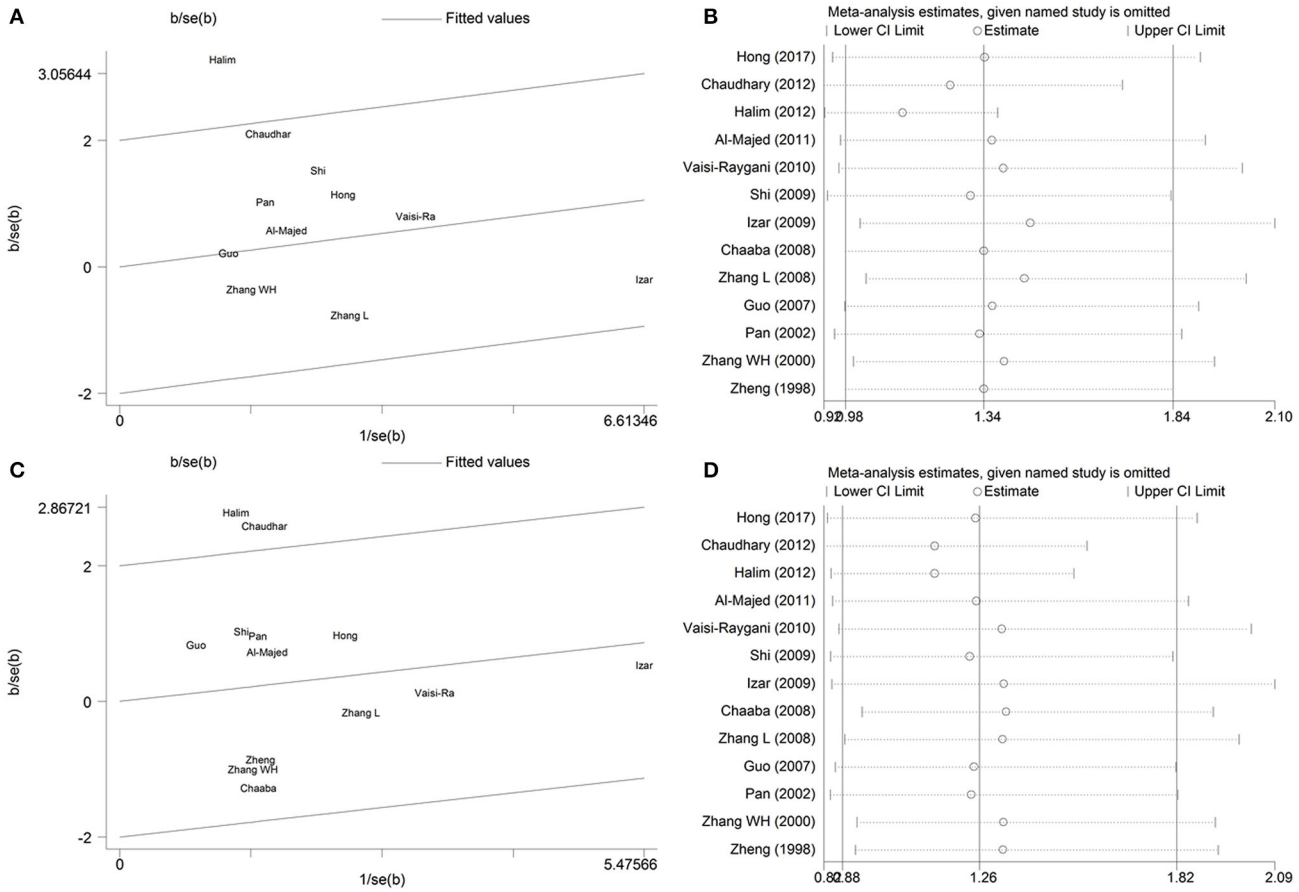

FIGURE 4 | Galbraith plot analysis and sensitivity analysis of the association between *APOE* gene polymorphism and the risk of coronary artery diseases in type 2 diabetes patients under the genetic model of ε2 allele vs. ε3 allele **(A,B)** and ε2/ε2+ε2/ε3 vs. ε3/ε3 **(C,D)**. For sensitivity analysis, open circle indicates the pooled ORs, horizontal lines represent the 95% CIs, given named study is omitted.

Heterogeneity across studies is common in meta-analysis of genetic association study (Munafo and Flint, 2004). Heterogeneity should be taken into consideration in the interpretation of the meta-analysis results. However, one of the strengths in this meta-analysis was the lack of significant heterogeneity in all genetic models except the genetic model of ε2 allele vs. ε3 allele. Between-study heterogeneity can be attributed to the potential differences such as the definition of disease, ethnicity, genotyping methods and sample size in the included studies. To explore the potential sources of heterogeneity under the genetic model of ε2 allele vs. ε3 allele, Galbraith plot analysis and sensitivity analysis were employed to detect whether there were outliers that could be the potential sources of heterogeneity between studies. The study conducted by Halim et al was considered as the main contributors to between-study heterogeneity. The heterogeneity was effectively decreased after omitting the study. The frequency of *APOE*-ε3 allele was nearly 95% in Halim's study, whereas lower than 90% in other studies (Zhang et al., 2008; Izar et al., 2009; Chaudhary et al., 2012; Hong et al., 2017). Consequently, the heterogeneity can be due to the distinct frequency of *APOE* ε2/ε3/ε4 polymorphisms among the included studies. Although Halim's study caused the substantial heterogeneity in the genetic model of ε2 allele vs. ε3 allele, the pooled effect was still insignificant after removing it.

There are several limitations in this meta-analysis that should be noted. First, the included studies were limited to only English or Chinese languages in our research and some eligible studies may be published in other languages, which would cause bias of the results. Second, all the included studies in this meta-analysis were the type of retrospective case-control studies, which may result in some selection bias. Third, publication bias existed in the following three genetic models: ε3/ε4 vs. ε3/ε3; ε3/ε4+ε4/ε4 vs. ε3/ε3; ε4 allele vs. ε3 allele. However, by using the trim and fill method, the recalculated ORs and their 95% CIs did not change, which indicated the stability and robustness of meta-analysis results. Last but not the least, T2DM complicated with CAD is a multifactorial disease caused by both genetic and environmental factors. The *APOE*-environment interactions should be considered. For example, the study by Talmud et al. has found that the impact of the *APOE*-ε4 on the risk of CAD appeared to be restricted to smokers (Talmud et al., 2004).

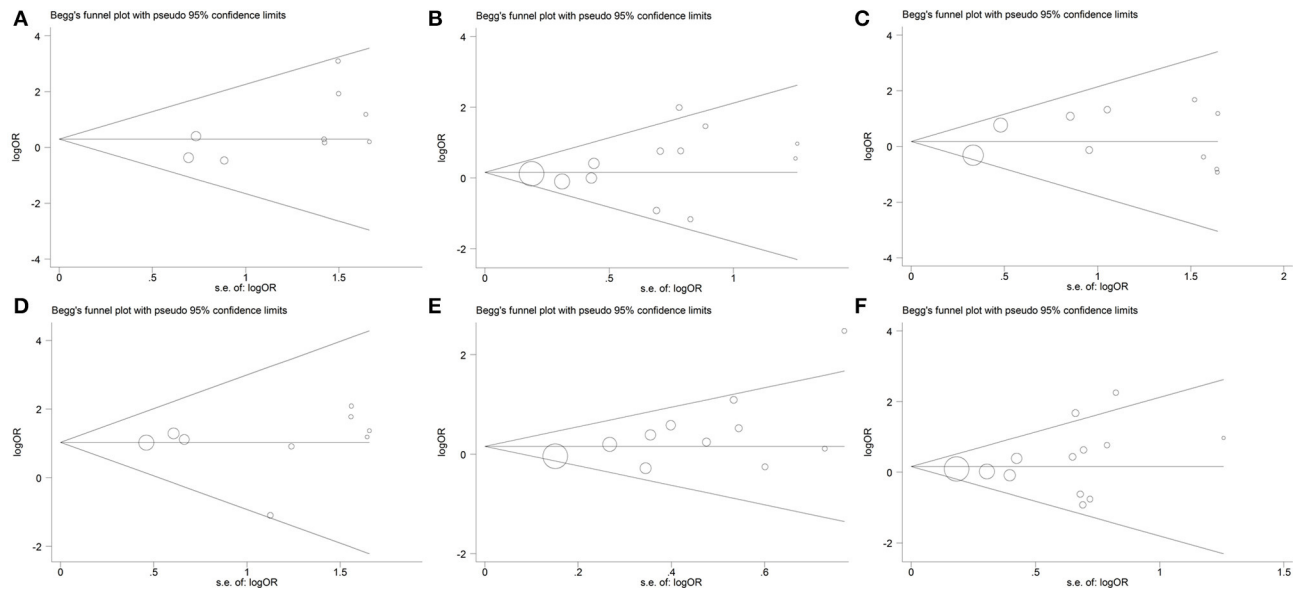

FIGURE 5 | Begg's funnel plot for the association between *APOE* gene polymorphism and the risk of coronary artery diseases in type 2 diabetes patients under the genetic model of ε2/ε2 vs. ε3/ε3 **(A)**, ε2/ε3 vs. ε3/ε3 **(B)**, ε2/ε4 vs. ε3/ε3 **(C)**, ε4/ε4 vs. ε3/ε3 **(D)**, ε2 allele vs. ε3 allele **(E),** and ε2/ε2+ε2/ε3 vs. ε3/ε3 **(F)**. Size of the open circles is proportional to the weight of studies.

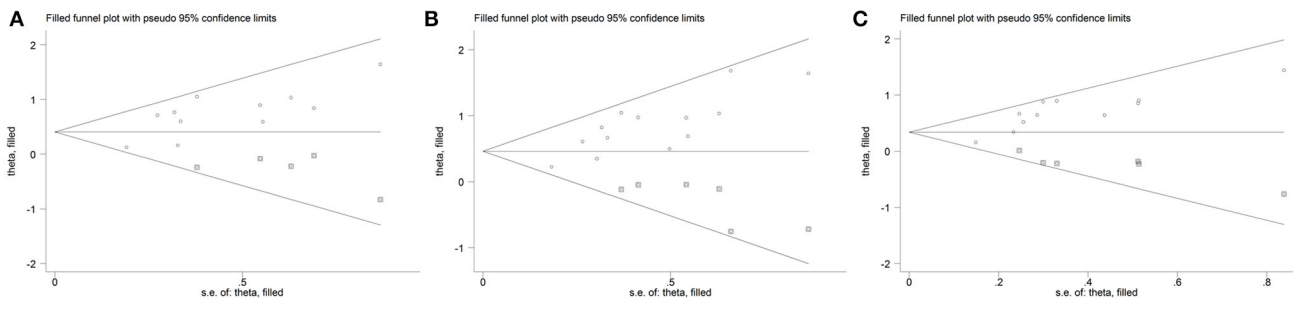

FIGURE 6 | Funnel plot with trim and fill method for the association between *APOE* gene polymorphism and the risk of coronary artery diseases in type 2 diabetes patients under the genetic model of ε3/ε4 vs. ε3/ε3 **(A)**, ε4/ε4+ε3/ε4 vs. ε3/ε3 **(B),** and ε4 allele vs. ε3 allele **(C)**. Circle represents the included studies; Square represents the possibly missing studies.

In conclusion, we observed a significant association between the *APOE* gene ε4 mutation and an increased risk of CAD in patients with T2DM, while the ε2 variation had null association with this disease. Taking into account the above limitations, more studies with larger sample size and incorporated with gene-environment interactions are needed to definitively determine the association between the *APOE* gene ε2/ε3/ε4 polymorphisms and the risk of CAD in patients with T2DM.

AUTHOR CONTRIBUTIONS

Conceived and designed the study: J-QL and HR. Performed the search: J-QL, HR, M-ZL. Analyzed the data: J-QL and HR. Contributed reagents/material/analysis tools: J-QL, HR, M-ZL, PX, P-FF, and D-XX. Wrote and review the manuscript: J-QL, HR and HB. Reference collection, data management, statistical analyses, paper writing, and study design: J-QL and HR.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2017.01031/full#supplementary-material

REFERENCES

Al-Majed, H. T., Qasem, J. A., Al-Sherifi, A. K., Al-Attar, A. A., Qasem, A. A., and Abdullah, S. A. (2011). Association between apolipoprotein E-polymorphism and Ischemic heart disease patients with or without type 2 diabetes mellitus: a preliminary study in Kuwait. *Arch. Iran. Med.* 14, 385–388. doi: 011146/AIM.006

Anthopoulos, P. G., Hamodrakas, S. J., and Bagos, P. G. (2010). Apolipoprotein E polymorphisms and type 2 diabetes: a meta-analysis of 30 studies including 5423 cases and 8197 controls. *Mol. Genet. Metab.* 100, 283–291. doi: 10.1016/j.ymgme.2010.03.008

Begg, C. B., and Mazumdar, M. (1994). Operating characteristics of a rank correlation test for publication bias. *Biometrics* 50, 1088–1101. doi: 10.2307/2533446

Bennet, A. M., Di Angelantonio, E., Ye, Z., Wensley, F., Dahlin, A., Ahlbom, A., et al. (2007). Association of apolipoprotein E genotypes with lipid levels and coronary risk. *JAMA* 298, 1300–1311. doi: 10.1001/jama.298.11.1300

Chaaba, R., Attia, N., Hammami, S., Smaoui, M., Ben Hamda, K., Mahjoub, S., et al. (2008). Association between apolipoprotein E polymorphism, lipids, and coronary artery disease in Tunisian type 2 diabetes. *J. Clin. Lipidol.* 2, 360–364. doi: 10.1016/j.jacl.2008.08.441

Chaudhary, R., Likidlilid, A., Peerapatdit, T., Tresukosol, D., Srisuma, S., Ratanamaneechat, S., et al. (2012). Apolipoprotein E gene polymorphism: effects on plasma lipids and risk of type 2 diabetes and coronary artery disease. *Cardiovasc. Diabetol.* 11:36. doi: 10.1186/1475-2840-11-36

Duval, S., and Tweedie, R. (2000). Trim and fill: a simple funnel-plot-based method of testing and adjusting for publication bias in meta-analysis. *Biometrics* 56, 455–463. doi: 10.1111/j.0006-341X.2000.00455.x

Egger, M., Davey Smith, G., Schneider, M., and Minder, C. (1997). Bias in meta-analysis detected by a simple, graphical test. *BMJ* 315:629.

Emerging Risk Factors, C., Sarwar, N., Gao, P., Seshasai, S. R., Gobin, R., Kaptoge, S., et al. (2010). Diabetes mellitus, fasting blood glucose concentration, and risk of vascular disease: a collaborative meta-analysis of 102 prospective studies. *Lancet* 375, 2215–2222. doi: 10.1016/S0140-6736(10)60484-9

Freitas Lima, L. C., Braga, V. A., do Socorro de França Silva, M., Cruz, J. C., Sousa Santos, S. H., de Oliveira Monteiro, M. M., et al. (2015). Adipokines, diabetes and atherosclerosis: an inflammatory association. *Front. Physiol.* 6:304. doi: 10.3389/fphys.2015.00304

Guo, J. J., Ju, J., and Xu, X. H. (2007). Association of polymorphisms of apolipoprotein E gene and High-sensitive C-reactive protein with type 2 diabetes with coronary heart disease. *Shaanxi Med. J.* 36, 1613–1616. doi: 10.3969/j.issn.1000-7377.2007.12.010

Halim, E. F., Reda, A. A., Hendi, A. A., Zaki, S. A., Essa, E. S., and Khalifa, A. S. (2012). Apolipoprotein E gene variants as a risk factor for coronary artery disease in type 2 diabetic Egyptian patients. *Egypt. J. Immunol.* 19, 1–10.

Higgins, J. P., Thompson, S. G., Deeks, J. J., and Altman, D. G. (2003). Measuring inconsistency in meta-analyses. *BMJ* 327, 557–560.doi: 10.1136/bmj.327.7414.557

Hong, G. Q., Cai, J. L., and Chen, J. P. (2017). Association of genetic polymorphisms of apolipoprotein E gene and type-2 diabetic mellitus complicated with coronary heart disease in Chaoshan patients. *Int. Med. Health Guid. News* 23, 792–794. doi: 10.3760/cma.j.issn.1007-1245.2017.06.004

Izar, M. C., Helfenstein, T., Ihara, S. S., Relvas, W. G., Santos, A. O., Fischer, S. C., et al. (2009). Association of lipoprotein lipase D9N polymorphism with myocardial infarction in type 2 diabetes: the genetics, outcomes, and lipids in type 2 diabetes (GOLD) study. *Atherosclerosis* 204, 165–170. doi: 10.1016/j.atherosclerosis.2008.08.006

Jayashankar, C. A., Andrews, H. P., Vijayasarathi, Pinnelli, V. B., Shashidharan, B., Nithin Kumar, H. N., et al. (2016). Serum uric acid and low-density lipoprotein cholesterol levels are independent predictors of coronary artery disease in Asian Indian patients with type 2 diabetes mellitus. *J. Nat. Sci. Biol. Med.* 7, 161–165. doi: 10.4103/0976-9668.184703

Jenkins, A. J., Rowley, K. G., Lyons, T. J., Best, J. D., Hill, M. A., and Klein, R. L. (2004). Lipoproteins and diabetic microvascular complications. *Curr. Pharm. Des.* 10, 3395–3418. doi: 10.2174/1381612043383188

Larifla, L., Armand, C., Bangou, J., Blanchet-Deverly, A., Numeric, P., Fonteau, C., et al. (2017). Association of APOE gene polymorphism with lipid profile and coronary artery disease in Afro-Caribbeans. *PLoS ONE* 12:e0181620. doi: 10.1371/journal.pone.0181620

Mohan, V., Deepa, R., Rani, S. S., Premalatha, G., and Chennai Urban Population, S. (2001). Prevalence of coronary artery disease and its relationship to lipids in a selected population in south india: the chennai urban population study (CUPS No. 5). *J. Am. Coll. Cardiol.* 38, 682–687. doi: 10.1016/S0735-1097(01)01415-2

Munafo, M. R., and Flint, J. (2004). Meta-analysis of genetic association studies. *Trends Genet.* 20, 439–444. doi: 10.1016/j.tig.2004.06.014

Naito, R., and Miyauchi, K. (2017). Coronary artery disease and type 2 diabetes mellitus. *Int. Heart J.* 58, 475–480. doi: 10.1536/ihj.17-191

Pan, M., Zhu, J. H., Liu, Y., Pan, H. Y., Wang, H. M., Liang, S., et al. (2002). Association of apolipoprotein E with essential hypertension, NIDDM and coronary heart disease in Chinese population. *China Synth. Med.* 3, 1–3. Available online at: http://www.cqvip.com/QK/85550X/200201/6817449.html

Paneni, F., Beckman, J. A., Creager, M. A., and Cosentino, F. (2013). Diabetes and vascular disease: pathophysiology, clinical consequences, and medical therapy: part I. *Eur. Heart J.* 34, 2436–2443. doi: 10.1093/eurheartj/eht149

Raj, R., Bhatti, J. S., Badada, S. K., and Ramteke, P. W. (2015). Genetic basis of dyslipidemia in disease precipitation of coronary artery disease (CAD) associated type 2 diabetes mellitus (T2DM). *Diabetes Metab. Res. Rev.* 31, 663–671. doi: 10.1002/dmrr.2630

Shi, Y. R., Zhou, H. Y., and Zhang, Y. (2009). Relationship of gene polymorphisms of fatty acid binding protein-2 and apolipoprotein E with coronary heart disease in type-2 diabetic patients. *J. Xi'an Jiaotong Univ.* 30, 85–88. Available online at: http://d.wanfangdata.com.cn/Periodical/xaykdxxb200901022

Song, Y., Stampfer, M. J., and Liu, S. (2004). Meta-analysis: apolipoprotein E genotypes and risk for coronary heart disease. *Ann. Intern. Med.* 141, 137–147. doi: 10.7326/0003-4819-141-2-200407200-00013

Stroup, D. F., Berlin, J. A., Morton, S. C., Olkin, I., Williamson, G. D., Rennie, D., et al. (2000). Meta-analysis of observational studies in epidemiology: a proposal for reporting. Meta-analysis Of Observational Studies in Epidemiology (MOOSE) group. *JAMA* 283, 2008–2012. doi: 10.1001/jama.283.15.2008

Sumi, S., Ramachandran, S., RamanKutty, V., Patel, M. M., Anand, T. N., Mullasari, A. S., et al. (2017). *ENPP1* 121Q functional variant enhances susceptibility to coronary artery disease in South Indian patients with type 2 diabetes mellitus. *Mol. Cell. Biochem.* 435, 67–72. doi: 10.1007/s11010-017-3057-2

Talmud, P. J., Lewis, S. J., Hawe, E., Martin, S., Acharya, J., Marmot, M. G., et al. (2004). No APOEepsilon4 effect on coronary heart disease risk in a cohort with low smoking prevalence: the Whitehall II study. *Atherosclerosis* 177, 105–112. doi: 10.1016/j.atherosclerosis.2004.06.008

Thakkinstian, A., McEvoy, M., Minelli, C., Gibson, P., Hancox, B., Duffy, D., et al. (2005). Systematic review and meta-analysis of the association between β2-adrenoceptor polymorphisms and asthma: a HuGE review. *Am. J. Epidemiol.* 162, 201–211. doi: 10.1093/aje/kwi184

Vaisi-Raygani, A., Rahimi, Z., Tavilani, H., and Pourmotabbed, T. (2010). Butyrylcholinesterase K variant and the APOE-ε4 allele work in synergy to increase the risk of coronary artery disease especially in diabetic patients. *Mol. Biol. Rep.* 37, 2083–2091. doi: 10.1007/s11033-009-9666-4

Wang, L., Lin, P., Ma, A., Zheng, H., Wang, K., Li, W., et al. (2015a). C-peptide is independently associated with an increased risk of coronary artery disease in T2DM subjects: a cross-sectional study. *PLoS ONE* 10:e0127112. doi: 10.1371/journal.pone.0127112

Wang, Y. L., Sun, L. M., Zhang, L., Xu, H. T., Dong, Z., Wang, L. Q., et al. (2015b). Association between Apolipoprotein E polymorphism and myocardial infarction risk: a systematic review and meta-analysis. *FEBS Open Bio.* 5, 852–858. doi: 10.1016/j.fob.2015.10.006

Wei, X., Ma, X., Lu, R., Bai, G., Zhang, J., Deng, R., et al. (2014). Genetic variants in PCSK1 gene are associated with the risk of coronary artery disease in type 2 diabetes in a Chinese Han population: a case control study. *PLoS ONE* 9:e87168. doi: 10.1371/journal.pone.0087168

Wei, Y., Guo, H., The, E., Che, W., Shen, J., Hou, L., et al. (2015). Persistent lipid abnormalities in statin-treated coronary artery disease patients with and without diabetes in China. *Int. J. Cardiol.* 182, 469–475. doi: 10.1016/j.ijcard.2015.01.024

Wu, Z. R., Chen, Z. C., Fu, M. X., Chen, J. Y., Han, L. Y., Zhou, L. H., et al. (2015). Polymorphism of apolipoprotein E gene and type 2 diabetic patients

with coronary heart disease among Chinese Han population: a meta-analysis. *J. Clin. Exp. Med.* 14, 982–985. doi: 10.3969/j.issn.1671-4695.2015.012.008

Xu, H., Li, H., Liu, J., Zhu, D., Wang, Z., Chen, A., et al. (2014). Meta-analysis of apolipoprotein E gene polymorphism and susceptibility of myocardial infarction. *PLoS ONE* 9:e104608. doi: 10.1371/journal.pone.0104608

Xu, M., Zhao, J., Zhang, Y., Ma, X., Dai, Q., Zhi, H., et al. (2016). Apolipoprotein E gene variants and risk of coronary heart disease: a meta-analysis. *Biomed Res. Int.* 2016:3912175. doi: 10.1155/2016/3912175

Yin, Y. W., Qiao, L., Sun, Q. Q., Hu, A. M., Liu, H. L., Wang, Q., et al. (2014). Influence of apolipoprotein E gene polymorphism on development of type 2 diabetes mellitus in Chinese Han population: a meta-analysis of 29 studies. *Metab. Clin. Exp.* 63, 532–541. doi: 10.1016/j.metabol.2013.12.008

Yin, Y. W., Sun, Q. Q., Zhang, B. B., Hu, A. M., Liu, H. L., Wang, Q., et al. (2013). Association between apolipoprotein E gene polymorphism and the risk of coronary artery disease in Chinese population: evidence from a meta-analysis of 40 studies. *PLoS ONE* 8:e66924. doi: 10.1371/journal.pone.0066924

Zhang, L., Qi, X. Y., Wang, Y., Jia, Z. L., and Xie, Y. (2008). A study on the association between polymorphism of apolipoprotein E gene and type 2 diabetic patients with coronary heart disease. *Acta. Acad. Med. CPAF* 17, 572–575. Available online at: http://d.g.wanfangdata.com.cn/Periodical_wjyxyxb200807007.aspx

Zhang, M. D., Gu, W., Qiao, S. B., Zhu, E. J., Zhao, Q. M., and Lv, S. Z. (2014a). Apolipoprotein E gene polymorphism and risk for coronary heart disease in the Chinese population: a meta-analysis of 61 studies including 6634 cases and 6393 controls. *PLoS ONE* 9:e95463. doi: 10.1371/journal.pone.0095463

Zhang, W. H., Zhang, G. W., Zhang, X., Fang, Y. M., Xu, Z. F., and Zhang, A. Z. (2000). Relationship between Apo E gene polymorphism and type 2 diabetes mellitus with its cardiovascular complication in Chinese. *Med. J. Chin. Civ. Adm* 12, 206–209. Available online at: http://www.cnki.com.cn/Article/CJFDTOTAL-ZMYX200004008.htm

Zhang, Y., Hu, C., Hong, J., Zeng, J., Lai, S., Lv, A., et al. (2014b). Lipid profiling reveals different therapeutic effects of metformin and glipizide in patients with type 2 diabetes and coronary artery disease. *Diabetes Care* 37, 2804–2812. doi: 10.2337/dc14-0090

Zhang, Y., Tang, H. Q., Peng, W. J., Zhang, B. B., and Liu, M. (2015). Meta-analysis for the association of apolipoprotein E epsilon2/epsilon3/epsilon4 polymorphism with coronary heart disease. *Chin. Med. J.* 128, 1391–1398. doi: 10.4103/0366-6999.156803

Zheng, Y. M., Sun, R., Li, X. Y., Zhu, D., Gao, M., Zhao, L. B., et al. (1998). Relationship between ApoE gene polymorphism and type 2 diabetes mellitus with its cardiovascular complications in Chinese. *Chin. J. Endocrinol. Metab.* 14, 11–14.

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frontiers in Genetics

REVIEW
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The Role of MicroRNAs in Arterial Stiffness and Arterial Calcification. An Update and Review of the Literature

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Arterial stiffness is an independent risk factor for fatal and non-fatal cardiovascular events, such as systolic hypertension, coronary artery disease, stroke, and heart failure. Moreover it reflects arterial aging which in many cases does not coincide with chronological aging, a fact that is in large attributed to genetic factors. In addition to genetic factors, **microRNAs (miRNAs)** seem to largely affect arterial aging either by advancing or by regressing arterial stiffness. MiRNAs are small RNA molecules, ∼22 nucleotides long that can negatively control their target gene expression posttranscriptionally. Pathways that affect main components of stiffness such as fibrosis and calcification seem to be influenced by up or downregulation of specific miRNAs. Identification of this aberrant production of miRNAs can help identify epigenetic changes that can be therapeutic targets for prevention and treatment of vascular diseases. The present review summarizes the specific role of the so far discovered miRNAs that are involved in pathways of arterial stiffness.

Keywords: miRNAs, arterial stiffness, vascular calcification, arterial aging

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INTRODUCTION

Arterial stiffness is a characteristic feature of normal arterial aging, but is also associated with accelerated cardiovascular disorders including systolic hypertension, coronary artery disease, stroke, and heart failure, irrespective of other risk factors, such as smoking, dyslipidemia, and diabetes mellitus (Laurent et al., 2006; Mitchell et al., 2010; Shirwany and Zou, 2010; Parthenakis et al., 2017). Another age-related process is arterial calcification, which in turn is a known risk predictor that increases morbidity and mortality in cardiovascular diseases (Goettsch and Aikawa, 2013; Jiang et al., 2017). MicroRNAs (miRNAs) are small (19–25 nucleotides long) non-coding RNAs that downregulate their target gene expression post-transcriptionally via mRNA degradation or translational repression (Pan et al., 2010; Vickers et al., 2014). They are widely studied in recent years and their role in cardiovascular dysfunction is to some extent revealed (Pan et al., 2010; Vickers et al., 2014). Identification of over- or underproduction of miRNAs could be therapeutic targets for prevention and treatment of vascular diseases. The objective of this review is to summarize studies of the most prominent miRNAs that affect major pathways of arterial stiffness and calcification. We searched PubMed for original articles on miRNAs and arterial stiffness or arterial calcification and we also used reviews affiliated to the subject and reviewed their references.

MiRNAs AND ARTERIAL STIFFNESS

Arterial stiffness results from complicated interactions between multiple components of the vessel wall, including extracellular matrix (ECM) composition, vascular smooth muscle cell (VSMC), and endothelial dysfunction. Collagen and elastin are the most important structural proteins of ECM and key regulators of arterial stiffness as they are responsible for blood vessels' strength and elasticity (Zieman et al., 2005; Wagenseil and Mecham, 2012). Reconstruction of ECM, notably increased levels of aberrant types of collagen and reduction of elastin, appears to be the most important mechanism contributing to arterial stiffness (Zieman et al., 2005; Wagenseil and Mecham, 2012; Fleenor and Berrones, 2015). Matrix metalloproteases (MMPs) are endopeptidases which degrade all kinds of ECM proteins. Thus they play a significant role in arterial stiffness via regulating collagen and elastin levels in ECM (Galis and Khatri, 2002; Zieman et al., 2005; Van Doren, 2015). Moreover, advanced glycation end products (AGEs) contribute to arterial stiffness through cross-linking with ECM proteins, including collagen, which reduces vessel's flexibility (Zieman et al., 2005; Fleenor and Berrones, 2015; Xiaomei et al., 2017). Furthermore, many hormones and cytokines are involved in aortic stiffness, such as **angiotensin II (Ang II)** which promotes arterial stiffness through regulating signaling pathways that result in altered ECM accumulation and increased vascular tone (Rodríguez-Vita et al., 2005; Zieman et al., 2005). Apart from structural abnormalities, VSMC proliferation, migration and calcification, as well as impaired endothelium-dependent dilation through paracrine molecules such as nitric oxide (NO) and endothelin are, also, implicated in the development of arterial stiffness (Zieman et al., 2005; Fleenor and Berrones, 2015). Some of the most prominent miRNAs that affect arterial stiffness through the mechanisms mentioned above are described in the subsequent sections (see **Table 1**).

MiRNAs AFFECTING EXTRACELLULAR MATRIX PROTEINS

MiR-181b, which seems to decrease with age, plays critical role in ECM remodeling and regulating vascular stiffness and systolic blood pressure (Hori et al., 2017). Chronic down-regulation of miR-181b expression with age was associated with activation of TGF-β (transforming growth factor-beta) signaling in the VSMCs (Hori et al., 2017). TGF-β can initiate multiple effects in the vessels including phenotypic modulation of the VECs (vascular endothelial cells) and VSMCs (Hori et al., 2017). It is also associated with induction of gene expression such as collagen I and III, stimulating the production of ECM (Hori et al., 2017). Furthermore, it also stimulates plasminogen activator-inhibitor production, which inhibits breakdown of ECM, resulting in increased ECM (Hori et al., 2017). **MiR-181b** inhibits TGF-β signaling pathway by directly inhibiting TGF-βi (transforming growth factor, beta induced) which encodes a protein that interacts with collagen playing a role in cell-collagen interactions (Hori et al., 2017). Similarly, **miR-599** suppresses VSMC proliferation and migration and, also, regulates ECM composition by inhibiting type I, type V collagen and proteoglycan via directly repressing TGF-β2 (Xie et al., 2015). Interestingly, **miR-145** suppresses TGFβ-dependent ECM accumulation and fibrosis by targeting TGFβ receptor II (TGFBR2), while promoting TGFβ-induced smooth muscle cell differentiation (Ning et al., 2015). It seems that a specific miRNA has a selective effect on TGFβ signaling and may facilitate unique downstream events (Ning et al., 2015). Increased expression of a miR-145 mimic in smooth muscle cells resulted in a decrease in TGFβ readout genes, SERPINE1 (PAI-1), and SMAD7 and led to reduced expression of elastin, collagens, and the matrix crosslinking genes, Lox (*Lysyl* oxidase) and Lox1 (Ning et al., 2015). On the other hand, both TGFβ signaling and miR-145 promote the differentiated phenotype of VSMC and contractile gene expressions, such as a-SMA (smooth muscle α-actin) and SM22α (Ning et al., 2015; see **Figure 1**).

MiR-29 is considered to be one of the most important miRNAs in regulating ECM composition, as it is known to affect the synthesis of many types of collagen, elastin, MMP-2 and other ECM proteins (Kriegel et al., 2012; Zhu et al., 2013). **MiR-29** reduces fibrosis in scleroderma by repressing type I, III, and IV of collagen, while downregulation of miR-29 from TGF-β promotes renal and cardiac fibrosis (Kriegel et al., 2012; Wang B et al., 2012; Zhu et al., 2013). Interestingly enough, however, other studies demonstrated that miR-29 and miR-15 reduce elastin levels during aortic development in the mouse (Ott et al., 2011; Zhu et al., 2013; see **Figure 1**). Similarly, Zhang et al. showed **elastin** levels could be increased by inhibiting miR-29a, while collagen levels remained unaffected (Zhang et al., 2012).

MiRNAs AFFECTING CYTOSKELETAL PATHWAYS, METALLOPROTEINASES, NITRIC OXIDE, AND ADHESION MOLECULES

The miR-203 expression is increased in aged mouse aorta (Nicholson et al., 2016). Overexpression of miR-203 decreases tyrosine kinase Src and attenuates extracellular signal regulated kinase (ERK) signaling resulting in an insufficient vascular smooth muscle Src-dependent cytoskeletal remodeling which leads to increased arterial stiffness (Min et al., 2012; Gao Y. Z. et al., 2014; Nicholson et al., 2016). Furthermore, miR-765 could accelerate arterial stiffness via knocking down apelin (APLN) and enhancing MMP-2 and MMP-9 (Liao et al., 2015). **APLN** reduces vascular tone through antagonizing Ang II and increasing eNOS activity (Anea et al., 2010; Liao et al., 2015). Also, **both miR-765 and miR-155** lower NO levels by direct eNOS gene downregulation regardless **APLN** intervention (Sun et al., 2012; Liao et al., 2015). **MiR-155**, also, mediates TNFα-induced impairment of endothelium-dependent vasodilation (Sun et al., 2012). Moreover, miR-1185 accelerates arterial stiffness though upregulating VCAM-1 (vascular cell adhesion molecule 1) and E-Selectin (Deng et al., 2017). Adhesion molecules increase arterial stiffness by stimulating MMP activity

TABLE 1 | miRNAs influencing biological pathways of arterial stiffness.

miRNA	Arterial stiffness	Function
miRNAs AFFECTING EXTRACELLULAR MATRIX PROTEINS		
mir-181b	Decreases	Inhibits TGF-β signaling and ECM production
mir-599	Decreases	Inhibits TGF-β signaling and ECM production
mir-145	Decreases	Inhibits TGF-β signaling and ECM production
mir-29	Decreases	Supresses collagen I, III and IV, but also elastin levels
miRNAs AFFECTING CYTOSKELETAL PATHWAYS, METALLOPROTEINASES, NITRIC OXIDE, AND ADHESION MOLECULES		
mir-203	Increases	Defective Src-dependent cytoskeletal remodeling
mir-765	Increases	Reduces apelin and eNOS activity and increases MMP-2, MMP9
mir-155	Increases	Inhibits eNOS activity
mir-1185	Increases	Upregulates VCAM-1 and E-Selectin
mir-126, mir-223	Decrease	Inhibit VCAM-1 and ICAM-1
miRNAs INDUCING ARTERIAL STIFFNESS THROUGH ANGIOTENSIN II		
mir-21	Increases	Ang II increases mir-21 through osteopontin and mir-21 increases fibroblast survival and ECM deposition
mir-19b	Decreases	Ang II represses mir-19b and upregulates CTGF
mir-181a	Decreases	Inhibits Ang II-induced osteopontin expression
mir-130a	Increases	Ang II upregulates mir-130a and VSMC proliferation
mir-155	Decreases	Inhibits Ang II-induced VSMC proliferation

ECM, extracellular matrix; TGF-b, transforming growth factor-b; MMP, matrix metalloproteinase; eNOS, endothelial nitric oxide synthase; Ang II, angiotensin II; CTGF, connective tissue growth factor.

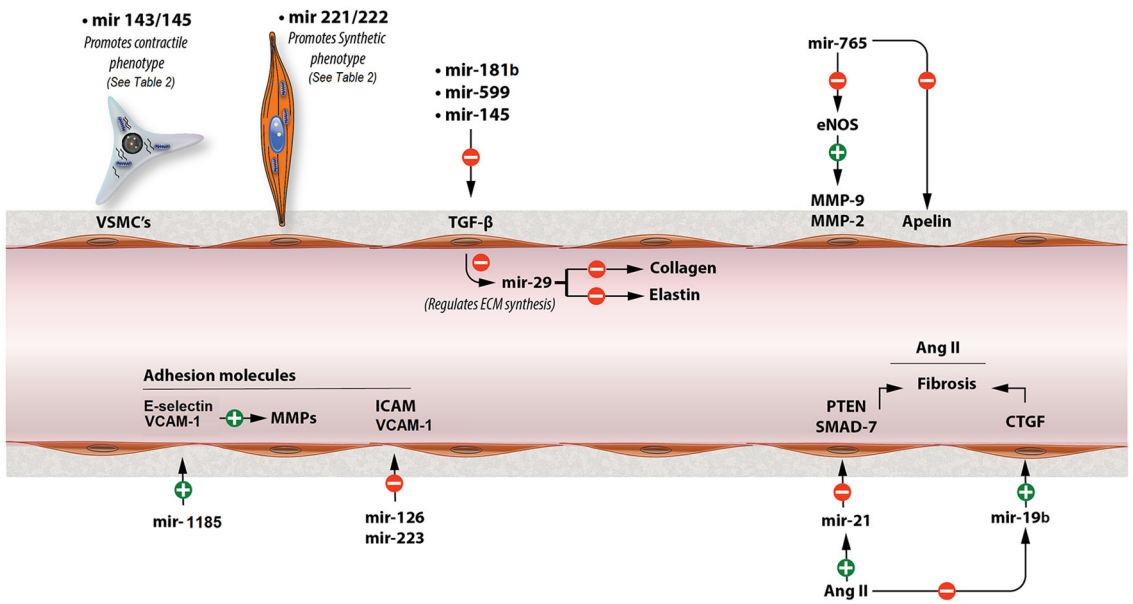

FIGURE 1 | Major miRNAs that contribute to the pathogenesis of arterial stiffness. This figure shows the mechanisms of major miRNAs involved in arterial stiffness through modulating TGF-β signaling, Ang II, adhesion molecules, MMP activity, and VSMC phenotypic switch. ECM, extracellular matrix; TGF-b, transforming growth factor-b; MMP, matrix metalloproteinase; eNOS, endothelial nitric oxide synthase; Ang II, angiotensin II; CTGF, connective tissue growth factor.

and, also, affecting vascular tone (Galis and Khatri, 2002; Zieman et al., 2005; Deng et al., 2017). Conversely, miR-126 and miR-223 inhibit VCAM-1 and ICAM-1 (intercellular adhesion molecule 1) activities, respectively, regulating vascular inflammation (Harris et al., 2008; Tabet et al., 2014; see **Figure 1**).

MiRNAs AND ARTERIAL STIFFNESS-CLINICAL STUDIES

Arterial stiffness is most precisely measured by pulse wave velocity (PWV; Laurent et al., 2006). In a study, downregulation of **miR-21** was related to decreased levels of arterial stiffness as

estimated by PWV in hypertensive patients at baseline and 1 year after antihypertensive treatment (Parthenakis et al., 2017). Interestingly, the effect on arterial stiffness was not correlated with patients' blood pressure levels (Parthenakis et al., 2017).

MiRNAs INDUCING ARTERIAL STIFFNESS THROUGH ANG II

MiR-21 plays an important role in Ang II-mediated fibrosis pathways (Laurent et al., 2006; Lorenzen et al., 2015). In cardiac fibrosis, osteopontin **(OPN)** that is activated by Ang II upregulates **miR-21** through transcription factor AP-1. **MiR-21**, then, increases fibrosis through enhancing fibroblast activity and ECM accumulation. In this process, **miR-21** downregulates PTEN (phosphatase and tensin homolog) and SMAD7, while increases ERK and AKT signaling pathways (Lorenzen et al., 2015; see **Figure 1**). Inversely, **miR-124** exerts an antifibrotic role by reducing pulmonary vascular fibroblast activation by repressing its target gene PTBP1 (polypyrimidine tract-binding protein 1) and subsequently affecting PTEN pathway (Wang et al., 2014).

PTEN is, also, the target of miR-19b-mediated cardiac fibroblast proliferation and migration (Zhong et al., 2016). However, in another study it was demonstrated that Ang II downregulated miR-19b expression and upregulated connective tissue growth factor (CTGF) expression in cultured cardiomyocytes via an AT1R-dependent pathway, increasing cardiac fibrosis (Gao S. et al., 2014; see **Figure 1**). All these effects of Ang II were abolished by telmisartan, which is an AT1R (Ang II type 1 receptor) antagonist (Gao S. et al., 2014). CTGF is associated with fibrosis through regulating ECM arrangement (Finckenberg et al., 2003; Gao S. et al., 2014). Finally, Ang II promotes VSMC proliferation by upregulating miR-130a which, in turn, decreases GAX expression, the growth arrest-specific homeobox which exerts an inhibitory role on VSMCs proliferation (Wu et al., 2011). On the contrary, **miR-155** overexpression was found to inhibit Ang II-induced viability and proliferation of mouse VSMCs by inducing G1 cell cycle arrest and downregulating the expression of AT1R in VSMCs (Yang et al., 2014).

OPN is a protein expessed in many cells, such as osteoblasts and macrophages but it is normally absent from vessel walls. However, under certain conditions, like injury of the wall or presence of atherosclerosis OPN is overexpressed and contributes to the progression of the inflammatory process (Giachelli et al., 1993; Weintraub et al., 2000; Abe et al., 2008; Remus et al., 2013). **MiR-181a** represses Ang II-mediated OPN increased levels in VSMC as well as Ang II-mediated VSMC adhesion to collagen due to decreased OPN and β1 integrin expression (Weintraub et al., 2000; Remus et al., 2013).

MiRNAs AFFECTING ARTERIAL STIFFNESS THROUGH VASCULAR SMOOTH MUSCLE CELL FUNCTION

Vascular smooth muscle cell (VSMC) is the most prevalent type of cell in vasculature and exert multiple functions influencing arterial stiffness (Sehgel et al., 2013, 2015; Fleenor and Berrones, 2015). They are essential regulators of vascular tone through modulating vasoconstriction and vasodilation (Sehgel et al., 2015; Xu F. et al., 2015). Their main characteristic that contributes to the pathogenesis of arterial stiffness is their ability to switch between two distinguishing phenotypes, a contractile (differentiated) and a proliferative (dedifferentiated) form (Davis-Dusenbery et al., 2011b; Kee et al., 2014). The contractile phenotype is defined as a quiescent state and is characterized of reduced ECM arrangement, suspended proliferation and migration and the expression of some marker genes, such as smooth muscle a-actin (a-SMA), SM22a, smooth muscle myosin heavy chain (MYH11), and myosin light chain kinase (MYLC; Davis-Dusenbery et al., 2011b; Kee et al., 2014; Xu F. et al., 2015). Conversely, dedifferentiated or synthetic phenotype induces VSMC proliferation, migration, and ECM accumulation resulting in an increase in arterial stiffness (Davis-Dusenbery et al., 2011b; Kee et al., 2014). There seems to be a plethora of miRNAs associated with VSMC proliferation, migration and phenotypic switch in literature and the most prominent of them are listed below (see **Table 2**).

CRITICAL ROLE OF BMP, TGF-B, AND PDGF-BB

Bone morphogenetic protein (BMP) and transforming growth factor-b (TGF-b) are the major signaling pathways that enhance the contractile phenotype, while, on the other hand, platelet-derived growth factor-BB (PDGF-BB) is the primary pathway inducing the proliferative phenotype (Chan et al., 2010). **MiR-24**, after induced by PDGF-BB, promotes VSMC proliferation through repressing Tribbles-like protein-3 (Trb3), which in turn downregulates SMAD transporters and BMP-TGF-b pathways (Chan et al., 2010). Similarly, miR-26a downregulates TGF-b and promotes VSMC proliferation and migration through suppressing the expression of SMAD-1 (Leeper et al., 2011). Inhibition of **miR-26a**, accelerated VSMC differentiation with a pronounced rise in both MYH11 expression and a-SMA and, also demonstrated increased levels of SMAD-1 and SMAD-4 inhibiting VSMC proliferation and migration (Leeper et al., 2011). Furthermore, PDGF-BB increases the expression of miR-541, which, in turn, promotes VSMC proliferation and invasion by directly repressing IRF7 (Interferon regulatory factor 7; Yang et al., 2016). Also, PDGF-BB can stimulate HIF-1a (hypoxia inducible factor-1a) expression to induce miR-21 upregulation which then inhibits TPM1 expression promoting VSMC proliferation (Wang et al., 2011). On the other hand, low levels of miR-96, which is negatively regulated by BMP4, increase Trb3 and promotes the contractile phenotype (Kim et al., 2014). **MiR-96** is modulated only by BMP signaling and is not affected by PDGF-BB (Kim et al., 2014).

KLF4 AND MYOCARDIN

CArG box is a part of DNA that controls the expression of gene markers promoted by the contractile phenotype. Serum response factor (SRF) and myocardin **(Myocd)** promote contractile gene

TABLE 2 | Role of miRNAs in VSMC phenotypic switch.

miRNA	Synthetic phenotype	Contractile phenotype	Function
mir-143/145	−	+	TGF-β and BMP activate mir-143/145, which in turn downregulate KLF4 and CD40
mir-133	−	+	Decreases KLF4
mir-1	−	+	Decreases KLF4 and Pim-1
mir-24	+	−	Represses Trb3 and decreases TGF-β and BMP
mir-26a	+	−	Supresses SMAD1 and TGF-β
mir-541	+	−	Represses IRF7
mir-96	+	−	Represses Trb3. Downregulated by BMP4
mir-221/222	+	−	Inhibit p27, p57, c-kit and repress myocardin
mir-223	+	−	Represses myocardin by downregulating mef2c
mir-195	−	+	Represses Cdc42, CCND1, FGF1, IL-1b, IL-6, IL-8
mir-10a	−	+	Represses HDAC4
mir-21, mir-146a	+	−	Inhibit Notch/Jag1 pathway
mir-21	+	−	Inhibits tropomyosin 1
mir-18a-5p	−	+	Represses syndescan4 and increases SMAD2
mir-23b	−	+	Supresses urokinase-type plasminogen activator, SMAD3, FoxO4, MMP-9
mir-663	−	+	Reduces MMP-9
mir-25	−	+	Represses CDK6
mir-142-5p	+	−	Downregulates BTG3 and increases cyclin D3
mir-365	−	+	Decreases cyclin D1
mir-638	−	+	Decreases cyclin D1 and NOR1
mir-141, mir-490-3p	−	+	Repress PAPP-A
mir-155	+	−	Downregulates eNOS expression
mir-135b-5p, mir-499a-3p	+	−	Repress mefc2
let-7d, mir-15b/16	−	+	Decrease KRAS and YAP

TGF-β, transforming growth factor-b; BMP, bone morphogenetic protein; KLF4, Krüppel-like factor-4; Trb3, Tribbles-like protein-3; IRF7, Interferon regulatory factor 7; HDAC4, histone deacetylase 4; BTG3, B cell translocation gene 3; PAPP-A, Pregnancy-associated plasma protein-A; eNOS, endothelial nitric oxide synthase; mefc2, myocyte enhancer factor 2C.

expression via enhancing CArG box activity, while Krüppel-like factor-4 (KLF4) has the opposite effect (Davis-Dusenbery et al., 2011a). TGF-β and BMP4-mediated upregulation of miR-143 and miR-145 through the CArG box downregulate KLF4 which leads to activation of contractile genes and induction of contractile phenotype (Cordes et al., 2009; Xin et al., 2009; Davis-Dusenbery et al., 2011a; Zhang et al., 2016; see **Figure 1**). **MiR-143/145** are considered to be among the most important miRNAs affecting VSMC phenotype switch by regulating crucial transcription factors, including KLF4 and Myocd. Moreover, **miR-133** indirectly decreases the activity of KLF4 via downregulating the transcription factor Sp-1 (Torella et al., 2011). Suppression of CD40, a type I transmembrane glycoprotein receptor, is another mechanism through which miR-145 contributes to the contractile phenotype of VSMC and inhibits TNF-α -mediated VSMC proliferation (Guo et al., 2016). Finally, miR-143/145 deficiency negatively regulate vascular tone through impaired vasodilation which is considered to result from increased VSMC switch to the dedifferentiated phenotype (Noratal et al., 2012). Similarly to **miR-143/145**, **miR-1** represses KLF4 and promotes VSMC differentiation from mouse embryonic stem cells (ESC; Xie et al., 2011). Also, Myocd induces miR-1 expression in human VSMCs and, on the other hand, miR-1 mediates **Myocd**-dependent inhibition of VSMC proliferation partly through the down-regulation of Pim-1, a kinase, which promotes the synthetic phenotype (Chen et al., 2011). Conversely, miR-221/222 promote proliferation of VSMCs partly by inhibiting p27(Kip1) and p57(Kip2) which inhibit VSMC proliferation (Liu et al., 2009, 2012; Chistiakov et al., 2015; see **Figure 1**). Moreover, PDGF-induced miR-221 has the same effect through another mechanism including downregulation of c-kit and Myocd (Davis et al., 2009). The expression of Myocd is also repressed by miR-223 via downregulating mef2c (Rangrez et al., 2012).

MiRNAs AFFECTING VSMC FUNCTION THROUGH NF-κB PATHWAY

Myocd induces differentiation and contractile phenotype of VSMCs and is, also, associated with inhibition of VSMC proliferation through downregulating NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) (p65)-dependent cell cycle progression (Tang et al., 2008; Chen et al., 2011). Similarly, miR-195 promotes contractile phenotype by suppressing Cdc42, CCND1, FGF1 and proinflammatory biomarkers, such as IL-1b, IL-6, and IL-8, which are also implicated in NFkB and p38 MARK (mitogen-activated protein kinases) pathway (Wang Y. S. et al., 2012). Another study, however, demonstrated that miR-10a enhances VSMC

differentiation from ESCs through NF-κB-miR-10a-HDAC4 signaling pathway (Huang et al., 2010). miR-10a mediates VSMC differentiation through repressing HDAC4 (histone deacetylase 4) and preventing its antimyogenic effects, as HDAC4 overxpression reduces VSMC markers, including MYH11 and a-SMA (Huang et al., 2010).

MiRNAs TARGETING MMP-9, TNF-A, CYCLIN D, PPAP-A, AND ENOS ACTIVITY

MiR-23b inhibits VSMC switch to proliferative phenotype via suppressing urokinase-type plasminogen activator, SMAD3 and transcription factor forkhead boxO4 (FoxO4; Iaconetti et al., 2015). Through downregulating FoxO4, **miR-23b** might, also, reduce MMP-9 (matrix metalloproteinase 9) levels and TNF-a induced VSMC migration (Iaconetti et al., 2015). MMP-9 is also reduced by **miR-663** by inhibiting JunB/myosin light chain 9 pathway which results in increasing VSMC contractile phenotype and marker genes (Korff et al., 2013; Li et al., 2013b). Also, miR-25 inhibits VSMC proliferation via directly downregulating the CDK6 gene, a cell cycle regulator, and, on the other hand, **miR-25** is repressed in TNF-a-mediated VSMC dedifferentiated phenotypic switch (Qi et al., 2015). **MiR-142-5p** promotes the synthetic phenotype by supressing B cell translocation gene 3 (BTG3) that results in increased levels of cyclin D3 and other cell cycle related genes (Kee et al., 2013). On the other hand, **miR-365** promotes contractile phenotype through negatively regulating cyclin D1 (Zhang et al., 2014). **MiR-638** has the same effect by targeting the NOR1/cyclin D pathway and repressing the expression of both NOR1 and cyclin D1 which exert a pro-proliferative effect (Li et al., 2013a). Furthermore, **miR-141** and **miR-490-3p** inhibit VSMC proliferation through repressing PAPP-A (Pregnancy-associated plasma protein-A; Sun et al., 2013; Zhang Y. et al., 2015). PAPP-A is a metalloproteinase correlated with VSMC proliferation via increasing the proteolysis of IGF-binding protein-4 (IGFBP-4; Sun et al., 2013; Zhang J. et al., 2015). Apart from regulating nitric oxide (NO) production that results in vascular relaxation, endothelial nitric oxide synthases (eNOS) significantly decreases proliferation and migration of VSMCs and functions as a pro-apoptotic protein (Zhang J. et al., 2015). **MiR-155** promotes the proliferative phenotype through downregulating eNOS expression in VSMCs (Zhang J. et al., 2015).

MiRNAs TARGETING NOTCH PATHWAY, SYNDECAN4, MEF2C, KRAS, AND YAP

The Notch signaling pathway, which is important in intercellular signaling communication, can, also, affect VSMC phenotypic switch (Cao et al., 2015). **Mir-146a** and miR-21 enhance VSMC proliferation via inhibiting Notch2/Jag1 pathway as they downregulate Notch2 and Jag1, respectively (Cao et al., 2015). **MiR-18a-5p** promotes contractile phenotype and increases a-SMA and SM22α protein amounts by repressing its target gene syndecan4 (Kee et al., 2014). Knockdown of syndecan4 increases **SMAD2** expression in VSMCs, which in turn promotes VSMC differentiation by inducing protein expression and promoter activity of SM22α (Kee et al., 2014). Likewise, let-7d and **miR-15b/16** attenuate VSMC proliferation through decreasing KRAS and YAP expression, respectively (Yu et al., 2011; Xu F. et al., 2015). Both KRAS and YAP positively regulate signaling pathways that result in increased VSMC proliferation and migration (Yu et al., 2011; Xu F. et al., 2015). On the other hand, miR-135b-5p and miR-499a-3p promote VSMCs proliferation and migration by directly repressing MEF2C (myocyte enhancer factor 2C) expression (Xu Z. et al., 2015).

MiRNAs AND ARTERIAL CALCIFICATION

Vascular calcification is highly associated with cardiovascular diseases, such as diabetes mellitus, chronic kidney disease, and atherosclerosis, and increases cardiovascular mortality (Goettsch and Aikawa, 2013). Arterial calcification leads to arterial stiffening and hypertension, due to transdifferentiation of VSMCs to a chondrocyte or osteoblast-like phenotype and elastic fiber degradation (Jiang et al., 2017). Here, we mention the major miRNAs that contribute to arterial calcification (see **Table 3**).

MiRNAs AFFECTING ARTERIAL CALCIFICATION THROUGH METALOPROTEINASES (MMP-2)

One of the pathways of vascular calcification is through dysregulation of matrix metalloproteinase-2 (MMP-2) which is a gelatinase that has an important role in matrix degradation and vascular remodeling (Jiang et al., 2017; see **Figure 2**). Upregulation of MMP2 expression and activity is shown in VSMCs and animal calcification models, which mediate elastin degradation, resulting in the production of soluble elastin peptides and stimulation of TGF-β1 signaling pathway from the ECM of vessel walls (Jiang et al., 2017). MMP2 upregulation also contributes to bone morphogenetic protein 2 (BMP2) expression in a β-glycerophosphate-induced VSMC calcification model that could increase **Runx** and **Msx2** expression and promote VSMC calcification (Jiang et al., 2017). MMP2 is a direct target gene of miR-29b-3p with a negative correlation between them (Jiang et al., 2017). Overexpression of miR-29b-3p in rat VSMCs resulted in MMP2 downregulation at the protein level and this could lead to decreased arterial and VSMCs calcification (Goettsch and Aikawa, 2013).

MiRNAs AFFECTING ARTERIAL CALCIFICATION THROUGH CALCIUM DEPOSITION AND OSTEOBLAST DIFFERENTIATION

MiR-29a/b, also, decreases VSMC calcification by repressing ADAMTS-7 (disintegrin and metalloproteinase with thrombospondin motifs-7; Du et al., 2012; see **Figure 2**). ADAMTS-7 mediates VSMC calcification via cartilage oligomeric matrix protein (COMP) degradation and increased levels of

TABLE 3 | Major miRNAs involved in arterial calcification.

miRNA	Arterial calcification	Function
miRNAs AFFECTING ARTERIAL CALCIFICATION THROUGH METALOPROTEINASES		
mir-29b-3p	Decreases	Represses MMP-2
miRNAs AFFECTING ARTERIAL CALCIFICATION THROUGH CALCIUM DEPOSITION AND OSTEOBLAST DIFFERENTIATION		
mir-29a/b	Decreases	Supresses ADAMTS-7
mir-30b-c	Decreases	Supresses RUNX2
mir-133a	Decreases	Supresses RUNX2
mir-204	Decreases	Supresses RUNX2
mir-205	Decreases	Supresses RUNX2 and SMAD1
mir-32	Increases	Induces BMP2, RUNX2, OPN, MGP, ALP
mir-2861, mir-3960	Increase	Repress HDAC5 and Hoxa2 and increase RUNX2
mir-297a	Decreases	Downregulates FGF23
miRNAs AFFECTING ARTERIAL CALCIFICATION THROUGH SMOOTH MUSCLE CELL OSTEOGENIC TRANSDIFFERENTIATION		
mir-125b	Decreases	Supresses osterix
mir-135a	Decreases	Downregulates KLF4/STAT3 pathway
mir-221/222	Increase	Alter Enpp1 and Pit-1 and regulate Pi and PPi levels

BMP-2, bone morphogenetic protein-2; ADAMTS-7, disintegrin and metalloproteinase with thrombospondin motifs-7; RUNX2, Runt-related transcription factor 2; OPN, osteopontin; MGP, matrix GLA protein; ALP, Alkaline phosphatase; HDAC5, histone deacetylase 5; Hoxa2, Homeobox A2; FGF23, fibroblast growth factor 23; Enpp1, ectonucleotide phosphodiesterase 1; Pit-1, Pi cotransporter-1; Pi, inorganic phosphate; PPi, pyrophosphate.

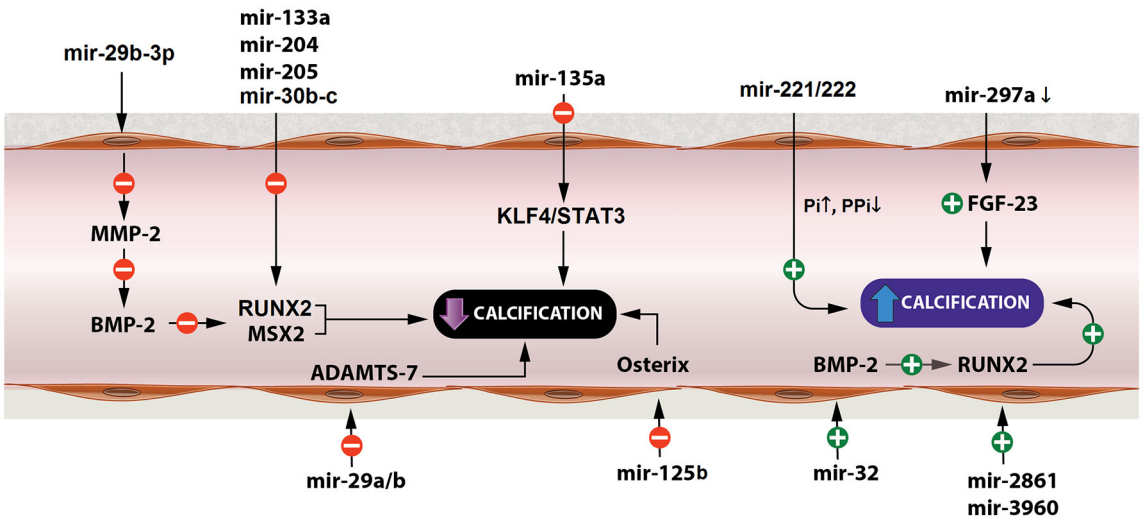

FIGURE 2 | miRNAs affecting arterial calcification. miRNAs play a significant role in arterial calcification by regulating key factors of the calcification process, such as BMP-2, RUNX, ADAMTS-7, osterix, and Pi levels. BMP-2, bone morphogenetic protein-2; RUNX, Runt-related transcription factor 2; ADAMTS-7, disintegrin and metalloproteinase with thrombospondin motifs-7; Pi, inorganic phosphate.

BMP2, SMAD proteins and Runx2 which result in increased mineralization (Du et al., 2012). COMP is a glycoprotein that prevents osteochondrogenic transdifferentiation of VSMCs partly by inhibiting BMP2 (Du et al., 2012).

Runx2 is a major transcription factor related to osteoblast transdifferentiation of VSMCs (Balderman et al., 2012). In VSMCs, Runx2 is not expressed under normal conditions but is overproduced in response to procalcifying stimuli, such as inflammation, oxidant stress, and bone morphogenetic protein-2 (BMP-2; Balderman et al., 2012). Runx2 is expressed in osteoblast-like VSMCs and upregulation of Runx2 drives VSMC transition to osteoblast-like cells (Balderman et al., 2012). Furthermore, osteoblast differentiation is, also, strongly associated with BMP-SMAD signaling pathways which subsequently interfere with Runx2 to enhance the overall process of bone formation (Qiao et al., 2014). **MiR-30b-c** suppress Runx2 protein expression preventing VSMC calcification (Balderman et al., 2012). On the other hand, BMP-2 downregulates miR-30b-c and, thereby, increases Runx2 expression promoting calcification of SMCs through

a Smad-independent pathway (Balderman et al., 2012). **MiR-133a** and **miR-204** are, also, negative regulators of osteogenic differentiation of VSMCs and inhibit arterial calcification via direct suppression of Runx2 (Cui et al., 2012; Liao et al., 2013). **MiR-205** reduces calcification by targeting SMAD-1, in addition to Runx2, suppressing the expression of both of them (Qiao et al., 2014). Conversely, miR-32 increases mouse VSMC calcification by inducing the expression of BMP2, Runx2, osteopontin (OPN), bone-specific phosphoprotein matrix GLA protein (MGP), and ALP activity (Liu et al., 2017). **MiR-32** enhances Runx2 expression and activity by decreasing PTEN (phosphatase and tensin homolog) levels and subsequently activating the PI3K-Akt (phosphatidylinositol 3-kinase/protein kinase B) signaling pathway in mouse VSMCs (Liu et al., 2017). Likewise, miR-2861 and miR-3960 enhance arterial calcification and osteoblastic formation of VSMCs through repressing histone deacetylase 5 (HDAC5) and Homeobox A2 (Hoxa2), respectively, which, in turn, result in increased levels of Runx2 (Kanzler et al., 1998; Kang et al., 2005; Xia et al., 2015; see **Figure 2**).

Moreover, fibroblast growth factor 23 (FGF23) modulates phosphorus levels, negatively regulates Klotho and is positively correlated to vascular calcification (Zheng et al., 2016). When Klotho is suppressed (like in rats with vascular calcification) FGF23 could enhance hyperphosphate-induced vascular calcification (Zheng et al., 2016). FGF23 is a potential target of **miR-297a** (Zheng et al., 2016). Low levels of miR-297a upregulate FGF23 and as a consequence suppresses Klotho resulting in further enhancement on vascular calcification (Zheng et al., 2016; see **Figure 2**).

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Also, miR-125b modulates arterial calcification by targeting transcription factor SP7 (osterix), since inhibition of endogenous miR-125b in calcified HCASMCs (human coronary artery smooth muscle cells) increases Runx2 and promotes osteoblastic transdifferentiation (Goettsch et al., 2011). Similarly, downregulated miR-135a resulted in elevated levels of osteocalcin and induced calcification (Lin et al., 2016). Moreover, miR-135a seems to be a potential osteogenic differentiation suppressor in senescent VSMCs involving, at least partially, the KLF4/STAT3pathway as it decreases STAT3 expression through directly downregulating KLF4 (Fukuyo et al., 2014; Lin et al., 2016). Other miRNAs that promote arterial calcification and osteoblastic differentiation of VSMCs are miR-221 and miR-222, which have to act cooperatively (Mackenzie et al., 2014). This function is independent of Runx2 and Msx2 and seems to be induced by altered Enpp1 (ectonucleotide phosphodiesterase 1) and Pit-1 (Pi cotransporter-1) expressions that regulate cellular inorganic phosphate (Pi) and pyrophosphate (PPi) levels (Mackenzie et al., 2014; see **Figure 2**).

CONCLUSIONS

MiRNAs are a novel class of non-coding RNAs that regulate gene expression. Extensive research during the last decade confirmed the association of miRNAs with cardiovascular diseases. MiRNAs seem to play a significant role in arterial stiffness and calcification through modulating critical pathways and molecules such as TGF-β and BMP signaling, Ang II, MMP activity, Runx, and phenotypic switch of VSMC. Thus, they may be used as therapeutic targets or diagnostic markers in the future to decrease arterial stiffness and prevent the development of cardiovascular diseases. However, it is more than obvious that the molecular biology and pathophysiology is very complex. As mentioned above, many miRNAs might have the same target gene (e.g., Runx2 is suppressed by miR-30b-c but enhanced by miR-32), while a single miRNA might exert multiple functions by targeting more than one genes and affecting different pathways with opposing results (e.g., miR-29, miR-19b and their role in fibrosis). Futhermore, miR-145, one of the most important miRNAs in cardiovascular pathophysiology, decreases arterial stiffness by inhibiting TGF-b signaling while, on the contrary, TGF-b activates miR-145 to promote the contractile phenotype of VSMCs and reduce arterial stiffness as well. Targeting TGF-b through miR-145 might have controversial results. To conclude, additional clinical and laboratory research should be continued for the establishment of miRNAs as treatment targets and biomarkers of cardiovascular diseases. Their emerging role is promising and could result in reducing overall cardiovascular morbidity and mortality in the near future.

AUTHOR CONTRIBUTIONS

SN and MP: Conceived and designed the work, collected the data, and drafted the article; MY: Contributed in drafting the manuscript and critical revision; PZ: Contributed in drafting the manuscript, critical revision, and final approval of the version to be published.

REFERENCES

Abe, K., Nakashima, H., Ishida, M., Miho, N., Sawano, M., Soe, N. N., et al. (2008). Angiotensin II–induced osteopontin expression in vascular smooth muscle cells involves Gq/11, Ras, ERK, Src and Ets-1. *Hypertens. Res.* 31, 987–998. doi: 10.1291/hypres.31.987

Anea, C. B., Ali, M. I., Osmond, J. M., Sullivan, J. C., David, W., Merloiu, A. M., et al. (2010). MMP-2 and MMP-9 dysfunction underlie vascular stiffness incircadian clock mutant mice. *Arterioscler. Thromb. Vasc. Biol.* 30, 2535–2543. doi: 10.1161/ATVBAHA.110.214379

Balderman, J. A. F., Lee, H. Y., Mahoney, C. E., Handy, D. E., White, K., Annis, S., et al. (2012). Bone morphogenetic protein-2 decreases microRNA-30b and microRNA-30c to promote vascular smooth muscle cell calcification. *J. Am. Heart Assoc.* 1:e003905. doi: 10.1161/JAHA.112.003905

Cao, J., Zhang, K., Zheng, J., and Dong, R. (2015). MicroRNA-146a and−21 cooperate to regulate vascular smooth muscle cell proliferation via

modulation of the Notch signaling pathway. *Mol. Med. Rep.* 11, 2889–2895. doi: 10.3892/mmr.2014.3107

Chan, M., Hilyard, A., Wu, C., Davis, B., Hill, N., Lal, A., et al. (2010). Molecular basis for antagonism between PDGF and the TGFbeta family of signalling pathways by control of miR-24 expression. *EMBO J.* 29, 559–573. doi: 10.1038/emboj.2009.370

Chen, J., Yin, H., Jiang, Y., Radhakrishnan, S. K., Huang, Z.-P., Li, J., et al. (2011). Induction of microRNA-1 by myocardin in smooth muscle cells inhibits cell proliferation. *Arterioscler. Thromb. Vasc. Biol.* 31, 368–375. doi: 10.1161/ATVBAHA.110.218149

Chistiakov, D. A., Sobenin, I. A., Orekhov, A. N., and Bobryshev, Y. V. (2015). Human miR-221/222 in physiological and atherosclerotic vascular remodeling. *Biomed. Res.* 2015:354517. doi: 10.1155/2015/354517

Cordes, K. R., Sheehy, N. T., White, M., Berry, E., Sarah, U., Muth, A. N., et al. (2009). miR-145 and miR-143 regulate smooth muscle cell fate decisions. *Nature* 460, 705–710. doi: 10.1038/nature08195

Cui, R.-R., Li, S.-J., Liu, L.-J., Yi, L., Liang, Q.-H., Zhu, X., et al. (2012). MicroRNA-204 regulates vascular smooth muscle cell calcification *in vitro* and *in vivo*. *Cardiovasc. Res.* 96, 320–329. doi: 10.1093/cvr/cvs258

Davis, B. N., Hilyard, A. C., Nguyen, P. H., Lagna, G., and Hata, A. (2009). Induction of MicroRNA-221 by platelet-derived growth factor signaling is critical for modulation of vascular smooth muscle phenotype. *J. Biol. Chem.* 284, 3728–3738. doi: 10.1074/jbc.M808788200

Davis-Dusenbery, B. N., Wu, C., and Hata, A. (2011b). Micro-managing vascular smooth muscle cell differentiation and phenotypic modulation. *Arterioscler. Thromb. Vasc. Biol.* 31, 2370–2377. doi: 10.1161/ATVBAHA.111.226670

Davis-Dusenbery, B. N., Chan, M. C., Reno, K. E., Weisman, A. S., Layne, M. D., Lagna, G., et al. (2011a). Down-regulation of Kruppel-like Factor-4 (KLF4) by microRNA-143/145 is critical for modulation of vascular smooth muscle cell phenotype by transforming growth factor-β and bone morphogenetic protein 4. *J. Biol. Chem.* 286, 28097–28110. doi: 10.1074/jbc.M111.236950

Deng, H., Song, Z., Xu, H., Deng, X., Zhang, Q., Chen, H., et al. (2017). MicroRNA-1185 promotes arterial stiffness though modulating VCAM-1 and E-selectin expression. *Cell. Physiol. Biochem.* 41, 2183–2193. doi: 10.1159/000475576

Du, Y., Gao, C., Liu, Z., Wang, L., Liu, B., He, F., et al. (2012). Upregulation of a disintegrin and metalloproteinase with thrombospondin motifs-7 by miR-29 repression mediates vascular smooth muscle calcification. *Arterioscler. Thromb. Vasc. Biol.* 32, 2580–2588. doi: 10.1161/ATVBAHA.112.300206

Finckenberg, P., Inkinen, K., Ahonen, J., Merasto, S., Louhelainen, M., Vapaatalo, H., et al. (2003). Angiotensin II induces connective tissue growth factor gene expression via calcineurin-dependent pathways. *Am. J. Pathol.* 163, 355–366. doi: 10.1016/S0002-9440(10)63659-0

Fleenor, B. S., and Berrones, A. J. (2015). *Arterial Stiffness: Implications and Interventions*. Cham: Springer.

Fukuyo, S., Yamaoka, K., Sonomoto, K., Oshita, K., Okada, Y., Saito, K., et al. (2014). IL-6-accelerated calcification by induction of ROR2 in human adipose tissue-derived mesenchymal stem cells is STAT3 dependent. *Rheumatology* 53, 1282–1290. doi: 10.1093/rheumatology/ket496

Galis, Z. S., and Khatri, J. J. (2002). Matrix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly. *Circ. Res.* 90, 251–262. doi: 10.1161/hh0302.105345

Gao, S., Liu, T. W., Wang, Z., Jiao, Z. Y., Cai, J., Chi, H. J., et al. (2014). Downregulation of MicroRNA-19b contributes to angiotensin II-induced overexpression of connective tissue growth factor in cardiomyocytes. *Cardiology* 127, 114–120. doi: 10.1159/000355429

Gao, Y. Z., Saphirstein, R. J., Yamin, R., Suki, B., and Morgan, K. G. (2014). Aging impairs smooth muscle-mediated regulation of aortic stiffness: a defect in shock absorption function? *Am. J. Physiol. Heart Circ. Physiol.* 307, H1252–H1261. doi: 10.1152/ajpheart.00392.2014

Giachelli, C. M., Bae, N., Almeida, M., Denhardt, D. T., Alpers, C. E., Schwartz, S. M., et al. (1993). Osteopontin is elevated during neointima formation in rat arteries and is a novel component of human atherosclerotic *Plaques* 92, 1686–1696. doi: 10.1172/JCI116755

Goettsch, C., and Aikawa, E. (2013). "Role of MicroRNAs in cardiovascular calcification, calcific aortic valve disease," ed E. Aikawa (InTech). doi: 10.5772/55326. Available online at: https://www.intechopen.com/books/calcific-aortic-valve-disease/role-of-micrornas-in-cardiovascular-calcification

Goettsch, C., Rauner, M., Pacyna, N., Hempel, U., Bornstein, S. R., and Hofbauer, L. C. (2011). MiR-125b regulates calcification of vascular smooth muscle cells. *Am. J. Pathol.* 179, 1594–1600. doi: 10.1016/j.ajpath.2011.06.016

Guo, X., Li, D., Chen, M., Chen, L., Zhang, B., Wu, T., et al. (2016). miRNA-145 inhibits VSMC proliferation by targeting CD40. *Sci. Rep.* 6:35302. doi: 10.1038/srep35302

Harris, T. A., Yamakuchi, M., Ferlito, M., Mendell, J. T., and Lowenstein, C. J. (2008). MicroRNA-126 regulates endothelial expression of vascular cell adhesion molecule 1. *Proc. Natl. Acad. Sci. U.S.A.* 105, 1516–1521. doi: 10.1073/pnas.0707493105

Hori, D., Dunkerly-eyring, B., Nomura, Y., Biswas, D., Steppan, J., Henao-mejia, J., et al. (2017). miR-181b regulates vascular stiffness age dependently in part by regulating TGF- β signaling. *PLoS ONE* 12:e0174108. doi: 10.1371/journal.pone.0174108

Huang, H., Xie, C., Sun, X., Ritchie, R. P., Zhang, J., and Chen, Y. E. (2010). miR-10a contributes to retinoid acid-induced smooth muscle cell differentiation. *J. Biol. Chem.* 285, 9383–9389. doi: 10.1074/jbc.M109.095612

Iaconetti, C., De Rosa, S., Polimeni, A., Sorrentino, S., Gareri, C., Carino, A., et al. (2015). Down-regulation of miR-23b induces phenotypic switching of vascular smooth muscle cells *in vitro* and *in vivo*. *Cardiovasc. Res.* 107, 522–533. doi: 10.1093/cvr/cvv141

Jiang, W., Zhang, Z., Yang, H., Lin, Q., Han, C., and Qin, X. (2017). The involvement of miR-29b-3p in arterial calcification by targeting matrix metalloproteinase-2. *Biomed Res.* 2017:6713606 doi: 10.1155/2017/6713606

Kang, J. S., Alliston, T., Delston, R., and Derynck, R. (2005). Repression of Runx2 function by TGF-beta through recruitment of class II histone deacetylases by Smad3. *EMBO J.* 24, 2543–2555. doi: 10.1038/sj.emboj.7600729

Kanzler, B., Kuschert, S. J., Liu, Y. H., and Mallo, M. (1998). Hoxa-2 restricts the chondrogenic domain and inhibits bone formation during development of the branchial area. *Development* 125, 2587–2597.

Kee, H. J., Kim, G. R., Cho, S. N., Kwon, J. S., Ahn, Y., Kook, H., et al. (2014). MiR-18a-5p microRNA increases vascular smooth muscle cell differentiation by downregulating syndecan4. *Korean Circ. J.* 44, 255–263. doi: 10.4070/kcj.2014.44.4.255

Kee, H. J., Park, S., Kwon, J.-S., Choe, N., Ahn, Y., Kook, H., et al. (2013). B cell translocation gene, a direct target of miR-142-5p, inhibits vascular smooth muscle cell proliferation by down-regulating cell cycle progression. *FEBS Lett.* 587, 2385–2392. doi: 10.1016/j.febslet.2013.06.005

Kim, S., Hata, A., and Kang, H. (2014). Down-regulation of miR-96 by bone morphogenetic protein signaling is critical for vascular smooth muscle cell phenotype modulation. *J. Cell. Biochem.* 115, 889–895. doi: 10.1002/jcb.24730

Korff, T., Pfisterer, L., and Schorpp-Kistner, M. (2013). MiR-663 and the miRaculous vascular smooth muscle phenotypic switch. *Circ. Res.* 113, 1102–1105. doi: 10.1161/CIRCRESAHA.113.302578

Kriegel, A. J., Liu, Y., Fang, Y., Ding, X., and Liang, M. (2012). The miR-29 family: genomics, cell biology, and relevance to renal and cardiovascular injury. *Physiol. Genomics* 44, 237–244. doi: 10.1152/physiolgenomics.00141.2011

Laurent, S., Cockcroft, J., Van Bortel, L., Boutouyrie, P., Giannattasio, C., Hayoz, D., et al. (2006). Expert consensus document on arterial stiffness: methodological issues and clinical applications. *Eur. Heart J.* 27, 2588–2605. doi: 10.1093/eurheartj/ehl254

Leeper, N. J., Raiesdana, A., Kojima, Y., Chun, H. J., Azuma, J., Kundu, R. K., et al. (2011). MicroRNA-26a is a novel regulator of vascular smooth muscle cell function. *J. Cell. Physiol.* 226, 1035–1043. doi: 10.1002/jcp.22422

Li, P., Zhu, N., Yi, B., Wang, N., Chen, M., You, X., et al. (2013b). MicroRNA-663 regulates human vascular smooth muscle cell phenotypic switch and vascular neointimal formation. *Circ. Res.* 113, 1117–1127. doi: 10.1161/CIRCRESAHA.113.301306

Li, P., Liu, Y., Yi, B., Wang, G., You, X., Zhao, X., et al. (2013a). MicroRNA-638 is highly expressed in human vascular smooth muscle cells and inhibits PDGF-BB-induced cell proliferation and migration through targeting orphan nuclear receptor NOR1. *Cardiovasc. Res.* 99, 185–193. doi: 10.1093/cvr/cvt082

Liao, X. B., Zhang, Z. Y., Yuan, K., Liu, Y., Feng, X., Cui, R. R., et al. (2013). MiR-133a modulates osteogenic differentiation of vascular smooth muscle cells. *Endocrinology* 154, 3344 −3352. doi: 10.1210/en.2012-2236

Liao, Y. C., Wang, Y. S., Hsi, E., Chang, M. H., You, Y. Z., and Juo, S. H. H. (2015). MicroRNA-765 influences arterial stiffness through modulating apelin expression. *Mol. Cell. Endocrinol.* 411, 11–19. doi: 10.1016/j.mce.2015.04.006

Lin, L., He, Y., Xi, B.-L., Zheng, H.-C., Chen, Q., Li, J., et al. (2016). miR-135a suppresses calcification in senescent VSMCs by regulating KLF4/STAT3 pathway. *Curr. Vasc. Pharmacol.* 14, 211–218. doi: 10.2174/1570161113666150722151817

Liu, J., Xiao, X., Shen, Y., Chen, L., Xu, C., Zhao, H., et al. (2017). MicroRNA-32 promotes calcification in vascular smooth muscle cells: implications as a novel marker for coronary artery calcification. *PLoS ONE* 12:e0174138. doi: 10.1371/journal.pone.0174138

Liu, X., Cheng, Y., Zhang, S., Lin, Y., Yang, J., and Zhang, C. (2009). A necessary role of miR-221 and miR-222 in vascular smooth muscle cell proliferation and neointimal hyperplasia. *Circ. Res.* 104, 476–487. doi: 10.1161/CIRCRESAHA.108.185363

Liu, X., Cheng, Y., Yang, J., Xu, L., and Zhang, C. (2012). Cell-specific effects of miR-221/222 in vessels: molecular mechanism and therapeutic application. *J. Mol. Cell. Cardiol.* 52, 242–255. doi: 10.1016/j.yjmcc.2011.11.008

Lorenzen, J. M., Schauerte, C., Hübner, A., Kölling, M., Martino, F., Scherf, K., et al. (2015). Osteopontin is indispensible for AP1-mediated angiotensin II-related miR-21 transcription during cardiac fibrosis. *Eur. Heart J.* 36, 2184–2196. doi: 10.1093/eurheartj/ehv109

Mackenzie, N. C. W., Staines, K. A., Zhu, D., Genever, P., and Macrae, V. E. (2014). miRNA-221 and miRNA-222 synergistically function to promote vascular calcification. *Cell Biochem. Funct.* 32, 209–216. doi: 10.1002/cbf.3005

Min, J., Reznichenko, M., Poythress, R. H., Gallant, C. M., Vetterkind, S., Li, Y., et al. (2012). Src modulates contractile vascular smooth muscle function via regulation of focal adhesions. *J. Cell. Physiol.* 227, 3585–3592. doi: 10.1002/jcp.24062

Mitchell, G. F., Hwang, S. J., Vasan, R. S., Larson, G., Pencina, M. J., Hamburg, N. M., et al. (2010). Arterial stiffness and cardiovascular events: the Framingham Heart Study. *Circulation* 121, 505–511. doi: 10.1161/CIRCULATIONAHA.109.886655

Nicholson, C. J., Seta, F., Lee, S., and Morgan, K. G. (2016). MicroRNA-203 mimics age-related aortic smooth muscle dysfunction of cytoskeletal pathways. *J. Cell. Mol. Med.* 21, 81–95. doi: 10.1111/jcmm.12940

Ning, Z., Koenig, S. N., Trask, A. J., Lin, C.-H., Hans, C. P., Garg, V., et al. (2015). mir145 regulates TGFBR2 expression and matrix synthesis in vascular smooth muscle cells. *Circ. Res.* 116, 23–34. doi: 10.1161/CIRCRESAHA.115.303970

Noratal, G. D., Pinnal, C., Zappellal, F., Elia, L., Salal, A., Condorelli, G., et al. (2012). Microrna 143-145 deficiency impairs vascular function. *Int. J. Immunopathol. Pharmacol.* 25, 467–474. doi: 10.1177/039463201202500216

Ott, C. E., Grünhagen, J., Jäger, M., Horbelt, D., Schwill, S., Kallenbach, K., et al. (2011). MicroRNAs differentially expressed in postnatal aortic development downregulate elastin via 39′ UTR and coding-sequence binding sites. *PLoS ONE* 6:e16250. doi: 10.1371/journal.pone.0016250

Pan, Z., Lu, Y., and Yang, B. (2010). MicroRNAs: a novel class of potential therapeutic targets for cardiovascular diseases. *Acta Pharmacol. Sin.* 31, 1–9. doi: 10.1038/aps.2009.175

Parthenakis, F., Marketou, M., Kontaraki, J., Patrianakos, A., Nakou, H., Touloupaki, M., et al. (2017). Low levels of microRNA-21 are a marker of reduced arterial stiffness in well-controlled hypertension. *J. Clin. Hypertens.* 19, 235–240. doi: 10.1111/jch.12900

Qi, L., Zhi, J., Zhang, T., Cao, X., Sun, L., Xu, Y., et al. (2015). Inhibition of microRNA-25 by tumor necrosis factor alpha is critical in the modulation of vascular smooth muscle cell proliferation. *Mol. Med. Rep.* 11, 4353–4358. doi: 10.3892/mmr.2015.3329

Qiao, W., Chen, L., and Zhang, M. (2014). MicroRNA-205 regulates the calcification and osteoblastic differentiation of vascular smooth muscle cells. *Cell. Physiol. Biochem.* 33, 1945–1953. doi: 10.1159/000362971

Rangrez, A. Y., M'Baya-Moutoula, E., Metzinger-Le Meuth, V., Hénaut, L., Djelouat MS el, I., Benchitrit, J., et al. (2012). Inorganic phosphate accelerates the migration of vascular smooth muscle cells: evidence for the involvement of miR-223. *PLoS ONE* 7:e47807. doi: 10.1371/journal.pone.0047807

Remus, E. W., Lyle, A. N., Weiss, D., Landàzuri, N., Weber, M., Searles, C., et al. (2013). miR181a protects against angiotensin II-induced osteopontin expression in vascular smooth muscle cells. *Atherosclerosis* 228, 168–174. doi: 10.1016/j.atherosclerosis.2013.01.037

Rodríguez-Vita, J., Sánchez-López, E., Esteban, V., Rupérez, M., Egido, J., and Ruiz-Ortega, M. (2005). Angiotensin II activates the Smad pathway in vascular smooth muscle cells by a transforming growth factor-β-independent mechanism. *Circulation* 111, 2509–2517. doi: 10.1161/01.CIR.0000165133.84978.E2

Sehgel, N. L., Zhu, Y., Sun, Z., Trzeciakowski, J. P., Hong, Z., Hunter, W. C., et al. (2013). Increased vascular smooth muscle cell stiffness: a novel mechanism for aortic stiffness in hypertension. *Am. J. Physiol. Heart Circ. Physiol.* 305, H1281–H1287. doi: 10.1152/ajpheart.00232.2013

Sehgel, N. L., Vatner, S. F., and Meininger, G. A. (2015). "Smooth muscle cell stiffness syndrome"-Revisiting the structural basis of arterial stiffness. *Front. Physiol.* 6:335. doi: 10.3389/fphys.2015.00335

Shirwany, N. A., and Zou, M. (2010). Arterial stiffness: a brief review. *Acta Pharmacol. Sin.* 31, 1267–1276. doi: 10.1038/aps.2010.123

Sun, H. X., Zeng, D. Y., Li, R. T., Pang, R. P., Yang, H., Hu, Y. L., et al. (2012). Essential role of microRNA-155 in regulating endothelium-dependent vasorelaxation by targeting endothelial nitric oxide synthase. *Hypertension* 60, 1407–1414. doi: 10.1161/HYPERTENSIONAHA.112.197301

Sun, Y., Chen, D., Cao, L., Zhang, R., Zhou, J., Chen, H., et al. (2013). MiR-490-3p modulates the proliferation of vascular smooth muscle cells induced by ox-LDL through targeting PAPP-A. *Cardiovasc. Res.* 100, 272–279. doi: 10.1093/cvr/cvt172

Tabet, F., Vickers, K. C., Cuesta Torres, L. F., Wiese, C. B., Shoucri, B. M., Lambert, G., et al. (2014). HDL-transferred microRNA-223 regulates ICAM-1 expression in endothelial cells. *Nat. Commun.* 5, 1–14. doi: 10.1038/ncomms4292

Tang, R.-H., Zheng, X.-L., Callis, T. E., Stansfield, W. E., He, J., Baldwin, A. S., et al. (2008). Myocardin inhibits cellular proliferation by inhibiting NF-kappaB(p65)-dependent cell cycle progression. *Proc. Natl. Acad. Sci. U.S.A.* 105, 3362–3367. doi: 10.1073/pnas.0705842105

Torella, D., Iaconetti, C., Catalucci, D., Ellison, G. M., Leone, A., Waring, C. D., et al. (2011). MicroRNA-133 controls vascular smooth muscle cell phenotypic switch *in vitro* and vascular remodeling *in vivo*. *Circ. Res.* 109, 880–893. doi: 10.1161/CIRCRESAHA.111.240150

Van Doren, S. R. (2015). Matrix metalloproteinase interactions with collagen and elastin. *Matrix Biol.* 44–46, 224–231. doi: 10.1016/j.matbio.2015.01.005

Vickers, K. C., Rye, K.-A., and Tabet, F. (2014). MicroRNAs in the onset and development of cardiovascular disease. *Clin. Sci.* 126, 183–194. doi: 10.1042/CS20130203

Wagenseil, J. E., and Mecham, R. P. (2012). Elastin in large artery stiffness and hypertension. *J. Cardiovasc. Transl. Res.* 5, 264–273. doi: 10.1007/s12265-012-9349-8

Wang, B., Komers, R., Carew, R., Winbanks, C. E., Xu, B., Herman-Edelstein, M., et al. (2012). Suppression of microRNA-29 Expression by TGF- 1 Promotes Collagen Expression and Renal Fibrosis. *J. Am. Soc. Nephrol.* 23, 252–265. doi: 10.1681/ASN.2011010055

Wang, D., Zhang, H., Li, M., Frid, M. G., Flockton, A. R., McKeon, B. A., et al. (2014). MicroRNA-124 controls the proliferative, migratory, and inflammatory phenotype of pulmonary vascular fibroblasts. *Circ. Res.* 114, 67–78. doi: 10.1161/CIRCRESAHA.114.301633

Wang, M., Li, W., Chang, G. Q., Ye, C. S., Ou, J. S., Li, X. X., et al. (2011). MicroRNA-21 regulates vascular smooth muscle cell function via targeting tropomyosin 1 in arteriosclerosis obliterans of lower extremities. *Arterioscler. Thromb. Vasc. Biol.* 31, 2044–2053. doi: 10.1161/ATVBAHA.111.229559

Wang, Y. S., Wang, H.-Y. J., Liao, Y.-C., Tsai, P.-C., Chen, K.-C., Cheng, H.-Y., et al. (2012). MicroRNA-195 regulates vascular smooth muscle cell phenotype and prevents neointimal formation. *Cardiovasc. Res.* 95, 517–526. doi: 10.1093/cvr/cvs223

Weintraub, A. S., Schnapp, L. M., Lin, X., and Taubman, M. B. (2000). Osteopontin deficiency in rat vascular smooth muscle cells is associated with an inability to adhere to collagen and increased apoptosis. *Lab. Invest.* 80, 1603–1615. doi: 10.1038/labinvest.3780171

Wu, W.-H., Hu, C.-P., Chen, X.-P., Zhang, W.-F., Li, X.-W., Xiong, X.-M., et al. (2011). MicroRNA-130a mediates proliferation of vascular smooth muscle cells in hypertension. *Am. J. Hypertens.* 24, 1087–1093. doi: 10.1038/ajh.2011.116

Xia, Z. Y., Hoo, Y., Xie, P. L., Tang, S. Y., Luo, X. H., Liao, E. Y., et al. (2015). Runx2/miR-3960/miR-2861 positive feedback loop is responsible for osteogenic transdifferentiation of vascular smooth muscle cells. *Biomed. Res.* 2015:624037. doi: 10.1155/2015/624037

Xiaomei, R., Ren, L., Wei, Q., Shao, H., Chen, L., and Liu, N. (2017). Advanced glycation end-products decreases expression of endothelial nitric oxide synthase through oxidative stress in human coronary artery endothelial cells. *Cardiovasc. Diabetol.* 16, 52. doi: 10.1186/s12933-017-0531-9

Xie, B., Zhang, C., Kang, K., and Jiang, S. (2015). MiR-599 inhibits vascular smooth muscle cells proliferation and migration by targeting TGFB2. *PLoS ONE* 10:e0141512. doi: 10.1371/journal.pone.0141512

Xie, C., Huang, H., Sun, X., Guo, Y., Hamblin, M., Ritchie, R. P., et al. (2011). MicroRNA-1 regulates smooth muscle cell differentiation by repressing Kruppel-like factor 4. *Stem Cells Dev.* 20, 205–210. doi: 10.1089/scd.2010.0283

Xin, M., Small, E. M., Sutherland, L. B., Qi, X., McAnally, J., Plato, C. F., et al. (2009). MicroRNAs miR-143 and miR-145 modulate cytoskeletal dynamics and responsiveness of smooth muscle cells to injury. *Genes Dev.* 23, 2166–2178. doi: 10.1101/gad.1842409

Xu, F., Shufian, A., Ahmed, I., Kang, X., Hu, G., Liu, F., et al. (2015). MicroRNA-15b/16 attenuates vascular neointima formation by promoting the contractile phenotype of vascular smooth muscle through targeting YAP. *Arterioscler. Thromb. Vasc. Biol.* 35, 2145–2152. doi: 10.1161/ATVBAHA.115.305748

Xu, Z., Han, Y., Liu, J., Jiang, F., Hu, H., Wang, Y., et al. (2015). MiR-135b-5p and MiR-499a-3p Promote Cell Proliferation and Migration in Atherosclerosis by Directly Targeting MEF2C. *Sci. Rep.* 5:12276. doi: 10.1038/srep12276

Yang, F., Xu, Z., Duan, S., and Luo, M. (2016). MicroRNA-541 promotes the proliferation of vascular smooth muscle cells by targeting IRF7. *Am. J. Transl. Res.* 8, 506–515.

Yang, L.-X., Liu, G., Zhu, G.-F., Liu, H., Guo, R.-W., Qi, F., et al. (2014). MicroRNA-155 inhibits angiotensin II-induced vascular smooth muscle cell proliferation. *J. Renin Angiotensin Aldosterone Syst.* 15, 109–116. doi: 10.1177/1470320313503693

Yu, M.-L., Wang, J.-F., Wang, G.-K., You, X.-H., Zhao, X.-X., Jing, Q., et al. (2011). Vascular smooth muscle cell proliferation is influenced by let-7d microRNA and its interaction with KRAS. *Circ. J.* 75, 703–709. doi: 10.1253/circj.CJ-10-0393

Zhang, J., Zhao, F., Yu, X., Lu, X., and Zheng, G. (2015). MicroRNA-155 modulates the proliferation of vascular smooth muscle cells by targeting endothelial nitric oxide synthase. *Int. J. Mol. Med.* 35, 1708–1714. doi: 10.3892/ijmm.2015.2181

Zhang, P., Huang, A., Ferruzzi, J., Mecham, R. P., Starcher, B. C., Tellides, G., et al. (2012). Inhibition of MicroRNA-29 enhances elastin levels in cells haploinsufficient for elastin and in bioengineered vessels-brief report. *Arterioscler. Thromb. Vasc. Biol.* 32, 756–759. doi: 10.1161/ATVBAHA.111.238113

Zhang, P., Zheng, C., Ye, H., Teng, Y., Zheng, B., Yang, X., et al. (2014). MicroRNA-365 inhibits vascular smooth muscle cell proliferation through targeting cyclin D1. *Int. J. Med. Sci.* 11, 765–770. doi: 10.7150/ijms.8938

Zhang, Y., Chen, B., Ming, L., Qin, H., Zheng, L., Yue, Z., et al. (2015). MicroRNA-141 inhibits vascular smooth muscle cell proliferation through targeting PAPP-A. *Int. J. Clin. Exp. Pathol.* 8, 14401–14408.

Zhang, Y. N., Xie, B. D., Sun, L., Chen, W., Jiang, S. L., Liu, W., et al. (2016). Phenotypic switching of vascular smooth muscle cells in the "normal region" of aorta from atherosclerosis patients is regulated by miR-145. *J. Cell. Mol. Med.* 20, 1049–1061. doi: 10.1111/jcmm.12825

Zheng, S., Zhang, S., Song, Y., Guo, W., Zhai, W., Qiu, X., et al. (2016). MicroRNA-297a regulates vascular calcification by targeting fibroblast growth factor 23. *Iran. J. Basic Med. Sci.* 19, 1331–1336. doi: 10.22038/ijbms.2016.7920

Zhong, C., Wang, K., Liu, Y., Lv, D., Zheng, B., Zhou, Q., et al. (2016). miR-19b controls cardiac fibroblast proliferation and migration. *J. Cell. Mol. Med.* 20, 1191–1197. doi: 10.1111/jcmm.12858

Zhu, H., Luo, H., and Zuo, X. (2013). MicroRNAs: their involvement in fibrosis pathogenesis and use as diagnostic biomarkers in scleroderma. *Exp. Mol. Med.* 45, 1–6. doi: 10.1038/emm.2013.71

Zieman, S. J., Melenovsky, V., and Kass, D. A. (2005). Mechanisms, pathophysiology, and therapy of arterial stiffness. *Arterioscler. Thromb. Vasc. Biol.* 25, 932–943. doi: 10.1161/01.ATV.0000160548.78317.29

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REVIEW
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Age-related vascular stiffening: causes and consequences

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Arterial stiffening occurs with age and is closely associated with the progression of cardiovascular disease. Stiffening is most often studied at the level of the whole vessel because increased stiffness of the large arteries can impose increased strain on the heart leading to heart failure. Interestingly, however, recent evidence suggests that the impact of increased vessel stiffening extends beyond the tissue scale and can also have deleterious microscale effects on cellular function. Altered extracellular matrix (ECM) architecture has been recognized as a key component of the pre-atherogenic state. Here, the underlying causes of age-related vessel stiffening are discussed, focusing on age-related crosslinking of the ECM proteins as well as through increased matrix deposition. Methods to measure vessel stiffening at both the macro- and microscale are described, spanning from the pulse wave velocity measurements performed clinically to microscale measurements performed largely in research laboratories. Additionally, recent work investigating how arterial stiffness and the changes in the ECM associated with stiffening contributed to endothelial dysfunction will be reviewed. We will highlight how changes in ECM protein composition contribute to atherosclerosis in the vessel wall. Lastly, we will discuss very recent work that demonstrates endothelial cells (ECs) are mechano-sensitive to arterial stiffening, where changes in stiffness can directly impact EC health. Overall, recent studies suggest that stiffening is an important clinical target not only because of potential deleterious effects on the heart but also because it promotes cellular level dysfunction in the vessel wall, contributing to a pathological atherosclerotic state.

Keywords: endothelial cell, atherosclerosis, pulse wave velocity, atomic force microscopy, collagen, glycation, mechanics, mechanical properties

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Introduction

Cardiovascular diseases are the leading cause of death worldwide[1], and age is considered a primary risk factor (Benetos et al., 2002). Arteries stiffen with both age and disease suggesting that age-related arterial stiffening may contribute to cardiovascular pathologies (Benetos et al., 2002). Macroscale analysis of composite arterial mechanics are widely used in the clinic, however, the importance of layer-specific microscale mechanics in altering cellular function to contribute to cardiovascular diseases is less well-established. In this review, we will discuss the structure and composition of the artery and each of its layers during aging as well as methods used to measure the mechanical properties of the artery at both the macro- and microscales. Recent advances in our

[1]http://www.who.int/nmh/publications/ncd_report_full_en.pdf

knowledge of the effects of these mechanical changes on the cells within the vessel are discussed as well as limitations in the current clinical approaches to prevent and/or reverse vessel stiffening.

Artery Structure

Arteries are composite materials, containing multiple concentric layers, each with a distinct composition and function (**Figure 1**). The intima is the innermost artery layer and is a composite of two layers. The luminal layer, known as the basal lamina, is comprised of a thin basement membrane with a proteoglycan rich matrix and small amounts of collagen (Palotie et al., 1983; Kramer et al., 1984). Endothelial cells (ECs) attach to the intimal basement membrane and line the arterial lumen where they act, in part, as regulators of vascular homeostasis. The second intimal layer is musculoelastic and consists of elastin fibers, individual smooth muscle cells, and collagen (Stary et al., 1992). The intima is separated from the media, the inner layer of the artery, by fenestrated elastin fibers of the internal elastic lamina. Elastin within the internal elastic lamina is oriented longitudinally in the direction of luminal blood flow, while in the media it is oriented circumferentially (Farand et al., 2007). The media is composed of lamellar units that are composites of elastin fibers, circumferentially oriented vascular smooth muscle cell (VSMC) layers, collagen fibers, and a mucopolysaccharide viscoelastic gel, commonly referred to as a "ground substance" (Stary et al., 1992). The characteristic lamellae of the media comprise the majority of the arterial wall bulk and are responsible for its elastic properties, allowing for the artery to expand and contract with the blood pulse. The outermost artery layer is the adventitia, which is composed of circumferentially arranged, wavy collagen fibrils intermixed with elastin and is surrounded by loose connective tissue. Fibroblasts are dispersed within the adventitia, but are generally absent from the intima and media artery layers (Ross and Glomset, 1973).

Arterial Mechanics

Composite Structure

When analyzed as a composite material, large arteries exhibit a non-linear stress-strain pattern, and therefore, are best described in terms of an elastic modulus evaluated at a given physiological stress along the stress-strain curve, termed the incremental elastic modulus (Bergel, 1961). The extracellular matrix (ECM) proteins collagen and elastin account for approximately half of the vessel dry weight, and play a crucial role in artery mechanics (O'Connell et al., 2008). Overall, Type I and Type III collagens account for 60% of the artery wall, and elastin 30% (Rizzo et al., 1989; Powell et al., 1992). At low degrees of stretch, the compliant elastin fibers dominate the mechanics, while at higher levels of deformation, helically oriented collagen fibers are recruited (Roach and Burton, 1957; Schriefl et al., 2012). Collagen fibers are 100–1,000 times stiffer than elastin which causes a sharp increase in the incremental elastic modulus at higher levels of circumferential stretch (Dobrin, 1978; Wagenseil et al., 2005; Lasheras, 2007; Wagenseil and Mecham, 2012). Under physiological strain loads, the incremental elastic modulus is a function of strain and the combined contributions of elastin and collagen (Wagenseil and Mecham, 2012).

The distinct composition of each artery layer lends itself to layer-specific mechanical properties that can vary from person to person, and also between large and small arteries (Baynes and Thorpe, 1999; Fonck et al., 2009). For example, measured under axial stretch and using non-axisymmetric deformations, the media is significantly less compliant than the adventitia (Yu et al., 1993; Pandit et al., 2005). The media and adventitia also have different load bearing proportions. Under circumferential tension, the media bears ∼60% of the load and the adventitia bears 40%. Longitudinal tension is primarily assumed by the adventitia which bears ∼75% of the load (Lu et al., 2004). Given that the mechanical properties of each layer vary, it is important to note that the cells within each layer are exposed to and sense the properties of the layer within which they reside. As such, measurements and analysis of

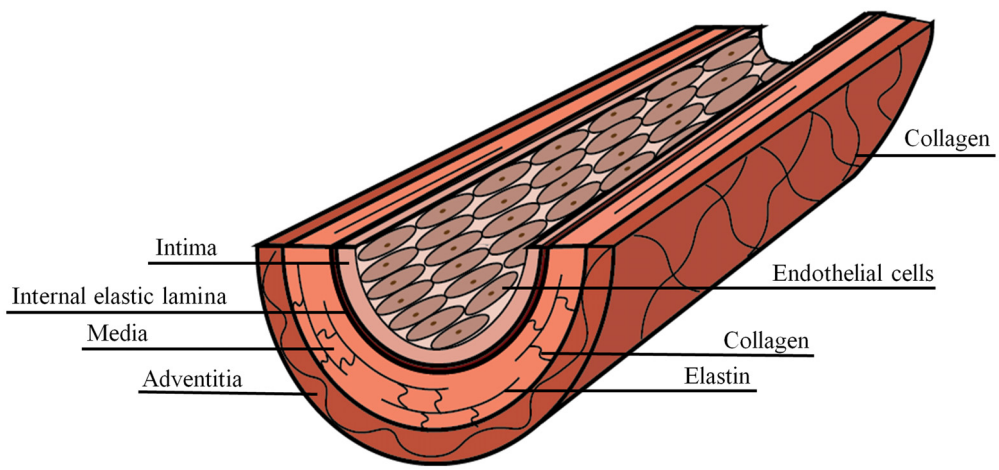


FIGURE 1 | Composite structure of the artery.

each individual layer is essential when considering cellular level mechanobiology.

Intima

The mechanical properties of the healthy intima are not well-established largely because artery mechanics are traditionally studied on the macroscale, where properties of the media and adventitia dominate. Several studies have analyzed the aorta as a two-layer construct consisting of a combined intima-media layer surrounded by the adventitial layer (Holzapfel et al., 2000; Lu et al., 2003). Cells sense their local mechanical properties on the order of five microns, suggesting that overall or composite layer artery mechanics are not representative of the microenvironment where ECs reside (Sen et al., 2005; Buxboim et al., 2010). Interestingly, recent evidence has shown that ECs are mechanosensitive to matrix stiffness and that increased intimal stiffness promotes endothelial dysfunction; therefore the mechanical properties of the intima alone may be important factors in cardiovascular disease progression and warrant further investigation (Reinhart-King et al., 2005; Huynh et al., 2011). In prior work, our laboratory utilized atomic force microscopy (AFM) to measure the mechanical stiffness of the denuded aortic bovine intima, a composite of surface collagen and underlying elastin (Buxboim et al., 2010; Peloquin et al., 2011). The healthy human intima has been reported to have an elastic modulus of 34.4 kPa and is considered a compliant material, with an elastic modulus similar to adipose tissue and lower than muscle (Lundkvist et al., 1996; Engler et al., 2004; Patel et al., 2005).

Media

Medial mechanics are dominated by elastin within the lamellar units at physiological pressures (Greenwald et al., 1997). When the artery is subjected to a transmural pressure, the elastin fibers possessing high entropically driven recoil properties are initially stretched, followed by the stretching of stiffer collagen fibers (Samila and Carter, 1981). The number of concentric lamellae layers in the media remains constant with age but scales with arterial radius and vessel wall tensional strength (Wolinsky and Glagov, 1967). The mechanical contribution of VSMCs dispersed within the aortic lamellae is still somewhat unclear. Contractile activation of VSMCs has been shown to increase the medial elastic modulus (Dobrin and Rovick, 1969; Tremblay et al., 2010), but separate studies have shown that lamellar mechanical properties are unchanged after VSMC activation with noradrenaline (Berry et al., 1975). Therefore, the exact contribution of the VSMC to the mechanical properties of the medial layer is not yet well-defined.

Adventitia

The mechanics of the adventitia are attributed to the collagen organization. In the zero-load state, the fibers assume a crimped morphology (Rezakhaniha et al., 2012). As an axial load is placed on the vessel, the collagen fibers deform and straighten, exhibiting their high tensile strength. In the inner adventitia, collagen fibers are oriented, thin, and intermixed with elastin allowing for vessel distension and protection against rupture, whereas the outer adventitia is primarily composed of thick, non-oriented collagen fibers that support the vessel (Chen et al., 2011).

Causes of Age-Related Arterial Stiffening

Changes in Elastin

Elastic fibers have an extremely low turnover rate *in vivo,* and this longevity allows for the accumulation of age-related changes caused by fragmentation, calcification, and MMP-degradation (Schlatmann and Becker, 1977). As elastin fibers decay, they lose functionality and shift load bearing onto stiffer collagen fibrils, which directly contributes to significant increases in arterial stiffness. Fatigue failure from pulsatile wall stress can cause elastin fragmentation throughout the lifetime (O'Rourke, 1976; Avolio et al., 1998; Greenwald, 2007). Calcium in the arterial wall also increases with age facilitating the direct binding of calcium ions to elastin fibers causing calcification (Urry, 1971; Urry and Ohnishi, 1974; Otto et al., 1999). Animal models that induce elevated elasto-calcinosis show increased medial elastin fragmentation and arterial stiffness (Elliott and McGrath, 1994; Gaillard et al., 2005).

Enzymatic degradation of elastin is mediated by matrix metalloproteinases which have low basal activity in healthy arteries to balance the absence of new elastin synthesis. With age, increased activity of the elastases MT1-MMP and MMP-2 has been observed, and MMP-2 has been found near fragmented elastin fibers within the aorta (Wang et al., 2003; Yasmin et al., 2005; Bonnema et al., 2007). The dysregulation of MMPs is already known to play a role in the cardiovascular pathologies hypertension and aneurysm (Thompson and Baxter, 1999; Yasmin et al., 2005). MMP-2 is found near fragmented elastin fibers within the aorta. Notably, even though the absolute elastin content in the aorta remains relatively stable with age, the elastin concentration decreases and is accompanied by a substantial increase in collagen concentration (Myers and Lang, 1946; Kanabrocki et al., 1960; Toda et al., 1980; Fonck et al., 2009). Age is also associated with changes on the amino acid scale that can contribute to decreased arterial compliance caused by a loss of elastin functionality. The compounds desmosine and isodesmosine are formed from four lysine amino acids and are critical for crosslinking elastin fibers to give them their elastic properties (Davis and Anwar, 1970). The concentrations of desmosine and isodesmosine and their crosslinks decrease with age (John and Thomas, 1972; Watanabe et al., 1996).

Changes in Collagen

In contrast to elastin, the collagen concentration in all three layers of the arterial wall increases with age, shifting the elastin:collagen balance that governs healthy arterial mechanics. Medial fibrosis occurs as a consequence of collagen fibers replacing VSMCs (Schlatmann and Becker, 1977). In general, in individuals over the age of 50, collagen redistributes within the media to bundle near lamellae units (Schlatmann and Becker, 1977; Greenberg, 1986). Recently it was shown that aged ECs have morphological changes resembling a VSMC phenotype and express smooth

muscle alpha actin and collagen I, indicating they may also deposit collagen that contributes to intimal thickening (Fleenor et al., 2012). Within the adventitia, collagen I and III deposition by fibroblasts increases with age and is accompanied by vessel stiffening (Fleenor et al., 2010).

In concert with increased collagen concentrations, collagen crosslinking by non-enzymatic glycation increases arterial stiffness with age (Sims et al., 1996; Schleicher et al., 1997). Glycation is a reaction between reducing sugars and proteins, and directly stiffens tissues in addition to producing deleterious end products. Advanced glycation end products (AGEs) accumulate through a Maillard reaction. Amino groups on proteins react with aldehydes or ketones on the reducing sugars to form shift bases that rearrange to Amadori products and are further modified to produce AGEs (Bakris et al., 2004; Sell and Monnier, 2012). The mechanism of AGE crosslinking *in vivo* is hypothesized to predominately occur between lysine residues on collagen and the AGE *N-ε-carboxy-methyllysine* (CML) (Ikeda et al., 1996; Schleicher et al., 1997). In addition to collagen crosslinking, AGEs are harmful to vascular health because they reduce nitric oxide availability, an important vasodilator used to maintain vascular tone that also has anti-inflammatory effectors on the endothelium (Bucala et al., 1991; De Caterina et al., 1995; Xu et al., 2003, 2005). Furthermore, AGEs interact with the receptor for advanced glycation end products (RAGE) to have downstream effects that include the production of reactive oxygen species, NF-κB inflammatory signaling, and endothelial hyperpermeability (Mullarkey et al., 1990; Wautier et al., 1996, 2001; Bierhaus et al., 1997; Park et al., 1998).

Techniques to Measure Vascular Stiffening

Given that age is a significant cardiovascular risk factor and that arterial stiffness is known to increase with age (Benetos et al., 2002), both macro- and microscale testing techniques have been developed to evaluate arterial mechanics. Arterial properties are measured on the macroscale to determine bulk mechanics, and on the microscale to determine layer-specific mechanics on the cellular level. Historically, greater emphasis has been placed on macroscale testing because it can be used clinically and is associated with cardiovascular events including aneurism, atherosclerosis, and hypertension (van Popele et al., 2001; Kaess et al., 2012; van Sloten et al., 2014). The emergence of ECM mechanics as an important cue affecting cell function has contributed to the development of new techniques to analyze microscale arterial properties.

Macroscale Mechanical Tests

Bulk mechanical testing on arteries is well-established and has been performed for over half a century (Hallock, 1934; Newman and Greenwald, 1978). Macroscale arterial mechanics are used clinically to predict the likelihood of cardiovascular disease risk (Blacher et al., 1999), and are also essential to the development of tissue engineered vascular grafts (Ravi and Chaikof, 2010). Several macroscale techniques for measuring the mechanical properties of intact arteries have been developed (**Figure 2**).

Tensile Testing

Tensile measurements are made by applying tension to a material until it reaches failure, producing a stress-strain curve. Artery samples are removed from the host, cut circumferentially or longitudinally, stored in temperature-controlled environments and tested within 48 h of surgery (Khanafer et al., 2013). As opposite ends of the sample are pulled vertically, the material enters the plastic regime and begins to exhibit necking, before ultimately reaching the failure point. The ultimate tensile strength, the elastic modulus (Young's Modulus), yield strength and the maximum elongation of the bulk material can be obtained from a tensile test stress-strain curve. For materials with non-linear stress-strain patterns, the incremental elastic modulus value can be calculated at specific locations along the stress-strain curve. Many studies treat the artery as an isotropic material and measure its properties uniaxially, although it should be noted that anisotropic materials are most accurately tested biaxially (Sacks, 2000; Vande Geest et al., 2005).

The most common method to determine the elastic modulus of an artery is based on the quotient of stress and strain derived from tensile testing. Despite the widespread use of tensile testing, there are more than four definitions of stress and strain in the literature, which leads to differences in the values obtained through mechanical testing results (Khanafer et al., 2013). The most common values used are engineering stress, which is defined as the force applied to the original cross-sectional area, and engineering strain, which is defined as the change in length over the original length (Khanafer et al., 2013). The Cauchy stress, or force per deformed area, is often used in tensile analysis of arteries (Khanafer et al., 2011; Walsh et al., 2014). The stretch ratio, or the final length over the initial length, is another commonly considered property in the mechanical characterization of arteries when the artery demonstrates a large deformation under tension (Khanafer et al., 2013). Given the various definitions of stress and strain, both the measurement technique and the associated analysis should be considered to accurately compare the reported mechanical properties.

Tensile testing has been performed on human arteries to characterize their mechanical changes in a number of conditions, including aging (Vande Geest et al., 2005; Haskett et al., 2010; Shafigh et al., 2013), as well as plaque (Walsh et al., 2014) and aneurism development (Khanafer et al., 2011). Most often, aortas, coronary arteries, and carotid arteries are used to study the differential mechanics of diseased states after autopsy because they are the dominant sites of disease development, and as the largest vessels, they are also the easiest to obtain (Glagov et al., 1988; Atienza, 2010; Karimi et al., 2013; Walsh et al., 2014). Healthy human coronary arteries have an elastic modulus of 1.5 MPa, which increases to 3.8 MPa in atherosclerotic vessels (Khanafer et al., 2011). These values are comparable to moduli for patients with aneurisms, where the circumferential elastic modulus is 3 MPa and the longitudinal elastic moduli is 2.3–3.7 MPa, when measured under physiological pressures (Khanafer et al., 2011). Interestingly, biaxial tensile testing has

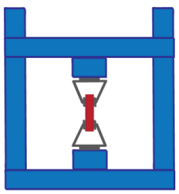
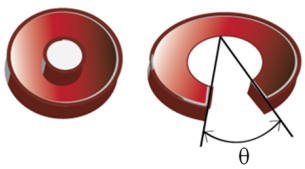
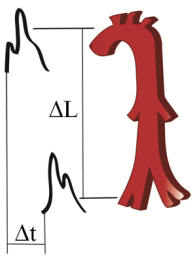
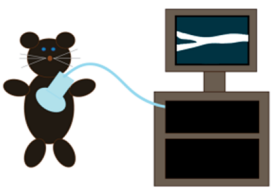
Technique	Diagram	Measurement
Tensile Testing		Bulk biological tissue mechanics
Opening Angle		Bulk residual mechanics of biological tissue
Pulse-Wave Velocity		Clinical measurement Bulk arterial system mechanics
Ultrasound		Clinical measurement Visual analysis of arterial distensibility and compliance

FIGURE 2 | Macroscale techniques to determine artery mechanics.

also shown that individuals over the age of 30 have stiffer abdominal aortas compared to younger patients (Vande Geest et al., 2005). These data suggest that stiffening may precede significant disease progression.

Tensile testing is a common technique for measuring human artery mechanics because it allows for straight-forward experimentation and simple calculations. However, the artery in particular is a complex viscoelastic composite material, and therefore, proper mechanical testing should incorporate these attributes (García et al., 2012; Walsh et al., 2014). Recent experiments have used biaxial testing devices to study blood vessels (Vande Geest et al., 2005; Zemánek et al., 2009; Haskett et al., 2010), which are more representative of *in vivo* mechanical loading. As examples, biaxial testing has been used to microscopically observe the failure point of artery rupture (Sugital and Matsumoto, 2013) and to show that the arterial wall is stiffer in the circumferential direction compared to the axial direction (Shafigh et al., 2013).

Opening Angle Measurements

The opening angle (OA) is a measure of the residual stress of an unloaded tissue and has been used as an indicator of artery composition and viscoelasticity (Fonck et al., 2007; Zhang et al., 2008; Wan and Gleason, 2013). The OA measurement was originally developed to understand the stress-free state of tissues (Chuong and Fung, 1986) but can also be used to elucidate the viscoelastic properties of an artery after tissue loading (Rehal et al., 2006). The OA of a blood vessel is the angle formed from the tip of the open sector to the midpoint of the interior wall after the intact vessel is cut in the radial direction (Zhang et al., 2008). OA measurements are commonly used to study diseased arteries, especially with respect to a hypertensive state, because they are a direct indicator of residual vessel stress. To understand the elastic properties of the vessel, it is important to characterize the stress-free reference state (Fung et al., 1979; Chuong and Fung, 1986).

As examples, OA measurements have been used to measure the anisotropic response of arteries in hypertensive, diabetic

and hypoxic conditions (Fung, 1990; Fung and Liu, 1991). Hypertension causes increased OA and conversely, arteries treated with a vasoconstrictor display a decreased OA (Han et al., 2006). OA is also correlated with systolic blood pressure (Matsumoto and Hayashi, 1996; Han et al., 2006) and with smaller-scale physiological changes. For example, increased OA correlates with an increase in VSMC proliferation and decreased OA values correlate with increased VSMC contraction (Han et al., 2006).

Opening angle measurements are often performed in concert with other mechanical tests, such as tensile testing (Williams et al., 2009; Tian et al., 2011), and in doing so have demonstrated a correlation between OA and arterial stiffness. For example, rabbit carotid arteries exhibit an average OA of 110°, which correlates with a tensile modulus of 2.3 MPa. When the artery is decellularized, the OA decreases to 70° which correlates with an increase in the tensile modulus to 3.5 MPa (Williams et al., 2009). OA analysis can also be performed after pre-stressing the arteries with a transmural pressure load to cause inflation and deflation and measuring the change in artery diameter and axial force (Matsumoto and Hayashi, 1996; Rachev and Greenwald, 2003). As an example, using OA combined with arterial pre-stress measurements, Matsumoto and Hayashi (1996) reported the mean circumferential stress of the thoracic aorta was 339 kPa for hypertensive rats compared to 263 kPa for normotensive controls, and that the circumferential strains were 0.72 and 0.61, respectively. Although taking an OA measurement is useful when analyzing the residual stress and strain properties of the arterial wall, it limits further testing on the uncut artery.

Pulse Wave Velocity Measurements

Pulse wave velocity (PWV) measurements are the primary clinical method used to assess arterial stiffening and to help predict CVD events (Laurent et al., 2006; Van Bortel et al., 2012; Protogerou et al., 2014). A PWV device determines the time for a pressure wave to propagate down a blood vessel by measuring in two separate areas of the body, usually the carotid and femoral arteries. The physical distance between the two catheters is measured on the surface of the body using a tape measure, and the velocity measurement is the ratio of distance traveled to elapsed time (van Popele et al., 2001; Laurent et al., 2006). Measurements along the aorta and aorto-iliac pathway are the most clinically relevant because the aorta and its branches are the first vessels that blood encounters out of the heart and PWV values using this pathway have been shown as predictive values for arterial stiffness (Laurent et al., 2006). The PWV value is directly correlated with the elastic modulus by the Moens–Korteweg equation, which takes into account the vessel radius and the wall thickness and density (Newman and Greenwald, 1978). PWVs are inversely correlated with artery distensibility and compliance (Cecelja and Chowienczyk, 2012). However, PWV does not take into account the differences in arterial stiffness between elastic and muscular parts of the vascular tree (Laurent et al., 2006).

Pulse wave velocity has many advantages over direct mechanical testing because it can be used for clinical applications, is non-invasive, and can directly lead to calculated elastic modulus values. Carotid-femoral PWV measurements are correlated with arterial stiffness and can be used to predict cardiovascular disease in patients (Laurent et al., 1994; Safar et al., 2003). Higher PWV measurements are associated with a higher risk of stroke, cardiovascular death, coronary heart disease (Sutton-Tyrrell et al., 2005) and hypertension (Cecelja and Chowienczyk, 2009). Patients with hypertension and high aortic PWV measurements have increased stenosis frequency, and a higher prevalence of atherosclerotic lesions in the aorta and lower extremities (Maarek et al., 1987). Hallock (1934) used PWV measurements to determine that PWV in the aorta increases from 4.1 m/s in patients ages 5–9 to 6.4 m/s in patients ages 35–44 and up to 10.5 m/s for patients over 65. Notably, PWV increases with age for both men and women (Hallock, 1934; van der-Heijden-Spek et al., 2000), and pulse pressure increases by 25% from ages 30 to 60 (Kelly et al., 1989). Using PWV and distensibility measurements, the 2001 Rotterdam Study demonstrated that stiffness is associated with atherosclerosis in both the carotid artery and the aorta (van Popele et al., 2001). The Rotterdam study also indicated that aortic stiffness alone can be used as a predictor of atherosclerosis because elevated aortic stiffness is associated with atherosclerosis throughout the entire arterial tree (van Popele et al., 2001). PWV has become a powerful method for predicting cardiovascular risk clinically, and in hypertensive patients, it is the best predictor of cardiovascular mortality regardless of age (Blacher et al., 1999).

Ultrasound Testing

In addition to PWV measurements, distensibility, and compliance measurements obtained using ultrasound are also used clinically to assess cardiovascular health. Ultrasound non-invasively images internal organs using sound waves and the associated reflected waves. Ultrasound and its accompanying image processing techniques provide a simple and visual determination of arterial mechanical properties. B-mode, or brightness-mode, ultrasound provides a two-dimensional cross-section of the vessel, whereas M-mode, or motion-mode, can record B-mode images in quick succession, providing a 'video' of the cross-sectional area. Pulse-Doppler ultrasound records images over time, but also employs the Doppler effect to record blood movement in the vessel. Due to the prevalence of ultrasound equipment, ultrasound is used extensively to produce non-invasive measurements of artery mechanics in both clinical and research settings.

Using ultrasound B- and M-modes, the expansion and contraction of the artery with the cardiac pulse can be measured to determine distensibility, which is a measure of artery elasticity (MacSweeney et al., 1992; Godia et al., 2007). Traditionally, the artery is found using B-mode detection, followed by capturing the movement in the vascular wall over time with M-mode ultrasound. The image of the artery walls is then traced to calculate distensibility from the image series (Godia et al., 2007). The distensibility coefficient is calculated as the relative change in artery diameter divided by the systolic pulse pressure (Reneman et al., 1986). A decrease in artery distensibility is directly correlated with increasing arterial stiffness, and therefore, indicates that distensibility can be used as a metric for cardiovascular disease (Gamble et al., 1994; Selzer et al., 2001).

The clinical definition of compliance is the ratio of blood volume to arterial blood pressure, although in practice, the compliance of an artery can be measured as the ratio of change in cross-sectional area to the change in pressure, which is known as the cross-sectional compliance (Reneman et al., 1986; Tozzi et al., 2000). The cross-sectional compliance value is equal to the distensibility coefficient multiplied by the cross-sectional area of the vessel (Reneman et al., 1986; Gamble et al., 1994). To obtain a compliance measurement, an artery can be examined under pulse-Doppler ultrasound to detect the artery wall movement (Reneman et al., 1986). The expansion and contraction of the vessel are normalized to the zero-load outer diameter and measured in conjunction with an ECG measurement that determines heart rate (Fonck et al., 2007). Techniques in ultrasound software can also assess other cardiovascular risk factors, such as wall strain (Godia et al., 2007), atherosclerotic plaque location, and intima-media thickness (Selzer et al., 2001; van Popele et al., 2001). Introducing microbubbles into the blood stream can allow for contrast enhanced ultrasound, where specific molecular targets in the vessel are imaged to study disease progression (Shim and Lindner, 2014).

Decreased arterial compliance is correlated with a higher risk of cardiovascular disease (Zieman et al., 2005). Using pulse-Doppler ultrasound, Reneman et al. (1986) showed that starting around age 30 the human carotid artery distensibility and cross-sectional compliance decrease. Human common carotid arteries have distensibility coefficients of $30*10^{-3}$/kPa at age 25 and this decreases linearly to approximately $18*10^{-3}$/kPa by age 60 (Reneman et al., 1986). The cross-sectional compliance also decreases linearly within this age range from $9*10^{-7}$ m^2/kPa to $6.5*10^{-7}$ m^2/kPa (Reneman et al., 1986). In addition to age-related effects, patients with increased serum cholesterol also have impaired radial artery compliance and distensibility (Giannattasio et al., 1996). A decrease in compliance can be attributed to altered arterial and blood flow pressures due to increases in wall thickening, collagen deposition, or elastin fragmentation (Zieman et al., 2005).

Microscale Mechanical Tests

While significant emphasis has been placed on macroscale measurements of vessel mechanics, less is known about the changes in the mechanical properties of the individual arterial layers or the ECM within these layers. Macroscale measurements generally treat the artery as a uniform material, and do not take into account that arteries are composite materials with three distinct layers. Mechanical analysis on the cell-scale is fundamental to the field of mechanobiology and our understanding of the mechanical cues driving cell behavior and function. Cell size is on the order of 10s of microns, and therefore macroscale mechanical tests do not necessarily measure the same properties being sensed locally by cells. Several approaches to measure microscale mechanical changes have been developed which measure layer-specific mechanical changes. Microscale tests are more relevant for studies of cellular mechanotransduction in the vessel wall and investigations of the cellular response to changes in arterial mechanics (**Figure 3**).

Atomic Force Microscopy

The use of AFM force indentation measurements for ECM mechanical testing in arteries is a relatively newer technique, and it allows for layer-specific mechanical properties to be determined. A cantilever with a micron-scale tip is brought in contact with a sample, and as the cantilever deflects, a laser beam focused on the back of the cantilever is also deflected, providing information regarding the depth of indentation (Binnig et al., 1986; Mann, 2007). The two primary AFM modes are 'contact mode,' where the cantilever tip is dragged along the sample surface, or 'AC (tapping)' mode, where the tip oscillates near its resonant frequency close to the sample surface and intermittent indentations are recorded (Zhong et al., 1993). Most commonly, AFM is used to "image" samples, creating a topographical map, by dragging the cantilever across a sample (Hurley, 2009). AFM tip materials, geometries, and sizes are dependent on the specific application. AFM indentations for force measurements can use tips with radii from ∼5 to 50 nm, allowing for nanoscale resolution (Hurley, 2009). Spherical beads of varying diameters up to the microscale can also be placed on the AFM cantilever for force indentation measurements (Radmacher, 1997; Costa and Yin, 1999). AFM tips can also be functionalized with biological sensors that limit the interaction area with the material (Leckband, 2011; Chtcheglova and Hinterdorfer, 2013).

There has been an increasing interest in the technique of AFM to obtain force measurements on biological materials (Huynh et al., 2011; Peloquin et al., 2011; Weisbrod et al., 2013). AFM force measurement records the deflection of the cantilever as it indents into the sample material to produce a force versus indentation curve (Binnig et al., 1986). Data can be fit to standard mathematical models such as the Hertz model, to provide mechanical properties such as the elastic modulus at the indentation location (Hertz, 1881; Mahaffy et al., 2000; Peloquin et al., 2011). Within the vasculature, AFM has been used to measure local stiffness at different locations within an artery, as well as the mechanical contributions of specific ECM components, namely collagen and elastin (Beenakker et al., 2012). AFM has also been used to study the mechanics of atherosclerotic plaques and fibrous caps (Hayenga et al., 2011; Marzec et al., 2014), as well the effects of age and diet on intima stiffness (Huynh et al., 2011; Peloquin et al., 2011; Weisbrod et al., 2013).

Importantly, the values found using AFM are orders of magnitude smaller than those found using tensile testing. In porcine aortas, AFM testing showed the tunica adventitia had an elastic modulus range of 0.7–391 kPa and that the pulmonary arteries have a stiffer adventitia that ranges between 2.3 and 1,130 kPa (Grant and Twigg, 2013). Notably, AFM testing of the porcine medial layer indicates it is orders of magnitude stiffer than the adventitia, and has an elastic modulus ranging from 1 to 30 MPa (Beenakker et al., 2012). Furthermore, Lundkvist et al. (1996), reported the intima stiffness of healthy human arteries is 34.4 kPa when measured using AFM, while tensile testing

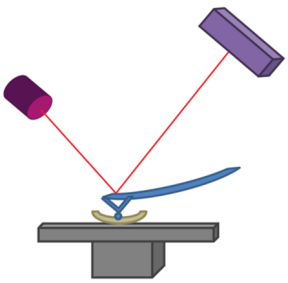
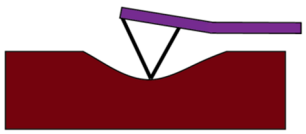
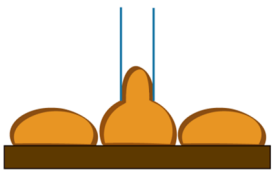
Technique	Diagram	Measurement
Atomic Force Microscopy		Sub-cellular mechanics Cellular mechanics Local biological tissue mechanics
Nanoindentation		Cellular mechanics Local biological tissue mechanics
Micropipette Aspiration		Cellular mechanics Local biological tissue mechanics

FIGURE 3 | Microscale techniques to determine artery mechanics.

on the composite artery indicates an elastic modulus ranging between 1.5 and 3.8 MPa (Khanafer et al., 2011; Karimi et al., 2013). Bulk mechanics typically provide values of the stiffest layer, the media, and therefore these data underscore the importance of analyzing mechanical properties of individual arterial layers using microscale techniques, especially when studying behaviors of mechanosensitive cells.

Atomic force microscopy has also been used to study cell and sub-cellular mechanics using live samples. AFM can be used to study cellular mechanics, including EC stiffness (Kusche-Vihrog et al., 2011; Lee et al., 2011). It was suggested that cells adopt the relative stiffness values of their matrix; therefore, cell-stiffness may be a measure of vascular health (Byfield et al., 2009). On the subcellular level, AFM has been used to study the properties of the plasma membrane, ECM, and cytoskeleton (Kasas and Dietler, 2008; Lu et al., 2008). When performing sub-cellular analysis, AFM tips can be modified to create bio-specific molecular sensors that are used for studying surface receptor interactions and measuring adhesion molecule stiffness (Leckband, 2011; Sarangapani et al., 2011). Simultaneous topography and recognition imaging (TREC) is a recent advancement in AFM technology, where a ligand is functionalized to the AFM cantilever tip to provide simultaneous measurements of material topography and cell-ligand interactions (Preiner et al., 2009). A recent study using TREC with a VE-cadherin-Fc modified AFM tip showed VE-cadherin forms ellipsoid clusters and suggests the protein is bound to actin filaments (Chtcheglova and Hinterdorfer, 2013). Because AFM can be used to determine artery mechanics at the cellular and subcellular levels, and its applications continue to develop, it is expected to have increased prevalence in the mechanobiology field.

Nanoindentation

Nanoindentation measurements use a nano-scale tip to indent into a material and generate a load versus displacement curve that can be used to calculate material mechanics, such as stiffness. Unlike AFM which records cantilever displacements, in nanoindentation, an external load is applied to the indenter tip to push the tip into the surface (Mann, 2007). Displacement in nanoindentation is measured directly by the cantilever's depression into the sample surface, unlike the deflected laser measurement used by the AFM. Whereas older studies relied on imaging the tip depression in the sample to determine the area of contact, current nanoindentation methods can estimate the area of contact based on a well-defined tip geometry, such as a triangular Berkovich or spheroid tip (Oliver and Pharr, 2004). Nanoindentation allows for micro- and nanoscale measurements, with a force range of 1 μN to 500 mN, and a displacement range of 1 nm to 20 μm (Fischer-Cripps, 2002; Ebenstein and Pruitt, 2006). Nanoindentors have indentation depths on the

nanometer scale (Fischer-Cripps, 2002); however, their contact area with the material can be hundreds to thousands of nanometers wide (Hurley, 2009). These length scales bridge the gap between more sensitive AFM measurements and macroscale testing.

Material hardness is calculated using nanoindentation by dividing the load on the tip during indentation by the contact area, and the plastic unloading stiffness is the slope of the load versus displacement curve at a given point (Oliver and Pharr, 2004). Different mathematical models are used to fit nanoindentation data and include the Hertz model (Hertz, 1881; Johnson, 1982), the Oliver-Pharr model (Oliver and Pharr, 1992, 2004), and the Johnson–Kendall–Roberts (JKR) adhesion model (Johnson et al., 1971). The choice of analysis model is based on the properties of the tip-sample interaction, taking into account the shape of the indentation curve and any discontinuities in the curve (Mann, 2007). For biological tissues and hydrated biomaterials, significant adhesion between the tip and sample must be accounted for when determining mechanical properties with nanoindentaiton (Kohn and Ebenstein, 2013).

Many biological materials have been characterized using nanoindentation, including bone and cartilage as well as biomimetic materials such as mollusk shells and spider silk (Ebenstein and Pruitt, 2006). Arterial tissue probed with nanoindentation indicates that porcine elastic aortas have elastic moduli between 60 and 70 kPa (Hemmasizadeh et al., 2012). The technique has also been used to measure the mechanical properties of rat arteries, where the effective elastic moduli ranged from 22 to 37 kPa (Hanna et al., 2013). The stiffness of carotid plaques has also been measured using nanoindentation (Ebenstein et al., 2009). The arterial stiffness values obtained with nanoindentation are comparable to AFM measurements, and both are orders of magnitude lower than the bulk vessel stiffness.

Although nanoindentation and AFM can provide similar information, nanoindentation is a more direct measurement technique for local mechanical property determination. Both methods produce force versus indentation curves to calculate mechanical properties such as the elastic modulus, however, the AFM has increased complexity that relies not only on the indentation parameters, but also on a laser deflection measurement. However, AFM is advantageous for smaller scale biological applications and has greater versatility in the information it can provide.

Micropipette Aspiration

Micropipette aspiration is a less complex technique to measure the mechanics of cells and tissues when compared to AFM and nanoindentation. During testing, a pipette uses suction to pull one end of a cell or matrix into a 0.5–0.8 mm glass tube (Sato et al., 2000; Matsumoto et al., 2002). The distance the material travels into the pipette is tracked using light microscopy (Shao et al., 1998) and is directly related to the material stiffness. Suction forces as low as 1 nN were used to measure the elastic modulus of ECs, which were reported to be 0.5 kPa (Hochmuth, 2000) and increased with increasing substrate stiffness (Byfield et al., 2009).

Soft biological tissue mechanics are measured using pipette aspiration by applying an aspiration pressure on the inside of the pipette to deform the tissue (Aoki et al., 1997). Based on the pipette properties and the degree of deformity, the tissue elastic modulus is calculated. Micropipette aspiration has been used to measure the ECM properties of porcine and rabbit aortas (Sato et al., 2000; Matsumoto et al., 2002; Ohashi et al., 2005). The rabbit aortic arch has an elastic modulus between 40 and 50 kPa compared to an elastic modulus of ∼40 kPa for the thoracic aorta (Matsumoto et al., 2002). The elastic moduli measured using micropipette aspiration are similar to the values obtained using AFM and nanoindentation. Although micropipette aspiration is relatively simple to perform and is well-characterized, it is a lesser used technique for testing the mechanics of *ex vivo* samples. Newer mechanical testing techniques such as nanoindentation and AFM have become more popular likely because of their versatility in measuring cellular and sub-cellular properties.

Effects of Vascular Mechanics on Atherosclerosis

Arterial Stiffening Promotes Vascular Diseases

The mechanical properties of arteries are altered with both age and disease primarily due to changes in the ECM. Vessel stiffness measurements using PWV and ultrasound are widely used in the clinic as indicators of vascular health, but the importance of vascular stiffening, especially on the microscale, in promoting disease is not well-understood (O'Rourke et al., 2002). Recent data suggest numerous cell types respond to matrix stiffness and that stiffness can promote stem cell differentiation, tissue morphogenesis, and tumor malignancy (Paszek and Weaver, 2004; Sieminski et al., 2004; Paszek et al., 2005; Engler et al., 2006). As such, it is likely that the stiffening, which occurs in the vasculature with age and disease, promotes cell dysfunction.

During atherosclerosis, cholesterol permeates into the blood vessel wall and must be retained there to elicit the inflammatory reaction resulting in plaque formation (Libby, 2002). Age-related changes in the arteries caused by ECM rearrangements directly contribute to increased LDL accumulation but they also alter endothelial and VSMC function to contribute to atherogenesis (Tada and Tarbell, 2004; Rudijanto, 2007; Kwon et al., 2008; Huynh et al., 2011). Stiffer arteries have been shown to exhibit increased permeability *in vivo*, and therefore are thought to have greater cholesterol uptake and atherosclerotic plaque initiation (Huynh et al., 2011).

Animal studies by Kothapalli et al. (2012) recently demonstrated that arterial stiffening and ECM crosslinking precede cardiovascular disease development. Treatment with the lysyl oxidase inhibitor BAPN to prevent collagen crosslinking reduced arterial stiffness and decreased atherosclerotic plaque size ∼50% in ApoE mice. A decrease in arterial stiffness reduced the deposition of a pro-atherogenic fibronectin and collagen ECM by VSMCs and limited monocyte binding. It is also known that media calcification precedes atherosclerotic plaque development,

and there is a strong relationship between arteriosclerosis and the calcification and degeneration of elastic fibers in arteries (Blumenthal et al., 1944; Shekhonin et al., 1985). Both human clinical trials using PWV macroscale measurements and animal studies using AFM microscale measurements of the arterial intima have demonstrated that hypertension is preceded by vessel stiffening (Kothapalli et al., 2012; Quevedo, 2012). Furthermore, in hypertensive patients, increased arterial stiffness is associated with increased mortality rates (Laurent et al., 2001). Mounting evidence suggests that arterial stiffness, measured on both macro- and microscales are causes of vascular pathologies, rather than consequences as previously hypothesized.

Mechanical Cues Affect Endothelial Cells to Promote Atherogenesis

Endothelial cells are known to be mechanosensitive, and are important regulators of vascular health and atherogenesis. The effect of flow on EC behavior has been widely studied, and it is well-established that ECs align in the direction of flow, that laminar flow promotes an atheroprotective endothelium, and that atherosclerotic plaques develop predominantly in regions of disturbed flow (Caro et al., 1969; Zarins et al., 1983; Levesque and Nerem, 1985; Tzima et al., 2005). The ECM composition has also been shown to alter EC behavior including increased RhoA activity, ICAM-1 expression, proliferation, morphology, and adhesion (Ingber et al., 1990; Tzima et al., 2001, 2002; Young et al., 2007) Recently, it has become apparent that ECs also respond to ECM mechanical cues where increased matrix stiffness has deleterious effects on endothelial NO production and barrier integrity (Huynh et al., 2011; Kohn et al., 2015).

An important function of the vascular endothelium in atherosclerosis prevention is acting as a protective barrier against cholesterol accumulation and leukocyte transmigration into the intima (Schwenke and Carew, 1989). Using AFM, our group has shown the arterial intima stiffens with age, and that in response to increased ECM matrix stiffness, EC-cell junction size and permeability increase both *in vitro* and *in vivo* (Huynh et al., 2011). Mechanistically, increased substrate stiffness activates the Rho/Rho kinase pathway to increase cellular contractile forces and compromise cell-cell junction integrity (Huynh et al., 2011; Krishnan et al., 2011). Recent work by Szulcek et al. (2013) demonstrated the complexity of endothelial monolayer regulation. RhoA is involved in both monolayer gap formation and closure depending on its subcellular localization. Immune cell infiltration into the intima has important implications for driving atherosclerotic plaque formation, and the increased EC-cell junction size in response to substrate stiffness facilitates leukocyte transmigration by the predominant paracellular route (Huynh et al., 2011; Stroka et al., 2013). Notably, the endothelial expression of VCAM-1, ICAM-1, and E-selectin were unaffected by substrate stiffness *in vitro*, suggesting the changes were largely driven by mechanics. In addition to the endothelial response to matrix mechanics compromising the arterial barrier against leukocyte transmigration, recent studies show that leukocytes also respond to substrate mechanics. Leukocytes exhibit elevated transmigration behavior with increasing stiffness and biphasic migration speeds (Hayenga and Aranda-Espinoza, 2013; Stroka et al., 2013). Interestingly, AGEs can promote increased adhesion molecule expression in ECs, suggesting that arterial stiffening caused by collagen glycation may have the synergistic effects of increased substrate mechanics and increased adhesion molecule expression contributing to leukocyte transmigration.

Arterial Stiffening Alters VSMC Behavior to Promote Atherosclerosis

A phenotypic switch from a contractile to a motile, synthetic phenotype in VSMCs is characteristic of VSMCs found in atherosclerotic lesions. During atherogenesis, VSMCs migrate from the media into the intima where their proliferation and ECM secretions increase plaque size (Rudijanto, 2007). Interestingly, VSMCs are durotactic, preferentially migrating in the direction of increased substrate stiffness, but their maximum migration speed depends on both matrix stiffness and ECM chemical cues (Peyton and Putnam, 2005; Isenberg et al., 2009; Sazonova et al., 2014). A proliferative phenotype was observed by VSMCs cultured on stiff collagen fibrils when compared to compliant collagen gels, even though β_1 integrin mediated cell-ECM interactions were comparable on both substrates (McDaniel et al., 2007). Similar to migration, VSMC proliferation is also dependent on ECM ligand density, but it has been demonstrated that increased matrix stiffness is the dominant factor affecting proliferation (Vatankhah et al., 2014). In addition to ECM cues, VSMC proliferation and migration is also stimulated by platelet derived growth factor (PDGF) released by ECs and macrophages during atherogenesis. Interestingly, increased matrix stiffness enhances VSMC proliferation and migration induced by PDGF signaling (Brown et al., 2010; Huynh et al., 2013).

Interventions for Age-Related Cardiovascular Stiffening

Stiffening of the arteries with age is well-established, and it is now becoming apparent that arterial stiffness contributes to cardiovascular diseases; therefore, preventing or reversing stiffening may be one method to improve cardiovascular health. Lifestyle changes and pharmacological interventions are two strategies for decreasing arterial stiffness (**Table 1**). Exercise is the most well-studied lifestyle intervention to improve arterial compliance.

Aerobic Exercise Reduces Macroscale Arterial Stiffening

Numerous human studies have demonstrated that habitually active adults have more compliant arteries when compared to their age-matched sedentary peers and that moderate aerobic exercise interventions successfully increased macroscale arterial compliance in healthy aged populations (Tanaka et al., 2000; Seals et al., 2008; Santos-Parker et al., 2014). Brisk walking can improve arterial compliance in as little as 3 months in healthy middle-aged men (Tanaka et al., 2000). However, in aged individuals with hypertension the same gains in arterial compliance were not achieved by a short-term aerobic exercise intervention, suggesting that existing cardiovascular pathologies may limit the benefits of exercise on arterial stiffness (Ferrier et al., 2001). The mechanism by which exercise restores arterial compliance is not fully understood and appears to vary with the type of exercise. Mouse

TABLE 1 | Selected interventions with human studies to reduce arterial stiffness.

Category	Intervention	Study	Outcome	Reference
Lifestyle	Aerobic exercise	Brisk walking in healthy sedentary male adults	Increased central arterial compliance	Tanaka et al. (2000)
Pharmaceutical AGE blocker	Aminoguanidine	Phase 3 clinical trial	Early termination due to safety and efficacy	Freedman et al. (1999) and Thornalley (2003)
Pharmaceutical AGE breaker	Alagebrium chloride (ALT-711)	Phases 2 and 3 clinical trials	No increase in cardiovascular health (Lifetime Risk Score) Clinical trials terminated due to finances	Oudegeest-Sander et al. (2013); clinicaltrials.gov
Pharmaceutical AGE breaker	TRC4186	Phase 1 clinical trial	Safety, tolerance, and pharmacokinetics established	Chandra et al. (2009)

studies show that voluntary wheel running decreases Type I collagen levels in the media and adventitia of old mice, while studies involving swimming and treadmill running did not show changes in arterial collagen composition (Nosaka et al., 2003; Maeda et al., 2005; Fleenor et al., 2010). Exercise may increase collagen turnover, thus preventing the accumulation of AGEs. However, while increased markers of collagen synthesis and turnover have been found in response to exercise for cardiac, tendon, and bone collagens, studies with arterial collagen still need to be completed (Langberg et al., 1999; Wallace et al., 2000; Radauceanu et al., 2007). It is important to note that deposition of collagen in bone and tendon in response to exercise is indicative of beneficial strengthening while collagen deposition in the arteries contributes to pathological stiffening, therefore, it is possible that the effects of exercise on collagen composition may be tissue specific.

Pharmacological Prevention of AGE Accumulation

Pharmacological interventions for arterial stiffening primarily fall into two categories: AGE blockers and AGE breakers. Several chemical compounds to prevent arterial stiffening by inhibiting AGE accumulation have been studied, but thus far, none have made it past clinical trials. Aminoguanidine was the first well-studied inhibitor of AGEs. Early studies in diabetic rats showed that it was able to inhibit AGE formation by blocking carbonyl reactivity on early glycation products even when animals were fed high glucose diets (Brownlee et al., 1986). Later studies with an aged, non-diabetic rat model showed aminoguanidine prevented arterial stiffening in 24-month old, end-of-life rats without altering collagen content (Corman et al., 1998). Despite the early promise of aminoguanidine, clinical trials were terminated when it caused impaired liver functionality and the initiation of lupus-like illnesses in patients (Freedman et al., 1999). An alternative AGE inhibitor, 2,3 diaminophenazine (2,3 DAP) did not make it past pre-clinical toxicity tests (Soulis et al., 1999). ALT-946 and OPB-9195 are other examples of AGE blockers that possess similar hydrazine structures to aminoguanidine, that have not yet reached clinical trials. Initial data indicates that ALT-946 is more effective and less toxic than aminoguanidine (Forbes et al., 2001; Wada et al., 2001). The failure of the AGE inhibitors mentioned here, and others, to succeed in clinical trials more than 15 years after initial studies with aminoguanidine, highlights the difficulty of safely and effectively reducing AGE accumulation *in vivo*.

Pharmacological Breaking of AGE Crosslinks

The second class of pharmaceutical interventions used to overcome arterial stiffness are compounds that break AGE crosslinks after they have been formed. The most widely studied of these is the thiazelium based ALT-711 (alagebrium chloride), which was discovered as a more stable form of the very first AGE breaker, PTB (Vasan et al., 1996). These nucleophilic compounds break carbon–carbon bonds between adjacent carbonyl groups in crosslinked proteins (Vasan et al., 1996; Kim and Spiegel, 2013). ALT-711 showed efficacy *in vitro* and *in vivo* at cleaving AGE crosslinks. The resulting collagen fragmentation increased the elasticity of cardiac and arterial tissue (Vasan et al., 1996; Wolffenbuttel et al., 1998; Little et al., 2005). The early success of ALT-711 led to more than 12 combined Phases 2 and 3 clinical trials[2]. Despite the potential for ALT-711, a randomized factorial study comparing the interaction between ALT-711 and exercise in older (over age 70) sedentary adults showed no improvement in arterial stiffness measured with PWV. Cardiovascular health measured using the Lifetime Risk Score (LRS) improved with exercise, but not the drug intervention (Oudegeest-Sander et al., 2013). All ongoing clinical trials for ALT-711 were terminated in 2009 by Synvista Therapeutics Inc. for financial reasons.[2] The safety, pharmacokinetics, and tolerance of another AGE crosslink breaking compound, TRC4186, were established during a successful phase 1 clinical trial and published in 2009 (Chandra et al., 2009). However, the necessary phase 2 and 3 clinical trials showing efficacy and continued safety have not been completed. Studies on a new, safer AGE breaker, C36 (3-benzyloxycarbonylmethyl-4-methyl-thiazol-3-ium bromide) showed decreased systemic arterial stiffness and improved collagen composition in diabetic rats (Cheng et al., 2007). There currently are no AGE blockers or breakers on the market today, indicating the difficulty of successfully and safely overcoming the effects of tissue stiffening that occur with age.

Future Perspectives

As the contribution of arterial stiffening to cardiovascular diseases comes into better focus, new studies will need to rely on

[2]http://clinicaltrials.gov/

macroscale measurement techniques, and expand the microscale understanding of how stiffening of each arterial layer contributes to disease. Recently, Weisbrod et al. (2013) have shown that intimal stiffening precedes hypertension and Huynh et al. (2011) showed that intimal stiffening leads to compromised endothelium integrity. These new findings complement existing literature with macroscale measurements that emphasize stiffening of the adventitial and medial layers. A particular challenge will be to develop microscale tools that can measure intimal stiffening *in vivo* for use in human clinical diagnoses and to contribute to a better understanding of the underlying mechanisms that govern the relationship between arterial stiffening and cardiovascular disease.

Author Contributions

JK and ML collected and analyzed relevant literature, wrote the manuscript, and created the figures and table. CR designed the research topic and wrote the manuscript.

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References

Aoki, T., Ohashi, T., Matsumoto, T., and Sato, M. (1997). The pipette aspiration applied to the local stiffness measurement of soft tissues. *Ann. Biomed. Eng.* 25, 581–587. doi: 10.1007/BF02684197

Atienza, J. M. (2010). Response of human coronary arteries at different mechanical conditions. *Conf. Proc. IEEE Eng. Med. Biol. Soc.* 41, 3585–3588. doi: 10.1109/IEMBS.2010.5627456

Avolio, A., Jones, D., and Tafazzoli-Shadpour, M. (1998). Quantification of alterations in structure and function of elastin in the arterial media. *Hypertension* 32, 170–175. doi: 10.1161/01.HYP.32.1.170

Bakris, G. L., Bank, A. J., Kass, D. A., Neutel, J. M., Preston, R. A., and Oparil, S. (2004). Advanced glycation end-product cross-link breakers: a novel approach to cardiovascular pathologies related to the aging process. *Am. J. Hypertens.* 172(Pt 2), 23S–30S. doi: 10.1016/j.amjhyper.2004.08.022

Baynes, J. W., and Thorpe, S. R. (1999). Role of oxidative stress in diabetic complications: a new perspective on an old paradigm. *Diabetes Metab. Res. Rev.* 48, 1–9. doi: 10.2337/diabetes.48.1.1

Beenakker, J. W. M., Ashcroft, B. A., Lindeman, J. H. N., and Oosterkamp, T. H. (2012). Mechanical properties of the extracellular matrix of the aorta studied by enzymatic treatments. *Biophys. J.* 102, 1731–1737. doi: 10.1016/j.bpj.2012.03.041

Benetos, A., Waeber, B., Izzo, J., Mitchell, G., Resnick, L., Asmar, R., et al. (2002). Influence of age, risk factors, and cardiovascular and renal disease on arterial stiffness: clinical applications. *Am. J. Hypertens.* 15, 1101–1108. doi: 10.1016/S0895-7061(02)03029-7

Bergel, D. (1961). The dynamic elastic properties of the arterial wall. *J. Physiol.* 156, 458–469. doi: 10.1113/jphysiol.1961.sp006687

Berry, C. L., Greenwald, S. E., and Rivett, J. F. (1975). Static mechanical properties of the developing and mature rat aorta. *Cardiovasc. Res.* 9, 669–679. doi: 10.1093/cvr/9.5.669

Bierhaus, A., Chevion, S., Chevion, M., Hofmann, M., Quehenberger, P., Illmer, T., et al. (1997). Advanced glycation end product-induced activation of NF-kappaB is suppressed by alpha-lipoic acid in cultured endothelial cells. *Diabetes Metab. Res. Rev.* 46, 1481–1490. doi: 10.2337/diabetes.46.9.1481

Binnig, G., Quate, C. F., and Gerber, C. (1986). Atomic force microscope. *Phys. Rev. Lett.* 56, 930–933. doi: 10.1103/PhysRevLett.56.930

Blacher, J., Asmar, R., Djane, S., London, G. M., and Safar, M. E. (1999). Aortic pulse wave velocity as a marker of cardiovascular risk in hypertensive patients. *Hypertension* 33, 1111–1117. doi: 10.1161/01.HYP.33.5.1111

Blumenthal, H. T., Lansing, A. I., and Wheeler, P. A. (1944). Calcification of the media of the human aorta and its relation to intimal arteriosclerosis, ageing and disease. *Am. J. Pathol.* 20, 665–687.

Bonnema, D. D., Webb, C. S., Pennington, W. R., Stroud, R. E., Leonardi, A. E., Clark, L. L., et al. (2007). Effects of age on plasma matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs). *J. Card. Fail.* 13, 530–540. doi: 10.1016/j.cardfail.2007.04.010

Brown, X. Q., Bartolak-Suki, E., Williams, C., Walker, M. L., Weaver, V. M., and Wong, J. Y. (2010). Effect of substrate stiffness and PDGF on the behavior of vascular smooth muscle cells: implications for atherosclerosis. *J. Cell. Physiol.* 225, 115–122. doi: 10.1002/jcp.22202

Brownlee, M., Vlassara, H., Kooney, A., Ulrich, P., and Cerami, A. (1986). Aminoguanidine prevents diabetes-induced arterial wall protein cross-linking. *Science* 232, 1629–1632. doi: 10.1126/science.3487117

Bucala, R., Tracey, K. J., and Cerami, A. (1991). Advanced glycosylation products quench nitric oxide and mediate defective endothelium-dependent vasodilatation in experimental diabetes. *J. Clin. Invest.* 87, 432–438. doi: 10.1172/JCI115014

Buxboim, A., Rajagopal, K., Brown, A. E. X., and Discher, D. E. (2010). How deeply cells feel: methods for thin gels. *J. Phys. Condens. Matter.* 22:194116. doi: 10.1088/0953-8984/22/19/194116

Byfield, F. J., Reen, R. K., Shentu, T. P., Levitan, I., and Gooch, K. J. (2009). Endothelial actin and cell stiffness is modulated by substrate stiffness in 2D and 3D. *J. Biomech.* 42, 1114–1119. doi: 10.1016/j.jbiomech.2009.02.012

Caro, C. G., Fitz-Gerald, J. M., and Schroter, R. C. (1969). Arterial wall shear and distribution of early atheroma in man. *Nature* 223, 1159–1160. doi: 10.1038/2231159a0

Cecelja, M., and Chowienczyk, P. (2009). Dissociation of aortic pulse wave velocity with risk factors for cardiovascular disease other than hypertension: a systematic review. *Hypertension* 54, 1328–1336. doi: 10.1161/HYPERTENSIONAHA.109.137653

Cecelja, M., and Chowienczyk, P. (2012). Role of arterial stiffness in cardiovascular disease. *JRSM Cardiovasc. Dis.* 1, 1–10. doi: 10.1258/cvd.2012.012016

Chandra, K. P., Shiwalkar, A., Kotecha, J., Thakkar, P., Srivastava, A., Chauthaiwale, V., et al. (2009). Phase 2 clinical studies of the advanced glycation-end product (AGE)-breaker TRC4186: safety, tolerability and pharmokinetics in healthy subjects. *Clin. Drug. Investig.* 29, 399–375. doi: 10.2165/11315260-000000000-00000

Chen, H., Liu, Y., Slipchenko, M. N., Zhao, X., Cheng, J. X., and Kassab, G. S. (2011). The layered structure of coronary adventitia under mechanical load. *Biophys. J.* 101, 2555–2562. doi: 10.1016/j.bpj.2011.10.043

Cheng, G., Wang, L. L., Long, L., Liu, H. Y., Cui, H., Qu, W. S., et al. (2007). Beneficial effects of C36, a novel breaker of advanced glycation endproducts cross-links, on the cardiovascular system of diabetic rats. *Br. J. Pharmacol.* 152, 1196–1206. doi: 10.1038/sj.bjp.0707533

Chtcheglova, L. A., and Hinterdorfer, P. (2013). Atomic force microscopy functional imaging on vascular endothelial cells. *Methods Mol. Biol.* 931, 331–344. doi: 10.1007/978-1-62703-056-4-16

Chuong, C. J., and Fung, Y. C. (1986). On residual stresses in arteries. *J. Biomech. Eng.* 108, 189–192. doi: 10.1115/1.3138600

Corman, B., Duriez, M., Poitevin, P., Heudes, D., Bruneval, P., Tedgui, A., et al. (1998). Aminoguanidine prevents age-related arterial stiffening and cardiac hypertrophy. *Proc. Natl. Acad. Sci. U.S.A.* 95, 1301–1306. doi: 10.1073/pnas.95.3.1301

Costa, K. D., and Yin, F. C. P. (1999). Analysis of indentation: implications for measuring mechanical properties with atomic force microscopy. *J. Biomech. Eng.* 121, 462–471. doi: 10.1115/1.2835074

Davis, N. R., and Anwar, R. A. (1970). On the mechanism of formation of desmosine and isodesmosine cross-links of elastin. *J. Am. Chem. Soc.* 92, 3778–3782. doi: 10.1021/ja00715a040

De Caterina, R., Libby, P., Peng, H. B., Thannickal, V. J., Rajavashisth, T. B., Gimbrone, M. A., et al. (1995). Nitric oxide decreases cytokine-induced endothelial activation: nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. *J. Clin. Invest.* 96, 60–68. doi: 10.1172/JCI118074

Dobrin, P. B. (1978). Mechanical properties of arterises. *Physiol. Rev.* 58, 265–324.

Dobrin, P. B., and Rovick, A. (1969). Influence of vascular smooth muscle on contractile mechanics and elasticity of arteries. *Am. J. Physiol.* 217, 1644–1651. doi: 10.1152/advan.00088.2014.

Ebenstein, D. M., Coughlin, D., Chapman, J., Li, C., and Pruitt, L. A. (2009). Nanomechanical properties of calcification, fibrous tissue, and hematoma from atherosclerotic plaques. *J. Biomed. Mater. Res.* 91, 1028–1037. doi: 10.1002/jbm.a.32321

Ebenstein, D. M., and Pruitt, L. A. (2006). Nanoindentation of biological materials. *NanoToday* 1, 26–33. doi: 10.1016/S1748-0132(06)70077-9

Elliott, R. J., and McGrath, L. T. (1994). Calcification of the human thoracic aorta during aging. *Calcif. Tissue Int.* 54, 268–273. doi: 10.1007/BF00295949

Engler, A. J., Griffin, M. A., Sen, S., Bönnemann, C. G., Sweeney, H. L., and Discher, D. E. (2004). Myotubes differentiate optimally on substrates with tissue-like stiffness: pathological implications for soft or stiff microenvironments. *J. Cell Biol.* 166, 877–887. doi: 10.1083/jcb.200405004

Engler, A. J., Sen, S., Sweeney, H. L., and Discher, D. E. (2006). Matrix elasticity directs stem cell lineage specification. *Cell* 126, 677–689. doi: 10.1016/j.cell.2006.06.044

Farand, P., Garon, A., and Plante, G. E. (2007). Structure of large arteries: orientation of elastin in rabbit aortic internal elastic lamina and in the elastic lamellae of aortic media. *Microvasc. Res.* 73, 95–99. doi: 10.1016/j.mvr.2006.10.005

Ferrier, K. E., Waddell, T. K., Gatzka, C. D., Cameron, J. D., Dart, A. M., and Kingwell, B. A. (2001). Aerobic exercise training does not modify large-artery compliance in isolated systolic hypertension. *Hypertension* 38, 222–226. doi: 10.1161/01.HYP.38.2.222

Fischer-Cripps, A. C. (2002). *Nanoindentation*. New York: Springer Science+Business Media, Inc. doi: 10.1007/978-0-387-22462-6

Fleenor, B. S., Marshall, K. D., Durrant, J. R., Lesniewski, L. A., and Seals, D. R. (2010). Arterial stiffening with ageing is associated with transforming growth factor-β1-related changes in adventitial collagen: reversal by aerobic exercise. *J. Physiol.* 588, 3971–3982. doi: 10.1113/jphysiol.2010.194753

Fleenor, B. S., Marshall, K. D., Rippe, C., and Seals, D. R. (2012). Replicative aging induces endothelial to mesenchymal transition in human aortic endothelial cells: potential role of inflammation. *J. Vasc. Res.* 49, 59–64. doi: 10.1159/000329681

Fonck, E., Feigl, G. G., Fasel, J., Sage, D., Unser, M., Rüfenacht, D. A., et al. (2009). Effect of aging on elastin functionality in human cerebral arteries. *Stroke* 40, 2552–2556. doi: 10.1161/STROKEAHA.108.528091

Fonck, E., Prod'hom, G., Roy, S., Augsburger, L., Rufenacht, D. A., and Stergiopulos, N. (2007). Effect of elastin degradation on carotid wall mechanics as assessed by a constituent-based biomechanical model. *Am. J. Physiol. Heart. Circ. Physiol.* 292, H2754–H2763. doi: 10.1152/ajpheart.01108.2006

Forbes, J. M., Soulis, T., Thallas, V., Panagiotopoulos, S., Long, D. M., Vasan, S., et al. (2001). Renoprotective effects of a novel inhibitor of advanced glycation. *Diabetologia* 44, 108–114. doi: 10.1007/s001250051587

Freedman, B. I., Wuerth, J. P., Cartwright, K., Bain, R. P., Dippe, S., Hershon, K., et al. (1999). Design and baseline characteristics for the aminoguanidine clinical trial in overt type 2 diabetic nephropathy (ACTION II). *Control. Clin. Trials* 20, 493–510. doi: 10.1016/S0197-2456(99)00024-0

Fung, Y. C. (1990). *Biomechanics: Stress, Strain and Growth*. New York: Springer. doi: 10.1007/978-1-4419-6856-2

Fung, Y. C., Fronek, K., and Patitucci, P. (1979). Pseudoelasticity of arteries and the choice of its mathematical expression. *Am. J. Phys.* 237, H620–H631.

Fung, Y. C., and Liu, S. Q. (1991). Changes of zero-stress state of rat pulmonary arteries in hypoxic hypertension. *J. Appl.Phys.* 70, 2455–2470.

Gaillard, V., Casellas, D., Seguin-Devaux, C., Schohn, H., Dauça, M., Atkinson, J., et al. (2005). Pioglitazone improves aortic wall elasticity in a rat model of elastocalcinotic arteriosclerosis. *Hypertension* 46, 372–379. doi: 10.1161/01.HYP.0000171472.24422.33

Gamble, G., Zorn, J., Sanders, G., MacMahon, S., and Sharpe, N. (1994). Estimation of arterial stiffness, compliance, and distensibility from M-mode ultrasound measurements of the common carotid artery. *Stroke* 25, 11–16. doi: 10.1161/01.STR.25.1.11

García, A., Martínez, M. A., and Peña, E. (2012). Viscoelastic properties of the passive mechanical behavior of the porcine carotid artery: influence of proximal and distal positions. *Biorheology* 49, 271–288. doi: 10.3233/BIR-2012-0606

Giannattasio, C., Mangoni, A. A., Failla, M., Carugo, S., Stella, M. L., Stefanoni, P., et al. (1996). Impaired radial artery compliance in normotensive subjects with familial hypercholesterolemia. *Atherosclerosis* 124, 249–260. doi: 10.1016/0021-9150(96)05834-0

Glagov, S., Zarins, C., Giddens, D. P., and Ku, D. N. (1988). Hemodynamics and atherosclerosis. Insights and perspectives gained from studies of human arteries. *Arch. Pathol. Lab. Med.* 112, 1018–1031.

Godia, E. C., Madhok, R., Pittman, J., Trocio, S., Ramas, R., Cabral, D., et al. (2007). Carotid artery distensibility: a reliability study. *J. Ultrasound Med.* 26, 1157–1165.

Grant, C. A., and Twigg, P. C. (2013). Pseudostatic and dynamic nanomechanics of the tunica adventitia in elastic arteries using atomic force microscopy. *ACS Nano* 7, 456–464. doi: 10.1021/nn304508x

Greenberg, S. R. (1986). The association of medial collagenous tissue with atheroma formation in the aging human aorta as revealed by a special technique. *Histol. Histopathol.* 1, 323–326.

Greenwald, S. E. (2007). Ageing of the conduit arteries. *J. Pathol.* 211, 157–172. doi: 10.1002/path.2101

Greenwald, S. E., Moore, J. E., Rachev, A., Kane, T. P., and Meister, J. J. (1997). Experimental investigation of the distribution of residual strains in the artery wall. *J. Biomech. Eng.* 119, 438–444. doi: 10.1115/1.2798291

Hallock, P. (1934). Arterial elasticity in man in relation to age as evaluated by the pulse wave velocity method. *Arch. Intern. Med. (Chic.)* 54, 770–798. doi: 10.1001/archinte.1934.00160170113010

Han, H. C., Marita, S., and Ku, D. N. (2006). Changes of opening angle in hypertensive and hypotensive arteries in 3-day organ culture. *J. Biomech.* 39, 2410–2418. doi: 10.1016/j.jbiomech.2005.08.003

Hanna, M. A., Taylor, C. R., Chen, B., La, H. S., Maraj, J. J., Kilar, C. R., et al. (2013). Structural remodeling of coronary resistance arteries: effects of age and exercise training. *J. Appl. Phys.* 117, 616–623. doi: 10.1152/japplphysiol.01296.2013

Haskett, D., Johnson, G., Zhou, A., Utzinger, U., and Vande Geest, J. (2010). Microstructural and biomechanical alterations of the human aorta as a function of age and location. *Biomech. Model. Mechan.* 9, 725–736. doi: 10.1007/s10237-010-0209-7

Hayenga, H. N., and Aranda-Espinoza, H. (2013). Stiffness increases mononuclear cell transendothelial migration. *Cell. Mol. Bioeng.* 6, 253–265. doi: 10.1007/s12195-013-0284-9

Hayenga, H. N., Trache, A., Trzeciakowski, J., and Humphrey, J. D. (2011). Regional atherosclerotic plaque properties in ApoE−/− mice quantified by atomic force, immunofluorescence, and light microscopy. *Res. Paper J. Vasc. Res.* 48, 495–504. doi: 10.1159/000329586

Hemmasizadeh, A., Autieri, M., and Darvish, K. (2012). Multilayer material properties of aorta determined from nanoindentation tests. *J. Mech. Behav. Biomed. Mater.* 15, 199–207. doi: 10.1016/j.jmbbm.2012.06.008

Hertz, H. (1881). Tiber die beriihrung fester elastischer korper (On the contact of elastic solids). *J. Reine Angew Mathematik* 95, 156–171.

Hochmuth, R. M. (2000). Micropipette aspiration of living cells. *J. Biomech.* 33, 15–22. doi: 10.1016/S0021-9290(99)00175-X

Holzapfel, G. A., Gasser, T. C., and Ogden, R. W. (2000). A new constitutive framework for arterial wall mechanics and a comparative study of material models. *J. Elast.* 61, 1–48. doi: 10.1023/A:1010835316564

Hurley, D. C. (2009). "Contact resonance force microscopy techniques for nanomechanical measurements: introduction," in *Applied Scanning Probe Methods XI: Scanning Probe Microscopy Techniques*, eds P. Avouris, B. Bhushan, D. Bimberg, K. von Klitzing, H. Sakaki, and R. Wiesendanger (Berlin: Springer-Verlag), 99–100.

Huynh, J., Bordeleau, F., Kraning-Rush, C. M., and Reinhart-King, C. A. (2013). Substrate stiffness regulates PDGF-induced circular dorsal ruffle formation through MLCK. *Cell Mol. Bioeng.* 6, 138–147. doi: 10.1007/s12195-013-0278-7

Huynh, J., Nishimura, N., Rana, K., Peloquin, J. M., Califano, J. P., Montague, C. R., et al. (2011). Age-related intimal stiffening enhances endothelial permeability and leukocyte transmigration. *Sci. Transl. Med.* 3, 112–122. doi: 10.1126/scitranslmed.3002761

Ikeda, K., Higashi, T., Sano, H., Jinnouchi, Y., Yoshida, M., Araki, T., et al. (1996). N (epsilon)-(carboxymethyl)lysine protein adduct is a major immunological epitope in proteins modified with advanced glycation end products of the Maillard reaction. *Biochemistry* 35, 8075–8083. doi: 10.1021/bi9530550

Ingber, D. E., Prusty, D., Frangioni, J. V., Cragoe, E. J., Lechene, C., and Schwartz, M. A. (1990). Control of intracellular pH and growth by fibronectin in capillary endothelial cells. *J. Cell Biol.* 110, 1803–1811. doi: 10.1083/jcb.110.5.1803

Isenberg, B. C., DiMilla, P. A., Walker, M., Kim, S., and Wong, J. Y. (2009). Vascular smooth muscle cell durotaxis depends on substrate stiffness gradient strength. *Biophys. J.* 97, 1313–1322. doi: 10.1016/j.bpj.2009.06.021

John, R., and Thomas, J. (1972). Chemical compositions of elastins isolated from aortas and pulmonary tissues of humans of different ages. *Biochem. J.* 127, 261–269.

Johnson, K. L. (1982). One hundred years of Hertz contact. *P. I. Mech. Eng.* 196, 363–378. doi: 10.1243/PIME-PROC-1982-196-039-02

Johnson, K. L., Kendall, K., and Roberts, A. D. (1971). Surface energy and contact of elastic solids. *Proc. R. Soc. A Math. Phy.* 324, 301–313. doi: 10.1098/rspa.1971.0141

Kaess, B. M., Rong, J., Larson, M. G., Hamburg, N. M., Vita, J. A., Levy, D., et al. (2012). Aortic stiffness, blood pressure progression, and incident hypertension. JAMA-J. *Am. Med. Assoc.* 308, 875–881. doi: 10.1001/2012.jama.10503

Kanabrocki, E. L., Fels, I. G., and Kaplan, E. (1960). Calcium, cholesterol and collagen levels in human aortas. *J. Gerontol.* 15, 383–387. doi: 10.1093/geronj/15.4.383

Karimi, A., Navidbakhsh, M., Shojaei, A., and Faghihi, S. (2013). Measurement of the uniaxial mechanical properties of healthy and atherosclerotic human coronary arteries. *Mater. Sci. Eng. C Mater. Biol. Appl.* 33, 2550–2554. doi: 10.1016/j.msec.2013.02.016

Kasas, S., and Dietler, G. (2008). Probing nanomechanical properties from biomolecules to living cells. *Pflugers. Arch.* 456, 13–27. doi: 10.1007/s00424-008-0448-y

Kelly, R., Hayward, C., Avolio, A., and O'Rourke, M. (1989). Non-invasive determination of age-related changes in the human arterial pulse. *Circulation* 80, 1652–1659. doi: 10.1161/01.CIR.80.6.1652

Khanafer, K., Duprey, A., Zainal, M., Schlict, M., Williams, D., and Berguer, R. (2011). Determination of the elastic modulus of ascending thoracic aortic aneurysm at different ranges of pressure using uniaxial tensile testing. *J. Thorac. Cardiovasc. Surg.* 142, 682–686. doi: 10.1016/j.jtcvs.2010.09.068

Khanafer, K., Schlicht, M. S., and Berguer, R. (2013). How should we measure and report elasticity in aortic tissue? *Eur. J. Vasc. Endovasc. Surg.* 45, 332–339. doi: 10.1016/j.ejvs.2012.12.015

Kim, T., and Spiegel, D. A. (2013). The unique reactivity of N-phenacyl-derived thiazolium salts toward α-dicarbonyl compounds. *Rejuvenation Res.* 16, 43–50. doi: 10.1089/rej.2012.1370

Kohn, J. C., and Ebenstein, D. M. (2013). Eliminating adhesion errors in nanoindentation of compliant polymers and hydrogels. *J. Mech. Behav. Biomed. Mater.* 20, 316–326. doi: 10.1016/j.jmbbm.2013.02.002

Kohn, J. C., Zhou, D. W., Bordeleau, F., Zhou, A. L., Mason, B. N., Mitchell, M. J., et al. (2015). Effects of matrix stiffness on endothelial cell response to fluid shear stress. *Biophys. J.* 108, 471–478. doi: 10.1016/j.bpj.201412.023

Kothapalli, D., Liu, S. L., Bae, Y. H., Monslow, J., Xu, T., Hawthorne, E. A., et al. (2012). Cardiovascular protection by ApoE and ApoE-HDL Linked to suppression of ECM gene expression and arterial stiffening. *Cell Rep.* 2, 1259–1271. doi: 10.1016/j.celrep.2012.09.018

Kramer, R. H., Bensch, K. G., Davison, P. M., and Karasek, M. A. (1984). Basal lamina formation by cultured microvascular endothelial cells. *J. Cell Biol.* 99, 692–698. doi: 10.1083/jcb.99.2.692

Krishnan, R., Klumpers, D. D., Park, C. Y., Rajendran, K., Trepat, X., van Bezu, J., et al. (2011). Substrate stiffening promotes endothelial monolayer disruption through enhanced physical forces. *Am. J. Physiol. Cell Physiol.* 300, C146–C154. doi: 10.1152/ajpcell.00195.2010

Kusche-Vihrog, K., Urbanova, K., Blanqué, A., Wilhelmi, M., Schillers, H., Kliche, K., et al. (2011). C-Reactive protein makes human endothelium stiff and tight. *Hypertension* 57, 231–237. doi: 10.1161/HYPERTENSIONAHA.110.163444

Kwon, G. P., Schroeder, J. L., Amar, M. J., Remaley, A. T., and Balaban, R. S. (2008). Contribution of macromolecular structure to the retention of low-density lipoprotein at arterial branch points. *Circulation* 117, 2919–2927. doi: 10.1161/CIRCULATIONAHA.107.754614

Langberg, H., Skovgaard, D., Petersen, L. J., Bulow, J., and Kjaer, M. (1999). Type I collagen synthesis and degradation in peritendinous tissue after exercise determined by microdialysis in humans. *J. Physiol.* 521(Pt 1), 299–306. doi: 10.1111/j.1469-7793.1999.00299.x

Lasheras, J. C. (2007). The biomechanics of arterial aneurysms. *Annu. Rev. Fluid Mech.* 39, 293–319. doi: 10.1146/annurev.fluid.39.050905.110128

Laurent, S., Boutouyrie, P., Asmar, R., Gautier, I., Laloux, B., Guize, L., et al. (2001). Aortic stiffness is an independent predictor of all-cause and cardiovascular mortality in hypertensive patients. *Hypertension* 37, 1236–1241. doi: 10.1161/01.HYP.37.5.1236

Laurent, S., Caviezel, B., Beck, L., Girerd, X., Billaud, E., Boutouyrie, P., et al. (1994). Carotid artery distensibility and distending pressure in hypertensive humans. *Hypertension* 23, 878–883. doi: 10.1161/01.HYP.23.6.878

Laurent, S., Cockcroft, J., Van Bortel, L., Boutouyrie, P., Giannattasio, C., Hayoz, D., et al. (2006). Expert consensus document on arterial stiffness: methodological issues and clinical applications. *Eur. Heart J.* 27, 2588–2605. doi: 10.1093/eurheartj/ehl254

Leckband, D. E. (2011). Novel functions and binding mechanisms of carbohydrate-binding proteins determined by force measurements. *Curr. Protein Pept. Sci.* 12, 743–759. doi: 10.2174/138920311798841735

Lee, S. Y., Zaske, A. M., Novellino, T., Danila, D., Ferrari, M., Conyers, J., et al. (2011). Probing the mechanical properties of TNF-α stimulated endothelial cell with atomic force microscopy. *Int. J. Nanomedicine* 6, 179–195. doi: 10.2147/IJN.S12760

Levesque, M. J., and Nerem, R. M. (1985). The elongation and orientation of cultured endothelial cells in response to shear stress. *J. Biomech. Eng.* 107, 341–347. doi: 10.1115/1.3138567

Libby, P. (2002). Inflammation in atherosclerosis. *Nature* 420, 868–874. doi: 10.1038/nature01323

Little, W. C., Zile, M. R., Kitzman, D. W., Hundley, W. G., O'Brien, T. X., and Degroof, R. C. (2005). The effect of alagebrium chloride (ALT-711), a novel glucose cross-link breaker, in the treatment of elderly patients with diastolic heart failure. *J. Card. Fail* 11, 191–195. doi: 10.1016/j.cardfail.2004.09.010

Lu, L., Oswald, S. J., Ngu, H., and Yin, F. C. (2008). Mechanical properties of actin stress fibers in living cells. *Biophys. J.* 95, 6060–6071. doi: 10.1529/biophysj.108.133462

Lu, X., Pandit, A., and Kassab, G. S. (2004). Biaxial incremental homeostatic elastic moduli of coronary artery: two-layer model. *Am. J. Physiol. Heart Circ. Physiol.* 287, H1663–H1669. doi: 10.1152/ajpheart.00226.2004

Lu, X., Yang, J., Zhao, J. B., Gregersen, H., and Kassab, G. S. (2003). Shear modulus of porcine coronary artery: contributions of media and adventitia. *Am. J. Physiol. Heart Circ. Physiol.* 285, H1966–H1975. doi: 10.1152/ajpheart.00357.2003

Lundkvist, A., Lilleodden, E., Siekhaus, W., Kinney, J., Pruitt, L., and Balooch, M. (1996). Viscoelastic properties of healthy human artery measured in saline solution by AFM-based indentation technique. *MRS Proc.* 436. doi: 10.1557/PROC-436-353

Maarek, B., Simon, A. C., Levenson, J., Pithois-Merli, I., and Bouthier, J. (1987). Heterogeneity of the atherosclerotic process in systemic hypertension poorly controlled by drug treatment. *Am. J. Cardiol.* 59, 414–417. doi: 10.1016/0002-9149(87)90947-7

MacSweeney, S. T., Young, G., Greenhalgh, R. M., and Powell, J. T. (1992). Mechanical properties of the aneurismal aorta. *Br. J. Surg.* 79, 1281–1284. doi: 10.1002/bjs.1800791211

Maeda, S., Iemitsu, M., Miyauchi, T., Kuno, S., Matsuda, M., and Tanaka, H. (2005). Aortic stiffness and aerobic exercise: mechanistic insight from microarray analyses. *Med. Sci. Sports Exerc.* 37, 1710–1716. doi: 10.1249/01.mss.0000175052.37087.f8

Mahaffy, R. E., Shih, C. K., MacKintosh, F. C., and Käs, J. (2000). Scanning probe-based frequency-dependent microrheology of polymer gels and biological cells. *Phys. Rev. Lett.* 85, 880–883. doi: 10.1103/PhysRevLett.85.880

Mann, A. B. (2007). "Nanomechanical properties of solid surfaces and thin films," in *Springer Handbook of Nanotechnology*, ed. B. Bhushan (New York, NY: Springer Science+Business Media, Inc.), 1137–1166.

Marzec, K. M., Wrobel, T. P., Rygula, A., Maslak, E., Jasztal, A., Fedorowicz, A., et al. (2014). Visualization of the biochemical markers of atherosclerotic plaque with the use of Raman, IR and AFM. *J. Biophotonics* 7, 744–756. doi: 10.1002/jbio.201400014

Matsumoto, T., Abe, H., Ohashi, T., Kato, Y., and Sato, M. (2002). Local elastic modulus of atherosclerotic lesions of rabbit thoracic aortas measured by pipette aspiration method. *Physiol. Meas.* 23, 635–648. doi: 10.1088/0967-3334/23/4/304

Matsumoto, T., and Hayashi, K. (1996). Stress and strain distribution in hypertensive and normotensive rat aorta considering residual strain. *J. Biomech. Eng.* 118, 62–73. doi: 10.1115/1.2795947

McDaniel, D. P., Shaw, G. A., Elliott, J. T., Bhadriraju, K., Meuse, C., Chung, K.-H., et al. (2007). The stiffness of collagen fibrils influences vascular smooth muscle cell phenotype. *Biophys. J.* 92, 1759–1769. doi: 10.1529/biophysj.106.089003

Mullarkey, C. J., Edelstein, D., and Brownlee, M. (1990). Free radical generation by early glycation products: a mechanism for accelerated atherogenesis in diabetes. *Biochem. Biophys. Res. Commun.* 173, 932–939. doi: 10.1016/S0006-291X(05)80875-7

Myers, V. C., and Lang, W. W. (1946). Some Chemical changes in the human thoracic aorta accompanying the aging process. *J. Gerontol.* 1(Pt 14), 441–444. doi: 10.1093/geronj/1.4_Part_1.441

Newman, D. L., and Greenwald, S. E. (1978). "Validity of the Moens–Korteweg equation," in *Arterial System*, eds R. D. Bauer and R. Busse (New York: Springer), 109–115.

Nosaka, T., Tanaka, H., Watanabe, I., Sato, M., and Matsuda, M. (2003). Influence of regular exercise on age-related changes in arterial elasticity: mechanistic insights from wall compositions in rat aorta. *Can. J. Appl. Physiol.* 28, 204–212. doi: 10.1139/h03-016

O'Connell, M. K., Murthy, S., Phan, S., Xu, C., Buchanan, J., Spilker, R., et al. (2008). The three-dimensional micro- and nanostructure of the aortic medial lamellar unit measured using 3D confocal and electron microscopy imaging. *Mat. Biol.* 27, 171–181. doi: 10.1016/j.matbio.2007.10.008

O'Rourke, M. F. (1976). Pulsatile arterial haemodynamics in hypertension. *Aust. N. Z. J. Med.* 6, 40–48. doi: 10.1111/j.1445-5994.1976.tb03322.x

O'Rourke, M. F., Staessen, J. A., Vlachopoulos, C., Duprez, D., and Plante, G. E. (2002). Clinical applications of arterial stiffness; definitions and reference values. *Am. J. Hypertens.* 15, 426–444. doi: 10.1016/S0895-7061(01)02319-6

Ohashi, T., Abe, H., Matsumoto, T., and Sato, M. (2005). Pipette aspiration technique for the measurement of nonlinear and anisotropic mechanical properties of blood vessel walls under biaxial stretch. *J. Biomech.* 38, 2248–2256. doi: 10.1016/j.jbiomech.2004.09.019

Oliver, W. C., and Pharr, G. M. (1992). An improved technique for determining hardness and elastic modulus using load and displacement sensing indentation experiments. *J. Mat. Res.* 7, 1564–1583. doi: 10.1557/JMR.1992.1564

Oliver, W. C., and Pharr, G. M. (2004). Measurement of hardness and elastic modulus by instrumented indentation: advances in understanding and refinements to methodology. *J. Mater. Res.* 19, 3–20. doi: 10.1557/jmr.2004.19.1.3

Otto, C. M., Lind, B. K., Kitzman, D. W., Gersh, B. J., and Siscovick, D. S. (1999). Association of aortic-valve sclerosis with cardiovascular mortality and morbidity in the elderly. *N. Engl. J. Med.* 341, 142–147. doi: 10.1097/00132586-200002000-00022

Oudegeest-Sander, M. H., Rikkert, M. G., Smits, P., Thijssen, D. H. J., van Dijk, A. P. J., Levine, B. D., et al. (2013). The effect of an advanced glycation end-product crosslink breaker and exercise training on vascular function in older individuals: a randomized factorial design trial. *Exp. Gerontol.* 48, 1509–1517. doi: 10.1016/j.exger.2013.10.009

Palotie, A., Tryggvason, K., Peltonen, L., and Seppä, H. (1983). Components of subendothelial aorta basement membrane. Immunohistochemical localization and role in cell attachment. *Lab. Invest.* 49, 362–370.

Pandit, A., Lu, X., Wang, C., and Kassab, G. S. (2005). Biaxial elastic material properties of porcine coronary media and adventitia. *Am. J. Physiol. Heart Circ. Physiol.* 288, H2581–H2587. doi: 10.1152/ajpheart.00648.2004

Park, L., Raman, K. G., Lee, K. J., Lu, Y., Ferran, L. J., Chow, W. S., et al. (1998). Suppression of accelerated diabetic atherosclerosis by the soluble receptor for advanced glycation endproducts. *Nat. Med.* 4, 1025–1031. doi: 10.1038/2012

Paszek, M. J., and Weaver, V. M. (2004). The tension mounts: mechanics meets morphogenesis and malignancy. *J. Mammary Gland Biol. Neoplasia* 9, 325–342. doi: 10.1007/s10911-004-1404-x

Paszek, M. J., Zahir, N., Johnson, K. R., Lakins, J. N., Rozenberg, G. I., Gefen, A., et al. (2005). Tensional homeostasis and the malignant phenotype. *Cancer Cell* 8, 241–254. doi: 10.1016/j.ccr.2005.08.010

Patel, P. N., Smith, C. K., and Patrick, C. W. (2005). Rheological and recovery properties of poly(ethylene glycol) diacrylate hydrogels and human adipose tissue. *J. Biomed. Mater. Res. A* 73, 313–319. doi: 10.1002/jbm.a.30291

Peloquin, J., Huynh, J., Williams, R. M., and Reinhart-King, C. A. (2011). Indentation measurements of the subendothelial matrix in bovine carotid arteries. *J. Biomech.* 44, 815–821. doi: 10.1016/j.jbiomech.2010.12.018

Peyton, S. R., and Putnam, A. J. (2005). Extracellular matrix rigidity governs smooth muscle cell motility in a biphasic fashion. *J. Cell. Physiol.* 204, 198–209. doi: 10.1002/jcp.20274

Powell, J. T., Vine, N., and Crossman, M. (1992). On the accumulation of D-aspartate in elastin and other proteins of the ageing aorta. *Atherosclerosis* 97, 201–208. doi: 10.1016/0021-9150(92)90132-Z

Preiner, J., Ebner, A., Chtcheglova, L., Zhu, R., and Hinterdorfer, P. (2009). Simultaneous topography and recognition imaging: physical aspects and optimal imaging conditions. *Nanotechnology* 20. doi: 10.1088/0957-4484/20/21/215103

Protogerou, A. D., Papaioannou, T. G., and Vlachopoulos, C. (2014). Arterial stiffness mapping : a better navigation to Ithaca? *J. Am. Coll. Cardiol.* 63, 1748–1750. doi: 10.1016/j.jacc.2014.01.044

Quevedo, K. K. (2012). Aortic stiffness, blood pressure progression, and incident hypertension. *Cardiol. Rev.* 28, 875–881. doi: 10.1001/2012.jama.10503

Rachev, A., and Greenwald, S. E. (2003). Residual strains in conduit arteries. *J. Biomech.* 36, 661–670. doi: 10.1016/S0021-9290(02)00444-X

Radauceanu, A., Moulin, F., Djaballah, W., Marie, P. Y., Alla, F., Dousset, B., et al. (2007). Residual stress ischaemia is associated with blood markers of myocardial structural remodelling. *Eur. J. Heart Fail.* 9, 370–376. doi: 10.1016/j.ejheart.2006.09.010

Radmacher, M. (1997). Measuring the elastic properties of biological samples with the AFM. IEEE *Eng. Med. Biol.* 16, 47–57. doi: 10.1109/51.582176

Ravi, S., and Chaikof, E. L. (2010). Biomaterials for vascular tissue engineering. *Regen. Med.* 5, 107–120. doi: 10.2217/rme.09.77

Rehal, D., Guo, X. M., Lu, X., and Kassab, G. S. (2006). Duration of no-load state affects opening angle of porcine coronary arteries. *Am. J. Physiol. Heart Circul. Physiol.* 290, H1871–H1878. doi: 10.1152/ajpheart.00910.2005

Reinhart-King, C. A., Dembo, M., and Hammer, D. A. (2005). The dynamics and mechanics of endothelial cell spreading. *Biophys. J.* 89, 676–689. doi: 10.1529/biophysj.104.054320

Reneman, R. S., van Merode, T., Hick, P., Muytjens, A. M., and Hoeks, A. P. (1986). Age-related changes in carotid artery wall properties in men. *Ultrasound Med. Biol.* 12, 465–471. doi: 10.1016/0301-5629(86)90218-8

Rezakhaniha, R., Agianniotis, A., Schrauwen, J. T. C., Griffa, A., Sage, D., Bouten, C. V. C., et al. (2012). Experimental investigation of collagen waviness and orientation in the arterial adventitia using confocal laser scanning microscopy. *Biomech. Model. Mechanobiol.* 11, 461–473. doi: 10.1007/s10237-011-0325-z

Rizzo, R. J., McCarthy, W. J., Dixit, S. N., Lilly, M. P., Shively, V. P., Flinn, W. R., et al. (1989). Collagen types and matrix protein content in human abdominal aortic aneurysms. *J. Vasc. Surg.* 10, 365–73. doi: 10.1016/0741-5214(89)90409-6

Roach, M. R., and Burton, A. C. (1957). The reason for the shape of the distensibility curves of arteries. *Can. J. Biochem. Physiol.* 35, 681–690. doi: 10.1139/o57-080

Ross, R., and Glomset, J. A. (1973). Atherosclerosis and the arterial smooth muscle cell: proliferation of smooth muscle is a key event in the genesis of the lesions of atherosclerosis. *Science* 180, 1332–1339. doi: 10.1126/science.180.4093.1332

Rudijanto, A. (2007). The role of vascular smooth muscle cells on the pathogenesis of atherosclerosis. *Acta Med. Indones.* 39, 86–93.

Sacks, M. S. (2000). Biaxial mechanical evaluation of planar biological materials. *J. Elasticity* 61, 199–246. doi: 10.1023/A:1010917028671

Safar, M. E., Levy, B. I., and Struijker-Boudier, H. (2003). Current perspectives on arterial stiffness and pulse pressure in hypertension and cardiovascular disease. *Circulation* 107, 2864–2869. doi: 10.1161/01.CIR.0000069826.36125.B4

Samila, Z. J., and Carter, S. A. (1981). The effect of age on the unfolding of elastin lamellae and collagen fibers with stretch in human carotid arteries. *Can. J. Physiol. Pharmacol.* 59, 1050–1057. doi: 10.1139/y81-160

Santos-Parker, J. R., LaRocca, T. J., and Seals, D. R. (2014). Aerobic exercise and other healthy lifestyle factors that influence vascular aging. *Adv. Physiol. Educ.* 38, 296–307.

Sarangapani, K. K., Marshall, B. T., McEver, R. P., and Zhu, C. (2011). Molecular stiffness of selectins. *J. Biol. Chem.* 286, 9567–9576. doi: 10.1074/jbc.M110.196485

Sato, M., Matsumoto, T., and Ohashi, T. (2000). An application of the pipette technique to the measurement of local mechanical properties of aortic walls. *Hum. Biomech.* 38, 147–154. doi: 10.1007/978-4-431-66967-8_19

Sazonova, O. V., Isenberg, B. C., Herrmann, J., Lee, K. L., Purwada, A., Valentine, A. D., et al. (2014). Extracellular matrix presentation modulates vascular smooth muscle cell mechanotransduction. *Matrix Biol.* 41, 36–43. doi: 10.1016/j.matbio.2014.11.001

Schlatmann, T. J. M., and Becker, A. E. (1977). Histologic changes in the normal aging aorta: Implications for dissecting aortic aneurysm. *Am. J. Cardiol.* 39, 13–20. doi: 10.1016/S0002-9149(77)80004-0

Schleicher, E. D., Wagner, E., and Nerlich, A. G. (1997). Increased accumulation of the glycoxidation product N(epsilon)- (carboxymethyl)lysine in human tissues in diabetes and aging. *J. Clin. Invest.* 99, 457–468. doi: 10.1172/JCI119180

Schriefl, A. J., Zeindlinger, G., Pierce, D. M., Regitnig, P., and Holzapfel, G. A. (2012). Determination of the layer-specific distributed collagen fibre orientations in human thoracic and abdominal aortas and common iliac arteries. *J. R. Soc. Interface* 9, 1275–1286. doi: 10.1098/rsif.2011.0727

Schwenke, D. C., and Carew, T. E. (1989). Initiation of atherosclerotic lesions in cholesterol-fed rabbits. II. Selective retention of LDL vs. selective increases in LDL permeability in susceptible sites of arteries. *Arterioscler. Thromb. Vasc. Biol.* 9, 908–918. doi: 10.1161/01.ATV.9.6.908

Seals, D. R., Desouza, C. A., Donato, A. J., and Tanaka, H. (2008). Habitual exercise and arterial aging. *J. Appl. Physiol.* 105, 1323–1332. doi: 10.1152/japplphysiol.90553.2008

Sell, D. R., and Monnier, V. M. (2012). Molecular basis of arterial stiffening: role of glycation-a mini-review. *Gerontology* 58, 227–237. doi: 10.1159/000334668

Selzer, R. H., Mack, W. J., Lee, P. L., Kwong-Fu, H., and Hodis, H. N. (2001). Improved common carotid elasticity and intima-media thickness measurements from computer analysis of sequential ultrasound frames. *Atherosclerosis* 154, 185–193. doi: 10.1016/S0021-9150(00)00461-5

Sen, S., Subramanian, S., and Discher, D. E. (2005). Indentation and adhesive probing of a cell membrane with AFM: theoretical model and experiments. *Biophys. J.* 89, 3203–3213. doi: 10.1529/biophysj.105.063826

Shafigh, M., Fatouraee, N., and Seddighi, A. S. (2013). Determining the biomechanical properties of human intracranial blood vessels through biaxial tensile test and fitting them to a hyperelastic model. *Eng. Solid Mech.* 1, 43–56. doi: 10.5267/j.esm.2013.08.003

Shao, J. Y., Ting-Beall, H. P., and Hochmuth, R. M. (1998). Static and dynamic lengths of neutrophil microvilli. *Proc. Natl. Acad. Sci. U.S.A.* 95, 6797–6802. doi: 10.1073/pnas.95.12.6797

Shekhonin, B. V., Domogatsky, S. P., Muzykantov, V. R., Idelson, G. L., and Rukosuev, V. S. (1985). Distribution of type I, III, IV and V collagen in normal and atherosclerotic human arterial wall: immunomorphological characteristics. *Coll. Relat. Res.* 5, 355–368. doi: 10.1016/S0174-173X(85)80024-8

Shim, C. Y., and Lindner, J. R. (2014). Cardiovascular molecular imaging with contrast ultrasound: principles and applications. *Korean Circ. J.* 44, 1–9. doi: 10.4070/kcj.2014.44.1.1

Sieminski, A. L., Hebbel, R. P., and Gooch, K. J. (2004). The relative magnitudes of endothelial force generation and matrix stiffness modulate capillary morphogenesis in vitro. *Exp. Cell Res.* 297, 574–584. doi: 10.1016/j.yexcr.2004.03.035

Sims, T. J., Rasmussen, L. M., Oxlund, H., and Bailey, A. J. (1996). The role of glycation cross-links in diabetic vascular stiffening. *Diabetologia* 39, 946–951. doi: 10.1007/s001250050536

Soulis, T., Sastra, S., Thallas, V., Mortensen, S. B., Wilken, M., Clausen, J. T., et al. (1999). A novel inhibitor of advanced glycation end-product formation inhibits mesenteric vascular hypertrophy in experimental diabetes. *Diabetologia* 42, 472–479. doi: 10.1007/s001250051181

Stary, H. C., Blankenhorn, D. H., Chandler, A. B., Glagov, S., Insull, J. Jr., Richardson, M., et al. (1992). A definition of the intima of human arteries and of its atherosclerosis-prone regions. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis. *Circulation* 85, 391–405. doi: 10.1161/01.CIR.85.1.391

Stroka, K. M., Hayenga, H. N., and Aranda-Espinoza, H. (2013). Human neutrophil cytoskeletal dynamics and contractility actively contribute to trans-endothelial migration. *PLoS ONE* 8:e61377. doi: 10.1371/journal.pone.0061377

Sugital, S., and Matsumoto, T. (2013). Novel biaxial tensile test for studying aortic failure phenomena at a microscopic level. *Biomed. Eng. Online* 12, 3. doi: 10.1186/1475-925X-12-3

Sutton-Tyrrell, K., Najjar, S. S., Boudreau, R. M., Venkitachalam, L., Kupelian, V., Simonsick, E. M., et al. (2005). Elevated aortic pulse wave velocity, a marker of arterial stiffness, predicts cardiovascular events in well-functioning older adults. *Circulation* 111, 3384–3390. doi: 10.1161/CIRCULATIONAHA.104.483628

Szulcek, R., Beckers, C. M. L., Hodzic, J., de Wit, J., Chen, Z., Grob, T., et al. (2013). Localized RhoA GTPase activity regulates dynamics of endothelial monolayer integrity. *Cardiovasc. Res.* 99, 471–482. doi: 10.1093/cvr/cvt075

Tada, S., and Tarbell, J. M. (2004). Internal elastic lamina affects the distribution of macromolecules in the arterial wall: a computational study. *Am. J. Physiol. Heart Circ. Physiol.* 287, H905–H913. doi: 10.1152/ajpheart.00647.2003

Tanaka, H., Dinenno, F. A., Monahan, K. D., Clevenger, C. M., DeSouza, C. A., and Seals, D. R. (2000). Aging, habitual exercise, and dynamic arterial compliance. *Circulation* 102, 1270–1275. doi: 10.1161/01.CIR.102.11.1270

Thompson, R. W., and Baxter, B. T. (1999). MMP inhibition in abdominal aortic aneurysms. Rationale for a prospective randomized clinical trial. *Ann. N. Y. Acad. Sci.* 878, 159–178. doi: 10.1111/j.1749-6632.1999.tb07682.x

Thornalley, P. J. (2003). Use of aminoguanidine (Pimagedine) to prevent the formation of advanced glycation endproducts. *Arch. Biochem. Biophys.* 419, 31–40. doi:10.1016/j.abb.2003.08.013

Tian, L., Lammers, S. R., Kao, P. H., Reusser, M., Stenmark, K. R., Hunter, K. S., et al. (2011). Linked opening angle and histological and mechanical aspects of the proximal pulmonary arteries of healthy and pulmonary hypertensive rats and calves. *Am. J. Physiol. Heart Circ. Physiol.* 301, H1810–1818. doi: 10.1152/ajpheart.00025.2011

Toda, T., Tsuda, N., Nishimori, I., Leszczynski, D. E., and Kummerow, F. A. (1980). Morphometrical analysis of the aging process in human arteries and aorta. *Acta Anat. (Basel).* 106, 35–44. doi: 10.1159/000145167

Tozzi, P., Corno, A., and Hayoz, D. (2000). Definition of arterial compliance. *Am. J. Physiol Heart C* 278, H1407.

Tremblay, D., Cartier, R., Mongrain, R., and Leask, R. L. (2010). Regional dependency of the vascular smooth muscle cell contribution to the mechanical properties of the pig ascending aortic tissue. *J. Biomech.* 43, 2228–2251. doi: 10.1016/j.jbiomech.2010.04.018

Tzima, E., Del Pozo, M. A., Kiosses, W. B., Mohamed, S. A., Li, S., Chien, S., et al. (2002). Activation of Rac1 by shear stress in endothelial cells mediates both cytoskeletal reorganization and effects on gene expression. *EMBO J.* 21, 6791–6800. doi: 10.1093/emboj/cdf688

Tzima, E., Del Pozo, M. A., Shattil, S. J., Chien, S., and Schwartz, M. A. (2001). Activation of integrins in endothelial cells by fluid shear stress mediates Rho-dependent cytoskeletal alignment. *EMBO J.* 20, 4639–4647. doi: 10.1093/emboj/20.17.4639

Tzima, E., Irani-Tehrani, M., Kiosses, W. B., Dejana, E., Schultz, D. A., Engelhardt, B., et al. (2005). A mechanosensory complex that mediates the endothelial cell response to fluid shear stress. *Nature* 437, 426–431. doi: 10.1038/nature03952

Urry, D. W. (1971). Neutral sites for calcium ion binding to elastin and collagen: a charge neutralization theory for calcification and its relationship to atherosclerosis. *Proc. Natl. Acad. Sci. U.S.A.* 68, 810–814. doi: 10.1073/pnas.68.4.810

Urry, D. W., and Ohnishi, T. (1974). Calcium ion binding to carbonyls of elastin hexapeptide. *Bioinorg. Chem.* 3, 305–313. doi: 10.1016/S0006-3061(00)82010-4

Van Bortel, L. M., Laurent, S., Boutouyrie, P., Chowienczyk, P., Cruickshank, J. K., De Backer, T., et al. (2012). Expert consensus document on the measurement of aortic stiffness in daily practice using carotid-femoral pulse wave velocity. *J. Hypertens.* 30, 445–448. doi: 10.1097/HJH.0b013e32834fa8b0

van der-Heijden-Spek, J. J., Staessen, J. A., Fagard, R. H., Hoeks, A. P., Struijker-Boudier, H. A., and van Bortel, L. M. (2000). The effect of age on brachial artery wall properties differs from the aorta and is gender dependent: a population study. *Hypertension* 35, 637–642. doi: 10.1161/01.HYP.35.2.637

van Popele, N. M., Grobbee, D. E., Bots, M. L., Asmar, R., Topouchian, J., Reneman, R. S., et al. (2001). Association between arterial stiffness and atherosclerosis: the rotterdam study. *Stroke* 32, 454–460. doi: 10.1161/01.STR.32.2.454

van Sloten, T. T., Schram, M. T., van den Hurk, K., Dekker, J. M., Nijpels, G., Henry, R. M., et al. (2014). Local stiffness of the carotid and femoral artery is associated with incident cardiovascular events and all-cause mortality: the Hoorn study. *J. Am. Coll. Cardiol.* 63, 1739–1747. doi: 10.1016/j.jacc.2013.12.041

Vande Geest, J. P., Sacks, M. S., and Vorp, D. A. (2005). Age dependency of the biaxial biomechanical behavior of human abdominal aorta. *J. Biomech. Eng.* 126, 815–822. doi: 10.1115/1.1824121

Vasan, S., Zhang, X., Kapurniotu, A., Bernhagen, J., Teichberg, S., Basgen, J., et al. (1996). An agent cleaving glucose-derived protein crosslinks in vitro and in vivo. *Nature* 382, 275–278. doi: 10.1038/382275a0

Vatankhah, E., Prabhakaran, M. P., Semnani, D., Razavi, S., Zamani, M., and Ramakrishna, S. (2014). Phenotypic modulation of smooth muscle cells by chemical and mechanical cues of electrospun tecophilic/gelatin nanofibers. *ACS Appl. Mater. Interfaces* 6, 4089–4101. doi: 10.1021/am405673h

Wada, R., Nishizawa, Y., Yagihashi, N., Takeuchi, M., Ishikawa, Y., Yasumura, K., et al. (2001). Effects of OPB-9195, anti-glycation agent, on experimental diabetic neuropathy. *Eur. J. Clin. Invest.* 31, 513–520. doi: 10.1046/j.1365-2362.2001.00826.x

Wagenseil, J. E., and Mecham, R. P. (2012). Elastin in large artery stiffness and hypertension. *J. Cardiovasc. Transl. Res.* 5, 264–273. doi: 10.1007/s12265-012-9349-8

Wagenseil, J. E., Nerurkar, N. L., Knutsen, R. H., Okamoto, R. J., Li, D. Y., and Mecham, R. P. (2005). Effects of elastin haploinsufficiency on the mechanical behavior of mouse arteries. *Am. J. Physiol. Heart Circ. Physiol.* 289, H1209–H1217. doi: 10.1152/ajpheart.00046.2005

Wallace, J. D., Cuneo, R. C., Lundberg, P. A., Rosén, T., Jørgensen, J. O. L., Longobardi, S., et al. (2000). Responses of markers of bone and collagen turnover to exercise, growth hormone (GH) administration, and GH withdrawal in trained adult males. *J. Clin. Endocrinol. Metab.* 85, 124–133. doi: 10.1210/jc.85.1.124

Walsh, M. T., Cunnane, E. M., Mulvihill, J. J., Akyildiz, A. C., Gijsen, F. J. H., and Holzapfel, G. A. (2014). Uniaxial tensile testing approaches for characterisation of atherosclerotic plaques. *J. Biomech.* 47, 793–804. doi: 10.1016/j.jbiomech.2014.01.017

Wan, W., and Gleason, R. L. Jr. (2013). Dysfunction in elastic fiber formation in fibulin-5 null mice abrogates the evolution in mechanical response of carotid arteries during maturation. *Am. J. Physiol. Heart Circ. Physiol.* 304, H674–H686. doi: 10.1152/ajpheart.00459.2012

Wang, M., Takagi, G., Asai, K., Resuello, R. G., Natividad, F. F., Vatner, D. E., et al. (2003). Aging increases aortic MMP-2 activity and angiotensin II in nonhuman primates. *Hypertension* 41, 1308–1316. doi: 10.1161/01.HYP.0000073843.56046.45

Watanabe, M., Sawai, T., Nagura, H., and Suyama, K. (1996). Age-related alteration of cross-linking amino acids of elastin in human aorta. *Tohoku J. Exp. Med.* 180, 115–130. doi: 10.1620/tjem.180.115

Wautier, J. L., Zoukourian, C., Chappey, O., Wautier, M. P., Guillausseau, P. J., Cao, R., et al. (1996). Receptor-mediated endothelial cell dysfunction in diabetic vasculopathy. Soluble receptor for advanced glycation end products blocks hyperpermeability in diabetic rats. *J. Clin. Invest.* 97, 238–243. doi: 10.1172/JCI118397

Wautier, M. P., Chappey, O., Corda, S., Stern, D. M., Schmidt, A. M., and Wautier, J. L. (2001). Activation of NADPH oxidase by AGE links oxidant stress to altered gene expression via RAGE. *Am. J. Physiol. Endocrinol. Metab.* 280, E685–E694.

Weisbrod, R. M., Shiang, T., Al Sayah, L., Fry, J. L., Bajpai, S., Reinhart-King, C. A., et al. (2013). Arterial stiffening precedes systolic hypertension in diet-induced obesity. *Hypertension* 62, 1105–1110. doi: 10.1161/HYPERTENSIONAHA.113.01744

Williams, C., Liao, J., Joyce, E. M., Wang, B., Leach, J. B., Sacks, M. S., et al. (2009). Altered structural and mechanical properties in decellularized rabbit carotid arteries. *Acta Biomaterialia* 5, 993–1005. doi: 10.1016/j.actbio.2008.11.028

Wolffenbuttel, B. H., Boulanger, C. M., Crijns, F. R., Huijberts, M. S., Poitevin, P., Swennen, G. N., et al. (1998). Breakers of advanced glycation end products restore large artery properties in experimental diabetes. *Proc. Natl. Acad. Sci. U.S.A.* 95, 4630–4634. doi: 10.1073/pnas.95.8.4630

Wolinsky, H., and Glagov, S. (1967). A lamellar unit of aortic medial structure and function in mammals. *Circ. Res.* 20, 99–111. doi: 10.1161/01.RES.20.1.99

Xu, B., Chibber, R., Ruggiero, D., Kohner, E., Ritter, J., and Ferro, A. (2003). Impairment of vascular endothelial nitric oxide synthase activity by advanced glycation end products. *FASEB J.* 17, 1289–1291. doi: 10.1096/fj.02-0490fje\r02-0490fje

Xu, B., Ji, Y., Yao, K., Cao, Y. X., and Ferro, A. (2005). Inhibition of human endothelial cell nitric oxide synthesis by advanced glycation end-products but not glucose: relevance to diabetes. *Clin. Sci. (Lond.)* 109, 439–446. doi: 10.1042/CS20050183

Yasmin, McEniery, C. M., Wallace, S., Dakham, Z., Pulsalkar, P., Maki-Petaja, K., et al. (2005). Matrix metalloproteinase-9 (MMP-9), MMP-2, and serum elastase activity are associated with systolic hypertension and arterial stiffness. *Arterioscler. Thromb. Vasc. Biol.* 25, 372–378. doi: 10.1161/01.ATV.0000151373.33830.41

Young, E. W. K., Wheeler, A. R., and Simmons, C. A. (2007). Matrix-dependent adhesion of vascular and valvular endothelial cells in microfluidic channels. *Lab. Chip.* 7, 1759–1766. doi: 10.1039/b712486d

Yu, Q., Zhou, J., and Fung, Y. C. (1993). Neutral axis location in bending and Young's modulus of different layers of arterial wall. *Am. J. Physiol.* 265, H52–H60.

Zarins, C. K., Giddens, D. P., Bharadvaj, B. K., Sottiurai, V. S., Mabon, R. F., and Glagov, S. (1983). Carotid bifurcation atherosclerosis. Quantitative correlation of plaque localization with flow velocity profiles and wall shear stress. *Circ. Res.* 53, 502–514. doi: 10.1161/01.RES.53.4.502

Zemánek, M., Burša, J., and Děták, M. (2009). Biaxial tension tests with soft tissues of arterial wall. *Eng. Mech.* 16, 3–11.

Zhang, W., Guo, X., and Kassab, G. S. (2008). A generalized Maxwell model for creep behavior of artery opening angle. *J. Biomech. Eng.* 130. doi: 10.1115/1.2979853

Zhong, Q., Inniss, D., Kjoller, K., and Elings, V. B. (1993). Fractured polymer/silica fiber surface studied by tapping mode atomic force microscopy. *Surf. Sci. Lett.* 290, L688–L692. doi: 10.1016/0167-2584(93)90906-Y

Zieman, S. J., Melenovsky, V., and Kass, D. A. (2005). Mechanisms, pathophysiology, and therapy of arterial stiffness. *Arterioscler. Thromb. Vasc. Biol.* 25, 932–943. doi: 10.1161/01.ATV.0000160548.78317.29

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Defects in Vascular Mechanics Due to Aging in Rats: Studies on Arterial Wave Properties from a Single Aortic Pressure Pulse

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Changes in vascular mechanics due to aging include elevated vascular impedance, diminished aorta distensibility, and an accelerated return of pulse wave reflection, which may increase the systolic workload on the heart. Classically, the accurate measurement of vascular mechanics requires the simultaneous recording of aortic pressure and flow signals. In practice, it is feasible to estimate arterial wave properties in terms of wave transit time (τ_w) and wave reflection index (RI) by using aortic pressure signal alone. In this study, we determined the τ_w and magnitudes of the forward ($|P_f|$) and backward ($|P_b|$) pressure waves in Long–Evans male rats aged 4 ($n = 14$), 6 ($n = 17$), 12 ($n = 17$), and 18 ($n = 24$) months, based on the measured aortic pressure and an assumed triangular flow (Q^{tri}). The pulsatile pressure wave was the only signal recorded in the ascending aorta by using a high-fidelity pressure sensor. The base of the unknown Q^{tri} was constructed using a duration, which equals to the ejection time. The timing at the peak of the triangle was derived using the fourth-order derivative of the aortic pressure waveform. In the 18-month-old rats, the ratio of τ_w to left ventricular ejection time (LVET) decreased, indicating a decline in the distensibility of the aorta. The increased $|P_b|$ associated with unaltered $|P_f|$ enhanced the RI in the older rats. The augmentation index (AI) also increased significantly with age. A significant negative correlation between the AI and τ_w/LVET was observed: $AI = -0.7424 - 0.9026 \times (\tau_w/LVET)$ ($r = 0.4901$; $P < 0.0001$). By contrast, RI was positively linearly correlated with the AI as follows: $AI = -0.4844 + 2.3634 \times RI$ ($r = 0.8423$; $P < 0.0001$). Both the decreased τ_w/LVET and increased RI suggested that the aging process may increase the AI, thereby increasing the systolic hydraulic load on the heart. The novelty of the study is that Q^{tri} is constructed using the measured aortic pressure wave to approximate its corresponding flow signal, and that calibration of Q^{tri} is not essential in the analysis.

Keywords: aging, aortic input impedance, arterial wave property, vascular impulse response, wave reflection factor, wave transit time

INTRODUCTION

Aging is known to be associated with deterioration in many structural and functional properties of aortas and large arteries, including dilated vessel diameter, increased wall thickness, diminished wall elasticity, and endothelial dysfunction (Lakatta and Yin, 1982; O'Rourke and Nichols, 2005). As age advances, the following histological alterations in the vasculature are observed: an increased rate of endothelial cell apoptosis, the degeneration of smooth muscles in the media, fragmentation of and decrease in the content of elastic fibers, and an increase in the number of irregularly arranged collagen fibers in the stroma (Lakatta, 1979; Yin, 1980; Lakatta and Yin, 1982; Chang et al., 1998). Collagen crosslinking by non-enzymatic glycation is also enhanced within the arterial wall (Sims et al., 1996; Schleicher et al., 1997). All these factors contribute to the age-related changes in the mechanical properties of the vasculature, including elevated arterial impedance, diminished aorta distensibility, and an accelerated return of pulse wave reflection (O'Rourke and Nichols, 2005). These changes in vascular mechanics are accelerated in the incidence of hypertension (Najjar et al., 2005; Franklin, 2006), coronary heart diseases (Mattace-Raso et al., 2006), congestive heart failure (Sutton-Tyrrell et al., 2005), and stroke (O'Leary et al., 1999) with advancing age.

The physical properties of the arterial system are reflected in the aortic input impedance (Z_i), which is the aortic pressure-flow relation in the frequency domain (McDonald, 1974; O'Rourke, 1982; Milnor, 1989; Wang et al., 2014a). While the aortic characteristic impedance (Z_c) is known (Nichols and O'Rourke, 2011), the wave separation method can be derived in the time domain to resolve the measured aortic pressure wave into its forward (P_f) and backward (P_b) components (Westerhof et al., 1972). The arterial wave transit time (τ_w) can be computed using the impulse response function, which is the time-domain equivalent of its input impedance in the frequency domain (Laxminarayan et al., 1978; Sipkema et al., 1980). Thus, the accurate measurement of arterial wave properties, including arterial τ_w and wave reflection magnitude (RM) or wave reflection index (RI), requires the simultaneous recording of aortic pressure and flow signals.

In practice, it is feasible to estimate the τ_w and magnitudes of the forward and backward pressure waves by using aortic pressure signal alone. Westerhof et al. (2006) provided a novel method to calculate the pressure wave reflection by using only the measured aortic pressure. Replacing the unknown flow signal with a triangular wave shape (Q^{tri}), they successfully resolved the measured aortic pressure wave into its components, P_f and P_b, to calculate the RM or RI. Chang et al. (2017) elaborated this concept by determining the arterial τ_w through vascular impulse response analysis. They discovered that the aortic impulse response is an effective method for the estimation of arterial τ_w by using a single pressure pulse recording with an assumed Q^{tri}.

In this study, we determined the age-related changes in arterial wave properties on the basis of the aortic pressure alone in Long–Evans male rats. The pulsatile pressure wave was the only signal recorded in the ascending aorta by using a high-fidelity pressure sensor. The timing at the peak of the Q^{tri} was derived using the fourth-order derivative of the aortic pressure waveform (Westerhof et al., 2006; Chang et al., 2017). On the basis of the measured aortic pressure and an assumed Q^{tri}, we calculated the arterial τ_w, magnitudes of the P_f and P_b waves, and augmentation index (AI) to delineate the age-related changes in the pulsatile component of the left ventricular (LV) afterload. The novelty of the study is that Q^{tri} is constructed using the measured aortic pressure wave to approximate its corresponding flow signal, and that calibration of Q^{tri} is not essential in the analysis.

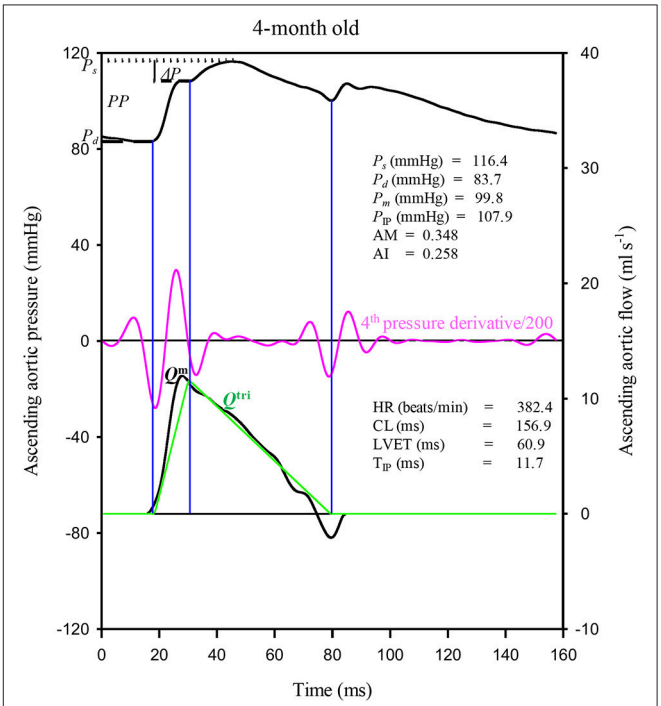

FIGURE 1 | Construction of an uncalibrated triangular flow (Q^{tri}) from the measured aortic pressure waveform in one rat aged 4 months as an example. The pressure and flow (Q^m) signals were simultaneously recorded in the ascending aorta under the anesthetized, open-chest condition. AM, augmentation magnitude, given by $\Delta P/(PP - \Delta P)$; AI, augmentation index, given by $\Delta P/PP$; CL, cardiac cycle length; HR, basal heart rate; LVET, left ventricular ejection time; P_S, systolic pressure; P_d, diastolic pressure; P_m, mean pressure; PP, pulse pressure; P_{IP}, pressure at inflection point; ΔP, given by $P_S - P_{IP}$; T_{IP}, time to inflection point.

Abbreviations: AI, Augmentation index; AM, augmentation magnitude; CL, cardiac cycle length (ms); HR, basal heart rate (beats min^{-1}); LVET, left ventricular ejection time (ms); Q^m, measured aortic flow wave (ml s^{-1}); Q^{tri}, uncalibrated triangular flow wave (ml s^{-1}); P_b, backward pressure wave (mmHg); P_d, diastolic aortic pressure (mmHg); P_f, forward pressure wave (mmHg); P_{IP}, pressure at inflection point (mmHg); P_m, mean aortic pressure (mmHg); P_{md}, mean diastolic pressure (mmHg); P_{ms}, mean systolic pressure (mmHg); PP, pulse pressure (mmHg); P_s, systolic aortic pressure (mmHg); ΔP, $P_s - P_{IP}$ (mmHg); RM, wave reflection magnitude; RI, wave reflection index; T_{IP}, time to inflection point (ms); Z_c, aortic characteristic impedance (mmHg s ml^{-1}); Z_i, aortic input impedance (mmHg s ml^{-1}); τ_w, wave transit time (ms).

METHODS

Animals and Catheterization

The effects of the aging process on the arterial mechanics were evaluated in specific pathogen-free Long–Evans male rats, aged 4 (n = 14), 6 (n = 17), 12 (n = 17), and 18 (n = 24) months. The rats were obtained from the colony maintained in the barrier facilities at the Laboratory Animal Center of the College of Medicine, National Taiwan University (Chang et al., 1998). All rats were allowed free access to Purina chow and water and were housed under 12 h light–dark cycles. The cages of the rats were examined periodically. Furthermore, the body weight (BW) of the rats was measured regularly to ensure the appropriate administration of the food. The experiment was conducted according to the *Guide for the Care and Use of Laboratory Animals*, and our study protocol was approved by the Animal Care and Use Committee of National Taiwan University (Chang et al., 1998).

The general surgical procedures and measurement of the cardiovascular variables in anesthetized rats were conducted as described previously (Chang et al., 1998). In brief, each rat was anesthetized with sodium pentobarbital (50 mg kg^{-1}, I.P.), placed on a heating pad, intubated, and ventilated using a rodent respirator (model 131; New England Medical Instruments, Medway, MA, USA) (Wu et al., 2011). A high-fidelity pressure catheter (model SPC 320, size 2 French; Millar Instruments, Houston, TX, USA) was used to measure the pulsatile ascending aortic pressure via the isolated carotid artery of the right side. The lead II ECG was recorded using a Gould ECG/Biotach amplifier (Cleveland, OH, USA). Selective aortic pressure signals from 5 to

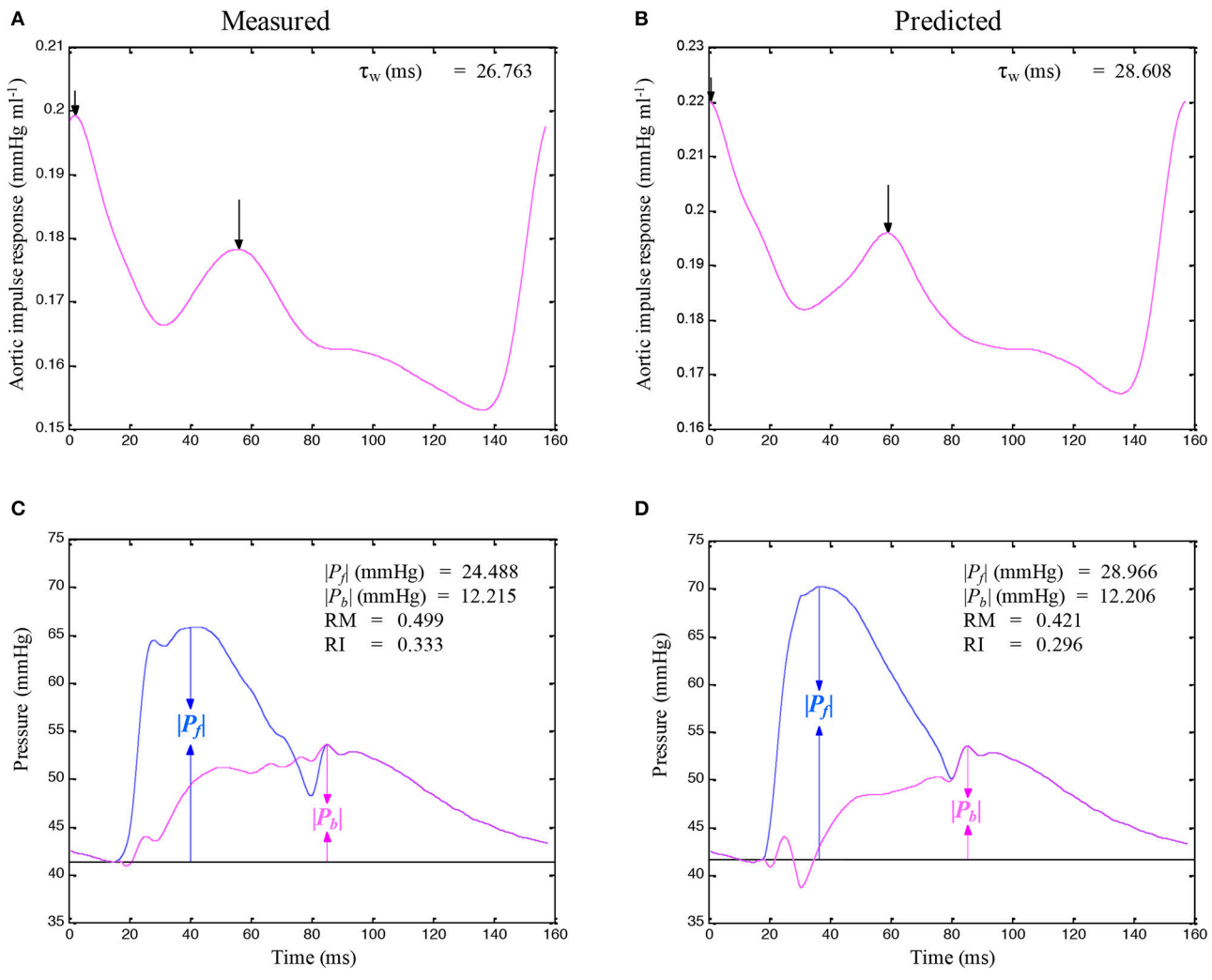

FIGURE 2 | Similarity between the measured aortic impulse response obtained from the measured pressure and Q^{m} **(A)** and the predicted aortic impulse response obtained from the measured pressure and Q^{tri} **(B)** in the same rat, which is shown in **Figure 1**. The measured P_f and P_b waves **(C)** and the predicted P_f and P_b waves **(D)** are also depicted. In (**C,D**), the amplitudes (peak - trough) of the P_f and P_b are represented by $|P_b|$ and $|P_f|$, respectively. P_f, forward pressure wave; P_b, backward pressure wave; RM, wave reflection magnitude, given by $|P_b|/|P_f|$; RI, wave reflection index, given by $|P_b|/(|P_f| + |P_b|)$; Q^{m}, measured aortic flow; Q^{tri}, uncalibrated triangular flow; τ_w, wave transit time.

TABLE 1 | Basic hemodynamic data measured in rats aged 4, 6, 12, and 18 months.

	BW	HR	CL	LVET	P_s	P_d	P_m	P_{ms}	P_{md}	P_{ms}/P_{md}
AGE (MONTHS)										
4 ($n = 14$)	374.1 ± 6.1	383.2 ± 6.1	157.1 ± 2.4	63.4 ± 0.7	139.7 ± 3.9	115.4 ± 2.7	128.8 ± 3.2	134.0 ± 3.5	125.2 ± 3.0	1.070 ± 0.005
6 ($n = 17$)	431.2 ± 8.9	372.8 ± 6.4	161.8 ± 2.9	66.7 ± 1.3	139.6 ± 3.5	116.9 ± 3.3	129.8 ± 3.3	134.7 ± 3.3	126.4 ± 3.3	1.066 ± 0.004
12 ($n = 17$)	482.1 ± 10.5	324.6 ± 9.2	187.5 ± 6.0	77.3 ± 1.8	139.1 ± 3.4	115.2 ± 2.6	128.2 ± 2.9	133.0 ± 3.1	124.8 ± 2.8	1.066 ± 0.003
18 ($n = 24$)	490.2 ± 9.3	307.9 ± 7.7	197.6 ± 4.8	82.4 ± 1.9	130.2 ± 3.1	100.9 ± 2.3	116.0 ± 2.6	122.2 ± 2.7	111.5 ± 2.4	1.096 ± 0.004
***P*-VALUE**										
4 vs. 6	<0.001	NS	NS	NS	NS	NS	NS	NS	NS	NS
4 vs. 12	<0.001	<0.001	<0.001	<0.001	NS	NS	NS	NS	NS	NS
4 vs. 18	<0.001	<0.001	<0.001	<0.001	NS	<0.005	<0.05	<0.05	<0.01	<0.001
6 vs. 12	<0.005	<0.001	<0.001	<0.001	NS	NS	NS	NS	NS	NS
6 vs. 18	<0.001	<0.001	<0.001	<0.001	NS	<0.001	<0.01	<0.05	<0.01	<0.001
12 vs. 18	*NS*	NS	NS	NS	NS	<0.005	<0.05	<0.05	<0.01	<0.001

All values are expressed as means ± standard error. BW, body weight (g); HR, heart rate (beats min^{-1}); CL, cardiac cycle length (ms); LVET, left ventricular ejection time (ms); P_s, systolic pressure (mmHg); P_d, diastolic pressure (mmHg); P_m, mean aortic pressure (mmHg); P_{ms}; mean systolic pressure (mmHg); P_{md}, mean diastolic pressure (mmHg); NS, not significant ($P > 0.05$).

TABLE 2 | Characteristics in relation to pulse wave reflection derived from aortic pressure waveform in rats aged 4, 6, 12, and 18 months.

	T_{IP}	P_{IP}	PP	ΔP	$\lvert P_f \rvert$	$\lvert P_b \rvert$	τ_w	τ_w/CL
AGE (MONTHS)								
4 ($n = 14$)	11.9 ± 0.4	129.2 ± 3.0	24.3 ± 1.5	10.4 ± 1.1	17.7 ± 1.0	10.9 ± 0.6	21.9 ± 1.3	0.140 ± 0.008
6 ($n = 17$)	11.5 ± 0.3	129.9 ± 3.1	22.8 ± 1.0	9.7 ± 0.8	16.4 ± 0.6	10.5 ± 0.4	21.7 ± 0.6	0.134 ± 0.003
12 ($n = 17$)	13.3 ± 0.5	127.5 ± 2.9	23.9 ± 1.1	11.6 ± 0.8	16.3 ± 0.8	10.9 ± 0.5	23.9 ± 0.6	0.129 ± 0.004
18 ($n = 24$)	14.6 ± 0.5	115.2 ± 2.6	29.4 ± 1.3	15.0 ± 0.9	18.2 ± 0.7	13.1 ± 0.6	22.5 ± 0.4	0.115 ± 0.003
***P*-VALUE**								
4 vs. 6	NS	NS	NS	NS	NS	NS	NS	NS
4 vs. 12	NS	NS	NS	NS	NS	NS	NS	NS
4 vs. 18	<0.001	<0.01	<0.05	<0.005	NS	<0.05	NS	<0.005
6 vs. 12	NS	NS	NS	NS	NS	NS	NS	NS
6 vs. 18	<0.001	<0.01	<0.005	<0.001	NS	<0.005	NS	<0.05
12 vs. 18	NS	<0.05	<0.01	<0.05	NS	<0.005	NS	NS

All values are expressed as means ± standard error. T_{IP}, Time to inflection point (ms); P_{IP}, pressure at the inflection point (mmHg); PP, pulse pressure (mmHg); ΔP, $P_s - P_{IP}$ (mmHg); $\lvert P_f \rvert$, magnitude of forward pressure wave (mmHg); $\lvert P_b \rvert$, magnitude of reflected pressure wave (mmHg); τ_w, arterial wave transit time (ms); NS, not significant ($P > 0.05$).

10 beats were averaged in the time domain, using the peak R wave of the ECG as a fiducial point.

To show the similarity between the measured and predicted arterial wave properties, the aortic pressure and flow (Q^m) signals were also simultaneously recorded in one rat aged 4 months, as an example, under the anesthetized, open-chest condition (**Figure 1**). The chest was opened through the second intercostal space on the right side. The pulsatile Q^m waveform was measured by using an electromagnetic flow probe (100 series, internal circumference 8 mm, Carolina Medical Electronics, King, NC, USA), which was positioned around the ascending aorta (Chang et al., 2017).

Construction of the Unknown Flow Wave by Using a Triangle

The unknown Q^{tri} was derived from the pressure waveform measured in the ascending aorta (Westerhof et al., 2006; Chang et al., 2017). The onset and termination of LV ejection were identified as the intersection of two tangential lines near the foot of the pressure wave (the first vertical blue lines in **Figures 1**, **3A,B**) and near the incisura (the third vertical blue lines in **Figures 1**, **3A,B**), respectively (Chang et al., 2015). The base of the Q^{tri} was constructed using a duration set equal to the ejection time, which is the time difference between the start and end points. The timing at the peak of the triangle was derived from the fourth-order derivative of the aortic pressure wave (the pink curves in **Figures 1**, **3A,B**; Westerhof et al., 2006; Chang et al., 2017). After ejection commenced, the first zero-crossing curve from above to below (the second vertical blue lines in **Figures 1**, **3A,B**) determined the peak of the triangle of blood flow, which was the inflection point of the pressure wave (Kelly et al., 1989; Westerhof et al., 2006; Chang et al., 2017). Thus, the uncalibrated Q^{tri} was approximated by a triangular shape (the green curves in **Figures 1**, **3A,B**) and

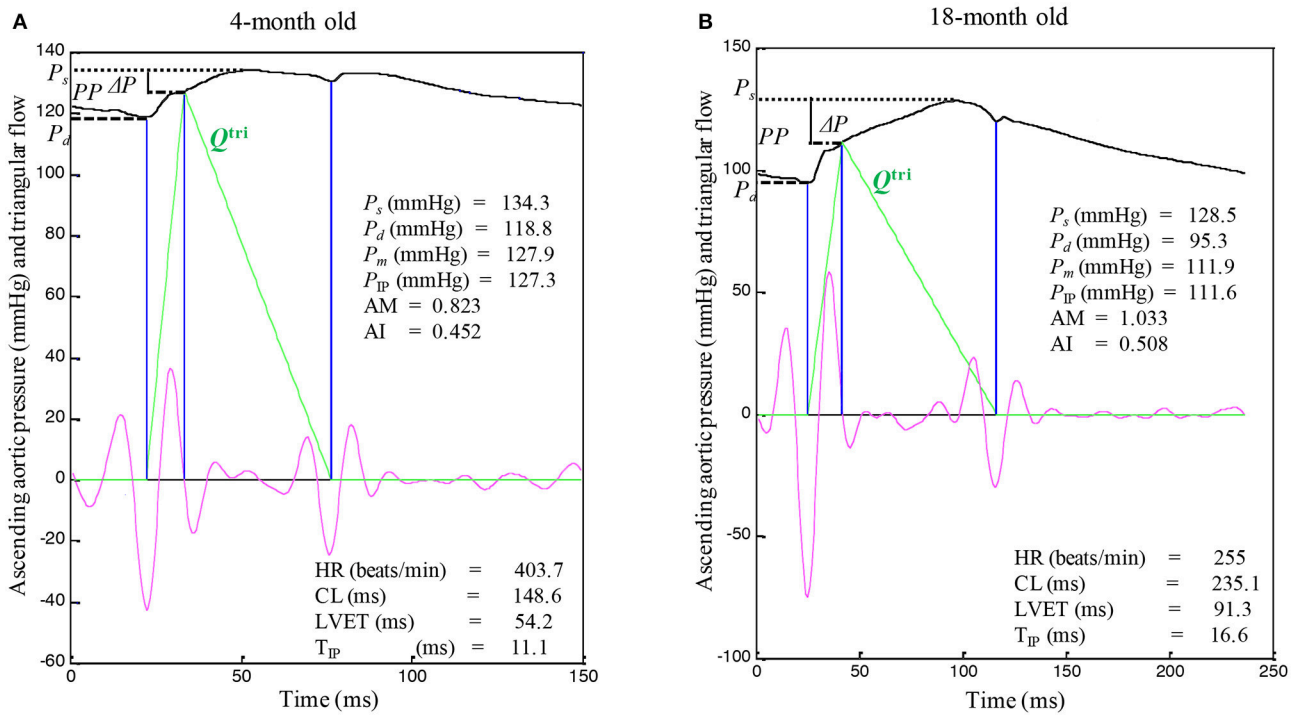

FIGURE 3 | An uncalibrated triangular flow (Q^{tri}) derived from the measured aortic pressure wave in rats aged 4 **(A)** and 18 months **(B)**. The 4-month old rat is the same one as shown in **Figure 1**. Only the pressure signal was recorded in the ascending aorta under the anesthetized, closed-chest condition. AM, augmentation magnitude, given by $\Delta P/(PP - \Delta P)$; AI, augmentation index, given by $\Delta P/PP$; CL, cardiac cycle length; HR, basal heart rate; LVET, left ventricular ejection time; P_S, systolic pressure; P_d, diastolic pressure; P_m, mean aortic pressure; PP, pulse pressure; P_{IP}, pressure at inflection point; ΔP, given by $P_S - P_{IP}$; T_{IP}, time to inflection point.

represented the corresponding flow wave of the aortic pressure signal.

After identifying the inflection point, the augmentation of pressure (ΔP) can be defined as the difference between the systolic pressure (P_s) and the pressure at the inflection point (P_{IP}): $\Delta P = P_s - P_{IP}$ (**Figures 1**, **3A,B**; Westerhof et al., 2006). The difference between P_s and the diastolic pressure (P_d) is the pulse pressure ($PP = P_s - P_d$). Thus, the augmentation magnitude (AM) is defined as the ratio of ΔP to the initial pressure rise ($PP - \Delta P$), given by AM = $\Delta P/(PP - \Delta P)$ (Westerhof et al., 2006). The AI is the pressure augmentation (ΔP) divided by the total pressure amplitude (PP), given by AI = $\Delta P/PP$.

Impulse Response Function Curve

A standard Fourier series expansion technique was performed to calculate the Z_i from the ratio of the ascending aortic pressure harmonics to the corresponding flow harmonics from either Q^m or Q^{tri} (McDonald, 1974; Milnor, 1989; Nichols and O'Rourke, 2011; Chang et al., 2015). The Z_c was calculated by averaging the high-frequency moduli of the Z_i data points from 4 to 10 harmonics (Wang et al., 2014a). The arterial τ_w was computed using the impulse response function curve (the pink lines in **Figures 2A,B**, **4A,B**; Sipkema et al., 1980; Latson et al., 1987), which was generated by using an inverse Fourier transformation of the Z_i after multiplying the first 12 harmonics by a Dolph–Chebychev weighting function with order 24 (Laxminarayan et al., 1978; Chang et al., 2015). One-half of the time difference between the appearance of the second reflected peak (long arrow) and the initial peak (short arrow) in the impulse response curve approximates the τ_w in the lower body circulation (Laxminarayan et al., 1978; Sipkema et al., 1980; Wu et al., 2012).

Arterial Wave Separation Analysis

The following equations were used to calculate the P_f and P_b from the measured aortic pressure wave (P_{ao}) in the time domain (Murgo et al., 1981; Chang et al., 2015):

$$P_f(t) = \frac{P_{ao}(t) + Z_c \times Q(t)}{2} \quad (1)$$

$$P_b(t) = \frac{P_{ao}(t) - Z_c \times Q(t)}{2} \quad (2)$$

The calculations of the P_f and P_b by using $Q(t)$ from either Q^m or Q^{tri} are depicted in **Figures 2C,D**, **4C,D**. The amplitudes (peak - trough) of the P_b and P_f are represented by $|P_b|$ and $|P_f|$, respectively. The aortic RM was then defined as the ratio of $|P_b|$ and $|P_f|$ (i.e., RM = $|P_b|/|P_f|$; Westerhof et al., 2006). The reflection index (RI) was calculated as RI = $|P_b|/(|P_f| + |P_b|)$.

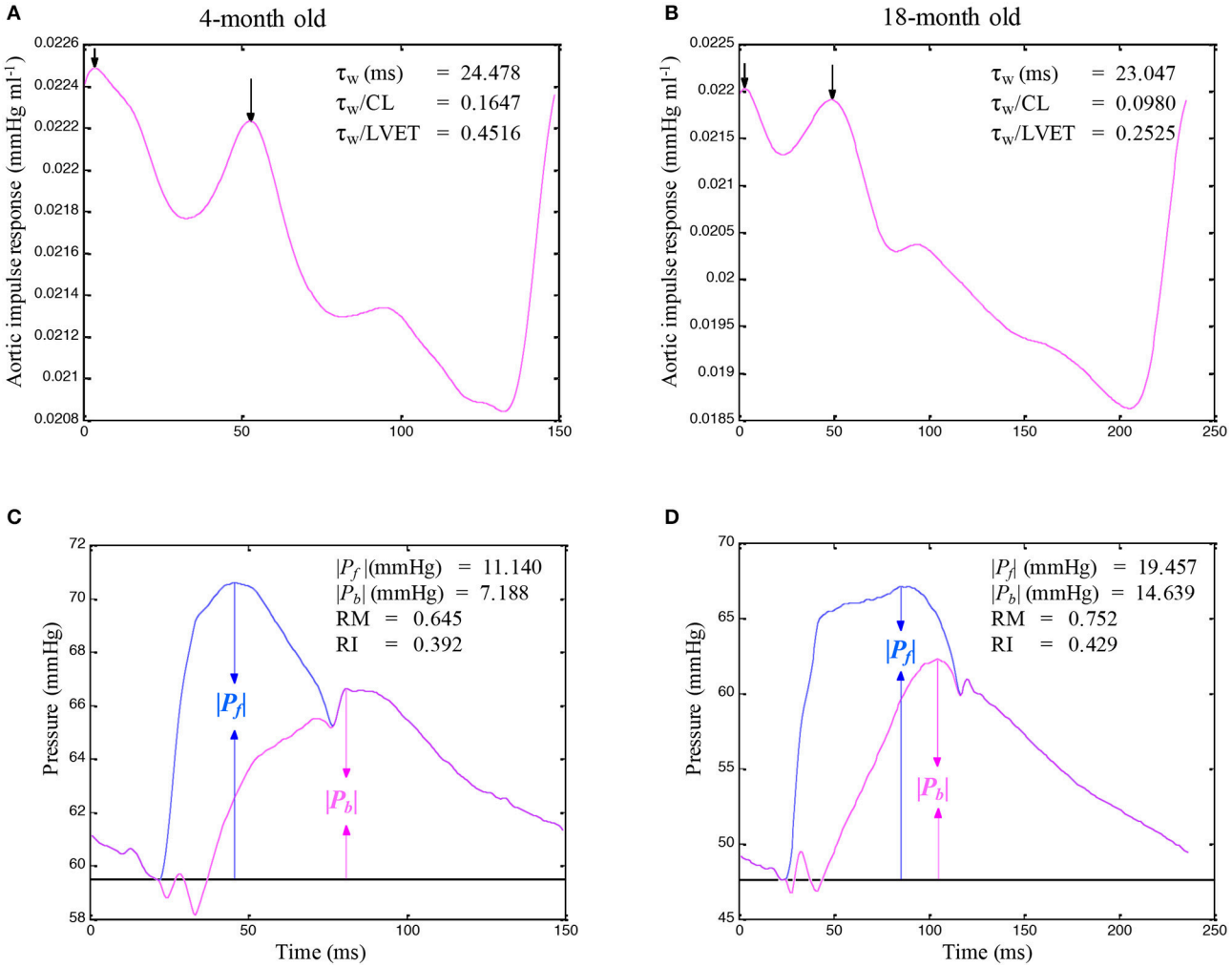

FIGURE 4 | Impulse response function curve obtained from the measured aortic pressure and an assumed triangular flow (Q^{tri}) in the same rats aged 4 **(A)** and 18 **(B)** months, which are shown in **Figure 3**. The P_f and P_b waves for the corresponding rats aged 4 **(C)** or 18 **(D)** months are also depicted. In **C** and **D**, the amplitudes (peak - trough) of the P_f and P_b are represented by $|P_b|$ and $|P_f|$, respectively. CL, cardiac cycle length; LVET, left ventricular ejection time; P_f, forward pressure wave; P_b, backward pressure wave; RM, wave reflection magnitude, given by $|P_b|/|P_f|$; RI, wave reflection index, given by $|P_b|/(|P_f| + |P_b|)$; τ_w, wave transit time.

Statistics

Results are expressed as means ± standard error. A one-way analysis of variance (ANOVA) was performed to determine the statistical significance of the results for multiple comparisons of the effect of the aging process on arterial wave properties (Wu et al., 2012). Statistical significance was assumed at the level of $P < 0.05$. In cases where the ANOVA results indicated that a hemodynamic variable differed significantly among age groups, Tukey's honest significant difference method was used to determine the groups of rats that exhibited divergent mean values for that variable (Wang et al., 2014b).

RESULTS

Table 1 presents the effect of age on BW, basal heart rate (HR), cardiac cycle length (CL), LV ejection time (LVET), and aortic pressure profile. In the rats, a significant increase in BW, decrease in HR, increase in CL, and prolongation in LVET were observed with an increase in age. However, the BW, HR, CL, and LVET did not significantly differ between the rats aged 12 and 18 months. The systolic blood pressure (P_s) did not change significantly as animals aged; however, diastolic (P_d), mean (P_m), mean systolic (P_{ms}), and mean diastolic (P_{md}) aortic pressures, and the P_{ms}/P_{md} ratio were significantly lower in 18-month-old rats than in the rats from other age groups.

Table 2 presents the pressure characteristics in relation to the pulse wave reflection derived from the aortic pressure signal in rats of different ages. Rats aged 18 months had markedly higher T_{IP} than did the rats aged 4 and 6 months. However, P_{IP} significantly decreased and ΔP increased in the 18-month-old rats compared with those in rats from other age groups. The PP values were markedly higher in the 18-month-old rats than

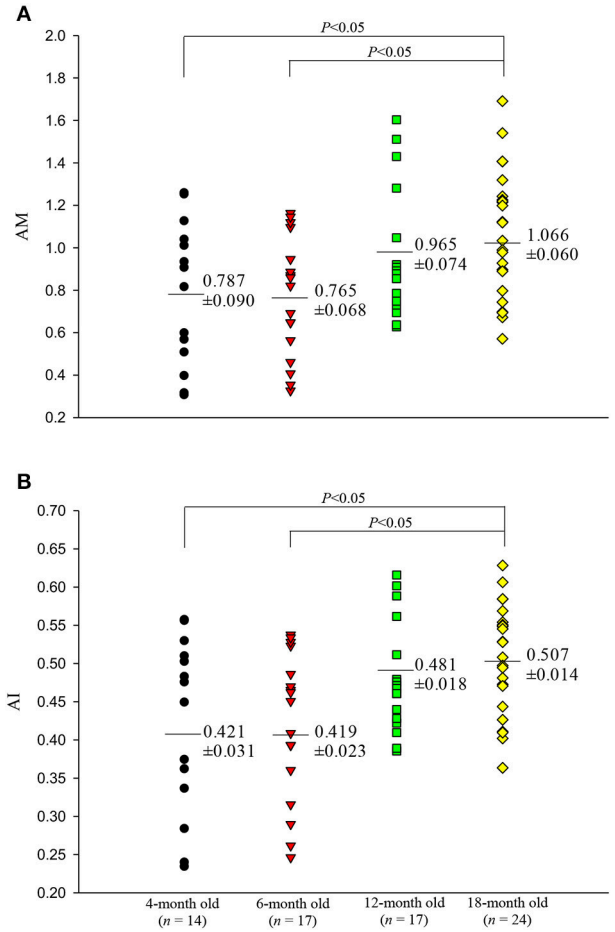

FIGURE 5 | Effects of aging on augmentation magnitude AM **(A)** and augmentation index AI **(B)**. The rats aged 18 months had significantly higher AM and AI than did those aged 4 and 6 months.

were those values in the 4-, 6-, and 12-month-old rats. Moreover, rats aged 18 months had significantly higher $|P_b|$, but not $|P_f|$ than did the rats in other age groups. Although, the arterial τ_w did not change significantly as animals aged, the τ_w/CL ratio was significantly lower in the rats aged 18 months than in the rats aged 4 and 6 months but not in those aged 12 months. No correlation between arterial τ_w and T_{IP} was observed.

Figure 1 illustrates the construction of an uncalibrated Q^{tri} from the measured pressure waveform in one rat aged 4 months as an example. The pressure and Q^m signals were simultaneously recorded in the ascending aorta under the anesthetized, open-chest condition. **Figure 2** depicts the similarity between the measured aortic impulse response obtained from the measured pressure and Q^m (A) and the predicted aortic impulse response obtained from the measured pressure and Q^{tri} (B) in the same rat. The measured P_f and P_b waves (C) and the predicted P_f and P_b waves (D) are also presented. Although, the Q^{tri} shape is an approximation that may differ from the actual Q^m shape, this approximation gave results close to those obtained with the measured Q^m.

Figures 3A,B illustrate the construction of an uncalibrated triangular flow from the measured pressure waveform in 4- and an 18-month old rats, respectively. The rat aged 4 months was the same one as shown in **Figure 1**. The pressure wave was the only signal recorded in the ascending aorta under the anesthetized, closed-chest condition. **Figure 4** depicts the aortic impulse responses obtained from the measured aortic pressure and Q^{tri} in the same 4- and 18-month old rats (**Figures 4A,B**, respectively). The P_f and P_b waves for the corresponding rats aged 4 (**Figure 4C**) or 18 (**Figure 4D**) months are also depicted.

Figures 5, **6** illustrate the effect of aging on the arterial wave properties in terms of the AM, AI, RM, and RI as well as the τ_w/LVET ratio. The AM (**Figure 5A**) and AI (**Figure 5B**) increased markedly in the 18-month-old rats compared with those in the 4- and 6-month-old rats. Moreover, the rats aged 18 months had significantly higher RM (**Figure 6A**) and RI (**Figure 6B**) values than did the rats aged 4 and 6 months. Although, the arterial τ_w did not change significantly as the rats aged (**Table 2**), the τ_w/LVET ratio was markedly lower in the 18-month-old rats than in the 4- and 6-month-old rats (**Figure 6C**).

Figure 7A illustrates relation between AI and τ_w/LVET, and **Figure 7C** depicts that between AI and RI, which were calculated from the measured aortic pressure and an assumed Q^{tri}. **Figure 7A** exhibits a significant inverse regression line for AI: $AI = -0.7424 - 0.9026 \times (\tau_w/LVET)$ ($r = 0.4901$; $P < 0.0001$). The regression equation of AI is given by $AI = -0.4844 + 2.3634 \times RI$ ($r = 0.8423$; $P < 0.0001$), and it is provided in **Figure 7C**. **Figure 7B** depicts relationship between PP and τ_w/LVET, and **Figure 7D** presents that between PP and RI. **Figure 7B** depicts an inverse regression line given by $PP = 44.0011 - 59.6778 \times (\tau_w/LVET)$ ($r = 0.5179$; $P < 0.0001$). The regression equation of PP is $PP = 1.8636 + 59.0396 \times RI$ ($r = 0.3363$; $P < 0.005$), and it is provided in **Figure 7D**.

Figure 8 illustrates relation between P_{ms}/P_{md} and τ_w/LVET, which exhibits a significant inverse regression line for P_{ms}/P_{md}: $(P_{ms}/P_{md}) = 1.1395 - 0.2030 \times (\tau_w/LVET)$ ($r = 0.4807$; $P < 0.0001$). However, the P_{ms}/P_{md} ratio has no correlation with the RI.

DISCUSSION

Earlier studies on the age-related changes of arterial mechanical properties in different species have shown that considerable changes occur in response to age. In humans, Gundel et al. (1981) measured the aortic pressure and flow signals to calculate Z_i and Z_c, demonstrating no relationship between age and Z_c. By contrast, Nichols et al. (1985) reported that aging process increased the steady and pulsatile components of the hydraulic load and the arterial wave reflections returned earlier with increasing age. However, Cox (1977) measured the carotid elastic modulus in rats of different ages, and suggested that the aging heart was somewhat compensated by a decrease in hydraulic load. In the present study, the older rats exhibited increased arterial stiffness and magnitude of the reflected pressure wave, which enhanced the systolic workload on the heart and contributed to

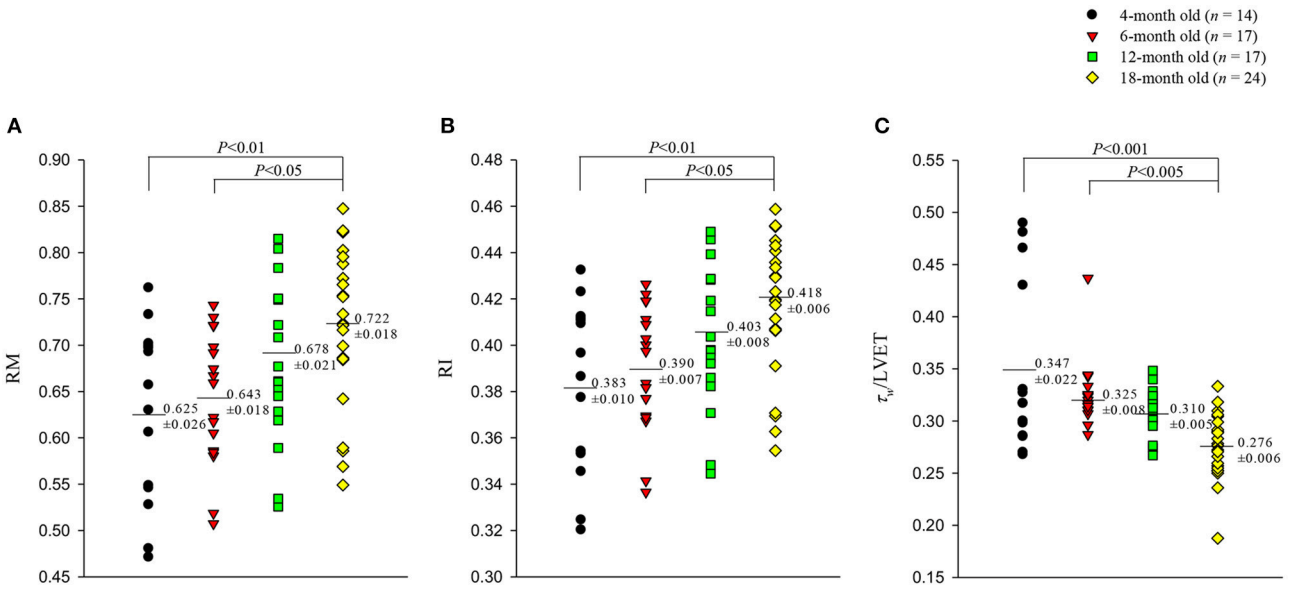

FIGURE 6 | Effects of aging on wave reflection magnitude RM **(A)**, wave reflection index RI **(B)**, and the ratio of wave transit time (τ_W) to left ventricular ejection period (LVET) **(C)**. The rats aged 18 months had significantly higher RM and RI than did those aged 4 and 6 months. Moreover, the τ_W/LVET ratio was markedly lower in the 18-month-old rats than in the 4- and 6-month-old rats.

a mismatch between the myocardial oxygen demand and supply.

In this study, the older rats exhibited a decline in HR and a prolongation in LVET. The decline in HR with age has also been described in other studies on rats (Bunag et al., 1990; Bunag and Teravainen, 1991). In the current study, significant declines in P_d and P_m but not P_s were observed in the 18-month-old rats. Although not reaching statistical significance, the rats aged 18 months had lower P_s than did the rats in the other age groups. The age-induced decline in the aortic pressure profile in the rats was consistent with the results of a previous report by Bunag et al. (1990).

The pulse wave velocity and traveling distance of pressure waves to reflection sites are the determinants of arterial τ_w. In this study, we analyzed the aortic impulse response to calculate the arterial τ_w and found that the calculation of arterial τ_w was influenced by the CL (**Figures 4A,B**). Although the arterial τ_w did not change significantly as animals aged (**Table 2**), the τ_w/CL ratio was significantly lower in the rats aged 18 months than in the rats aged 4 and 6 months but not in those aged 12 months. The decreased τ_w/CL ratio indicated a decline in the distensibility of the aorta in rats with advancing age. In the absence of any significant change in the $|P_f|$, the significant rise in the $|P_b|$ in senescence (**Table 2**) was responsible for the increased RM (**Figure 6A**) and RI (**Figure 6B**). Both the arterial RM and RI augmented by age in turn increased the intensity of the wave reflection from the peripheral circulation in the older rats.

For the calculation of AM and AI, defining an inflection point on the aortic pressure waveform is imperative (Westerhof et al., 2006; London and Pannier, 2010). In this study, the inflection point was identified by the first zero-crossing curve from positive to negative on the fourth derivative of the pressure signal during ventricular ejection (Kelly et al., 1989; Westerhof et al., 2006; Chang et al., 2017). With the inflection point determined, we found that the rats aged 18 months had higher ΔP values than did those in other age groups. Although PP also increased as the rats aged, the increased ΔP dominated the increased PP, leading to an augmentation in aortic AM and AI.

From the definition, AI depends on the overlap between the P_f and the P_b, which is determined by both the timing and magnitude of the reflected pressure wave (Westerhof et al., 2006; London and Pannier, 2010). The overlap between the P_f and the P_b depends on both the arterial τ_w and the LVET duration (London and Pannier, 2010). With a shortened τ_w, the reflected waves return earlier, thereby affecting the central arteries during systole rather than diastole. With a lengthened LVET, it is favorable for the reflected wave to return during systole. Thus, the decreased τ_w/LVET ratio may increase the overlap between the P_f and the P_b, thereby increasing the systolic workload on the heart and reducing aortic pressure during diastole (London and Pannier, 2010).

In the present study, the τ_w/LVET ratio was markedly smaller in the 18-month-old rats than in the rats from other age groups (**Figure 6C**). We found that the aortic AI was inversely affected by the arterial τ_w/LVET ratio; thus, the lower the arterial τ_w/LVET ratio, the higher the aortic AI (**Figure 7A**), which was consistent with those of a previous report by London and Pannier (2010). Although aging did not affect $|P_f|$, the older rats had increased $|P_b|$ (**Table 2**). Using simple linear regression analysis, we found that the aortic AI augmented by age was associated with the increased $|P_b|$: AI = 0.2423 + 0.0192 × $|P_b|$ (r = 0.5044; P < 0.0001), and had strong positive correlation with the RI

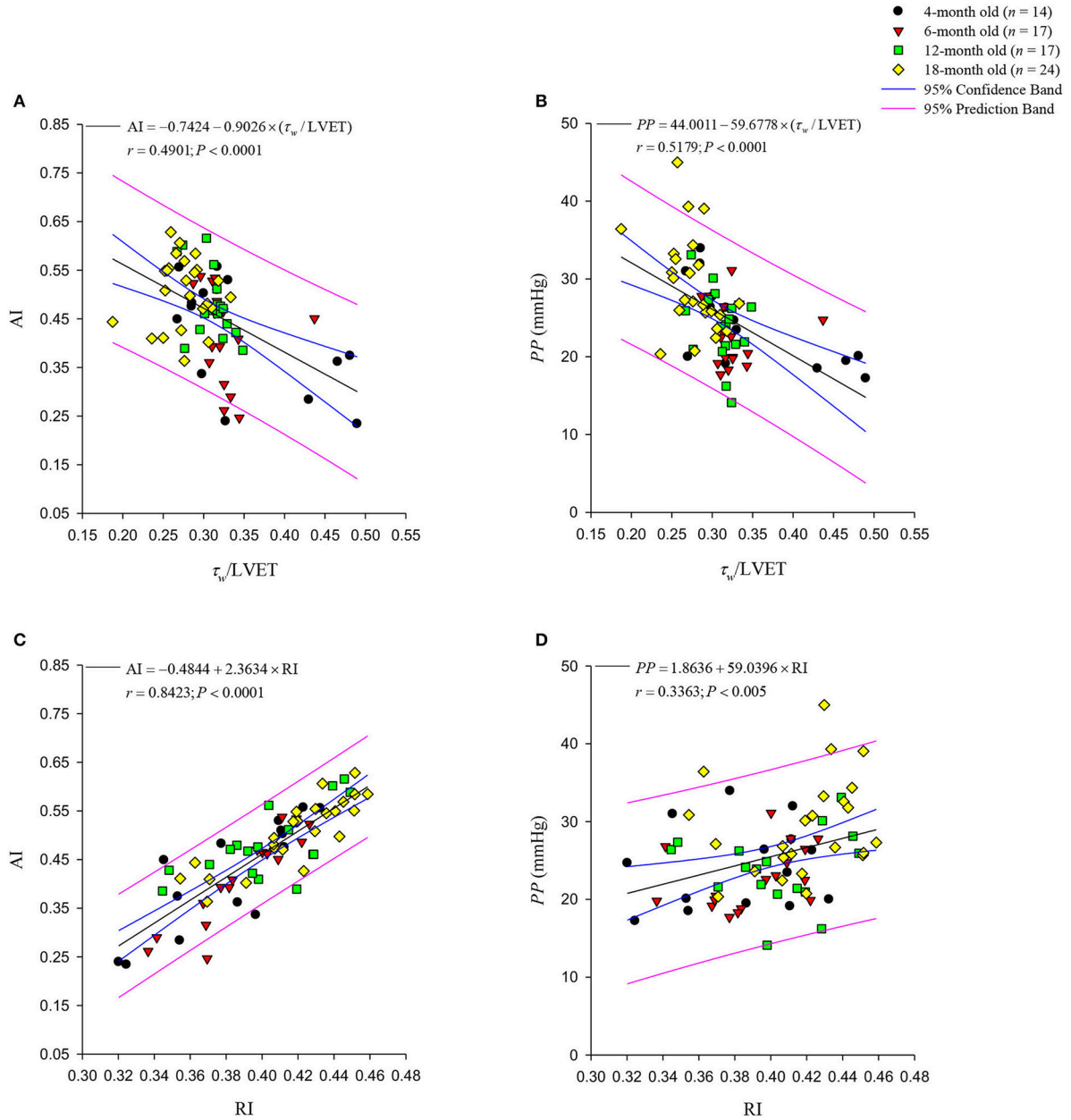

FIGURE 7 | Relationships between the augmentation index (AI) and the ratio of wave transit time (τ_W) to left ventricular ejection time (LVET) **(A)** and wave reflection index RI **(C)**. The aortic AI was significantly inversely related to the τ_W/LVET ratio. By contrast, the RI and the aortic AI were directly related. In **(B)**, the pulse pressure (*PP*) shows a negative linear correlation with the τ_W/LVET ratio. The positive regression line of *PP* with RI is depicted in **(D)**. The aortic AI had a closer correlation with the RI than the *PP* did.

(**Figure 7C**). As the arterial τ_w/LVET ratio decreased and the arterial RI increased with age, the aortic AI increased, thereby augmenting the systolic workload on the heart.

Similarly to AI, *PP* was augmented as arterial τ_w/LVET ratio decreased (**Figure 7B**) and arterial RI was increased (**Figure 7D**) in older rats. However, AI exhibited closer correlation with RI than *PP* did. These findings suggest that the aortic AI might be a better index describing the arterial wave properties than the aortic *PP*.

In considering the ventricular/vascular coupling, O'Rourke et al. (1984) suggested that the ascending aortic pressure wave includes two components: P_{ms}, which is relevant to LV performance as a pump, and P_{md}, which is relevant to LV perfusion. Thus, the augmented P_{ms}/P_{md} ratio may cause a mismatch between the myocardial oxygen demand and supply. In this study, the rats aged 18 months had lower P_{ms} than did the rats in the other age groups (**Table 1**). Although P_{md} also decreased as the rats aged, the decreased P_{md} dominated the decreased

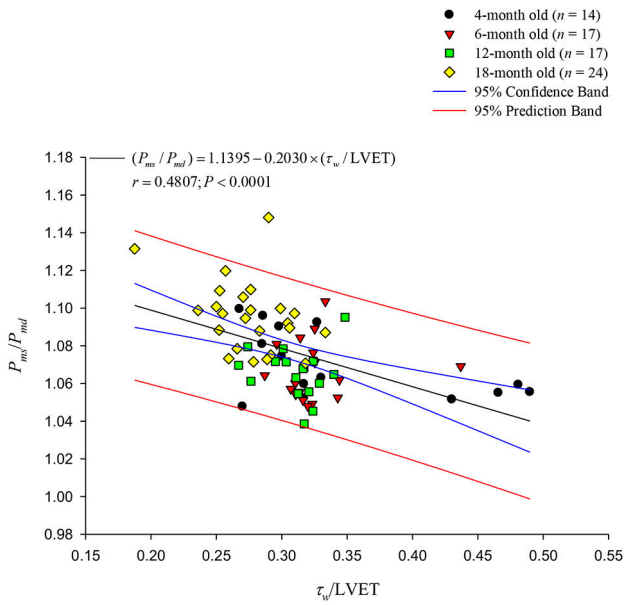

FIGURE 8 | Relationship between the P_{ms}/P_{md} ratio and the τ_W/LVET ratio. The aortic P_{ms}/P_{md} ratio was significantly inversely related to the τ_W/LVET ratio. LVET, left ventricular ejection time; P_{md}, mean diastolic pressure; P_{ms}, mean systolic pressure; τ_W, wave transit time.

P_{ms}, leading to an increase in P_{ms}/P_{md} ratio. The P_{ms}/P_{md} ratio augmented by age was associated with the diminished τ_W/LVET ratio (**Figure 8**), suggesting that as aging stiffened aortas, the older rats were accompanied with deterioration in the myocardial oxygen demand/supply ratio.

This study has several limitations. Because Z_i cannot be measured in conscious animals, evaluating the effects of pentobarbital-induced anesthesia on rats is impossible. The results reported here pertain only to the measurements made in anesthetized rats (Wu et al., 2012). This condition might have induced changes in the aortic pressure profiles and introduced reflex effects that are not observed under ordinary conditions (Wang et al., 2014a). The degree to which anesthesia influences the pulsatile hemodynamics in rats is not known with certainty. However, studies on other animals suggest that the effects are small in relation to the biological and experimental variability between animals (Cox, 1974). Moreover, the uncalibrated Q^{tri} was constructed using the measured aortic pressure wave to approximate the corresponding flow signal. Although the Q^{tri} is an approximation that may differ from the actual flow wave shape (Westerhof et al., 2006), the use of this concept to describe the arterial wave properties has been validated in studies by Westerhof et al. (2006) and Chang et al. (2017).

CONCLUSIONS

We determined the mechanical defects due to arterial aging on the basis of the measured aortic pressure and an assumed triangular flow. Because the $|P_f|$ was unaltered, the increase in $|P_b|$ enhanced the intensity of the wave reflection, thereby augmenting RI in the older rats. A reduction in the τ_W/CL ratio with age indicated a decline in the distensibility of the aorta, which resulted in arterial wave reflections returned earlier with increasing age. As the arterial τ_W/LVET ratio decreased and arterial RI increased with age, the aortic AI increased. With an increase in the P_{ms}/P_{md} ratio, the older rats were accompanied with deterioration in the myocardial oxygen demand/supply ratio. All these findings suggest that aging potentially impairs the pulsatile component of arterial mechanics, thereby increasing the systolic workload imposed on the heart.

PERSPECTIVES

Our contribution in this endeavor is to provide a path to consider the clinical application of the method estimating the arterial wave properties, based on the measured pressure alone. The advantage of the technique is that an assumed Q^{tri} is derived from the measured pressure and that the flow calibration is not essential in the analysis. Westerhof et al. (2006) suggested that the method can also be performed using the carotid pressure wave as a surrogate for the pressure measured in the ascending aorta. The carotid pressure can be obtained non-invasively by applanation tonometry (Van Bortel et al., 2001) or by using a transfer function on finger arterial pressure (Westerhof, 2005) or radial artery pressure (Chen et al., 1997). In large epidemiological studies, it is helpful to evaluate the arterial wave properties as a function of age by using a minimally non-invasive measurement on aortic pressure alone, because the construction of the unknown Q^{tri}, the separation of the aortic pressure waves, and the calculation of the arterial τ_W can be automated.

AUTHOR CONTRIBUTIONS

CC, RC, CW, and KC developed the concept of the study, designed the experiments, and wrote the manuscript. CC, RC, and SH performed the animal experiments, collected the data, and performed statistical analysis. MW, YC, HK, and LL provided advice on the surgical procedures used in the study. CW and KC interpreted the data, supervised this work, and critically revised the manuscript. All authors have read and approved the final manuscript.

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REFERENCES

Bunag, R. D., Krizsan, D., and Eriksson, L. (1990). Mediation of reflex tachycardia becomes exclusively 3-adrenergic in old Fischer 344 rats. *Mech. Aging Dev.* 52, 179–194.

Bunag, R. D., and Teravainen, T. L. (1991). Waning cardiovascular responses to adrenergic drugs in conscious aging rats. *Mech. Aging Dev.* 61, 313–326. doi: 10.1016/0047-6374(91)90063-6

Chang, K. C., Tsai, Y. F., Chow, C. Y., Peng, Y. I., and Chen, T. J. (1998). Age-related changes of arterial mechanical properties in rats: analysis using exponentially tapered T-tube model. *J. Gerontol. Biol. Sci.* 53A, B274–B280.

Chang, R. W., Chang, C. Y., Lai, L. C., Wu, M. S., Young, T. H., Chen, Y. S., et al. (2017). Determining arterial wave transit time from a single aortic pressure pulse in rats: vascular impulse response analysis. *Sci. Rep.* 7:40998. doi: 10.1038/srep40998

Chang, R. W., Chang, C. Y., Wu, M. S., Yu, H. Y., Luo, J. M., Chen,. Y. S., et al. (2015). Systolic aortic pressure-time area is a useful index describing arterial wave properties in rats with diabetes. *Sci. Rep.* 5:17293. doi: 10.1038/srep17293

Chen, C. H., Nevo, E., Fetics, B., Pak, P. H., Yin, F. C., Maughan, W. L., et al. (1997). Estimation of central aortic pressure waveform by mathematical transformation of radial tonometry pressure. Validation of generalized transfer function. *Circulation* 95, 1827–1836. doi: 10.1161/01.CIR.95.7.1827

Cox, R. H. (1974). Three-dimensional mechanics of arterial segments *in vitro* methods. *J. Appl. Physiol.* 36, 381–384.

Cox, R. H. (1977). Effects of age on the mechanical properties of rat carotid artery. *Am. J. Physiol.* 233, H256–H263.

Franklin, S. S. (2006). Hypertension in older people: part 1. *J. Clin. Hyperten.* 8, 444–449. doi: 10.1111/j.1524-6175.2006.05113.x

Gundel, W., Cherry, G., Rajagopalan, B., Tan, L. B., Lee, G., and Schultz, D. (1981). Aortic input impedance in man: acute response to vasodilator drugs. *Circulation* 63, 1305–1314. doi: 10.1161/01.CIR.63.6.1305

Kelly, R., Hayward, C., Avolio, A., and O'Rourke, M. F. (1989). Noninvasive determination of age-related changes in the human arterial pulse. *Circulation* 80, 1652–1659. doi: 10.1161/01.CIR.80.6.1652

Lakatta, E. G. (1979). Alterations in the cardiovascular system that occur in advanced age. *Fed. Proc.* 38, 163–167.

Lakatta, E. G., and Yin, F. C. P. (1982). Myocardial aging: functional alterations and related cellular mechanisms. *Am. J. Physiol.* 242, H927–H941.

Latson, T. W., Yin, F. C. P., and Hunter, W. C. (1987). "The effects of finite wave velocity and discrete reflection on ventricular loading," in *Ventricular/Vascular Coupling: Clinical, Physiological, and Engineering Aspects*, ed F. C. P. Yin (New York, NY: Springer-Verlag), 354–383.

Laxminarayan, S., Sipkema, P., and Westerhof, N. S. (1978). Characterization of the arterial system in the time domain. *IEEE Trans. Biomed. Eng.* 25, 177–184. doi: 10.1109/TBME.1978.326244

London, G. M., and Pannier, B. (2010). Arterial functions: how to interpret the complex physiology. *Nephrol. Dial. Transplant.* 25, 3815–3823. doi: 10.1093/ndt/gfq614

Mattace-Raso, F. U. S., Van Der Cammen, T. J. M., Hofman, A., Van Popele, N. N., Bos, M. L., Schalekamp, M. A., et al. (2006). Arterial stiffness and risk of coronary heart disease and stroke: the Rotterdam Study. *Circulation* 113, 657–663. doi: 10.1161/CIRCULATIONAHA.105.555235

McDonald, D. A. (1974). *Blood Flow in Arteries, 2nd Edn*. London: Edward Arnold.

Milnor, W. R. (1989). *Hemodynamics, 2nd Edn.* Baltimore, MD: Williams & Wilkins.

Murgo, J. P., Westerhof, N., Giolma, J. P., and Altobelli, S. A. (1981). Manipulation of ascending aortic pressure and flow with the Valsalva maneuver: relationship to input impedance. *Circulation* 63, 122–132. doi: 10.1161/01.CIR.63.1.122

Najjar, S. S., Scuteri, A., and Lakatta, E. G. (2005). Arterial aging: is it an immutable cardiovascular risk factor? *Hypertension* 46, 454–462. doi: 10.1161/01.HYP.0000177474.06749.98

Nichols, W. W., and O'Rourke, M. F. (2011). *McDonald's Blood Flow in Arteries, 6th Edn*. London: Edward Arnold.

Nichols, W. W., O'Rourke, M. F., Avolio, A. P., Yaginuma, T., Murgo, J. P., and Pepine, C. J., et al. (1985). Effects of age on ventricular-vascular coupling. *Am. J. Cardiol.* 55, 1179–1184. doi: 10.1016/0002-9149(85)90659-9

O'Leary, D. H., Polak, J. F., Kronmal, R. A., Manolio, T. A., Burke, G. L., and Wolfson, S. K. Jr. (1999). Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke in older adults. Cardiovascular Health Study Collaborative Research Group. *N. Engl. J. Med.* 340, 14–22. doi: 10.1056/NEJM199901073400103

O'Rourke, M. F. (1982). Vascular impedance in studies of arterial and cardiac function. *Physiol. Rev.* 62, 571–652.

O'Rourke, M. F., and Nichols, W. W. (2005). Aortic diameter, aortic stiffness, and wave reflection increase with age and isolated systolic hypertension. *Hypertension* 45, 652–658. doi: 10.1161/01.HYP.0000153793.84859.b8

O'Rourke, M. F., Yaginuma, T., and Avolio, A. P. (1984). Physiological and pathophysiological implications of ventricular/vascular coupling. *Ann. Biomed. Eng.* 12, 119–134. doi: 10.1007/BF02584226

Schleicher, E. D., Wagner, E., and Nerlich, A. G. (1997). Increased accumulation of the glycoxidation product N(epsilon)-(carboxymethyl)lysine in human tissues in diabetes and aging. *J. Clin. Invest.* 99, 457–468. doi: 10.1172/JCI119180

Sims, T. J., Rasmussen, L. M., Oxlund, H., and Bailey, A. J. (1996). The role of glycation cross-links in diabetic vascular stiffening. *Diabetologia* 39, 946–951. doi: 10.1007/BF00403914

Sipkema, P., Westerhof, N., and Randall, O. S. (1980). The arterial system characterized in the time domain. *Cardiovasc. Res.* 14, 270–279. doi: 10.1093/cvr/14.5.270

Sutton-Tyrrell, K., Najjar, S. S., Boudreau, R. M., Venkitachalam, L., Kupelian, V., Simonsick, E. M., et al. (2005). Elevated aortic pulse wave velocity, a marker of arterial stiffness, predicts cardiovascular events in well-functioning older adults. *Circulation* 111, 3384–3390. doi: 10.1161/CIRCULATIONAHA.104.483628

Van Bortel, L. M., Balkestein, E. J., van der Heijden-Spek, J. J., Vanmolkot, F. H., Staessen, J. A., Kragten, J. A., et al. (2001). Non-invasive assessment of local arterial pulse pressure: comparison of applanation tonometry and echo-tracking. *J. Hypertens.* 19, 1037–1044. doi: 10.1097/00004872-200106000-00007

Wang, C. H., Chang, R. W., Ko, Y. H., Tsai, P. R., Wang, S. S., Chen, Y. S., et al. (2014a). Prevention of arterial stiffening by using low-dose atorvastatin in diabetes is associated with decreased malondialdehyde. *PLoS ONE* 9:e90471. doi: 10.1371/journal.pone.0090471

Wang, C. H., Wu, E. T., Wu, M. S., Tsai, M. S., Ko, Y. H., Chang, R. W., et al. (2014b). Pyridoxamine protects against mechanical defects in cardiac aging in rats: studies on load dependence of myocardial relaxation. *Exp. Physiol.* 99, 1488–1498. doi: 10.1113/expphysiol.2014.082008

Westerhof, B. E. (2005). *Blood Pressure Analysis on Time Scales From Seconds to Days (dissertation)*. Amsterdam: University of Amsterdam.

Westerhof, B. E., Guelen, I., Westerhof, N., Karemaker, J. M., and Avolio, A. (2006). Quantification of wave reflection in the human aorta from pressure alone: a proof of principle. *Hypertension* 48, 595–601. doi: 10.1161/01.HYP.0000238330.08894.17

Westerhof, N., Sipkema, P., van den Bos, G. C., and Elzinga, G. (1972). Forward and backward waves in the arterial system. *Cardiovasc. Res.* 6, 648–656. doi: 10.1093/cvr/6.6.648

Wu, E. T., Liang, J. T., Wu, M. S., and Chang, K. C. (2011). Pyridoxamine prevents age-related aortic stiffening and vascular resistance in association with reduced collagen glycation. *Exp. Gerontol.* 46, 482–488. doi: 10.1016/j.exger.2011.02.001

Wu, M. S., Chang, C. Y., Chang, R. W., and Chang, K. C. (2012). Early return of augmented wave reflection impairs left ventricular relaxation in aged Fisher 344 rats. *Exp. Gerontol.* 47, 680–686. doi: 10.1016/j.exger.2012.06.006

Yin, F. C. P. (1980). "The aging vasculature and its effects on the heart," in *The Heart in Old Age: Its Function and Response to Stress,* ed M. L. Weisfeldt (New York, NY: Raven), 137-217.

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The Temporal Relationship between Arterial Stiffening and Blood Pressure Is Modified by Methotrexate Treatment in Patients with Rheumatoid Arthritis

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Background: The temporal relationship between arterial stiffness and blood pressure (BP) may vary depending on age and other clinical and demographic factors. Since both BP and arterial stiffness are also affected by inflammatory processes, we examined the temporal arterial stiffness-BP relationship in patients with rheumatoid arthritis (RA) treated with either methotrexate (MTX), an anti-rheumatic agent shown to reduce cardiovascular risk in meta-analyses, or other disease-modifying anti-rheumatic drugs (DMARDs).

Methods: Measurements of clinic and 24-h peripheral and central systolic and diastolic BP (SBP and DBP), and pulse wave velocity (PWV) were assessed in RA patients on stable treatment with either MTX ± other DMARDs (MTX group, $n = 41$, age 61 ± 14 years, 73% females) or other DMARDs (non-MTX group, $n = 18$, age 65 ± 13 years, 89% females). Measurements were performed at baseline and after 8 months. The temporal relationships were examined using cross-lagged path analysis with models that included age, sex, body mass index, prednisolone, and folic acid use and 28-joint disease activity score.

Results: There were significant differences in the temporal arterial stiffness-BP relationships between those in the MTX and DMARD groups. A higher PWV at baseline caused a significant increase in 6 out of 8 different measures of SBP at 8 months amongst those treated with DMARDs (standardized β, range = 0.54–0.66, $p < 0.003$ for each) and 3 out of 8 different measures of DBP (standardized β, range = 0.52–0.61, $p < 0.003$ for each) but was not associated with either SBP or DBP at 8 months amongst those treated with MTX. The difference in the effect of baseline PWV on 8-month BP between the 2 groups was also significant ($p < 0.003$) for 4 measures including clinic peripheral SBP (β = 7.0, 95% CI = 2.8–11.1 mmHg per 1 m/s higher baseline PWV; $p < 0.001$).

Conclusions: Higher arterial stiffness preceded increases in BP in subjects with RA treated with DMARDs, but these effects did not occur amongst those treated with MTX. The different effects were seen mostly in measures of SBP but were also present in some measures of DBP. Our findings suggest MTX may confer a protective effect against stiffness mediated increases in BP in patients with RA.

Keywords: methotrexate, disease-modifying anti-rheumatic drugs, blood pressure, arterial stiffness, pulse wave velocity, rheumatoid arthritis, cross-lagged path analysis

INTRODUCTION

Arterial stiffness and blood pressure (BP) are two well established independent risk factors for cardiovascular disease (CVD), yet both are also closely associated with each other (Mitchell, 2014). The relationship between the two parameters is likely to be bidirectional based on haemodynamic, vascular biology, and physiology principles, with both factors potentially capable of influencing one another. Thus, whilst arterial stiffening may cause increases in blood pressure due to a reduced ability to buffer the BP waveform, it is also possible that the increased damage to arteries from higher BP may lead to an increase in arterial stiffness (Mitchell, 2014). A stronger temporal effect of BP on arterial stiffness than vice-versa was recently observed in middle aged adults without hypertension followed for a period of 7 years (Chen et al., 2016). This was however in contrast to other studies, mostly cross-sectional and in older populations, that have generally found a stronger effect of arterial stiffness on BP (Najjar et al., 2008). The strength of each temporal relationship may therefore depend on the populations studied whereby age and other factors related to the underlying pathophysiology may determine the dominating causal effects. One such factor is represented by chronic systemic inflammation, a common feature in patients with autoimmune disease states such as rheumatoid arthritis (RA), a condition notoriously associated with a significant increase in cardiovascular morbidity and mortality when compared to the general population (Avina-Zubieta et al., 2008).

In addition to their known status as CVD risk factors in the general population, increased BP (Baghdadi et al., 2015) and increased arterial stiffness (Ikdahl et al., 2016) also increase the risk of CVD in RA patients. Methotrexate (MTX) is a commonly used disease-modifying anti-inflammatory drug (DMARD) in this population and observational studies have shown that MTX treatment is associated with a lower clinic BP and a reduced prevalence of hypertension in RA patients (Cuchacovich and Espinoza, 2009; Mangoni et al., 2017). Furthermore, recent meta-analyses have shown that the use of MTX in RA and other chronic inflammatory states is associated with a significantly lower risk of cardiovascular events, including myocardial infarction (Roubille et al., 2015). Since inflammation is also associated with arterial stiffening and elevated BP (Savoia and Schiffrin, 2006; Jain et al., 2014), use of methotrexate may influence the causal effects of elevated stiffness on increases in BP unlike other DMARDs, despite similar anti-inflammatory effects. However, the predominating temporal relationships between BP and arterial stiffness has not yet been assessed in the RA population.

In this medium term follow-up study of 8 months, we performed repeat assessments of arterial stiffness using pulse wave velocity (PWV) and both clinic and 24-h ambulatory blood pressure (peripheral and central) in a cohort of 59 subjects with a confirmed diagnosis of RA. We then determined the temporal associations between stiffness and BP in those subjects treated with methotrexate + DMARDs ($n = 41$) and in those not using methotrexate and treated only with DMARDs ($n = 18$).

METHODS

Study Design

We conducted a repeat cross-sectional study with measurements of BP and PWV at baseline and at 8 months follow-up. A cross-lagged panel approach (Selig and Little, 2012) was used for the analysis to determine the strength of the temporal relationships between baseline PWV and 8-month BP, and between baseline BP and 8-month PWV. In total, we recorded 16 measures of BP; 8 × SBP and × DBP, with each including clinic, day-time, night-time, 24-h, for both peripheral and central measures. **Figure 1** shows an example of the cross-lagged causal pathways for PWV and clinic peripheral SBP. The arrows indicate causal directions e.g., clinic peripheral SBP at baseline affecting clinic peripheral SBP and PWV at 8-month follow-up. Our primary interest was to determine the strength of the relationships between baseline PWV and follow-up BP (β_1) and the association between baseline BP and follow-up PWV (β_2).

Patient Recruitment and Ethical Approval

We studied a consecutive series of patients with stable RA, aged ≥18 years, recruited from the outpatient clinics of the Rheumatology Department at Flinders Medical Centre and the Repatriation General Hospital, in the Southern Health Local Heath Network, Adelaide, Australia. RA was diagnosed according to the 1987 American College of Rheumatology or the 2010 American College of Rheumatology/European League Against Rheumatism criteria (Aletaha et al., 2010). Study participants were classified as currently treated with MTX for at least 8 weeks (MTX group), or not taking MTX for at least 1 year or being MTX-naïve and treated with other synthetic and/or biologic DMARDs (non-MTX group). Exclusion criteria were atrial fibrillation, active cancer or current treatment with anti-cancer drugs, heart failure, and cognitive impairment.

The study (registered in the Australian New Zealand Clinical Trials Registry with the registration number

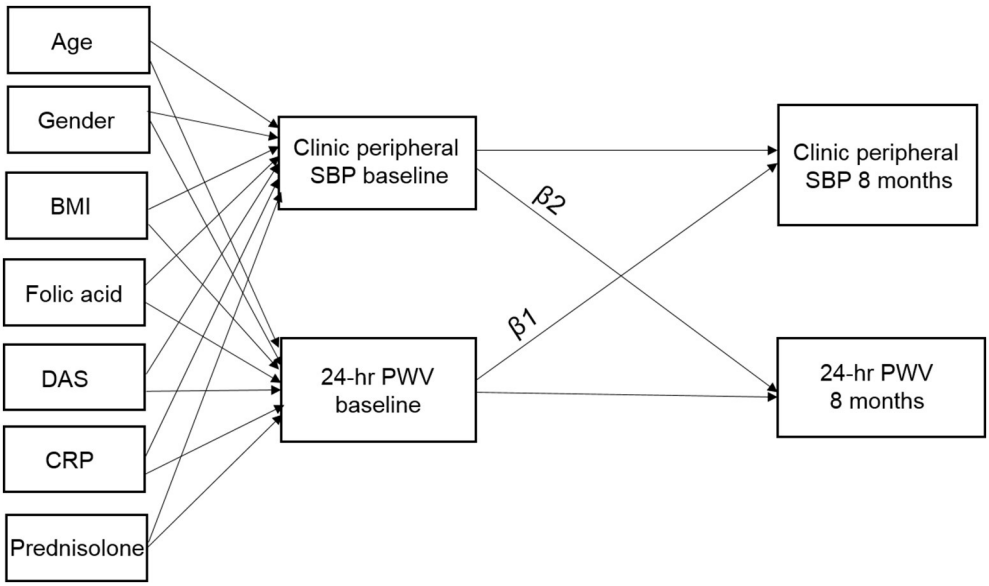

FIGURE 1 | β_1, cross-lagged path coefficient from baseline PWV to follow-up clinic peripheral systolic blood pressure (SBP); β_2, cross-lagged path coefficient from baseline clinic peripheral SBP to follow-up PWV. Baseline measures of age, gender, BMI, DAS, serum CRP, and use of folic acid and prednisolone are included as covariates. Arrows indicate hypothesized causal directions and when significant in the cross-lagged path analysis this provides an indication of causality.

ACTRN12616001366448) was approved by the Southern Adelaide Clinical Human Research Ethics Committee (Ethics Approval Number: 76.14). Each participant gave written consent before entering the study in accordance with the Declaration of Helsinki.

Clinic Peripheral and Central Blood Pressure

Clinic peripheral systolic (SBP) and diastolic (DBP) BP were measured in the morning, in a quite environment at room temperature, using the clinically validated automatic BP monitor (model UA-767PC; AND Medical, Sydney, Australia) according to current guidelines (Palatini et al., 1998; Baghdadi et al., 2015; Gabb et al., 2016). The average of the last two sitting BP measurements was calculated and used in analyses. Clinic central SBP and DBP was measured non-invasively using Pulse Wave Analysis (PWA, SphygmoCor version 7.1, AtCor Medical, Sydney, Australia; O'Rourke et al., 2001).

24-h Peripheral and Central Blood Pressure and PWV

Twenty-four hour, daytime (between 08:00 a.m. and 12:00 a.m.), and night-time (between 12:00 a.m. and 08:00 a.m.; Fagard et al., 1996) peripheral and central BP and PWV were measured using a validated ambulatory oscillometric BP monitor (Mobil-O-Graph PWA monitor, IEM, Stolberg, Germany; Jones et al., 2000; Westhoff et al., 2005; Franssen and Imholz, 2010; Wei et al., 2010). The central BP measurement, similar to that measured using the SphygmoCor device (Weiss et al., 2012), is based on the ARCSolver method, which determines aortic BP and PWV using the oscillometric BP technique (Wassertheurer et al., 2010; Weber et al., 2011).

Clinical and Demographic Characteristics

The following data were collected from patient interviews, medical questionnaires, clinical notes, and hospital administrative databases: age, gender, medical, and medication history, Stanford health assessment questionnaire (HAQ) (Pincus et al., 1983), pain visual analog scale (McCormack et al., 1988), global health score (Anderson et al., 2012), weight, height, body mass index (BMI), and the 28-joint disease activity score (DAS28) (Prevoo et al., 1995).

C-Reactive Protein

High-sensitivity C-reactive protein (CRP) was measured in serum by latex-enhanced immunoturbidimetry on an automated Modular PPE Analyzer (Roche Diagnostics; Pepys and Hirschfield, 2003).

Statistical Analysis

Each analysis was performed in Stata version 14.2 (StataCorp, Texas, USA) using commands for structural equation modeling (SEM) with maximum-likelihood estimation. For each of the 16 different BP outcome variables we fitted a separate SEM model with MTX treatment status as a separate strata (grouping) variable. The endogenous (dependent) variables in each model were baseline and follow-up BP, and baseline and follow-up PWV. Exogenous (independent) variables in each model were age, gender, BMI, DAS28, serum CRP, and use of folic acid and prednisolone which were used as predictors of the baseline measures of BP and PWV. Measurement errors between BP and PWV were also allowed to co-vary at each time-point

TABLE 1 | Baseline clinical and demographic characteristics of the MTX and the non-MTX groups.

	All subjects ($n = 59$)	Non-MTX group ($n = 18$)	MTX group ($n = 41$)	*P*-value[a]
Age (years)	62 ± 13	65 ± 13	61 ± 14	0.34
Females (%)	78.0	88.9	73.2	0.18
Body Mass Index (Kg/m^2)	27.8 ± 6.2	28.6 ± 6.3	27.5 ± 6.2	0.55
Current smoking (%)	13.6	5.6	17.1	0.23
Hypertension (%)	35.6	38.9	34.2	0.73
Diabetes (%)	10.2	22.2	4.9	0.06
Dyslipidaemia (%)	30.5	38.9	26.8	0.75
Previous cardiovascular event (%)	8.5	22.2	2.4	0.03
Chronic kidney disease (%)	3.4	5.6	2.4	0.52
Liver disease (%)	3.4	5.6	2.4	0.52
Depression (%)	22.0	38.9	14.6	0.04
RA duration (years)	11 [4, 24]	15 [8, 27]	9 [3, 23]	0.21
MTX dose (mg/week)	14.3 ± 5.2	14.3 ± 5.2	–	–
DAS28 score	3.0 ± 1.2	3.7 ± 1.0	2.6 ± 1.1	<0.001
C-reactive protein (mg/L)	1.90 [0.57, 6.0]	1.95 [0.74, 4.1]	1.90 [0.57, 6.7]	0.76
Stanford HAQ score	0.5 [0.0, 1.5]	1.065 [0.38, 1.75]	0.25 [0.00, 1.13]	0.06
Pain visual analog score	0.76 [0.20, 1.34]	0.90 [0.40, 1.52]	0.70 [0.08, 1.24]	0.13
Global health score	0.58 [0.08, 1.30]	0.10 [0.38, 1.40]	0.30 [0.04, 0.96]	0.03
Hydroxychloroquine (%)	25.4	22.2	26.8	0.49
Leflunomide (%)	5.1	0.0	7.3	0.55
Sulfasalazine (%)	15.3	22.2	12.2	0.43
Abatacept (%)	3.4	5.6	2.4	0.52
Rituximab (%)	0.0	0.0	0.0	1.00
Tocilizumab (%)	8.5	22.2	2.4	0.03
Adalimumab (%)	6.8	5.6	7.3	0.64
Etanercept (%)	23.7	33.3	19.5	0.32
Certolizumab pegol (%)	1.7	2.4	0.0	1.00
Golimumab (%)	5.1	0.0	7.3	0.55
Prednisolone (%)	33.9	33.3	34.1	0.95
Prednisolone daily dose (mg)	5 [4.75, 7.50]	5.00 [4.0, 5.0]	[5.00 4.5, 10.0]	0.89
Ibuprofen (%)	6.8	0.0	9.8	0.30
Aspirin* (%)	18.6	22.2	17.1	0.72
Antihypertensive drugs (%)	27.1	27.8	26.8	1.00
Folic acid (%)	54.2	5.6	75.6	<0.001
Fish oil (%)	37.3	27.8	41.5	0.39

*MTX, methotrexate; RA, rheumatoid arthritis; DAS, disease activity score 28; HAQ, health assessment questionnaire. *Daily dose 100 mg in all patients.*
[a]Using t-test, χ^2-test or Fishers Exact as appropriate. Figures are Mean ± SD, median[25th percentile, 75th percentile], or n(%).

(**Figure 1**). The specific coefficients of interest were the effect of baseline PWV on follow-up BP (β_1) and the effect of baseline BP on follow-up PWV (β_2). Stratifying by MTX treatment status allowed separate coefficient estimates for each group of subjects and also calculation of the difference (and confidence intervals) for each coefficient between the 2 groups. We also performed a formal Wald test of group invariance in each coefficient to formally test whether the 2 estimates for each coefficient were the same for both groups. Model fit was determined using standard SEM measures: the chi-square (χ^2) test of model fit vs. saturated fit for which non-significant values ($p > 0.05$) indicate acceptable fit, the comparative fit index (CFI) for which values >0.90 reflect acceptable model fit, and values >0.95 reflect excellent model fit, and the standardized root-mean-square residual (SRMSR) for which values of 0.05 or less reflect excellent model fit, while a value of <0.08 reflects a good fit. Given the 16 different measures of BP that were assessed, a Bonferroni correction was applied to the results and coefficients were only considered significant for $p < 0.003$. Similarly, we also calculated 99.7% confidence intervals (CI's).

RESULTS

Descriptive Statistics

Table 1 describes the baseline clinical and demographic characteristics of the patients in the MTX and the non-MTX groups. The mean (±SD) age of the population was 62 ± 13 and 78% were females. There were no differences between the

non-MTX group of subjects and those taking MTX in terms of other medication use, chronic diseases and disease scores except for a significant difference for the presence of depression (38.9 vs. 14.6%, respectively, $p = 0.04$), the use of folic acid (5.6 vs. 75.6%; $p < 0.001$), the DAS28 (3.7 ± 1.0 vs. 2.6 ± 1.1, $p < 0.001$), and the Global Health score [median (IQR) 0.1 (0.38, 1.40) vs. 0.3 (0.04, 0.96); $p = 0.03$].

Table 2 describes the correlations, means and standard deviations for 24 h PWV, clinic peripheral SBP and 24 h peripheral SBP at the baseline and 8-month assessments ($n = 59$). Across the 2 time points, there was a slight increase in both clinic and ambulatory BP but there was no significant change in PWV. Amongst the 16 different measures of BP, pairwise Pearson correlations between measures of baseline SBP and follow-up PWV were moderate ($r = 0.31$–0.54), correlations between baseline DBP and follow-up PWV were weak ($r = 0.06$–0.20), correlations between baseline PWV and follow-up SBP were moderate ($r = 0.40$–0.55), correlations between baseline PWV and follow-up DBP were weak to moderate ($r = -0.02$ to 0.44), whilst correlations between baseline and follow-up measures of PWV were high ($r = 0.91$).

TABLE 2 | Correlation matrix and descriptive statistics for PWV and clinic and 24 h peripheral SBP at baseline (0M) and 8-month (8M) assessments ($n = 59$).

	Clinic SBP 0M	24 h PWV 0M	Clinic SBP 8M	24 h PWV 8M
Clinic SBP 0M	1.00			
24 h PWV 0M	0.51	1.00		
Clinic SBP 8M	0.66	0.51	1.00	
24 h PWV 8M	0.54	0.91	0.55	1.00
N	59	59	59	59
Mean	126.1	9.10	127.7	9.08
SD	17.5	2.01	18.1	2.18
	24 h SBP 0M	**24 h PWV 0M**	**24 h SBP 8M**	**24 h PWV 8M**
Clinic SBP 0M	1.00			
24 h PWV 0M	0.53	1.00		
Clinic SBP 8M	0.56	0.40	1.00	
24 h PWV 8M	0.43	0.91	0.44	1.00
N	59	59	59	59
Mean	126.5	9.10	107.9	9.08
SD	12.7	2.01	15.41	2.18

SD, standard deviation.

Effect of PWV on BP (β_1 Path Coefficients)

Figure 2 shows the standardized estimates with 99.7% CI's for β_1 by DMARD treatment regime. For subjects in the non-MTX group, out of the 8 measures of SBP, all except 24 h peripheral SBP and night-time peripheral SBP showed a significant effect of baseline PWV on follow-up BP (standardized β, range = 0.54–0.66, $p < 0.003$ for each). Out of the 8 measures of DBP, 3 showed a significant effect of baseline PWV on follow-up BP (standardized β, range = 0.54–0.66, $p < 0.003$ for each) including night-time peripheral, 24-h central and night-time central DBP. There were no significant effects of baseline PWV on measures of either SBP or DBP amongst subjects using MTX.

Effect of BP on PWV (β_2 Path Coefficients)

Figure 3 shows the standardized estimates with 99.7% CI's for β_2 by type of DMARD. For both those subjects in the non-MTX group and those in the MTX group, there were no significant effects of baseline SBP or baseline DBP, on follow-up PWV.

Difference in Path Coefficients between Groups

Figure 4 shows the difference in the estimated effects of baseline PWV on increases in BP at 8 months (β_1) for the different treatments (DMARDs vs. MTX). There was a significant difference between groups in the estimated effects for clinic peripheral SBP, clinic central SBP, 24 h central SBP and night central SBP ($p < 0.003$ for each). The largest difference in the effect occurred for clinic peripheral SBP (β = 7.0, 95% CI = 2.8–11.1 mmHg per 1 m/s higher baseline PWV; $p < 0.001$). There were no significant differences between groups in the effects of baseline PWV on any of the measures of follow-up DBP. There were also no significant differences between groups in β_2 (effect of baseline BP on follow-up PWV) for any of the 16 measures of BP. The Wald test for group invariance in the estimated coefficients between the 2 groups showed a significant difference in the β_1 coefficient for clinic peripheral SBP ($p = 0.0004$), clinic central SBP ($p = 0.006$), day-time peripheral SBP ($p = 0.014$), 24 h central SBP ($p = 0.005$), day-time central SBP ($p = 0.045$), and night-time central SBP ($p = 0.013$). There was also a significant difference in the β_2 coefficient for clinic peripheral DBP ($p = 0.043$) and clinic central DBP ($p = 0.046$; **Figures 3**, **4**).

Model Fit Statistics

Table 3 describes the various indices of model fit for each of the 16 measures of BP. Overall, the indices indicated excellent model fit with the mean CFI = 0.956 and the mean SRMSR = 0.046. The mean χ^2 value for the fitted model vs. the saturated model was $\chi^2 = 44.0$ (28 df) ($p = 0.028$) indicating acceptable fit.

Changes in Heart Rate and CRP

Table 4 displays the changes between baseline and follow-up in average 24-h heart rate and log-transformed CRP for each of the 2 groups, as well as the associated differences in these changes between groups. There were no significant differences between baseline and follow-up for each group, and no significant difference in the change between groups.

DISCUSSION

In this repeat cross-sectional study of patients with stable RA, we examined the strength of the temporal relationships between arterial stiffness (PWV) and follow-up BP and between baseline BP and follow-up PWV. We observed evidence for a causal effect of arterial stiffness on increased SBP in subjects not treated with MTX. The effects were seen for both central and peripheral and for clinic and ambulatory SBP. The effects were similar

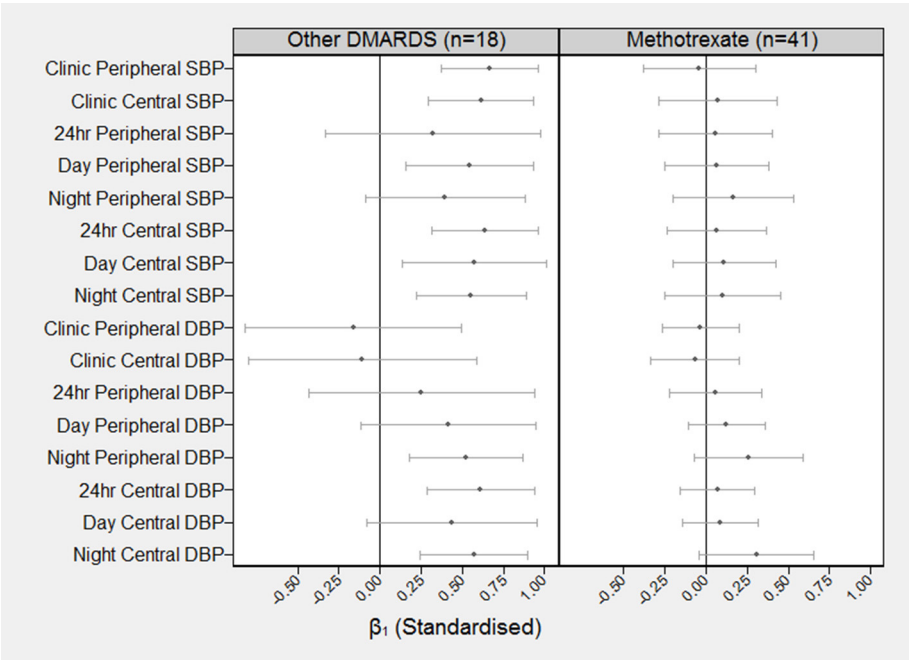

FIGURE 2 | Standardized coefficients (and 99.7% confidence intervals) describing the correlation between baseline PWV and BP at follow-up BP (β_1). The standardized β_1 can be interpreted as the number of SD increases in BP at 8 months for each one SD increase in PWV at baseline. For example, the standardized β_1 coefficient for baseline PWV on clinic peripheral SBP for the other DMARDS group is $\beta_1 = 0.66$ (99.7% CI = 0.37–0.96).

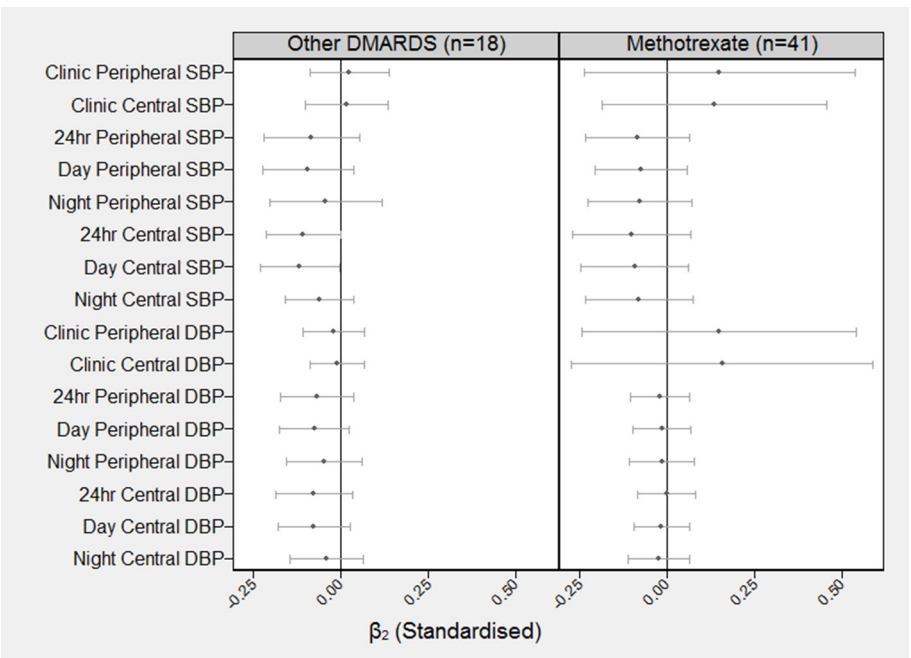

FIGURE 3 | Standardized coefficients (and 99.7% confidence intervals) describing the correlation between baseline BP at follow-up PWV (β_2). The standardized β_2 can be interpreted as the number of SD increases in PWV at 8 months for each one SD increase in BP at baseline. For example, the standardized β_2 coefficient for baseline BP on clinic peripheral PWV for the other DMARDS group is $\beta_2 = 0.02$ (99.7% CI = −0.09–0.14).

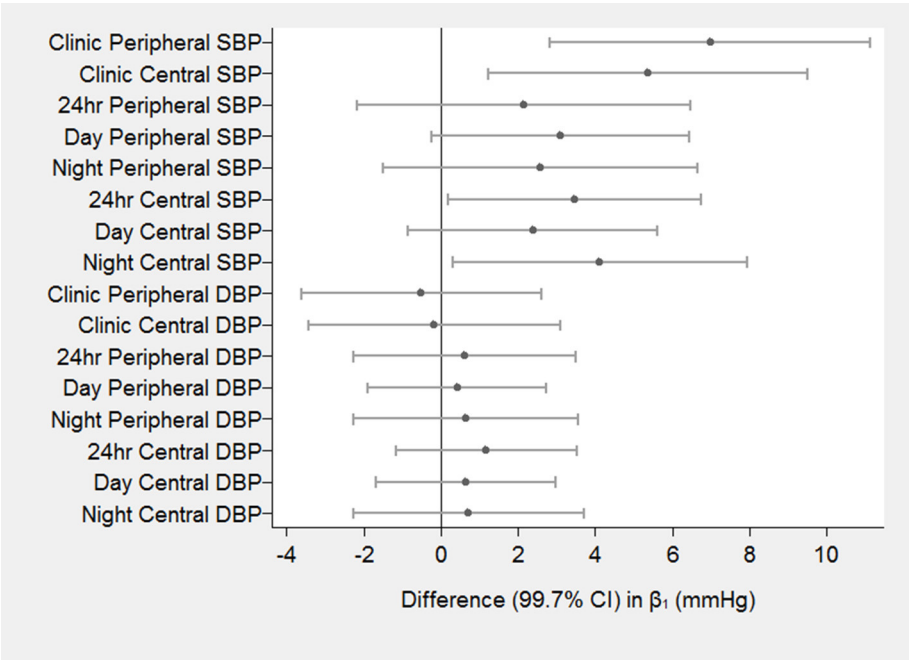

FIGURE 4 | Differences in the effects of baseline PWV on follow-up BP between DMARDS group and MTX group. The estimated effects (β) are the differences in increased blood pressure (in mmHg) at 8 months for a 1 m/s higher baseline PWV for DMARDS vs. MTX.

TABLE 3 | Indices of model fit for each of the 16 blood pressure models.

Blood pressure variable	χ^2 test (28 df)[a]	*p*-value	CFI[b]	SRMSR[v]
Clinic peripheral SBP	44.8	0.023	0.95	0.05
Clinic central SBP	38.9	0.082	0.97	0.05
24 h peripheral SBP	36.8	0.122	0.98	0.03
Day peripheral SBP	36.1	0.141	0.98	0.04
Night peripheral SBP	47.4	0.012	0.95	0.04
24 h central SBP	35.8	0.147	0.98	0.04
Day central SBP	34.3	0.190	0.98	0.04
Night central SBP	47.4	0.013	0.95	0.05
Clinic peripheral DBP	32.6	0.253	0.99	0.05
Clinic central DBP	37.1	0.116	0.97	0.06
24 h peripheral DBP	49.6	0.007	0.93	0.05
Day peripheral DBP	60.7	<0.001	0.91	0.06
Night peripheral DBP	51.1	0.005	0.93	0.04
24 h central DBP	55.8	0.001	0.93	0.05
Day central DBP	47.2	0.013	0.95	0.05
Night central DBP	47.9	0.011	0.94	0.04

[a] *Chi-squared test of fitted model vs. saturated fit with 28 degrees of freedom;*
[b] *CFI, Comparative fit index;*
[c] *SRMSR, Standardized Root Mean Square Residual.*

during the day and night and we also observed the same effect of PWV on night-time and 24 h central DBP amongst subjects not taking MTX. By contrast, none of the causal effects of increased stiffness on BP were observed amongst subjects treated with MTX medication. Since the differences in causal effects were predominantly related to systolic blood pressure, rather than diastolic pressure, this suggests the possible involvement of effects on stroke volume. Since we did not measure stroke volume directly, further studies are required to determine whether this parameter might explain, at least in part, the observed between-group differences.

Since inflammation is believed to have a primary influence in the pathophysiology of increased arterial stiffness (Jain et al., 2014), studying a population known to have high underlying levels of inflammation, and associated cardiovascular risk, provides a valuable opportunity to examine the extent to which inflammation and specific DMARDs may influence the stiffness-BP relationship. The 2 groups of subjects within our own study had similar values of PWV at baseline, which were in line with values normally seen for their age. Our results therefore suggest that arterial stiffness related increases in BP were prevented in those using MTX. If this finding were also to be observed in individuals without RA, it would support the idea that the effects of increased arterial stiffness may partly be due to underlying inflammation, and that MTX acts to disrupt these specific pathways more effectively than other DMARDs. Furthermore, MTX appears to exert additional vasculoprotective effects independent of reduced inflammation, through the accumulation of adenosine, a vasodilator, BP lowering, and stimulating nitric-oxide (NO) synthesis agent, and stimulation of 5′ AMP-activated protein kinase (AMPK) (Costa and Biaggioni, 1998; Tian and Cronstein, 2007; Schneider et al., 2015; Thornton et al., 2016). In this study we observed non-significant reductions in CRP, a measure of systemic inflammation, of ∼37% in each group, suggesting that systemic inflammation was not a major explanatory factor of the observed causal effects.

TABLE 4 | Twenty-four hour heart rate and CRP values at baseline and follow-up for each group and estimated changes.

	24-h heart rate			Change MTX vs. Non-MTX (p-value)[b]
	0 months	8 months	Change[a]	
Non-MTX ($n = 18$)	68.9 ± 10.7	69.1 ± 8.6	0.2 ± 1.7 ($p = 0.90$)	
MTX ($n = 41$)	71.2 ± 9.4	70.2 ± 9.6	−1.1 ± 1.1 ($p = 0.35$)	−1.30 ± 2.07 (0.53)
	Log CRP			
	0 months	8 months	Change[a]	
Non-MTX (n =1 8)	0.57 ± 1.31	0.40 ± 1.31	−0.21 ± 0.36 ($p = 0.56$)	
MTX ($n = 41$)	0.79 ± 1.67	0.49 ± 1.61	−0.30 ± 0.23 ($p = 0.19$)	−0.09 ± 0.43 ($p = 0.83$)

[a] *Adjusted marginal means using mixed effects linear regression model.*
[b] *From MTX × visit interaction term.*

Our results may not be generalizable to all individuals with RA, particularly younger patients. Our cohort was typical of the RA population and consisted of mostly middle-aged to older patients. Recently, the Bogalusa study, a 7 year longitudinal cohort study in a younger population of adults, used similar methods to ours to examine the dominant temporal relationship between arterial stiffness and BP. In this younger cohort they reported that elevated BP precedes increased arterial stiffness rather than vice-versa (Chen et al., 2016) and concluded that amongst younger adults the arterial wall may not yet be stiff enough to significantly influence BP.

Our study had a number of strengths. Firstly we used cross-lagged path analysis, a specific form of path analysis that simultaneously examines reciprocal, longitudinal relationships among a set of inter-correlated variables and allows better determination of causal relationships (Selig and Little, 2012). Overall, our models provided excellent levels of fit indicating that the proposed causal pathways were feasible for the given data. In addition we used a wide variety of BP measures including 24 h BP monitoring with repeat measurements every 20 min during the day and every 30 min during the night. This reduces measurement error and increases statistical power. We also collected clinical data on a large number of potential confounders and were able to demonstrate similarity between groups except for a few variables including DAS28 and use of folic acid that were adjusted for in our analysis.

There are several limitations to our study. The duration of follow-up in our study was relatively short with a mean of 8 months. Most of the evidence from other studies have been derived from cohorts with between 4 and 8 years average follow-up in which larger effects are likely to occur (Chen et al., 2016). However, the strength of the associations observed in these studies and our own study were similar. In addition, our sample size was relatively small and so we may have been underpowered to detect some of the smaller effects that were observed. Although, characteristic of a RA population, the study population was also fairly heterogeneous with some subjects receiving BP medications as well as different anti-inflammatory medications for the treatment of their RA. Although, we adjusted for the use of prednisolone, a more homogeneously treated population would allow us to better explore the associations without the risk of selection bias. Finally, although we tried to reduce the possibility of confounding and also used a structural equation modeling approach to better identify the most likely direction of causality, the results may still be influenced by residual confounding and reverse causality since not all subjects were free of cardiovascular disease at baseline. A fully randomized controlled trial in a disease free non-hypertensive population is required to determine both the true causal effects of PWV on BP and also whether MTX use can inhibit the damaging effects of increased stiffness.

In summary we have demonstrated that although elevated arterial stiffening preceded increases in BP in subjects with RA, these effects did not occur amongst those patients using MTX medication. The beneficial effects were seen mostly in SBP but were also apparent to some degree with DBP, particularly with regards to the more accurate assessment of 24-h blood pressures. These findings suggest MTX may confer a protective effect against stiffness mediated increases in BP in patients with RA.

AUTHOR CONTRIBUTIONS

RW performed the analysis, helped conceive the study design, provided Ph.D. supervision, performed the first draft of the manuscript and reviewed later versions. LB collected and formatted the data for the study as part of her Ph.D., performed ethics applications, obtained consent from patients and reviewed several versions of the manuscript. MS was involved in all aspects of the study design, provided clinical patients for the study, and reviewed the manuscript before submission. AM was involved in all aspects of the study design, Ph.D. supervision, helped draft the first version of the manuscript and provided several revisions of the manuscript.

REFERENCES

Aletaha, D., Neogi, T., Silman, A. J., Funovits, J., Felson, D. T., Bingham, C. O. III, et al. (2010). Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Arthritis Rheum.* 62, 2569–2581. doi: 10.1002/art.27584

Anderson, J., Caplan, L., Yazdany, J., Robbins, M. L., Neogi, T., Michaud, K., et al. (2012). Rheumatoid arthritis disease activity measures: American College of Rheumatology recommendations for use in clinical practice. *Arthritis Care Res.* 64, 640–647. doi: 10.1002/acr.21649

Avina-Zubieta, J. A., Choi, H. K., Sadatsafavi, M., Etminan, M., Esdaile, J. M., and Lacaille, D. (2008). Risk of cardiovascular mortality in patients with rheumatoid arthritis: a meta-analysis of observational studies. *Arthritis Rheum.* 59, 1690–1697. doi: 10.1002/art.24092

Baghdadi, L. R., Woodman, R. J., Shanahan, E. M., and Mangoni, A. A. (2015). The impact of traditional cardiovascular risk factors on cardiovascular outcomes in patients with rheumatoid arthritis: a systematic review and meta-analysis. *PLoS ONE* 10:e0117952. doi: 10.1371/journal.pone.0117952

Chen, W., Li, S., Fernandez, C., Sun, D., Lai, C. C., Zhang, T., et al. (2016). Temporal relationship between elevated blood pressure and arterial stiffening among middle-aged black and white adults: the bogalusa heart study. *Am. J. Epidemiol.* 183, 599–608. doi: 10.1093/aje/kwv274

Costa, F., and Biaggioni, I. (1998). Role of nitric oxide in adenosine-induced vasodilation in humans. *Hypertension* 31, 1061–1064. doi: 10.1161/01.HYP.31.5.1061

Cuchacovich, R., and Espinoza, L. R. (2009). Does TNF-alpha blockade play any role in cardiovascular risk among rheumatoid arthritis (RA) patients? *Clin. Rheumatol.* 28, 1217–1220. doi: 10.1007/s10067-009-1208-x

Fagard, R., Brguljan, J., Thijs, L., and Staessen, J. (1996). Prediction of the actual awake and asleep blood pressures by various methods of 24 h pressure analysis. *J. Hypertens.* 14, 557–563. doi: 10.1097/00004872-199605000-00003

Franssen, P. M., and Imholz, B. P. (2010). Evaluation of the Mobil-O-Graph new generation ABPM device using the ESH criteria. *Blood Press. Monit.* 15, 229–231. doi: 10.1097/MBP.0b013e328339be38

Gabb, G. M., Mangoni, A. A., Anderson, C. S., Cowley, D., Dowden, J. S., Golledge, J., et al. (2016). Guideline for the diagnosis and management of hypertension in adults - 2016. *Med. J. Aust.* 205, 85–89. doi: 10.5694/mja16.00526

Ikdahl, E., Rollefstad, S., Wibetoe, G., Olsen, I. C., Berg, I. J., Hisdal, J., et al. (2016). Predictive value of arterial stiffness and subclinical carotid atherosclerosis for cardiovascular disease in patients with rheumatoid arthritis. *J. Rheumatol.* 43, 1622–1630. doi: 10.3899/jrheum.160053

Jain, S., Khera, R., Corrales-Medina, V. F., Townsend, R. R., and Chirinos, J. A. (2014). Inflammation and arterial stiffness in humans. *Atherosclerosis* 237, 381–390. doi: 10.1016/j.atherosclerosis.2014.09.011

Jones, C. R., Taylor, K., Chowienczyk, P., Poston, L., and Shennan, A. H. (2000). A validation of the Mobil O Graph (version 12) ambulatory blood pressure monitor. *Blood Press. Monit.* 5, 233–238. doi: 10.1097/00126097-200008000-00007

Mangoni, A. A., Baghdadi, L. R., Shanahan, E. M., Wiese, M. D., Tommasi, S., Elliot, D., et al. (2017). Methotrexate, blood pressure and markers of arterial function in patients with rheumatoid arthritis: a repeated cross-sectional study. *Ther. Adv. Musc. Dis.* doi: 10.1177/1759720X17719850. [Epub ahead of print].

McCormack, H. M., Horne, D. J., and Sheather, S. (1988). Clinical applications of visual analogue scales: a critical review. *Psychol. Med.* 18, 1007–1019. doi: 10.1017/S0033291700009934

Mitchell, G. F. (2014). Arterial stiffness and hypertension. *Hypertension* 64,13–18. doi: 10.1161/HYPERTENSIONAHA.114.00921

Najjar, S. S., Scuteri, A., Shetty, V., Wright, J. G., Muller, D. C., Fleg, J. L., et al. (2008). Pulse wave velocity is an independent predictor of the longitudinal increase in systolic blood pressure and of incident hypertension in the Baltimore Longitudinal Study of Aging. *J. Am. Coll. Cardiol.* 51, 1377–1383. doi: 10.1016/j.jacc.2007.10.065

O'Rourke, M. F., Pauca, A., and Jiang, X. J. (2001). Pulse wave analysis. *Br. J. Clin. Pharmacol.* 51, 507–522. doi: 10.1046/j.0306-5251.2001.01400.x

Palatini, P., Frigo, G., Bertolo, O., Roman, E., Da Corta, R., and Winnicki, M. (1998). Validation of the A&D TM-2430 device for ambulatory blood pressure monitoring and evaluation of performance according to subjects' characteristics. *Blood Press. Monit.* 3, 255–260.

Pepys, M. B., and Hirschfield, G. M. (2003). C-reactive protein: a critical update. *J. Clin. Invest.* 111, 1805–1812. doi: 10.1172/JCI200318921

Pincus, T., Summey, J. A., Soraci, S. A. Jr., Wallston, K. A., and Hummon, N. P. (1983). Assessment of patient satisfaction in activities of daily living using a modified Stanford Health Assessment Questionnaire. *Arthritis Rheum.* 26, 1346–1353. doi: 10.1002/art.1780261107

Prevoo, M. L., van't Hof, M. A., Kuper, H. H., van Leeuwen, M. A., van de Putte, L. B., and van Riel, P. L. (1995). Modified disease activity scores that include twenty-eight-joint counts. Development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. *Arthritis Rheum.* 38, 44–48. doi: 10.1002/art.1780380107

Roubille, C., Richer, V., Starnino, T., McCourt, C., McFarlane, A., Fleming, P., et al. (2015). The effects of tumour necrosis factor inhibitors, methotrexate, non-steroidal anti-inflammatory drugs and corticosteroids on cardiovascular events in rheumatoid arthritis, psoriasis and psoriatic arthritis: a systematic review and meta-analysis. *Ann. Rheum. Dis.* 74, 480–489. doi: 10.1136/annrheumdis-2014-206624

Savoia, C., and Schiffrin, E. L. (2006). Inflammation in hypertension. *Curr. Opin. Nephrol. Hypertens.* 15, 152–158. doi: 10.1097/01.mnh.0000203189.57513.76

Schneider, H., Schubert, K. M., Blodow, S., Kreutz, C. P., Erdogmus, S., Wiedenmann, M., et al. (2015). AMPK dilates resistance arteries via activation of SERCA and BKCa channels in smooth muscle. *Hypertension* 66, 108–116. doi: 10.1161/HYPERTENSIONAHA.115.05514

Selig, J. P., and Little, T. D. (2012). "Autoregressive and cross-lagged panel analysis for longitudinal data," in *Handbook of Developmental Research Methods*, eds B. Laursen, T. D. Little, and N. A. Card (New York, NY: The Guildford Press), 265–278.

Thornton, C. C., Al-Rashed, F., Calay, D., Birdsey, G. M., Bauer, A., Mylroie, H., et al. (2016). Methotrexate-mediated activation of an AMPK-CREB-dependent pathway: a novel mechanism for vascular protection in chronic systemic inflammation. *Ann. Rheum. Dis.* 75, 439–448. doi: 10.1136/annrheumdis-2014-206305

Tian, H., and Cronstein, B. N. (2007). Understanding the mechanisms of action of methotrexate: implications for the treatment of rheumatoid arthritis. *Bull. NYU Hosp. Jt. Dis.* 65, 168–173.

Wassertheurer, S., Kropf, J., Weber, T., van der Giet, M., Baulmann, J., Ammer, M., et al. (2010). A new oscillometric method for pulse wave analysis: comparison with a common tonometric method. *J. Hum. Hypertens.* 24, 498–504. doi: 10.1038/jhh.2010.27

Weber, T., Wassertheurer, S., Rammer, M., Maurer, E., Hametner, B., Mayer, C. C., et al. (2011). Validation of a brachial cuff-based method for estimating central systolic blood pressure. *Hypertension* 58, 825–832. doi: 10.1161/HYPERTENSIONAHA.111.176313

Wei, W., Tolle, M., Zidek, W., and van der Giet, M. (2010). Validation of the mobil-O-Graph: 24 h-blood pressure measurement device. *Blood Press. Monit.* 15, 225–228. doi: 10.1097/MBP.0b013e328338892f

Weiss, W., Gohlisch, C., Harsch-Gladisch, C., Tolle, M., Zidek, W., and van der Giet, M. (2012). Oscillometric estimation of central blood pressure: validation of the Mobil-O-Graph in comparison with the SphygmoCor device. *Blood Press. Monit.* 17, 128–131. doi: 10.1097/MBP.0b013e328353ff63

Westhoff, T. H., Straub-Hohenbleicher, H., Schmidt, S., Tolle, M., Zidek, W., and van der Giet, M. (2005). Convenience of ambulatory blood pressure monitoring: comparison of different devices. *Blood Press. Monit.* 10, 239–242. doi: 10.1097/01.mbp.0000172711.82287.7f

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REVIEW
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The Aging Risk and Atherosclerosis: A Fresh Look at Arterial Homeostasis

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A considerable volume of research over the last decade has focused on understanding the fundamental mechanisms for the progression of atherosclerosis—the underlying cause for the vast majority of all cardiovascular (CVD)-related complications. Aging is the dominant risk factor for clinically significant atherosclerotic lesion formation, yet the heightened impact of aging on the disease is not accounted for by changes in traditional risk factors, such as lack of physical activity, smoking, hypertension, hyperlipidemia, or diabetes mellitus. This review will examine the pathological and biochemical processes of atherosclerotic plaque formation and growth, with particular focus on the aging risk vis-a-vis arterial homeostasis. Particular focus will be placed on the impact of a number of important contributors to arterial homeostasis including bone marrow (BM)-derived vascular progenitor cells, differential monocyte subpopulations, and the role of cellular senescence. Finally, this review will explore many critical observations in the way the disease process has been reassessed both by clinicians and researchers, and will highlight recent advances in this field that have provided a greater understanding of this aging-driven disease.

Keywords: atherosclerosis, cardiovascular disease, vascular repair, arterial homeostasis, aging, risk factors, bone marrow-derived vascular progenitor cells

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ATHEROSCLEROSIS: ANCIENT AND INFLAMMATORY

Atherosclerosis is defined by the formation and growth of plaques within the arterial lumen with concurrent loss of vascular elasticity. Eventually, this condition can lead to consequent blood flow reduction through the affected vessel, and has long been associated with CVD-related death. Around the year 1505, Leonardo da Vinci recorded his observations of a "parched and shrunk and withered … artery that feeds the heart," attributing this as the cause of death of an elderly man who passed away "without any movement or sign of anything amiss" (McCurdy, 1923). However, CVD remained a relatively uncommon cause of death until the dramatic surge in CVD mortality in the past 100 years, due primarily to marked and progressive increases in life expectancy, as a result of improved sanitation and public health, with a staggering reduction in communicable diseases and violent deaths. During this time period, numerous traditional lifestyle-associated CVD risk factors were identified and increased in prevalence, leading many to believe that atherosclerosis is an exclusively lifestyle-dependent modern disease. However, recent reports have challenged this view, demonstrating that mummies from various ancient societies—from as much as 5,300 years ago—exhibited a high prevalence of arterial changes compatible with atherosclerosis (Thompson et al., 2013; Clarke et al., 2014; Thomas et al., 2014; Zink et al., 2014). Additionally, a single nucleotide polymorphism (SNP) recently shown to be a genetic risk factor for CVD (minor allele of rs10757274 on chromosome 9p21) was detected in at least one of these

mummies, despite the fact that it was initially believed that this gene modification arose much later in history (Zink et al., 2014). Many putative mechanisms have been considered to account for the presence of CVD that far back in human history (Thompson et al., 2013; Thomas et al., 2014). In contrast, risk factors most commonly associated with disease in modern populations include modifiable risks—such as tobacco use, obesity, diet, and physical inactivity—and non-modifiable risks—including age, gender, numerous identified SNPs in germ cells, and race (Yusuf et al., 2004; Goldschmidt-Clermont et al., 2012). Algorithms designed to interpret these factors and provide relative individual risk scores [Framingham (D'Agostino et al., 2008), QRISK®2-2016 (Hippisley-Cox et al., 2008), ACC/AHA ASCVD Goff et al., 2014] have concluded that the strongest risk factor for atherosclerosis and CVD is indisputably age; using existing algorithms, simply increasing age—while holding all other variables constant—results in a marked increase in risk for CVD (Rauscher et al., 2003; Karra et al., 2005; Goldschmidt-Clermont et al., 2012; Madonna et al., 2016). The average annual rates of first CVD events for men increase over 20-fold or more between the age groups 35–44 (incidence of 3 per 1,000) and 85–94 (incidence of 74 per 1,000), while similar rates are observed in women ∼10 years later in life (Benjamin et al., 2017). Similarly, the average age of myocardial infarction is 65 and 72 for men and women, respectively (Members et al., 2016). These acute clinical events occurring at advanced ages often manifest in the absence of associated symptoms, and are frequently fatal (Myerburg and Junttila, 2012). However, atherosclerosis itself—as the principal mechanism for the majority of these events—is a chronic, inflammatory, progressive disease with initial manifestations beginning at young ages (McGill et al., 2000; Zieske et al., 2002; Hong, 2010). Evidence of the disease has been reported in multiple arterial regions in children under the age of 10, and in the aortas of individuals less than a year old (McGill et al., 2000; Hong, 2010). Advanced lesions of atherosclerosis have been detected in the arteries, including coronaries, of US military even at a young age (Enos et al., 1953; Webber et al., 2012). Importantly, although the results of these studies may not be directly comparable to the general population for reasons such as risk factor disparity between groups (notably smoking and obesity), they provide two important insights to disease prevalence over the past several decades: first, that disease prevalence has appreciably declined during this time, and, second, that onset of disease occurs at an early age and has potential to progress rapidly with age (Levy, 2012).

Historically, atherosclerosis was often viewed exclusively as the result of dyslipidemia. Both high-density lipoprotein (HDL) and low-density lipoprotein (LDL) play critical roles in the transport of cholesterol and have been implicated in atherosclerosis (Fisher et al., 2012; Ference et al., 2017). Specifically, normal levels of HDL and HDL-cholesterol (HDL-C)—often called the "good cholesterol"—are associated with a variety of antiatherogenic processes and reduced levels of CVD overall (Fisher et al., 2012; Rosenson et al., 2016). In contrast, elevated levels of LDL and LDL-cholesterol (LDL-C) have been implicated in atherosclerosis progression (Fisher et al., 2012; Ference et al., 2017). However, we now know that elevated circulating LDL alone is generally insufficient to account for the observed extent of disease and is responsible for at most half of arterial lesions of atherosclerosis in elderly people, while disease can be found even in the complete absence of hyperlipidemia (Libby et al., 2009; Wick and Grundtman, 2012). Near the end of the twentieth century, the notion that atherosclerosis was a fundamentally inflammatory disease began to gain popularity, catalyzed largely by pathologist Russel Ross' pioneering report, "Atherosclerosis—An Inflammatory Disease," and by the work of Peter Libby (Ross, 1999; Libby, 2002; Libby et al., 2002). The formation and progression of atherosclerotic plaques as we understand today largely support Ross' own "response-to-injury" hypothesis, implicating plaque formation as a consequence of focal endothelial cell (EC) injury and inflammation (Tuttolomondo et al., 2012; Wick and Grundtman, 2012). However, as more of the underlying mechanisms and pathways controlling atherosclerosis are identified, it becomes clear that this disease does not simply follow a single, forward direction of irreversible progression. In contrast, it is evident now that atherosclerosis is the result of a myriad of independent pathways and their complex interactions (Libby, 2016). These pathways involve a multitude of various players, including various subpopulations of immune competent cells such as monocytes and macrophages, bone marrow (BM)-derived progenitor cells, chemokines and receptors, and cellular and subcellular fates including protein aggregation, cellular senescence, and apoptosis. In the following sections, each of these various aspects of atherosclerosis—and the ever-present effect of aging on these pathways and players, and beyond—will be explored.

ARTERIAL HOMEOSTASIS, AGING, AND BALANCE BETWEEN INJURY AND REPAIR

Recently, it has been shown that genetic expression profiles and epigenetic modifications exert complex control and regulatory mechanisms for atherosclerosis (Karra et al., 2005; Dunn et al., 2014). Perhaps most critical to the understanding of this disease, are the recent findings that the most determinant factors for atherosclerosis progression are repair mechanisms, slowing or preventing the progression of disease, thus maintaining arterial homeostasis in spite of the presence of risk factors (Goldschmidt-Clermont, 2003). Specifically, a growing body of evidence indicates that vascular repair, effected by BM-derived competent vascular progenitor cells that are programmed by chemokines, cytokines, matrix proteins, and growth factors, serves to counter the effects of endothelial injury and dysfunction that would otherwise lead to atherosclerosis progression (Rauscher et al., 2003; Karra et al., 2005; Madonna et al., 2016). Using a well-established mouse model of atherosclerosis (severely hyperlipidemic, apolipoprotein E-deficient mice), it was shown that with aging, the capacity of BM-derived progenitor cells to repair arteries damaged by high lipid concentrations becomes impaired (Rauscher et al., 2003), and that the development of atherosclerotic lesions occurs contemporaneously with the loss of repair capacity, and not simply when the severe

hyperlipidemia begins (Karra et al., 2005). Other work has identified the accumulation of senescent cells that produce pro-inflammatory cytokines and matrix metalloproteinases (MMPs) within atherosclerotic sites, but not in adjacent surrounding tissues (Childs et al., 2016). Identification of these cells is generally dependent on the presence of $p16^{INK4A}$, a cyclin-dependent kinase inhibitor responsible for cell cycle arrest in senescent cells (Baker et al., 2011, 2016). The factors released by senescent cells constitute a senescence-associated secretory phenotype (SASP), and have been shown to propagate atherosclerosis, while the selective targeting and elimination of these senescent cells (referred to as senolysis) has been shown to slow atherosclerotic lesions growth through reduction of inflammatory and adhesion factors (Childs et al., 2017). Additional data suggests that BM-derived progenitor cells may protect from these effects through reduction of endothelial senescence (Rauscher et al., 2003; Baker et al., 2016; Childs et al., 2016). Another interesting potential source of arterial aging to have emerged only recently involves the formation and accumulation of improperly folded proteins (Ayyadevara et al., 2016). Although most commonly associated with neurodegenerative diseases such as Alzheimer's and Parkinson's disease, recent reports have indicated that dysfunctional processes of either protein production or degradation (resulting in aggregation) lead to proteotoxicity and have been linked to CVD (Willis and Patterson, 2013). Additional reports have confirmed the presence of upregulated protein aggregates in models of aging and hypertension, identifying proteins within these aggregates known to be involved with CVD, and suggesting a variety of causes for aggregation including cellular senescence (Ayyadevara et al., 2016). These findings may indicate a novel potential therapeutic target by examining the processes by which these protein aggregates form. Taken together, these results suggest that methods aimed at improving vascular repair, as an additional strategy to that of reducing vascular injury, may provide significantly heightened results in improving arterial homeostasis.

More recently, the crucial role of the BM in the maintenance of arterial homeostasis was further evidenced. Indeed, a novel impact of aging as a driver of cardiovascular disease risk was identified within hematopoietic progenitor cells. With aging, these cells have been shown to acquire somatic DNA mutations that provide competitive growth advantages and lead to an expanded pool of mutated clones (a process known as clonal hematopoiesis of intermediate potential, or CHIP; Steensma et al., 2015). A recent study examined over 8,000 individuals from case-control studies, and revealed that elderly carriers of CHIP had nearly double the risk of coronary heart disease when compared to non-carriers (Jaiswal et al., 2017). Moreover, this study demonstrated that the loss of function mutation of Tet2 (the second most commonly mutated gene in CHIP) led to accelerated atherosclerosis in mice, likely as a result of transcriptional modifications of associated macrophages (Jaiswal et al., 2017). Interestingly, the loss of Tet2 also resulted in a highly induced expression of interleukin-1 beta (IL-1β), a well-characterized proinflammatory cytokine known to be involved in the development of atherosclerotic plaques (Jaiswal et al., 2017; Ridker et al., 2017). IL-1β is also activated via inflammasomes containing NOD-like receptors (NLRs; Furman et al., 2017; Ridker et al., 2017). Moreover, examination of inflammasome expression profiles revealed that individuals with profiles resulting in constitutive expression of IL-1β present characteristics (arterial stiffness, elevated blood pressure, oxidative stress, and chronic levels of inflammatory cytokines) associated with CVD, while inhibition of IL-1β resulted in significant reductions in recurrent CVD (Furman et al., 2017; Ridker et al., 2017). In addition to Tet2, mutated genes identified in this work include *DNMT3A*, *ASXL1*, and *JAK2*, and are associated with various processes including DNA methylation, chromatin remodeling, and cell proliferation. In similar fashion, other reports have demonstrated the critical importance of epigenetic modification of various genes involved in atherosclerosis. Specifically, it was shown that the methylation of CpG islands in the promoter region of the gene encoding for estrogen receptor alpha (ERα) is not only strongly associated with aging, but is responsible for the proliferation of smooth muscle cells involved in the growth of atherosclerotic plaques (Post et al., 1999; Ying et al., 2000).

Another critical area of interest with regards to aging and arterial homeostasis as it pertains to atherosclerosis is that of specific monocyte subpopulations. As recently as the late 1980s, it was widely believed that monocytes presented as a single population of cells (Stansfield and Ingram, 2015). However, it was more recently demonstrated that these cells represent a heterogeneous mixture of cell subpopulations with distinct patterns in surface receptor expression as well as primary function in response to conditions such as atherosclerosis (Gordon and Taylor, 2005). In mice, these subsets are often characterized as being either "inflammatory" ($Ly6C^{hi}$) monocytes that play an innate roles in antimicrobial defense, or "resident" ($Ly6C^{low}$) monocytes that actively patrol lumenal surface of the vasculature, removing damaged cells and debris, and generally promoting arterial homeostasis (Geissmann et al., 2003; Woollard and Geissmann, 2010; Thomas et al., 2015). This patrolling behavior was first observed in the small vessels of the microcirculation, but has since been observed in larger vessels such as the carotid artery (Geissmann et al., 2003; Quintar et al., 2017). Importantly, it was shown that this patrolling behavior plays a potential role in endothelial protection, and that atherosclerosis can trigger an upregulation of the number of this subpopulation of monocytes (Quintar et al., 2017). In humans, monocyte subpopulations are divided into three main categories, and share many characteristics with those identified in mice: the "classical" or "inflammatory" monocytes ($CD14^{++}CD16^{-}$), the "non-classical" monocytes ($CD14^{+}CD16^{++}$) involved in arterial patrolling, and the more recently identified "intermediate" ($CD14^{++}CD16^{+}$) subpopulation of monocytes (Passlick et al., 1989; Geissmann et al., 2003; Woollard and Geissmann, 2010; Ziegler-Heitbrock et al., 2010; Stansfield and Ingram, 2015). Human studies of atherosclerosis have mirrored those in mice, demonstrating that non-classical $CD16^{+}$ monocytes are primarily associated with disease, and further suggest roles for monocyte patrolling in injury healing and homeostasis (Stansfield and Ingram, 2015). However, additional monocyte

subpopulations have also been implicated in atherosclerosis progression in both mice and humans (Hilgendorf et al., 2015; Stansfield and Ingram, 2015). For example, although some results have shown that intermediate monocyte levels serve as an independent predictor of acute cardiovascular events such as stroke and myocardial infarction, additional population studies have demonstrated that increased levels of circulating classical monocytes serve as a reliable predictor of cardiovascular events regardless of age, sex, or other known traditional risk factors (Hilgendorf et al., 2015). Additionally, classical Ly6C^{hi} monocytes in mice were shown to infiltrate the endothelium at sites of injury, and contribute to foam cell formation (Swirski et al., 2007; Stansfield and Ingram, 2015). Ultimately, each of these subpopulations play varying roles in the formation, progression, and also regression of atherosclerosis.

As our understanding of this complex disease advances, it has become clear that there are numerous competing forces of disease progression and repair. It appears likely therefore that disease progression is the result of both the accumulation of signals and processes which exacerbate atherosclerotic inflammation, and which limit the efficiency of repair mechanisms, as a consequence of aging and exposure to traditional risk factors (Rauscher et al., 2003; Goldschmidt-Clermont et al., 2012). Given these recent advances in the understanding of this disease, it is important to review the proposed process by which arterial plaques form, and the importance of the competing forces of inflammation and repair throughout.

FORMATION OF THE ATHEROSCLEROTIC PLAQUE: LOCAL AND DISTANT (ESPECIALLY BONE MARROW FUNCTION) MODIFIERS

Although risk factors for atherosclerotic disease systemically affect all regions of the vasculature, plaque formation is not uniformly distributed. Instead, atherosclerotic plaques typically occur at a number of reproducible focal sites such as the inner wall of vessel curvature, the lateral walls of bifurcations, and near branch points (Pan, 2009; Warboys et al., 2011). An elegant theory to explain this plaque distribution disparity relies on the relative amount of shear stress exerted on the ECs in contact with the blood as it traverses the dynamic lumen geometry. There are two dominant forces exerted by blood on the inner lining of ECs within the vasculature. These hemodynamic forces include transmural pressure (a force exerted radially across the vessel wall, **Figure 1A**) and shear stress (the frictional force applied to the ECs by blood tangential to the direction of flow, **Figure 1B**). The value of endothelial shear stress (ESS, sometimes called wall shear stress or WSS) is determined as a function of blood viscosity (η) and its shear rate ($^{\delta v}/_{\delta y}$, a measure of the rate at which blood velocity increases from the endothelium toward the center of the vessel, **Figure 1B**). Blood flow in regions of relatively straight, uniform diameter vessels exhibits nearly laminar flow, resulting in ESS-values of 20–70 dyne/cm^2 (2–7 Pa) (Chatzizisis et al., 2007; Chiu and Chien, 2011). However, at the aforementioned locations prone to atherosclerotic plaque formation, the laminar flow characteristics are disrupted, causing regions of oscillatory or reversed flow and subsequent reduction in ESS (<10 dyne/cm^2, **Figure 1C**). In these regions of disturbed flow, a number of events and responses to low shear stress occur that contribute to the process of atherosclerotic plaque formation.

Following the "response-to-injury" hypothesis of atherosclerosis formation and progression, conditions of low overall ESS have been shown to facilitate additional insults to the endothelium leading to dysfunction. One such path initiated by low ESS is the sustained activation of sterol regulatory elements binding proteins (SREBPs). Once activated, these endoplasmic reticulum (ER)-bound transcription factors contribute to a strong inflammatory response and increase the expression of genes that encode for the LDL receptor (LDLR) (Liu et al., 2002; Xiao et al., 2013). In addition, a large volume of research has described the involvement of the lectin-like oxidized-LDL (ox-LDL) receptor-1 (LOX-1) in the initiation and progression of atherosclerosis (Mehta et al., 2006; Mitra et al., 2011; Pirillo et al., 2013). LOX-1 has been reported to be responsible for the uptake and degradation of ox-LDL, and its expression has been shown *in vitro* to be upregulated by a number of atherosclerosis-related stimuli including shear stress, inflammatory cytokines [such as IL-1β and tumor necrosis factor alpha (TNFα)], and conditions such as hypertension (Mitra et al., 2011; Pirillo et al., 2013). Moreover, it was demonstrated that the deletion of LOX-1 reduced atherosclerosis in a LDLR knockout mouse model of disease (Mehta et al., 2007). Yet another change associated with low ESS is the shift in EC morphology from tightly packed cells aligned with the direction of blood flow to a damaged morphology with cuboidal cells demonstrating no observed preferential alignment, and resulting in leakier junctions between cells (Davies, 2009; Pan, 2009; Linton et al., 2015). This disorganized cell morphology is compounded by the loss of repair mechanisms affected by progenitor cells. As discussed previously, BM-derived progenitor repair cells play a critical role in protecting arteries against atherosclerosis. One reparative role played by these cells is the restoration of damaged endothelial tissue or engraftment into these damaged regions to reverse endothelial injury (Madonna et al., 2016). Turbulent flow may disrupt the interaction between repair-competent cells in the blood and the arterial wall, thus limiting the effectiveness of the repair process (**Figures 1D,E**) (Xu, 2009; Chiu and Chien, 2011). It is also possible that low ESS prevents the wash-out of senescent ECs and macrophages, thus hindering their replacement by fresh such cells (Childs et al., 2016). The net result of the up-regulation of LDLR and the increased cell permeability is a dramatic increase in levels of LDL infiltration in regions of low ESS (**Figure 2**, Stage I) (Chatzizisis et al., 2007). Additional pro-inflammatory pathways activated under conditions of low ESS include the mitogen-activated protein (MAP) kinase and nuclear factor κB (NF-κB) signaling pathways, while levels of miR-10a and its regulatory, anti-inflammatory effects on the NF-κB pathway are attenuated under these conditions (Fang et al., 2010; Bryan et al., 2014). Recently, this strictly dichotomous view of "low" and "high" ESS and its effect on the endothelium and atherosclerosis has

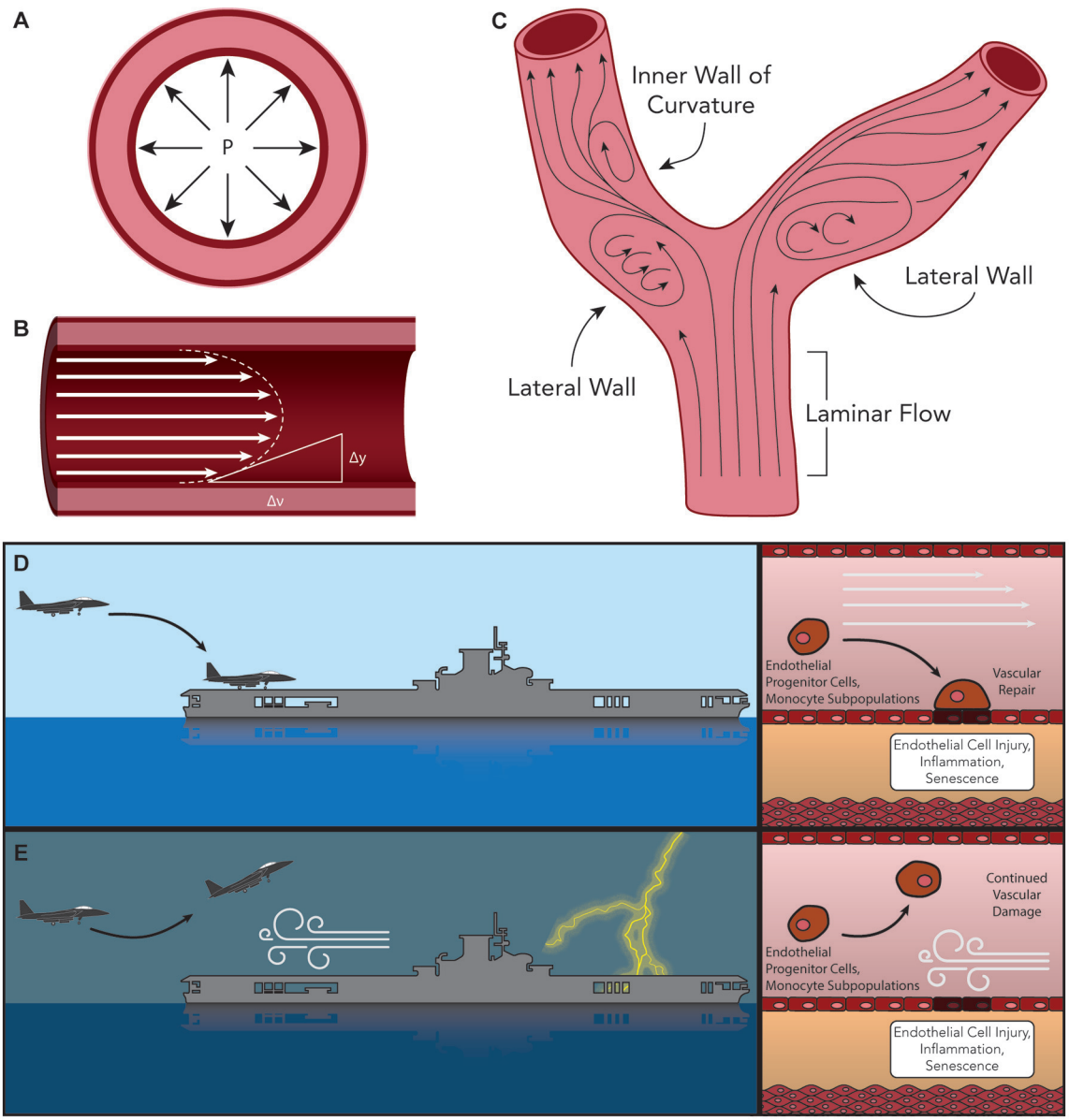

FIGURE 1 | Hemodynamic forces exerted on the arteries. **(A)** Trans-mural pressure exerted radially across the vessel wall. **(B)** Endothelial Shear Stress (ESS), determined as the product of blood viscosity (η) and its shear rate ($^{\delta v}/_{\delta y}$) such that ESS $= \eta \bullet (^{\delta v}/_{\delta y})$. **(C)** Typical sites of atherosclerotic plaque formation (lateral walls of bifurcations, inner wall of vessel curvature, and near branch points). Laminar flow in these regions is disrupted, causing oscillatory or reversed flow and subsequent low ESS. Comparison of the ability of bone marrow (BM)-derived vascular progenitor cells and other reparative factors to interact with injured endothelial cells under **(D)** laminar or **(E)** turbulent flow can be envisioned as the difference between landing a plane under calm or stormy conditions, respectively.

been revisited, and it is now suggested that—while physiological values of ESS appear to be atheroprotective—both "low" and "high" shear stress outside this physiological range may lead to atherosclerosis progression (Hung et al., 2015; Eshtehardi and Teng, 2016).

The combined net result of declining vascular repair mechanisms coupled with endothelial inflammation and the activation of ECs is the increased capture of circulating monocytes. This process, known as the leukocyte adhesion cascade, begins with the interaction of monocytes and displayed adhesion molecules in the regions of inflammation, leading to the stepwise rolling, firm adhesion, and transmigration of the monocyte into the vascular intima (**Figure 2**, Stage II) (Ley et al., 2007; Gerhardt and Ley, 2015). Monocyte recruitment is largely directed by the presence of EC-derived chemokines (including CCL5, CCL2, and CX_3CL1), their respective receptors (including CCR2, CXCR3, CX_3CR1, and others), and various adhesion molecules such as VCAM-1 and ICAM-1 (Geissmann

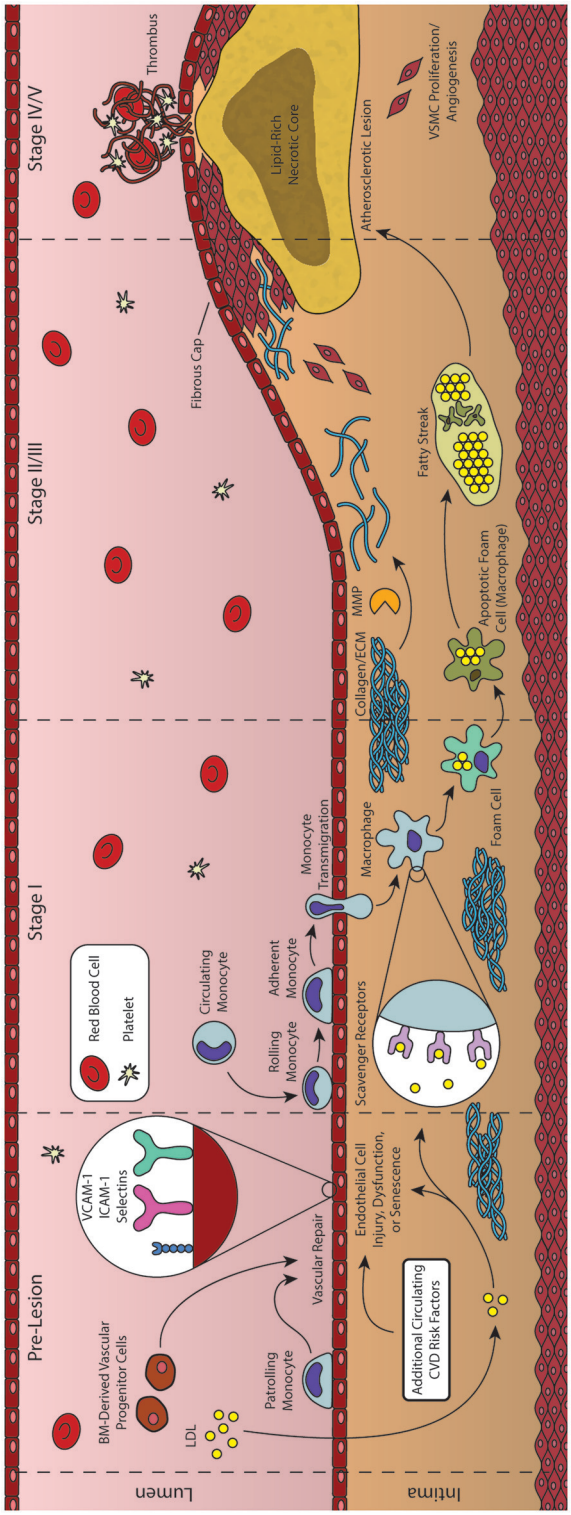

FIGURE 2 | Formation and progression of an atherosclerotic plaque. As an inflammatory disease, the initial stages of atherosclerosis involve an inflammatory insult to the endothelial cells lining the artery lumen. Bone marrow (BM)-derived progenitor cells have been shown to be critical in responding to vascular injury, effecting vascular repair and maintaining homeostasis. In the absence of vascular repair, injured endothelial cells begin to express adhesion molecules that facilitate the transmigration of monocytes into the vessel intima. These monocytes then differentiate into macrophages and begin engulfing lipid and lipid products, forming foam cells. As foam cells aggregate, they form the characteristic fatty streak, while many macrophages begin to undergo apoptosis. Inefficient clearance of apoptotic macrophages leads to secondary necrosis, resulting in a growing lipid-rich necrotic core. In response to the growing lesion, smooth muscle cells migrate to the intima, helping to form the overlying fibrous cap. Rupture of this cap can expose the necrotic core, leading to thrombus formation and subsequent acute cardiovascular events.

(Continued)

et al., 2003; Tacke et al., 2007; Tuttolomondo et al., 2012; van der Vorst et al., 2015). Following transmigration from the lumen into the intima, these monocytes undergo differentiation into macrophages capable of engulfing the growing pool of apoB-containing LDL and its modified variants (Ley et al., 2011). These differentiated macrophages identify and engulf LDL through expression of scavenger receptors such as oxidized LDL receptor 1 (LOX-1), scavenger receptor A (SRA), and CD36 (**Figure 2**, Stage II) (Yoshida and Kisugi, 2010; Ley et al., 2011).

Instructively, through the use of CD36/SRA double knockout mice, it has been shown that lipid-laden macrophages will still accumulate in atherosclerotic vessel walls, suggesting additional routes of lipid uptake (Manning-Tobin et al., 2009). Other proposed mechanisms include the capability of macrophages to accumulate LDL via receptor-independent pinocytosis, and the hydrolysis of free cholesterol from LDL aggregates (Barthwal et al., 2013; Bentzon et al., 2014). Cholesterol liberated from lipoproteins taken up by these pathways is transported within the macrophage to the ER where it is esterified to cholesteryl esters by acetyl-coenzyme A:cholesterol acetyltransferase 1 (ACAT1) and neutral cholesterol ester hydrolase (nCEH), while reverse cholesterol transport is controlled by ATP-binding cassette (ABC) transporters including ABCA1 and ABCG1 (Ley et al., 2011). Under healthy conditions, the liberated cholesterol is accepted by HDL particles primary via protein ApoA1, while modification of this protein can produce dysfunctional HDL and produce proatherogenic effects (Fisher et al., 2012). However, under atherosclerotic conditions, macrophages demonstrate both an increase in lipid uptake and inefficient efflux of cholesteryl esters, generating characteristic lipid-laden foam cells (**Figure 2**, Stage II) (Yu et al., 2013). Agonists for the Liver X Receptor (LXR) transcription factors have been proposed as potential therapeutic agents to improve cholesterol efflux and thus reduce foam cell formation, but have also been shown to induce liver steatosis (Levin et al., 2005; Hijmans et al., 2015). Moreover, while efforts have been made to generate synthetic agonists avoiding this effect, recent results have suggested that these agents do not function directly through cholesterol efflux (Kratzer et al., 2009; Kappus et al., 2014). It has been shown that such defects in lipid metabolism can cause ER stress and dysfunction, ultimately leading to apoptosis of the cell (Seimon et al., 2010). Compounding this problem, defects in cholesterol efflux also inhibit efficient efferocytosis (clearance of apoptotic cells by nearby macrophages), leading to an accumulation of apoptotic

cells in these regions. The growing population of apoptotic macrophages and foam cells generate the characteristic fatty streak associated with early atherosclerotic plaques (**Figure 2**, Stage III/IV) (Moore and Tabas, 2011; Van Vré et al., 2012). Apoptotic cells not cleared through phagocytosis then undergo secondary necrosis, releasing cellular debris along with additional oxidized lipids and pro-inflammatory cytokines that contribute to continued inflammation and a growing necrotic core of the more advanced atherosclerotic lesion (**Figure 2**, Stage IV) (Moore and Tabas, 2011; Bentzon et al., 2014).

Atherogenesis often ceases here, with many lesions progressing no further than the fatty streak, due in large part to various reparative mechanisms such as those initiated by BM-derived progenitor cells (Goldschmidt-Clermont et al., 2012). However, some lesions undergo additional changes within the vascular intima causing a pronounced growth of the plaque and remodeling of the vessel itself. Specifically, macrophages and foam cells within the fatty streak begin releasing inflammatory cytokines (IL-1β, IL-4, and TNFα) that interact with various growth factors [platelet-derived growth factor (PDGF), CD-40] to activate MMPs (Chistiakov et al., 2013). Interestingly, the role of IL-4 has been debated, with early studies demonstrating that IL-4 deficiency in a mouse model of atherosclerosis reduced lesion formation, while later studies found a lack of involvement of this cytokine regardless of disease induction method (Ramji and Davies, 2015). Once activated, these MMPs are responsible for remodeling and weakening the extracellular matrix (ECM) surrounding the vascular smooth muscle cells (VSMC) of the vessel. As a result of the weakened ECM, arterial cells begin to sense transmural pressure as a micro-angioplasty stretch with each pulse, stimulating growth factor release by components of the fatty streak that promote migration of these stretched VSMCs to the growing lesion (Rudijanto, 2007). Under healthy physiological conditions, mature VSMCs rarely undergo proliferation or migration, and exhibit a contractile phenotype (expressing α-smooth muscle actin and calponin) in which they control blood vessel diameter and blood flow through vasodilation or vasoconstriction (Rudijanto, 2007; Zhang et al., 2016). However, in response to injury, VSMCs undergo a transition to a non-contractile or synthetic phenotype, decreasing contractile marker expression, and generating collagen-rich fibrous ECM that eventually represents a major component of this newly formed fibroatheroma (**Figure 2**, Stage V) (Bentzon et al., 2014; Zhang et al., 2016). Complicating this process further, neither elevated levels of cholesterol nor extensive macrophage involvement are strictly required for this VSMC proliferation. As discussed previously, other mechanisms—such as epigenetic modification of specific genes associated with SMC proliferation—have been shown to be involved in the progression of atherosclerotic plaques (Post et al., 1999; Ying et al., 2000). In addition, deposition and growth of calcium granules may also occur, and has been shown to exhibit both stabilizing and destabilizing effects to the vulnerability of the fibrous cap depending on their extent and location (Li et al., 2007). Indeed vascular calcification, a condition pathognomonic for atherosclerosis, may be quantified in the coronary arteries using computed tomography (CT) to generate a coronary artery calcium (CAC) score that is used in conjunction with the Framingham Risk Score to improve prognostic value of coronary events (Yeboah et al., 2012; Maurovich-Horvat et al., 2014). Modulations of the ECM and proliferation of VSMCs are also required for arterial remodeling, which can be either constrictive or expansive (as a compensatory mechanism to counter atherosclerotic plaque growth) (Wentzel et al., 2012; Bentzon et al., 2014). These advanced plaques also possess the potential to undergo rupture or erosion, exposing the thrombogenic core to the blood flow of the vessel lumen (**Figure 2**, Stage VI). A common trait of plaques vulnerable to rupture is a thin covering of ECs, VSMCs, and ECM—termed thin-cap fibroatheromas (TFCAs) and possessing a cap of $<65\,\mu m$ in thickness (Virmani et al., 2000). Activation of MMPs from abundant macrophages present in the plaque may cause degradation of the ECM near the cap, destabilizing EC adhesion and allowing for exposure of the necrotic core, leading to subsequent thrombus formation in the lumen of the vessel. Although this is a rare fate for atherosclerotic plaques (most either do not progress to advanced stages or become stable plaques with caps $>65\,\mu m$), the subsequent formation of a thrombus from plaque rupture can cause complete occlusion of the local artery, with consequent myocardial infarction and/or sudden cardiac death. Another potential thrombogenic fate of an atherosclerotic plaque is that of plaque erosion, in which a thrombus is formed in the absence of rupture, often with a disrupted endothelium and an enhanced proliferation of VSMCs (Virmani et al., 1999; Braunwald, 2013). Although less common than plaque rupture, plaque erosion has been shown to be more common in women and the elderly, while the underlying mechanisms of plaque erosion are only beginning to be elucidated and still incompletely understood (Libby and Pasterkamp, 2015; Chandran et al., 2017). Regardless of the etiology of the plaque source, the resulting thrombus formed by these processes may also embolize and travel to a secondary vessel causing ischemia, myocardial infarction, or sudden coronary death (Davies, 2000; Bentzon et al., 2014). Alternatively, repeated cycles of clinically silent rupture of the cap followed by healing may contribute to overall plaque growth and luminal stenosis. Another potent source of plaque growth is intraplaque hemorrhage. As the atherosclerotic plaque grows, the region becomes progressively more inflamed as well as hypoxic, inducing angiogenesis and vasa vasorum into the plaque itself. These neovessels have been observed to be fragile and leaky, and are an important source of hemorrhage within the wall of the artery, contributing not only to further inflammation, but also plaque growth and vessel stenosis (Kolodgie et al., 2003). In this way, tissue ischemia caused by luminal stenosis can occur even in the absence of clinically significant rupture (Kovacic and Fuster, 2012).

CONTROL OF INFLAMMATION: A NOVEL THERAPEUTIC OPPORTUNITY TO RESTORE ARTERIAL HOMEOSTASIS

Atherosclerosis is the most significant human health problem globally. We know today that the disease does not follow

a simple, unidirectional progression, and is determined by a myriad of pathways, control mechanisms, and repair processes; these encompass multiple inflammatory molecules, BM-derived progenitor cells, a range of immune cells such as specific monocyte subpopulations, genetic mutations, and epigenetic modifications among numerous other participants both known and those yet to be discovered. Ultimately, however, the clinical result for an immensely large number of individuals is the formation and growth of vascular lesions with the potential to rupture, leading to life-threatening conditions. It is imperative to continue to evolve technological strategies to both predict and detect the formation, progression, and clinical status of these atherosclerotic plaques, while additional details are elucidated regarding the process of disease progression. One example of important progress has been made in the control of inflammation when inflammation is no longer promoting repair, but instead has taken a damaging role for the artery. It was recently reported that a monoclonal antibody against IL1-beta, when injected systemically to patient with CVD and high inflammatory index (CRP levels $\geq$ 2 mg/mL), is capable of reducing risk for coronary events, even with already reduced lipids and had no further effect on lipid levels (Ridker et al., 2017). The role of inflammation is increasingly established in the progression of arterial lesions, and it is useful to consider inflammation in the context of arterial homeostasis. Arterial repair is triggered and controlled by molecules that belong to inflammatory pathways. However, as was hypothesized and subsequently demonstrated in an animal model, the progression of atherosclerotic inflammation is modulated by the presence or the absence of an efficient repair process (Goldschmidt-Clermont et al., 2005). In the presence of BM vascular progenitor cells capable of arterial repair, the artery heals and inflammatory signals subside and vanish. However, as previously discussed, reductions in the availability of BM-derived vascular progenitor cells occurring as a consequence of aging or genetic susceptibility (exhaustion or dysfunction) result in a lack of arterial healing. These reductions can occur either because repair-capable cells are no longer produced effectively by the BM, because the produced cells have become dysfunctional, or a combination thereof. Consequently, inflammatory signals do not subside and vanish, and indeed are heightened to the point where they attract and support monocytes/macrophages and other immune competent cells that further enhance arterial injury (**Figure 3**). Hence, the maintenance of arterial homeostasis is a complex process that must balance injuries to the arterial wall (lipids, smoke, etc.), inflammatory processes required for triggering and supporting arterial repair, and the renewal of BM-derived vascular progenitor cells that are necessary for such repair.

The example of inflammation suppression in individuals with established CVD is important, as it proves that inflammation can contribute to the disease, and that the control of excessive inflammation via specific agents can lead to consequent reduction in CVD complications. However, this work addresses only one aspect of the overwhelmingly complex disease that is atherosclerosis. As discussed herein, the pathways and players involved in the regulation and progression of this ubiquitous disease are immensely diverse, and subject to continuous

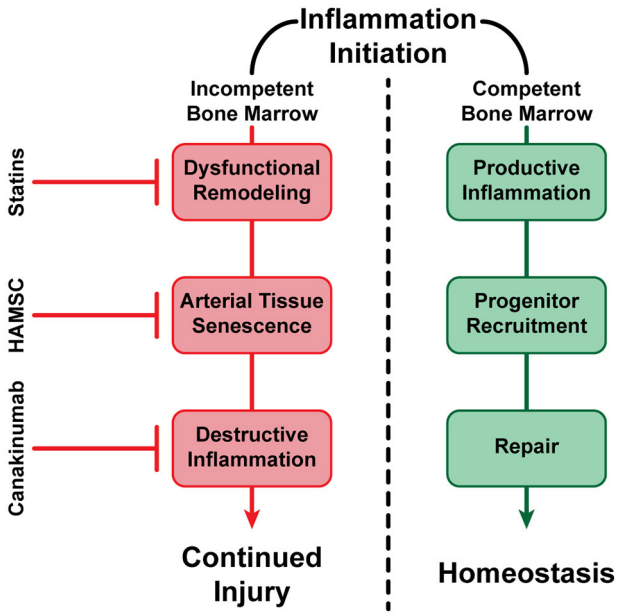

FIGURE 3 | A visualization of alternative fates of vascular inflammation. In the presence of healthy BM-derived vascular progenitor cells capable of arterial repair, inflammation subsides and vanishes (negative feedback loop), resulting in the maintenance of vascular homeostasis. In the absence of sufficient BM-derived vascular progenitor cells, or if such cells are no longer capable of arterial repair, inflammation continues or even expands, resulting in potential dysfunctional remodeling, continued cell senescence, and propagation of arterial injury (positive feedback loop). However, successful treatments have been established such as statin therapy, which has been shown to reduce circulating LDL-Cholesterol (LDL-C), increase HDL-Cholesterol (HDL-C), reducing inflammation and dysfunctional remodeling (Nozue et al., 2013); Human Allogeneic Mesenchymal Stem Cells (HAMSC) (Tompkins et al., 2017), and possibly other progenitor cells (Song et al., 2012), may be able to reverse arterial tissue senescence and improve arterial repair without affecting circulating lipids; and anti-inflammatory molecules such as Canakinumab have also been shown to reduce destructive inflammation without affecting circulating lipids (Ridker et al., 2017). Hence, our armamentarium to prevent, stabilize or even reverse atherosclerosis and its thromboembolic complications are becoming increasingly effective at promoting arterial homeostasis.

reassessment. In much the same way that inflammation has been shown to be involved in the repair of arterial damage or contribute to disease progression when excessive, each component of atherosclerosis may play a critical part depending on the context, while many such components may not yet be fully established. It is instructive that administration of adult mesenchymal stem cells to individuals suffering from aging frailty has been shown, in double blind placebo-controlled prospective randomized trial, to improve functional outcomes of patients, many of which being dependent on the integrity of the cardiovascular system (Tompkins et al., 2017). The concurrent approaches of taming excessive inflammation with specific antagonists, and augmenting the cellular capacity for arterial repair with selected stem cells, in addition to the previously developed armamentarium, may represent the strategies required to restore arterial homeostasis whenever lost, and help to reduce atherosclerosis and its dreadful complications during the twenty-first century.

AUTHOR CONTRIBUTIONS

All authors contributed equally to the drafting, editing, and final composition of the manuscript. TH: designed and prepared the corresponding figures for the manuscript.

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REFERENCES

Ayyadevara, S., Mercanti, F., Wang, X., Mackintosh, S. G., Tackett, A. J., Prayaga, S. V., et al. (2016). Age- and hypertension-associated protein aggregates in mouse heart have similar proteomic profiles. *Hypertension* 67, 1006–1013. doi: 10.1161/HYPERTENSIONAHA.115.06849

Baker, D. J., Childs, B. G., Durik, M., Wijers, M. E., Sieben, C. J., Zhong, J., et al. (2016). Naturally occurring p16(Ink4a)-positive cells shorten healthy lifespan. *Nature* 530, 184–189. doi: 10.1038/nature16932

Baker, D. J., Wijshake, T., Tchkonia, T., LeBrasseur, N. K., Childs, B. G., van de Sluis, B., et al. (2011). Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. *Nature* 479, 232–236. doi: 10.1038/nature10600

Barthwal, M. K., Anzinger, J. J., Xu, Q., Bohnacker, T., Wymann, M. P., and Kruth, H. S. (2013). Fluid-phase pinocytosis of native low density lipoprotein promotes murine M-CSF differentiated macrophage foam cell formation. *PLoS ONE* 8:e58054. doi: 10.1371/journal.pone.0058054

Benjamin, E. J., Blaha, M. J., Chiuve, S. E., Cushman, M., Das, S. R., Deo, R., et al. (2017). Heart disease and stroke statistics-—2017 update: a report from the american heart association. *Circulation* 135, e146–e603. doi: 10.1161/CIR.0000000000000485

Bentzon, J. F., Otsuka, F., Virmani, R., and Falk, E. (2014). Mechanisms of plaque formation and rupture. *Circ. Res.* 114, 1852–1866. doi: 10.1161/CIRCRESAHA.114.302721

Braunwald, E. (2013). Coronary plaque erosion: recognition and management. *JACC Cardiovasc. Imaging* 6, 288–289. doi: 10.1016/j.jcmg.2013.01.003

Bryan, M. T., Duckles, H., Feng, S., Hsiao, S. T., Kim, H. R., Serbanovic-Canic, J., et al. (2014). Mechanoresponsive networks controlling vascular inflammation. *Arterioscler. Thromb. Vasc. Biol.* 34, 2199–2205. doi: 10.1161/ATVBAHA.114.303424

Chandran, S., Watkins, J., Abdul-Aziz, A., Shafat, M., Calvert, P. A., Bowles, K. M., et al. (2017). Inflammatory differences in plaque erosion and rupture in patients with ST-segment elevation myocardial infarction. *J. Am. Heart Assoc.* 6:e005868. doi: 10.1161/JAHA.117.005868

Chatzizisis, Y. S., Coskun, A. U., Jonas, M., Edelman, E. R., Feldman, C. L., and Stone, P. H. (2007). Role of endothelial shear stress in the natural history of coronary atherosclerosis and vascular remodeling: molecular, cellular, and vascular behavior. *J. Am. Coll. Cardiol.* 49, 2379–2393. doi: 10.1016/j.jacc.2007.02.059

Childs, B. G., Baker, D. J., Wijshake, T., Conover, C. A., Campisi, J., and van Deursen, J. M. (2016). Senescent intimal foam cells are deleterious at all stages of atherosclerosis. *Science* 354, 472–477. doi: 10.1126/science.aaf6659

Childs, B. G., Gluscevic, M., Baker, D. J., Laberge, R. M., Marquess, D., Dananberg, J., et al. (2017). Senescent cells: an emerging target for diseases of ageing. *Nat. Rev. Drug. Discov.* 16, 718–735. doi: 10.1038/nrd.2017.116

Chistiakov, D. A., Sobenin, I. A., and Orekhov, A. N. (2013). Vascular extracellular matrix in atherosclerosis. *Cardiol. Rev.* 21, 270–288. doi: 10.1097/CRD.0b013e31828c5ced

Chiu, J. J., and Chien, S. (2011). Effects of disturbed flow on vascular endothelium: pathophysiological basis and clinical perspectives. *Physiol. Rev.* 91, 327–387. doi: 10.1152/physrev.00047.2009

Clarke, E. M., Thompson, R. C., Allam, A. H., Wann, L. S., Lombardi, G. P., Sutherland, M. L., et al. (2014). Is atherosclerosis fundamental to human aging? Lessons from ancient mummies. *J. Cardiol.* 63, 329–334. doi: 10.1016/j.jjcc.2013.12.012

D'Agostino, R. B. Sr., Vasan, R. S., Pencina, M. J., Wolf, P. A., Cobain, M., Massaro, J. M., et al. (2008). General cardiovascular risk profile for use in primary care: the framingham heart study. *Circulation* 117, 743–753. doi: 10.1161/CIRCULATIONAHA.107.699579

Davies, M. J. (2000). The pathophysiology of acute coronary syndromes. *Heart* 83, 361–366. doi: 10.1136/heart.83.3.361

Davies, P. F. (2009). Hemodynamic shear stress and the endothelium in cardiovascular pathophysiology. *Nat. Clin. Pract. Cardiovasc. Med.* 6, 16–26. doi: 10.1038/ncpcardio1397

Dunn, J., Qiu, H., Kim, S., Jjingo, D., Hoffman, R., Kim, C. W., et al. (2014). Flow-dependent epigenetic DNA methylation regulates endothelial gene expression and atherosclerosis. *J. Clin. Invest.* 124, 3187–3199. doi: 10.1172/JCI74792

Enos, W. F., Holmes, R. H., and Beyer, J. (1953). Coronary disease among united states soldiers killed in action in korea: preliminary report. *J. Am. Med. Assoc.* 152, 1090–1093. doi: 10.1001/jama.1953.03690120006002

Eshtehardi, P., and Teng, Z. (2016). Protective or destructive: high wall shear stress and atherosclerosis. *Atherosclerosis* 251, 501–503. doi: 10.1016/j.atherosclerosis.2016.05.046

Fang, Y., Shi, C., Manduchi, E., Civelek, M., and Davies, P. F. (2010). MicroRNA-10a regulation of proinflammatory phenotype in athero-susceptible endothelium *in vivo* and *in vitro*. *Proc. Natl. Acad. Sci. U.S.A.* 107, 13450–13455. doi: 10.1073/pnas.1002120107

Ference, B. A., Ginsberg, H. N., Graham, I., Ray, K. K., Packard, C. J., Bruckert, E., et al. (2017). Low-density lipoproteins cause atherosclerotic cardiovascular disease. 1. Evidence from genetic, epidemiologic, and clinical studies. A consensus statement from the European Atherosclerosis Society Consensus Panel. *Eur. Heart J.* 38, 2459–2472. doi: 10.1093/eurheartj/ehx144

Fisher, E. A., Feig, J. E., Hewing, B., Hazen, S. L., and Smith, J. D. (2012). High-density lipoprotein function, dysfunction, and reverse cholesterol transport. *Arterioscler. Thromb. Vasc. Biol.* 32, 2813–2820. doi: 10.1161/ATVBAHA.112.300133

Furman, D., Chang, J., Lartigue, L., Bolen, C. R., Haddad, F., Gaudilliere, B., et al. (2017). Expression of specific inflammasome gene modules stratifies older individuals into two extreme clinical and immunological states. *Nat. Med.* 23, 174–184. doi: 10.1038/nm.4267

Geissmann, F., Jung, S., and Littman, D. R. (2003). Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* 19, 71–82. doi: 10.1016/S1074-7613(03)00174-2

Gerhardt, T., and Ley, K. (2015). Monocyte trafficking across the vessel wall. *Cardiovasc. Res.* 107, 321–330. doi: 10.1093/cvr/cvv147

Goff, D. C. Jr., Lloyd-Jones, D. M., Bennett, G., Coady, S., D'Agostino, R. B. Sr., Gibbons, R., et al. (2014). 2013 ACC/AHA guideline on the assessment of cardiovascular risk: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. *J. Am. Coll. Cardiol.* 63(25 Pt B), 2935–2959. doi: 10.1016/j.jacc.2013.11.005

Goldschmidt-Clermont, P. J. (2003). Loss of bone marrow-derived vascular progenitor cells leads to inflammation and atherosclerosis. *Am. Heart J.* 146(4 Suppl.), S5–S12. doi: 10.1016/j.ahj.2003.09.002

Goldschmidt-Clermont, P. J., Creager, M. A., Losordo, D. W., Lam, G. K., Wassef, M., and Dzau, V. J. (2005). Atherosclerosis 2005: recent discoveries and novel hypotheses. *Circulation* 112, 3348–3353. doi: 10.1161/CIRCULATIONAHA.105.577460

Goldschmidt-Clermont, P. J., Dong, C., Seo, D. M., and Velazquez, O. C. (2012). Atherosclerosis, inflammation, genetics, and stem cells: 2012 update. *Curr. Atheroscler. Rep.* 14, 201–210. doi: 10.1007/s11883-012-0244-1

Gordon, S., and Taylor, P. R. (2005). Monocyte and macrophage heterogeneity. *Nat. Rev. Immunol.* 5, 953–964. doi: 10.1038/nri1733

Hijmans, B. S., Tiemann, C. A., Grefhorst, A., Boesjes, M., van Dijk, T. H., Tietge, U. J., et al. (2015). A systems biology approach reveals the physiological origin of hepatic steatosis induced by liver X receptor activation. *FASEB J.* 29, 1153–1164. doi: 10.1096/fj.14-254656

Hilgendorf, I., Swirski, F. K., and Robbins, C. S. (2015). Monocyte fate in atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* 35, 272–279. doi: 10.1161/ATVBAHA.114.303565

Hippisley-Cox, J., Coupland, C., Vinogradova, Y., Robson, J., Minhas, R., Sheikh, A., et al. (2008). Predicting cardiovascular risk in England and Wales: prospective derivation and validation of QRISK2. *BMJ* 336, 1475–1482. doi: 10.1136/bmj.39609.449676.25

Hong, Y. M. (2010). Atherosclerotic cardiovascular disease beginning in childhood. *Korean Circ. J.* 40, 1–9. doi: 10.4070/kcj.2010.40.1.1

Hung, O. Y., Brown, A. J., Ahn, S. G., Veneziani, A., Giddens, D. P., and Samady, H. (2015). Association of wall shear stress with coronary plaque progression and transformation. *Interv. Cardiol. Clin.* 4, 491–502. doi: 10.1016/j.iccl.2015.06.009

Jaiswal, S., Natarajan, P., Silver, A. J., Gibson, C. J., Bick, A. G., Shvartz, E., et al. (2017). Clonal hematopoiesis and risk of atherosclerotic cardiovascular disease. *N. Engl. J. Med.* 377, 111–121. doi: 10.1056/NEJMoa1701719

Kappus, M. S., Murphy, A. J., Abramowicz, S., Ntonga, V., Welch, C. L., Tall, A. R., et al. (2014). Activation of liver X receptor decreases atherosclerosis in Ldlr(-)/(-) mice in the absence of ATP-binding cassette transporters A1 and G1 in myeloid cells. *Arterioscler. Thromb. Vasc. Biol.* 34, 279–284. doi: 10.1161/ATVBAHA.113.302781

Karra, R., Vemullapalli, S., Dong, C., Herderick, E. E., Song, X., Slosek, K., et al. (2005). Molecular evidence for arterial repair in atherosclerosis. *Proc. Natl. Acad. Sci. U.S.A.* 102, 16789–16794. doi: 10.1073/pnas.0507718102

Kolodgie, F. D., Gold, H. K., Burke, A. P., Fowler, D. R., Kruth, H. S., Weber, D. K., et al. (2003). Intraplaque hemorrhage and progression of coronary atheroma. *N. Engl. J. Med.* 349, 2316–2325. doi: 10.1056/NEJMoa035655

Kovacic, J. C., and Fuster, V. (2012). Smoking gun theory: angiographically normal or mild coronary plaque as a cause of myocardial infarction. *Circulation* 126, 2918–2920. doi: 10.1161/CIRCULATIONAHA.112.150425

Kratzer, A., Buchebner, M., Pfeifer, T., Becker, T. M., Uray, G., Miyazaki, M., et al. (2009). Synthetic LXR agonist attenuates plaque formation in apoE$^{-/-}$ mice without inducing liver steatosis and hypertriglyceridemia. *J. Lipid. Res.* 50, 312–326. doi: 10.1194/jlr.M800376-JLR200

Levin, N., Bischoff, E. D., Daige, C. L., Thomas, D., Vu, C. T., Heyman, R. A., et al. (2005). Macrophage liver X receptor is required for antiatherogenic activity of LXR agonists. *Arterioscler. Thromb. Vasc. Biol.* 25, 135–142. doi: 10.1161/01.ATV.0000150044.84012.68

Levy, D. (2012). Combating the epidemic of heart disease. *JAMA* 308, 2624–2625. doi: 10.1001/jama.2012.164971

Ley, K., Laudanna, C., Cybulsky, M. I., and Nourshargh, S. (2007). Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat. Rev. Immunol.* 7, 678–689. doi: 10.1038/nri2156

Ley, K., Miller, Y. I., and Hedrick, C. C. (2011). Monocyte and macrophage dynamics during atherogenesis. *Arterioscler. Thromb. Vasc. Biol.* 31, 1506–1516. doi: 10.1161/ATVBAHA.110.221127

Li, Z. Y., Howarth, S., Tang, T., Graves, M., U-King-Im, J., and Gillard, J. H. (2007). Does calcium deposition play a role in the stability of atheroma? Location may be the key. *Cerebrovasc. Dis.* 24, 452–459. doi: 10.1159/000108436

Libby, P. (2002). Inflammation in atherosclerosis. *Nature* 420, 868–874. doi: 10.1038/nature01323

Libby, P. (2016). Assisted living in the atheroma: elderly macrophages promote plaques. *Cell Metab.* 24, 779–781. doi: 10.1016/j.cmet.2016.11.013

Libby, P., and Pasterkamp, G. (2015). Requiem for the 'vulnerable plaque'. *Eur. Heart J.* 36, 2984–2987. doi: 10.1093/eurheartj/ehv349

Libby, P., Ridker, P. M., and Hansson, G. K., Leducq Transatlantic Network on Atherothrombosis. (2009). Inflammation in atherosclerosis: from pathophysiology to practice. *J. Am. Coll. Cardiol.* 54, 2129–2138. doi: 10.1016/j.jacc.2009.09.009

Libby, P., Ridker, P. M., and Maseri, A. (2002). Inflammation and atherosclerosis. *Circulation* 105, 1135–1143. doi: 10.1161/hc0902.104353

Linton, M. F., Yancey, P. G., Davies, S. S., Jerome, W. G. J., Linton, E. F., and Vickers, K. C. (2015). "The role of lipids and lipoproteins in atherosclerosis (2015 update)," in *Endotext,* eds L. J. De Groot, G. Chrousos, K. Dungan, K. R. Feingold, A. Grossman, J. M. Hershman, C. Koch, M. Korbonits, R. McLachlan, M. New, J. Purnell, R. Rebar, F. Singer, and A. Vinik. (South Dartmouth, MA: MDText.com, Inc.).

Liu, Y., Chen, B. P., Lu, M., Zhu, Y., Stemerman, M. B., Chien, S., et al. (2002). Shear stress activation of SREBP1 in endothelial cells is mediated by integrins. *Arterioscler. Thromb. Vasc. Biol.* 22, 76–81. doi: 10.1161/hq0102.101822

Madonna, R., Novo, G., and Balistreri, C. R. (2016). Cellular and molecular basis of the imbalance between vascular damage and repair in ageing and age-related diseases: as biomarkers and targets for new treatments. *Mech. Ageing Dev.* 159, 22–30. doi: 10.1016/j.mad.2016.03.005

Manning-Tobin, J. J., Moore, K. J., Seimon, T. A., Bell, S. A., Sharuk, M., Alvarez-Leite, J. I., et al. (2009). Loss of SR-A and CD36 activity reduces atherosclerotic lesion complexity without abrogating foam cell formation in hyperlipidemic mice. *Arterioscler. Thromb. Vasc. Biol.* 29, 19–26. doi: 10.1161/ATVBAHA.108.176644

Maurovich-Horvat, P., Ferencik, M., Voros, S., Merkely, B., and Hoffmann, U. (2014). Comprehensive plaque assessment by coronary CT angiography. *Nat. Rev. Cardiol.* 11, 390–402. doi: 10.1038/nrcardio.2014.60

McCurdy, E. (1923). *Leonardo da Vinci's Note-Books: Arranged and Rendered into English*. New York, NY: Empire state book company.

McGill, H. C. Jr., McMahan, C. A., Herderick, E. E., Malcom, G. T., Tracy, R. E., and Strong, J. P. (2000). Origin of atherosclerosis in childhood and adolescence. *Am. J. Clin. Nutr.* 72(5 Suppl.), 1307S–1315S.

Mehta, J. L., Chen, J., Hermonat, P. L., Romeo, F., and Novelli, G. (2006). Lectin-like, oxidized low-density lipoprotein receptor-1 (LOX-1): a critical player in the development of atherosclerosis and related disorders. *Cardiovasc. Res.* 69, 36–45. doi: 10.1016/j.cardiores.2005.09.006

Mehta, J. L., Sanada, N., Hu, C. P., Chen, J., Dandapat, A., Sugawara, F., et al. (2007). Deletion of LOX-1 reduces atherogenesis in LDLR knockout mice fed high cholesterol diet. *Circ. Res.* 100, 1634–1642. doi: 10.1161/CIRCRESAHA.107.149724

Members, W. G., Mozaffarian, D., Benjamin, E. J., Go, A. S., Arnett, D. K., Blaha, M. J., et al. (2016). Heart disease and stroke statistics-2016 update: a report from the american heart association. *Circulation* 133, e38–e360. doi: 10.1161/CIR.0000000000000350

Mitra, S., Goyal, T., and Mehta, J. L. (2011). Oxidized, LDL, LOX-1 and atherosclerosis. *Cardiovasc. Drugs Ther.* 25, 419–429. doi: 10.1007/s10557-011-6341-5

Moore, K. J., and Tabas, I. (2011). Macrophages in the pathogenesis of atherosclerosis. *Cell* 145, 341–355. doi: 10.1016/j.cell.2011.04.005

Myerburg, R. J., and Junttila, M. J. (2012). Sudden cardiac death caused by coronary heart disease. *Circulation* 125, 1043–1052. doi: 10.1161/CIRCULATIONAHA.111.023846

Nozue, T., Yamamoto, S., Tohyama, S., Fukui, K., Umezawa, S., Onishi, Y., et al. (2013). Comparison of change in coronary atherosclerosis in patients with stable vs. unstable angina pectoris receiving statin therapy (from the Treatment With Statin on Atheroma Regression Evaluated by Intravascular Ultrasound With Virtual Histology [TRUTH] study). *Am. J. Cardiol.* 111, 923–929. doi: 10.1016/j.amjcard.2012.12.008

Pan, S. (2009). Molecular mechanisms responsible for the atheroprotective effects of laminar shear stress. *Antioxid. Redox Signal.* 11, 1669–1682. doi: 10.1089/ars.2009.2487

Passlick, B., Flieger, D., and Ziegler-Heitbrock, H. W. (1989). Identification and characterization of a novel monocyte subpopulation in human peripheral blood. *Blood* 74, 2527–2534.

Pirillo, A., Norata, G. D., and Catapano, A. L. (2013). LOX-1, OxLDL, and atherosclerosis. *Mediators Inflamm.* 2013:152786. doi: 10.1155/2013/152786

Post, W. S., Goldschmidt-Clermont, P. J., Wilhide, C. C., Heldman, A. W., Sussman, M. S., Ouyang, P., et al. (1999). Methylation of the estrogen receptor gene is associated with aging and atherosclerosis in the cardiovascular system. *Cardiovasc. Res.* 43, 985–991. doi: 10.1016/S0008-6363(99)00153-4

Quintar, A., McArdle, S., Wolf, D., Marki, A., Ehinger, E., Vassallo, M., et al. (2017). Endothelial protective monocyte patrolling in large arteries intensified by western diet and atherosclerosis. *Circ. Res.* 120, 1789–1799. doi: 10.1161/CIRCRESAHA.117.310739

Ramji, D. P., and Davies, T. S. (2015). Cytokines in atherosclerosis: key players in all stages of disease and promising therapeutic targets. *Cytokine Growth Factor Rev.* 26, 673–685. doi: 10.1016/j.cytogfr.2015.04.003

Rauscher, F. M., Goldschmidt-Clermont, P. J., Davis, B. H., Wang, T., Gregg, D., Ramaswami, P., et al. (2003). Aging, progenitor cell exhaustion, and atherosclerosis. *Circulation* 108, 457–463. doi: 10.1161/01.CIR.0000082924.75945.48

Ridker, P. M., Everett, B. M., Thuren, T., MacFadyen, J. G., Chang, W. H., Ballantyne, C., et al. (2017). Antiinflammatory therapy with canakinumab for atherosclerotic disease. *N. Engl. J. Med.* 377, 1119–1131. doi: 10.1056/NEJMoa1707914

Rosenson, R. S., Brewer, H. B. Jr., Ansell, B. J., Barter, P., Chapman, M. J., Heinecke, J. W., et al. (2016). Dysfunctional HDL and atherosclerotic cardiovascular disease. *Nat. Rev. Cardiol.* 13, 48–60. doi: 10.1038/nrcardio.2015.124

Ross, R. (1999). Atherosclerosis–an inflammatory disease. *N. Engl. J. Med.* 340, 115–126. doi: 10.1056/NEJM199901143400207

Rudijanto, A. (2007). The role of vascular smooth muscle cells on the pathogenesis of atherosclerosis. *Acta Med. Indones.* 39, 86–93.

Seimon, T. A., Nadolski, M. J., Liao, X., Magallon, J., Nguyen, M., Feric, N. T., et al. (2010). Atherogenic lipids and lipoproteins trigger CD36-TLR2-dependent apoptosis in macrophages undergoing endoplasmic reticulum stress. *Cell Metab.* 12, 467–482. doi: 10.1016/j.cmet.2010.09.010

Song, X., Ma, Q., Liu, X., Seo, P., Herderick, E., Webster, K., et al. (2012). Will periodic intravenous injections of conditioned bone marrow cells effectively reduce atherosclerosis? *Antioxid Redox. Signal.* 16, 85–91. doi: 10.1089/ars.2011.4139

Stansfield, B. K., and Ingram, D. A. (2015). Clinical significance of monocyte heterogeneity. *Clin. Transl. Med.* 4:5. doi: 10.1186/s40169-014-0040-3

Steensma, D. P., Bejar, R., Jaiswal, S., Lindsley, R. C., Sekeres, M. A., Hasserjian, R. P., et al. (2015). Clonal hematopoiesis of indeterminate potential and its distinction from myelodysplastic syndromes. *Blood* 126, 9–16. doi: 10.1182/blood-2015-03-631747

Swirski, F. K., Libby, P., Aikawa, E., Alcaide, P., Luscinskas, F. W., Weissleder, R., et al. (2007). Ly-6Chi monocytes dominate hypercholesterolemia-associated monocytosis and give rise to macrophages in atheromata. *J. Clin. Invest.* 117, 195–205. doi: 10.1172/JCI29950

Tacke, F., Alvarez, D., Kaplan, T. J., Jakubzick, C., Spanbroek, R., Llodra, J., et al. (2007). Monocyte subsets differentially employ CCR2, CCR5, and CX3CR1 to accumulate within atherosclerotic plaques. *J. Clin. Invest.* 117, 185–194. doi: 10.1172/JCI28549

Thomas, G. S., Wann, L. S., Allam, A. H., Thompson, R. C., Michalik, D. E., Sutherland, M. L., et al. (2014). Why did ancient people have atherosclerosis?: from autopsies to computed tomography to potential causes. *Glob. Heart* 9, 229–237. doi: 10.1016/j.gheart.2014.04.002

Thomas, G., Tacke, R., Hedrick, C. C., and Hanna, R. N. (2015). Nonclassical patrolling monocyte function in the vasculature. *Arterioscler. Thromb. Vasc. Biol.* 35, 1306–1316. doi: 10.1161/ATVBAHA.114.304650

Thompson, R. C., Allam, A. H., Lombardi, G. P., Wann, L. S., Sutherland, M. L., Sutherland, J. D., et al. (2013). Atherosclerosis across 4000 years of human history: the horus study of four ancient populations. *Lancet* 381, 1211–1222. doi: 10.1016/S0140-6736(13)60598-X

Tompkins, B. A., DiFede, D. L., Khan, A., Landin, A. M., Schulman, I. H., Pujol, M. V., et al. (2017). Allogenic mesenchymal stem cells ameliorate aging frailty a phase ii randomized, double-blinded, placebo-controlled clinical trial. *J. Gerontol. A Biol. Sci. Med. Sci.* 72, 1513–1522. doi: 10.1093/gerona/glx137

Tuttolomondo, A., Di Raimondo, D., Pecoraro, R., Arnao, V., Pinto, A., and Licata, G. (2012). Atherosclerosis as an inflammatory disease. *Curr. Pharm. Des.* 18, 4266–4288. doi: 10.2174/138161212802481237

van der Vorst, E. P., Döring, Y., and Weber, C. (2015). Chemokines and their receptors in Atherosclerosis. *J. Mol. Med.* 93, 963–971. doi: 10.1007/s00109-015-1317-8

Van Vré, E. A., Ait-Oufella, H., Tedgui, A., and Mallat, Z. (2012). Apoptotic cell death and efferocytosis in atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* 32, 887–893. doi: 10.1161/ATVBAHA.111.224873

Virmani, R., Burke, A. P., and Farb, A. (1999). Plaque rupture and plaque erosion. *Thromb. Haemost.* 82(Suppl. 1), 1–3.

Virmani, R., Kolodgie, F. D., Burke, A. P., Farb, A., and Schwartz, S. M. (2000). Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions. *Arterioscler. Thromb. Vasc. Biol.* 20, 1262–1275. doi: 10.1161/01.ATV.20.5.1262

Warboys, C. M., Amini, N., de Luca, A., and Evans, P. C. (2011). The role of blood flow in determining the sites of atherosclerotic plaques. *F1000 Med. Rep.* 3:5. doi: 10.3410/M3-5

Webber, B. J., Seguin, P. G., Burnett, D. G., Clark, L. L., and Otto, J. L. (2012). Prevalence of and risk factors for autopsy-determined atherosclerosis among US service members, 2001-2011. *JAMA* 308, 2577–2583. doi: 10.1001/jama.2012.70830

Wentzel, J. J., Chatzizisis, Y. S., Gijsen, F. J., Giannoglou, G. D., Feldman, C. L., and Stone, P. H. (2012). Endothelial shear stress in the evolution of coronary atherosclerotic plaque and vascular remodelling: current understanding and remaining questions. *Cardiovasc. Res.* 96, 234–243. doi: 10.1093/cvr/cvs217

Wick, G., and Grundtman, C. (2012). *Inflammation and Atherosclerosis.* Wien: Springer.

Willis, M. S., and Patterson, C. (2013). Proteotoxicity and cardiac dysfunction–Alzheimer's disease of the heart? *N. Engl. J. Med.* 368, 455–464. doi: 10.1056/NEJMra1106180

Woollard, K. J., and Geissmann, F. (2010). Monocytes in atherosclerosis: subsets and functions. *Nat. Rev. Cardiol.* 7, 77–86. doi: 10.1038/nrcardio.2009.228

Xiao, H., Lu, M., Lin, T. Y., Chen, Z., Chen, G., Wang, W. C., et al. (2013). Sterol regulatory element binding protein 2 activation of NLRP3 inflammasome in endothelium mediates hemodynamic-induced atherosclerosis susceptibility. *Circulation* 128, 632–642. doi: 10.1161/CIRCULATIONAHA.113.002714

Xu, Q. (2009). Disturbed flow-enhanced endothelial turnover in atherosclerosis. *Trends Cardiovasc. Med.* 19, 191–195. doi: 10.1016/j.tcm.2009.12.002

Yeboah, J., McClelland, R. L., Polonsky, T. S., Burke, G. L., Sibley, C. T., O'Leary, D., et al. (2012). Comparison of novel risk markers for improvement in cardiovascular risk assessment in intermediate-risk individuals. *JAMA* 308, 788–795. doi: 10.1001/jama.2012.9624

Ying, A. K., Hassanain, H. H., Roos, C. M., Smiraglia, D. J., Issa, J. J., Michler, R. E., et al. (2000). Methylation of the estrogen receptor-alpha gene promoter is selectively increased in proliferating human aortic smooth muscle cells. *Cardiovasc. Res.* 46, 172–179. doi: 10.1016/S0008-6363(00)00004-3

Yoshida, H., and Kisugi, R. (2010). Mechanisms of LDL oxidation. *Clin. Chim. Acta* 411, 1875–1882. doi: 10.1016/j.cca.2010.08.038

Yu, X. H., Fu, Y. C., Zhang, D. W., Yin, K., and Tang, C. K. (2013). Foam cells in atherosclerosis. *Clin. Chim. Acta* 424, 245–252. doi: 10.1016/j.cca.2013.06.006

Yusuf, S., Hawken, S., Ounpuu, S., Dans, T., Avezum, A., Lanas, F., et al. (2004). Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. *Lancet* 364, 937–952. doi: 10.1016/S0140-6736(04)17018-9

Zhang, Y. N., Xie, B. D., Sun, L., Chen, W., Jiang, S. L., Liu, W., et al. (2016). Phenotypic switching of vascular smooth muscle cells in the 'normal region' of aorta from atherosclerosis patients is regulated by miR-145. *J. Cell. Mol. Med.* 20, 1049–1061. doi: 10.1111/jcmm.12825

Ziegler-Heitbrock, L., Ancuta, P., Crowe, S., Dalod, M., Grau, V., Hart, D. N., et al. (2010). Nomenclature of monocytes and dendritic cells in blood. *Blood* 116, e74–e80. doi: 10.1182/blood-2010-02-258558

Zieske, A. W., Malcom, G. T., and Strong, J. P. (2002). Natural history and risk factors of atherosclerosis in children and youth: the PDAY study. *Pediatr. Pathol. Mol. Med.* 21, 213–237. doi: 10.1080/pdp.21.2.213.237

Zink, A., Wann, L. S., Thompson, R. C., Keller, A., Maixner, F., Allam, A. H., et al. (2014). Genomic correlates of atherosclerosis in ancient humans. *Glob. Heart* 9, 203–209. doi: 10.1016/j.gheart.2014.03.2453

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frontiers in Genetics

REVIEW
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Non coding RNAs in aortic aneurysmal disease

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An aneurysm is a local dilatation of a vessel wall which is >50% its original diameter. Within the spectrum of cardiovascular diseases, aortic aneurysms are among the most challenging to treat. Most patients present acutely after aneurysm rupture or dissection from a previous asymptomatic condition and are managed by open surgical or endovascular repair. In addition, patients may harbor concurrent disease contraindicating surgical intervention. Collectively, these factors have driven the search for alternative methods of identifying, monitoring and treating aortic aneurisms using less invasive approaches. Non-coding RNA (ncRNAs) are emerging as new fundamental regulators of gene expression. The small microRNAs have opened the field of ncRNAs capturing the attention of basic and clinical scientists for their potential to become new therapeutic targets and clinical biomarkers for aortic aneurysm. More recently, long ncRNAs (lncRNAs) have started to be actively investigated, leading to first exciting reports, which further suggest their important and yet largely unexplored contribution to vascular physiology and disease. This review introduces the different ncRNA types and focus at ncRNA roles in aorta aneurysms. We discuss the potential of therapeutic interventions targeting ncRNAs and we describe the research models allowing for mechanistic studies and clinical translation attempts for controlling aneurysm progression. Furthermore, we discuss the potential role of microRNAs and lncRNAs as clinical biomarkers.

Keywords: microRNAs, long non-coding RNAs, aneurysms, vascular cells, therapeutic targets, biomarkers

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Introduction

Arterial aneurysms (AAs) are asymmetrical dilatations of arterial wall, most commonly found in the infra-renal abdominal aorta (see **Figure 1**). Lesions develop when intraluminal pressures exceed the capacity of the arterial wall which has developed a localized weakness. It is estimated that abdominal aortic aneurysms (AAA) account for approximately 2–4% of all deaths in men aged 65 and over (Clouse et al., 1998; Booher and Eagle, 2011). Thoracic aortic

Abbreviations: AA, arterial aneurysm; AAA, abdominal aorta aneurysm; Ang II, angiotensin II; ANRIL, antisense non-coding RNA in the INK4 locus; ARF, alternate reading frame; BAV, bicuspid aortic valve; BRG, Brahma-related gene; DOCK: dedicator of cytokinesis; EC, endothelial cell; ECM, extracellular matrix; ELN, elastin; ERK, extracellular-signal-regulated kinase; HIF, hypoxia-inducible factor; JNK, c-Jun NH(2)-terminal kinase; KLF, Krüppel-like factor; lncRNA, long non coding RNA; miRNA, miR, microRNA; MMP, matrix metalloproteinases; ncRNA: non coding RNA; PPE, porcine pancreatic elastase; PTEN, Phosphatase and tensin homolog; rRNA, ribosomal RNA; SMC, smooth muscle cell; SnoRNA, small nucleolar RNA; SNP, single nucleotide polymorphism; TAA, thoracic aortic aneurysm; TIMP, tissue inhibitors of metalloproteinase; TGFβ, transforming growth factor β; TNFα, tumor necrosis factor α; tRNA, transfer RNA; VSMC, vascular smooth muscle cell.

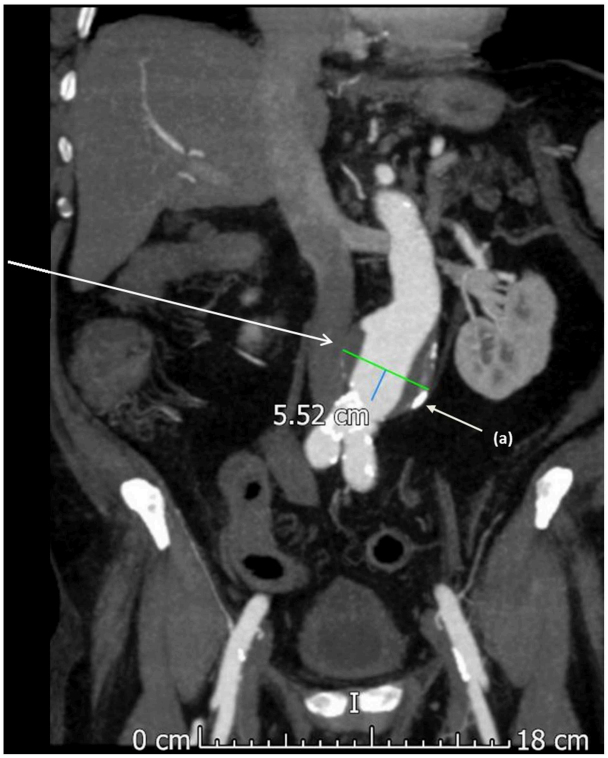

FIGURE 1 | A computed tomography (CT) scan with contrast showing an abdominal aortic aneurysm. This aneurysm was asymptomatic and found during routine surveillance scanning of a 67 year old male. Calcified atherosclerotic plaque is noted on the aneurysm wall and in the distal aorta (a).

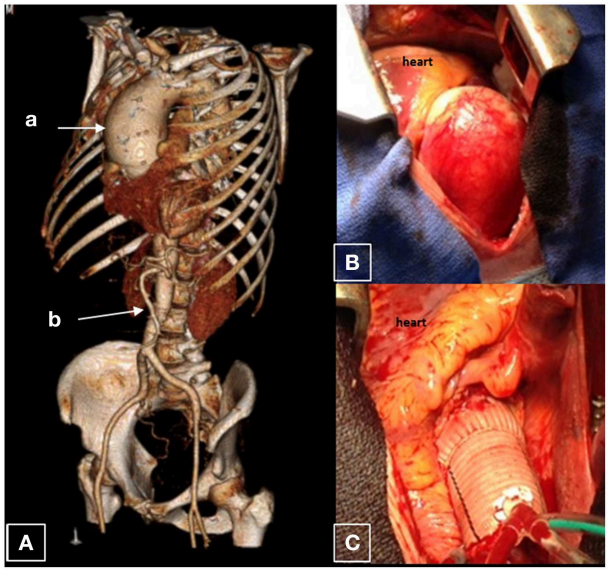

FIGURE 2 | Ascending aortic aneurysm. This is an aorta of a 37 year old male who was found to have a 7.7 cm saccular ascending aortic aneurysm associated with a leaking bicuspid aortic valve. The remainder of the aorta is normal caliber. **(A)** CT reconstruction of the whole aorta with ascending aortic aneurysm (a), with a normal caliber abdominal aorta (b). **(B)** Surgical view of this aneurysm; **(C)** Dacron graft after surgical replacement.

aneurysms (TAAs) (**Figures 2**, **3**) represent the most lethal site for a dilatation. They may remain asymptomatic for years and manifest as an acute rupture or dissection which can be fatal if left untreated. The prevalence of thoracic aneurysms in the United States is estimated at 10.4 per 100,000 people (Clouse et al., 1998). TAAs is a silent disease and 95% of cases present acutely from previously asymptomatic patients (Clouse et al., 1998). Thoracic aortic aneurysms are classified according to the segment of the dilatation. This anatomical classification is important because the etiology, natural history and treatment may differ for each segment.

The ascending thoracic aorta and the descending/abdominal aortas have different embryological aetiologies, pathological features and their evolution progresses differently (better described below—Section Mechanisms Underlying Abdominal Aortic Aneurysms). Aneurysms are much more common in the abdominal aorta than in the ascending aorta. Classically, atherosclerosis has been considered the underlying cause of abdominal aortic aneurysms (Lederle et al., 1997; Guo et al., 2001). Known cardiovascular risk factors mostly associated with AAA include smoking, increasing age, hypertension, hyperlipidaemia which predispose to atherosclerosis (Lederle et al., 1997; Coady et al., 1999). On the other hand, atherosclerosis is infrequently implicated in ascending thoracic aortic aneurysm development. Ascending thoracic aneurysms are associated with hypertension, connective tissue disease, bicuspid aortic valves, familial thoracic aneurysm syndrome, (Coady et al., 1999; Guo et al., 2001; Nuenninghoff et al., 2003; Demers et al., 2004).

This review focuses on non-coding (nc) RNAs as possible new mechanisms of diagnosis and monitoring of aneurysmal disease and novel therapeutic enabling to control the progression to dissection and rupture. The recent advancement in DNA and RNA sequencing techniques have led to the understanding that around 98% of the human genome contains regions of DNA which code for RNA molecules unable to produce proteins and hence termed ncRNAs. Far from being "junk RNA," many ncRNAs are proving to be very powerful regulators of gene expression, acting at both transcriptional and post-transcriptional level. ncRNAs are arbitrarily defined accordingly to their size, as small ncRNAs and long ncRNAs (lncRNAs). MicroRNAs (miRNAs, miRs) are the most popular small ncRNAs. Here, we will review miRNA and lncRNAs for their regulatory roles in vascular function, with special attention to their contribution to VSMC function and vascular degenerative disease. Finally, we will discuss the potential of ncRNAs as new therapeutic targets and clinical biomarkers to be employed in patients with aneurysms at different stage of evolution.

Non-Coding RNAs

About 2% of the eukaryotic genome contains protein coding genes and the remaining DNA was previously supposed to be "junk DNA," i.e., DNA devoid of any functional importance. In fact, large-scale analyses of mammalian transcriptomes have

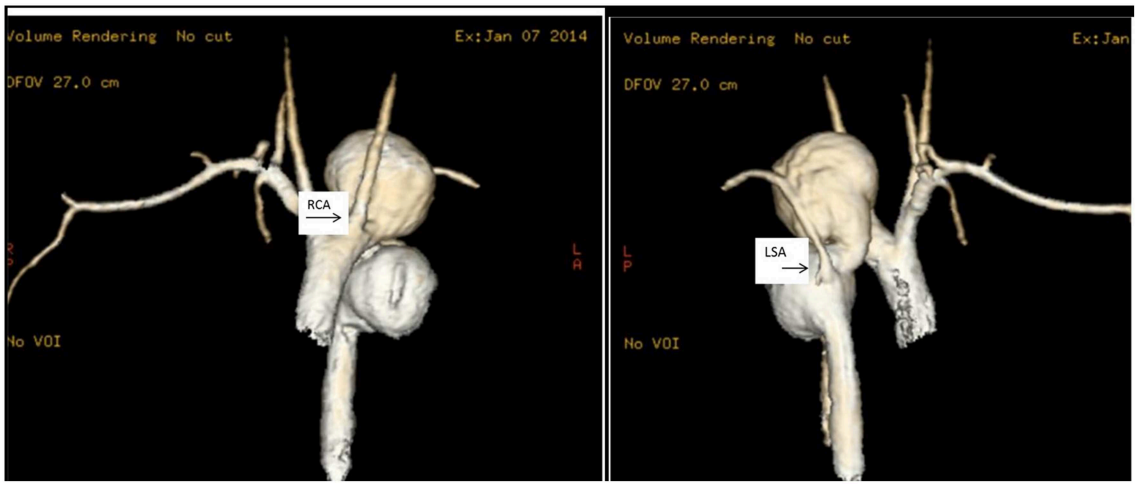

FIGURE 3 | Thoracic aorta aneurysms. MRI scans of the ascending aorta in a 17 year old girl with multiple thoracic aortic aneurysms. In (i), it is clearly visible a large saccular aneurysm of the aortic arch just distal to the right carotid artery (RCA) and a second large saccular aneurysm in the descending aorta. In (ii), the MRI scan shows the ascending and descending aorta. The left subclavian artery (LSA) comes off the descending aorta in between two large saccular aneurysms.

identified 60% of RNA molecules that are not translated into protein. However, the use of new sequencing technologies has identified that most of the genome is transcribed, producing a heterogeneous population of "noncoding RNA" (ncRNAs), which do not hold information for protein translation. Although ncRNAs do not code for proteins, they play different roles in regulating gene expression, thus impacting on cell functions. According to their size, ncRNA are classified as "small ncRNA" (20–200 nucleotides in their mature forms) and "long ncRNA" (more than 200nt and up to 100 kilobases). According to their function, ncRNAs can also be classified as "housekeeping" ncRNAs, which are constitutively expressed and crucial for normal function and cellular viability. These include tRNA, rRNA, SnoRNA. By contrast, the "regulatory" ncRNAs, which are important for the transcriptional regulation of genes. In recent years, several ncRNAs have been found to regulate vascular pathologies, including aortic aneurysm (thoracic and abdominal) disease.

MicroRNAs

MiRNAs are post transcriptional regulators of gene expression. They influence the stability and translation of messenger RNAs (mRNAs). MiRNA are encoded (often as polycistronic "clusters") in intergenic region of protein coding genes or by miRNA genes. MiRNA genes are transcribed by RNA pol lI as capped and polyadenylated primary miRNA transcripts (pri-mRNA). The pri mRNA is then processed in two independent steps by the enzymes Drosha and DICER. Dorsha/DGCR8 cleaves the pri-miRNA transcript to a 60–100 nucleotide long hairpin loop which is transported to the cytoplasm where it is further processed to mature miRNA, a single stranded of ∼22 nucleotides. This can be incorporated into the RNA-induced silencing complex (RISC) and mediates gene silencing by affecting the integrity and/or the protein synthesis capacity of the targeted mRNAs. A miRNA recognize its mRNA targets mRNAs by the semi complementarity between its "seed sequence" region and one or more "miRNA binding sites," usually located in the 3′ untranslated region (3′ UTR) of the mRNAs(Gregory et al., 2006).

Long Non Coding RNAs

The RNA transcripts that are longer than 200 nucleotides which are polyadenylated and are devoid of open reading frames are defined as lncRNAs. The functional annotation of mammalian cDNA (FANTOM3) project identified 35,000 of such non coding transcripts (Amaral et al., 2011). LncRNA are located and transcribed with the intergenic region of genome by the RNA PolI enzyme or in some cases by RNA pol III. The majority are transcribed as complex, interlaced networks of overlapping sense and antisense transcripts. On the basis of their genomic loci, long ncRNA (lncRNAs) can be classified onto: (1) Sense lncRNAs, transcribed from a locus overlapping with protein coding gene; (2) Antisense lncRNAs loci, that overlap with the antisense strand of the protein coding gene; (3) Bidirectional lncRNAs, located on the antisense strand of the protein coding gene; (4) Intronic lncRNAs, transcribed from introns to protein coding genes; (5) Long intergenic ncRNAs (lincRNAs), that are not in the proximity of protein coding gene. LncRNAs display high organ and cell specificity and are not well conserved across species. They can be located in the nucleus or in the cytosol and exert actions *in cis* and *in trans*. LncRNAs can regulate different aspects of transcription by playing different roles, including acting on transcription factors (TFs), RNA polII, mRNA processing, splicing and the epigenetic machinery (Kapranov et al., 2007; Broadbent et al., 2008; Mohammad et al., 2008). Additionally, lncRNAs can exert a post-transcriptional regulation of protein coding genes, for example by acting as antisenses inhibitors of mRNAs (Kornienko et al., 2013).

Exploratory studies performed on the lncRNA in the cardiovascular setting have so far identified a few lncRNA associated

with cardiovascular diseases. These include MALAT1 (metastasis associated lung adenocarcinoma transcript 1), LIPCAR (lincRNA predicting cardiac remodeling), CARL (cardiac apoptosis related lncRNA), MiAT (myocardial infarction–associated transcript), HCG22 (HLA complex group 22), Brave heart, CHRF (cardiac hypotrophy factor), ANRIL (antisense ncRNA in the INK4 locus), and HIF1A-AS1 (antisense hypoxia inducible factor 1 alpha antisense RNA), KCNQ1OT (KCNQ1 overlapping transcript), and SENCR (smooth muscle and endothelial cell-enriched migration/differentiation-associated lncRNA). Specific studies looking at lncRNAs in aortic aneurysm are still lacking (Skroblin and Mayr, 2014). Because of the high number of lncRNAs, research based on predefined lncRNA candidate is not ideal and next generation sequencing methods will be useful in exploring lncRNA which can be functionally related with aortic aneurysms and it might support the identification of lncRNAs to be investigated as biomarkers for diagnosing sporadic aortic aneurysms and to follow up aneurysm evolution.

Cellular Mechanisms and Signaling Pathways Underpinning Aneurysm Formation and Evolution

The currently available pharmacological approaches are unable to prevent aneurism progression. Although there is some success demonstrated with Angiotensin II (Ang II) receptor blockers in non-pathological aneurysms in Marfan's patients (Attenhofer Jost et al., 2014), this therapeutic approach is not proven to be effective enough to substitute the need for surgical therapy. This lack of effective therapies suggests the need for a better understanding the molecular mechanisms underpinning aneurysm pathology and progression. New mechanistic insight should provide better targets for therapeutic intervention. In this section, we'll describe the pathways and cellular mechanisms underlying aneurysm formation and evolution.

Mechanisms Underlying Abdominal Aortic Aneurysms

Abdominal aortic aneurysms have been linked to atherosclerosis and the pathophysiology of this aneurysm has been well studied (Guo et al., 2006; Shimizu et al., 2006). Histological analyses of human AAA samples revealed the three pathological hall marks of abdominal aorta aneurysms: leukocyte infiltration, degradation of extra cellular matrix (ECM) and VSMC depletion (Guo et al., 2006). Moreover, work on animal models (described below) confirmed this data and further indicated that abdominal aneurysm development involves local inflammatory responses leading to infiltration of macrophages, neutrophils, mast cells and T and B lymphocytes (Sun et al., 2007). The prominent inflammatory cells present in aortic *tunica media* and *adventitia* are macrophages. Macrophage infiltration is critical for abdominal aneurysm development, but its contribution has not been defined. CCR2 (C-C chemokine receptor 2) and myeloid differentiation factor are important for macrophage mediated response to inflammation. CCR2 deficiency in mouse models attenuates the severity of the induced aneurysm (Daugherty et al., 2010). Neutrophils are also present in experimental and human AAA tissue. L selectin adhesion molecule mediates the trafficking of neutrophils from plasma to the aortic wall and L selectin attenuates abdominal aneurysm formation in mouse models (Ishibashi et al., 2004).

The plasma concentration of certain cytokines, such as tumor necrosis factor alpha (TNF-α), increases in patients with abdominal aneurysm. However, TNF alpha receptor deficiency has no significant effect on Ang II- induced AAA formation in LDL receptor deficient atherosclerotic mice. Another cytokine that plays a critical role is transforming growth factor beta (TGF-β). Systemic blockade of TGF-β activity was found to increase Ang II-induced AAA (Wang et al., 2010).

The drug cyclosporin induces TGF- β expression and its administration stops aneurysm formation in the rat elastase and mouse calcium chloride models of AAA (Dai et al., 2011). Interleukin 1β, IL6, IL17, and IL23 are increased in AAA in human tissue as compared with healthy tissue (Szekanecz et al., 1994). AAA formation and progression is also mediated by various proteases, which degrade elastin and collagen present in the aortic wall. Matrix metalloproteinase-9 (MMP-9) is a zinc endopeptidase contributing to calcium chloride-induced aneurysm development in mice (Tanaka et al., 2009). C-Jun N terminal kinase (JNK) was found increased in abdominal aneurysms in murine models. Moreover, a JNK specific inhibitor prevented the aneurysms formation in these models (Yoshimura et al., 2005).

Thoracic Aorta Aneurysm Pathology and Mechanisms

Thoracic aortic aneurysm (TAA) is caused by proteoglycan accumulation, elastic fiber fragmentation, focal or diffuse VSMC degradation and loss. The pathological changes can result in excessive degradation of ECM components, leading to the loss of mechanical strength and integrity, aortic dilation, dissection, or rupture (El-Hamamsy and Yacoub, 2009). Histopathological findings of the aortic wall in patients with ascending aorta aneurysm reveal the presence of cystic medial necrosis or medial degeneration. The aneurysm is characterized by disruption of the lamellar organization of elastic fibers, cyst like lesions within the tunica media, pooling of proteoglycans, elastin and collagen fiber fragmentation and coagulative necrosis of VSMCs (El-Hamamsy and Yacoub, 2009). The NOTCH pathway is activated in fibroblasts and deregulated in VSMCs of patients with thoracic aneurysm. However, the Notch signaling is downregulated in the ascending aorta of patients with bicuspid aortic valve (BAV) and in abdominal aneurysms. Genetic variation in the *NOTCH1* gene appears to confer susceptibility to ascending aortic aneurysm formation in patients with BAV (McKellar et al., 2007). More research is needed to clarify the role of the NOTCH pathway in aneurysms and to elucidate the possibility of targeting it therapeutically.

NcRNAs in Aneurysms

NcRNAs have become a subject of great interest for vascular biology and medicine. The scientists interested in

aneurysm disease can combine cell and animal models and analyses on clinical samples for their ncRNA studies. Below, we report on the current knowledge and discuss the importance of research model optimization for future research.

Table 1 summarized the miRNAs currently known to be functionally involved and/or expressionally deregulated in AAs. **Table 2** summarized the lncRNAs that have been shown to be involved with Vbiology and proposes their link to AA.

Vascular Cell Biology

Cell culture experiments on human aortic VSMCs isolated from clinical tissue samples suggest the role of miRNAs in aortic aneurysm development. In this article we present the miRNAs studied in VSMCs. Similar studies on lncRNAs are still lacking. However, we suggest new roles for these ncRNA molecules based on the results of some pioneers studies in non-aneurysmal VSMCs (*vide infra* at LncRNAs).

miRNAs

The VSMC phenotype and behavior is strongly regulated by miRNAs. The phenotype of VSMCs can change between a contractile to a synthetic state and vice versa, and aortic aneurysm can be associated with a reduction of contractile VSMC number. Knockout of Dicer impairs miRNA biogenesis. In VSMCs, this induces embryonic lethality, showing abnormal development of vascular vessels. These findings suggest that miRNAs play important roles in proliferation, migration and phenotypic transformation of VSMCs. MiRNAs are additionally involved in VSMC biology and behavior, acting on cell apoptosis proliferation, and migration through modulating the expression of vasoactive molecules, cytokines, MMPs and growth factors.

The miR-29 family of miRs contains three members (miR-29a, miR-29b, and miR-29c) that are encoded by two separate *loci*, giving rise to bi-cistronic precursor miRs (miR-29a/b1 and miR-29b2/c). This family targets numerous gene transcripts that encode ECM proteins involved in fibrotic responses, including

TABLE 1 | microRNAs which could be involved with aneurysmal disease.

MiRNA	Up/down regulation	Target genes	Function regulated	Expression sites	References
miR143/145	Down	Klf4, myocardin, Elk-1,	Direct VSCM differentiation, repress VSCM proliferation	Murine cardiac progenitors Mice VSMCs	Cordes et al., 2009; Elia et al., 2009
miR29	up	ECM protein encoding genes (COL1A1, COL1A2, COL3A1, FBN1, ELN, MCL1, MMP2, MMP9)	Regulate ECM	Human aortic SMCs Murine models of experimental AAA	Chen et al., 2012; Maegdefessel et al., 2012
miR 205	up	TIMP3, RECK	Regulate ECM	Murine model of AAA	Kim et al., 2014
miR 26a	down	SMAD-1	Promotes vascular SMC proliferation while inhibitin g cellular differentiation and apoptosis, and alters TGF-β pathway signaling	Human aortic SMCs	Leeper et al., 2011
miR 21	up	PTEN	Modulate proliferation and apoptosis of VSMCs	Murine models of experimental AAA	Maegdefessel et al., 2012
miR 195	up	ELN, different COL isoforms, MMP2, MMP9	Regulate ECM	Mouse aorta SMCs	Zampetaki et al., 2014
miR 221/222	up	p27, p57, c-Kit	pro-proliferative, pro-migration, and anti-apoptotic effects, Promote a synthetic phenotype in VSMCs	Human aortic SMCs Aorta SMCs and ECs from rats	Davis et al., 2009; Liu et al., 2012

AAA, abdominal aortic aneurysm; c-kit, COL1A1, collagen type 1 alpha 1; EC, endothelial cell; ELK1, ETS domain-containing protein; ELN, elastin; FBN, fibrillin; KLF4, Kruppel-like factor 4; MCL1, myeloid cell leukemia 1; MMP, matrix metalloproteinase; PTEN, phosphatase and tensin homolog; SMAD-1, mothers against decapentaplegic homolog 1; RECK, reversion-inducing-cysteine-rich protein with kazal motifs; SMC, smooth muscle cell; TIMP3, metalloproteinase inhibitor 3; VSMC, vascular smooth muscle cell.

TABLE 2 | Long noncoding RNAs studied in vascular smooth muscle cells.

lncRNA	Class	Function regulated	References
ANRIL	NAT	Influences VSMC function by regulating CDKN2A, CDKN2B, DAB2IP, LRP1, LRPR, CNTN3 expression	Boucher et al., 2003; Elmore et al., 2009; Gretarsdottir et al., 2010; Bown et al., 2011; Congrains et al., 2012
SENCR	Antisense	Inhibitis of VSMC migration	Bell et al., 2014
LncAng362	Antisense	Reduces VSMC proliferation	Leung et al., 2013
HIF1A-AS1	Antisense	Pro-apoptotic and anti-proliferative effect on VSMCs	Wang et al., 2014

ANRIL, antisense non-coding RNA in the INK4 locus; SENCR, smooth muscle and endothelial cell-enriched migration/differentiation-associated lncRNA; HIF1A-AS1, antisense hypoxia inducible factor 1 alpha anti sense RNA; NAT, natural antisense transcript; LRP1, low density lipoprotein receptor-related protein 1; LRPR, low density lipoprotein receptor; CDKN, cyclin-dependent kinase inhibitors; DAB2IP, DAB2 interacting protein; CNTN3, contactin-3; VSMC, vascular smooth muscle cells.

several collagen isoforms (e.g., COL1A1, COL1A2, COL3A1), fibrillin-1, and elastin (ELN) (Boon et al., 2011).

Over-expression of miR-21 increases VSMC survival and proliferation (Song et al., 2012). By contrast, miR-133 reduces proliferation and migration of VSMC through repressing transcription factor Sp-1 (Torella et al., 2011). MiR-133 is highly expressed in the healthy vasculature, especially in VSMCs. It is anti-proliferative and upregulated when VSMCs are quiescent. Overexpression of miR-133 reduced VSMC proliferation *in vitro*; while anti-miR-133 increased VSMC proliferation (Torella et al., 2011). *In vitro* studies showed that ERK1/2 activation is partly responsible for miR-133 downregulation when VSMCs are primed for the phenotypic switch (Zhan et al., 2003; Torella et al., 2011).

MiR-143/145 have been extensively studied in vascular pathology. This cluster alters the SMC phenotypic switch and its expression is decreased by acute and chronic vascular stress. The two miRNAs cooperate in the regulation of VSMC fate and plasticity by targeting several transcription factors, such as KLF4, KLF5 myocardin and Elk-1 (Boettger et al., 2009). A study of Cordes et al. (2009) demonstrates the role of the miR-143/145 cluster in VSMC differentiation and the repression of their proliferation. Zampetaki *at al* focused on miR-195 in aortic aneurysmal disease, performing both *in vitro* and *in vivo* studies (Zampetaki et al., 2014). *In vitro* experiments on murine VSMCs confirmed the involvement of miR-195 in ECM homeostasis regulation. Proteomic analyses on the conditioned medium from VSMCs transfected with either pre-miR-195 or anti-miR-195 showed that miR-195 expression is reversely correlated with elastin, a direct target of this miRNA (Zampetaki et al., 2014). Elastin is fundamental for the maintenance of aortic mechanical integrity, allowing the vessel to resume its shape after stretching or contracting. Low elastin levels are typical of aneurysmal diseases. Recently, Kim et al. showed miR-205 is upregulated in abdominal aortic endothelial cells (ECs) and miR-205 stimulates MMP activity by targeting two endogenous MMP inhibitors, known as tissue inhibitor 3 of MMP (TIMP3) and reversion-inducing cysteine-rich protein with kazal motifs (RECK) (Kim et al., 2014).

The role or miR-26a has been studied by Leeper and collaborators, working on human aortic SMCs. They performed several assays to assess how transfection with either anti-miR or pre-miR affected VSMCs behavior. The decrease in miR-26a levels was associated with a reduction in VSMCs proliferation and migration, and a significant increase of H_2O_2-induced apoptosis, in comparison to control cells. They demonstrate the targeted action of this miR on SMAD-1 protein in VSMCs, and thus the effects on TGB-β pathway. All these effects could be fundamental in AA development (Leeper et al., 2011).

MiR-221/222 seems to have a cell specific effect in blood vessels. In a study by Liu et al., SMCs and ECs were isolated from murine aorta samples. The effects of miR-221/222 downregulation and upregulation were analyzed on each cell type studying their proliferation, migration and apoptosis. These miRNAs resulted to have a pro-proliferative, pro-migration and anti-apoptotic effect on VSMCs, while an anti-proliferative, anti-migration and pro-apoptotic effect on ECs. The action of miR-221/222 are mediated by directly suppressing c-kit and cyclin-dependent kinase inhibitors p27Kip1 and p57Kip2, three proteins involved in key processes as cell differentiation, proliferation, migration and apoptosis (Liu et al., 2012). miR-221/222 is up-regulated, while miR-133, miR-143/145, and miR-663 are down-regulated in response to PDGF treatment on VSMCs (Liu et al., 2012). MiR-221/222 is overexpressed in injured vascular wall SMCs in rat carotid arteries after angioplasty. Specific knockdown of miR-221 and miR-222 resulted in decreased VSMC proliferation *in vitro*. Knockdown also suppressed VSMC proliferation *in vivo* and decreased neointimal lesion formation after angioplasty in rat carotid arteries (Liu et al., 2010).

LncRNAs

As aforementioned, here we are reviewing the actions of a few lncRNAs in vascular cell biology at large since specific studies on lncRNAs in aneurysmal VSMCs are still lacking. We are confident that this gap will be soon filled by the pioneer researchers who have started lncRNA research programs in vascular medicine.

LncRNAs regulate gene expression, including chromatin remodeling, mRNA transcription and processing, and post-transcriptional pathways. It is important to explore whether lncRNAs could be involved in vascular disease through these mechanisms. Wang et al. showed that HIF1A-AS1 mediates the pro-apoptosis and anti-proliferative responses induced by *BRG1* gene (Brahma-related gene 1) in VSMCs (Wang et al., 2014). They also showed that BRG1 expression is significantly increased in the aortic media of TAA patients compared to the normal group, implying that BRG1 may play a role in development and progression of TAA. LncRNAs play critical roles in the regulation of cellular process such as cell growth and apoptosis (Wang et al., 2014). BRG1 was knocked down in VSMCs and a microarray was performed and the expression of HIF1A-AS1 was found to be regulated by BRG1. Overexpression of BRG1 in TAA and the interaction between *BRG1* and the lncRNA HIF1A-AS1 in VSMCs might provide clues to the molecular mechanisms involved in TAA. This should be pursued by using isolated VSMCs from TAA patients and appropriate animal models (Wang et al., 2014). The lncRNA HIF1A-AS1 plays an important role in the pathophysiology of VSMCs.

The Natural antisense (NATs) ANRIL, KCNQ1OT, and SENCR have been found expressed in VSMCs (Congrains et al., 2012). ANRIL, as discussed elsewhere in this review, also regulates the function of VSMCs. As explained below, the genomic region (9p21) coding for *ANRIL* also contains the *CDKN2A* and *CDKN2B* genes which encode the cell cycle regulators p16 (INK4a), p14 (ARF) and p15 (INK4b). The role of ANRIL in various types of aneurysms needs to be investigated. The 9p21 genotype had an influence on CDKN2A/CDKN2B/ANRIL expression levels in VSMCs, VSMC proliferation and VSMC content in atherosclerotic plaques. Analyses of VSMC primary cultures showed that the 9p21 risk genotype was associated with reduced expression of p16 (INK4a), p15 (INK4b) and ANRIL and with increased VSMC proliferation (Congrains et al., 2012).

RNA sequencing (RNA-seq) by Bell et al. on the human coronary artery SMCs showed 31 unannotated lncRNAs, including SENCR, which is enriched in vascular cells. SENCR is

transcribed antisense from the 5′ end of the *FLI1* gene and exists as two splice variants (Bell et al., 2014). SENCR has a cytoplasmic location (Bell et al., 2014). RNA-seq experiments in VSMCs after SENCR knockdown revealed decreased expression of myocardin and numerous smooth muscle contractile genes, while several promigratory genes were increased. Loss-of-function studies in scratch wound and Boyden chamber assays suggests SENCR as an inhibitor of VSMC migration (Bell et al., 2014).

Finally, several lncRNAs (Lnc Ang362, Lnc-Ang 162, LncAng 112, LncAng249, and LncANg219) were found to be expressionally regulated by Ang II in VSMCs (Leung et al., 2013). The lncRNA AngII362 also mediates the proliferation of VSMC by acting on the miR221/222 (Leung et al., 2013).

In vivo Aneurysm Models

In order to develop knowledge of ncRNAs that can be translated to clinical applications, it is important to devise "reproducible" models mimicking the features of human aneurysms, such as lesion in the elastic layer, inflammatory infiltrate within the adventitial and medial layers, and increased proteolytic activity within the aneurysm wall. Clinical relevant models of AA are still lacking, particularly for TAA. Consequently, there is large scope for improvement, as discussed below.

One of the first animal models employed for aneurysm studies is the blotchy mouse (Andrews et al., 1975). These mice have a mutation (*blotchy* allele) in the *Mottled* locus gene of the X chromosome, which leads to cross-linking abnormalities in elastin and collagen. These mice have a variety of connective tissue abnormalities, including spontaneous thoracic aneurysm formation and reduce tensile strength of the skin (Andrews et al., 1975). Aneurysms in this model are histopathologically similar to that observed in humans, with elastic fiber fragmentation and inflammatory infiltrate within the medial and adventitial layers (Andrews et al., 1975). To the best of our knowledge, this mouse model has not been employed for ncRNA studies. By contrast, another genetic model, the *Fibulin-4(R/R)* mice have been used for studying miR-29 expression by Boon et al. (2011). Fibulin proteins form bridges that stabilize the organization of extracellular matrix structures (Hanada et al., 2007). Hanada et al. have shown that reduced Fibulin-4 expression leads to dilatation of the ascending aorta, aneurysm formation, dissection of the aortic wall and thickened aortic valvular leaflets that are associated with aortic valve stenosis and insufficiency (Hanada et al., 2007). At the expressional level, the TGF-beta signaling appears perturbed (Hanada et al., 2007). Taken together, these pathological and expressional features suggest the relevance of *Fibulin-4(R/R)* for aneurysmal studies.

Mice with an homozygous Apolipoprotein E gene depletion (*ApoE KO*) are also predisposed to elastic fiber fragmentation as well as to atherosclerosis and atherosclerotic plaque formation (Anidjar et al., 1990). In addition to these genetic models, the delivery of exogenous substances has also been employed to trigger AAA in mice. Examples are the perivascular delivery of calcium chloride or proteases (collagenases and elastases), transient perfusion of elastase into infrarenal aorta, and prolonged systemic Ang II infusion (Daugherty and Cassis, 2004). These interventions can have been performed either in wild type mice or in atherosclerotic *ApoE KO* (Azuma et al., 2009).

One study investigated the role on miR-21 and miR-29 in mice with AAA (Maegdefessel et al., 2014a). They noted that miR-21 was associated with protective qualities against AAA development. They took antagomiR against miR-21 and lentiviral vector (LV) of pre-miR-21, which would down-regulate and up-regulate miR-21, respectively. With the antagomiR-21, they noted a marked increase in the development of AAA in murine models, whereas LV-mediated miR-21 overexpression significantly decreases AAA progression. Interestingly, they also noted that in samples that were exposed to high levels of nicotine to simulate the modifiable risk factor of smoking, there was a marked increase in miR-21, and eluded to the fact that this may be a "safety mechanism," in order to try and combat the negative effects of smoking (Maegdefessel et al., 2014a). In a different studies, The miR-21 was shown to prevent abdominal aortic aneurysms development by targeting PTEN (phosphatase and tensin homolog) (Ji et al., 2007). MiR-21 regulates growth and survival of VSMCs by decreasing the expression of PTEN and inducing expression of Bcl-2, resulting in pro-proliferative and anti-apoptotic effects in a carotid injury model in rats. In regards to aortic dilatation, miR-21 was significantly up-regulated in two established murine models of AAA disease. Among the miR-21 target genes that alter proliferation and apoptosis, PTEN was the only target gene to be significantly down-regulated at three different time points during aneurysm development and progression. miR-21 also targets multiple members of the dedicator of cytokinesis (DOCK) superfamily and modulates the activity of ras-related C3 botulinum toxin substrate 1 (Rac1) small GTPase that regulates VSMC phenotype (Ji et al., 2007).

The role of miR-29 in aneurysm formation and development of aortic dilation was confirmed by Maegdefessel et al. (2012). MiR-29 is known to be involved in the regulation of ECM within the vascular wall. Consequently, increased miR-29 expression would down-regulate ECM synthesis. In aneurismal formation, an increase in ECM is a protective quality, an attempt to increase the tensile strength of the vessel in order to prevent diameter expansion. The miR-29 family, which targets numerous gene transcripts that encode ECM proteins, is known to modulate gene expression during development and aging of the abdominal aorta and during the progression of aortic aneurysms (Boon et al., 2011; Maegdefessel et al., 2012, 2014a). Maegdefessel et al. (2012, 2014a) looked in particular at miR-29b, one of the family members of miR-29. In their murine models, they noted that there was an increase in ECM production when they inhibited miR-29b expression in their antimiR-29 mouse population. In addition to this, they also noted a concurrent decrease in MMP2 and MMP9. These findings were also seen in their human aneurysm/non-aneurysm tissue studied. This suggests a strong link with increased miR-29b expression and aneurismal development (Maegdefessel et al., 2014a). Interestingly, similar findings were also found by another team: Boon et al. (2011) looked at miR-29b in association with aneurysm formation and increasing age. Age is a significant risk factor for the development of AAA disease. They noted that vascular wall changes due to age are crucial to the development of AAA disease. In their study they found

increased levels of miR-29 in aging vessel walls. Knowing that miR-29 is associated with reduced ECM production and therefore increased AAA development, they inferred from their results that the age related risks associated with AAA development were due to increased miR-29 levels and reduced wall structural integrity (Boon et al., 2011). Inflammation is another key element in the AAA development and disease progression as it has strong correlations with vessel reconstruction and alteration (Shimizu et al., 2006). One study noted that miRNA expression may be linked to inflammatory responses within vasculature, in turn, affecting AAA development (Maegdefessel et al., 2014b). They looked at miR-23b-24-27b cluster in murine models, and attempted to find a correlation with up-regulated inflammatory mediators. They found that miR-24 had the most significant negative correlation with inflammatory mediators, looking in particular at chitinas 3-like1 (CH3L1). CH3L1 is a known mediator of inflammation and has been suggested as a novel biomarker of chronic inflammatory disease (Lee et al., 2011). They found that in murine AAA models miR-24 levels are inversely correlated with increased levels of CH3L1. Inferring from these results, we can speculate that miR-24 not only has protective qualities against the inflammatory processes involved in AAA disease, but it may also be used as a biomarker for the progression of the disease. Nonetheless, there were no significant changes in circulating miR-24 between small and large AAA. However, CH3L1 levels were significantly increased in large AAA compared with small, and that using both in conjunction may be a viable biomarker of disease progression (Maegdefessel et al., 2014b). These results were noted to correlate to those in *ex-vivo* human AAA tissue.

Finally, miR-712 is the murine homolog of the aforementioned MMP activator miR-205 (Kim et al., 2014). In addition, miR-712 was proved involved with aneurysm formation in mice (Kim et al., 2014).

Concerning lncRNAs, the aforementioned Lnc Ang362, LncAng 162, LncAng 112, LncAng249, and LncANg219 were found to have altered expression altered in the aorta of Ang II treated mice (Leung et al., 2013).

Research has timidly expanded to large animal models, which better mimic the human anatomy and allow for diagnostic (including *in vivo* imaging) and therapeutic approaches similar to what could be delivered to human patients. One study in particular looked at the use of porcine arterial tissues that have been *in vivo* manipulated with a mixture of collagenases and calcium chloride in order to replicate TAA as close as possible to the ones observed in patients (Eckhouse et al., 2013). Eckhouse et al., reproduced this model and demonstrated that under the conditions they used the outcome was predictable and consistent. In addition to this, they cited the importance of finding a large mammalian model that will accurately mimic human pathology. They propose that murine models previously used in TAA studies are only useful at the cellular level, as this data is not easily transferable to human models. We concur with this opinion. Their porcine model, on the other hand, was noted to have the mural structure and contents that are seen in human TAA tissue studies, i.e., cell contents, wall constituent configuration and protease levels (in particular MMPs). The importance of this study is that the development of a reproducible TAA model that can be used in any study, providing a level of standardization and transferability to the disease in humans (Riches et al., 2013). We are currently developing the model to be used in our own translational research projects.

Ex-vivo Aneurysm Models

A recent study co-authored by one (TGA) of us, used porcine arterial segments to create an *ex-vivo* model of aneurysm comparable to human AAA. Freshly isolated porcine arteries were pretreated with collagenase and elastase before being cultured under flow in a bioreactor for 12 days. This produced aneurysmal-like remodeling. VSMCs were harvested from the porcine aneurysmal vessels and further studied *in vitro* in comparisons with cells prepared from human end stage AAA disease. Cellular senescence was investigated using β-galactosidase staining and apoptosis was quantified using a fluorescence-based caspase 3 assay. The data on VSMC morphology, senescence and proliferation were comparable to that of end stage human AAA vessels (Riches et al., 2013). The authors propose this model can be used to examine temporal changes in VSMC biology in porcine and also human aortic vessels and to identify the molecular mechanisms conductive to or protective from aneurysmal remodeling (Riches et al., 2013). In the longer term, this may inform and functionally validate new therapeutic targets (including miRNAs and lncRNAs) as part of a translational pipeline preceding human interventional studies.

Work on Clinical Samples and Patient Population

In recent years miRNAs have been found to regulate vascular pathologies in aortic aneurysm (thoracic and abdominal disease).

Expressional Studies

Liao et al. performed microarray and systemic analysis of miRNA from the ascending aorta segments of TAA patients and normal aorta from organ donors (Liao et al., 2011). The resulting miRNAs were validated by qPCR and target genes were predicted TargetScan v5.1 and Mirind V5.1. The miRNAs which were validated as significantly upregulated in human TAA are miR-183*, −433, −553, −491-3p, −30c, and −338-5p. By contrast, miR-143, −145, −22, −24, −93 and −768-5p were lower in the TAA samples (Liao et al., 2011). Elia et al. also found that miR143 and 145 are down regulated in ascending aortic aneurysms (Elia et al., 2009). Loss of miR-143 and miR-145 expression induces structural modification of the aorta due to an incomplete differentiation of VSMC. This confirms a previous study which showed transition of VSMC from contractile to synthetic phenotype exists in media of TAA aorta, indicating that VSMC are less differentiated in TAA (Elia et al., 2009).

In another study, Jones et al. compared miRNA expression levels in aortic tissue specimens collected from patients with ascending TAAs and looked at the aneurysm progression (Jones et al., 2011). They observed decreased expression of miRs −1, −21, −29a, −133a, and −486 as compared with normal aortic specimens. A significant relationship between expression of miR-1, −21, −29a, and −133a and aortic diameter was identified; as aortic diameter increased, miR expression decreased (Jones et al., 2011). Through the use of a bioinformatics approach,

members of the matrix metalloproteinase (MMP) family, proteins involved in TAA development, were examined for putative miR binding sites. MMP2 and MMP9 were identified as potential targets for miR-29a and miR-133a, respectively, and MMP-2 was subsequently verified as a miR-29a target *in vitro* (Jones et al., 2011). Reduced miR29-b was also replicable in human aneurysmal tissue samples compared with controls (Maegdefessel et al., 2012).

Kumari et al. compared ascending TAA with descending TAA tissue using miRNA microarray and found that the expression level of miR-1, 30c-2*, miR-145, miR-204, miR-331-3p were upregulated in ascending TAA in comparison with descending TAA (Premakumari et al., 2014). Importantly this data suggests that these miR mayhave biological and clinical relevance in the context of TAAs and may provide significant targets for diagnostic/prognostic and therapeutic applications (Premakumari et al., 2014).

Pahl et al. compared miRNA expression levels using an affymetrix GeneChip miRNA array in human AAA vs. control human aorta tissue (Torella et al., 2011). Three miRNA were found upregulated in AAA: miR 181a*, miR 146a, miR21. While five miRNA miR 133b, miR 133a, miR 331-3p, miR 30c-2*, and miR204 were significantly down regulated in human AAA tissue. The down regulated miRs replicated well in the qPCR validation while the upregulated three miRs failed to replicate. In addition, they found that miR 133 is down regulated in human AAA tissue (Torella et al., 2011).

LncRNA expression has not yet been investigated in clinical samples. However, we expect that this gap will be soon filled, including by our group. Because of the high number of lncRNAs, next generation sequencing methods will be useful in developing these studies.

Genetic Studies

Genome-wide association studies (GWAS) is an approach used to identify associations between single nucleotide polymorphisms (SNP), variations in a single nucleotide within the DNA sequence, and their influence on health and disease. AA is a complex disease with important risk factors like male sex, cigarette smoking, a personal history of myocardial infarction and a heritability of 70% i.e., family history (Wahlgren et al., 2010). GWAS suggest a role of ANRIL in AA. There are five chromosomal regions in the human genome with the strongest supporting evidence of contribution to the genetic risk for AAA: (1) *ANRIL* (also known as *CDKN2BAS*) on chr9p221 locus is expressed in humans. 9p21.3. This locus is among the most strongly replicated regions for cardiovascular disease and is also associated with coronary artery disease. *ANRIL* encodes an antisense lncRNA that regulates expression of the cyclin-dependent kinase inhibitors *CDKN2A* and *CDKN2B;* (Congrains et al., 2012) (2) DAB2 interacting protein (*DAB2IP*), which encodes an inhibitor of cell growth and survival; (3) low density lipoprotein receptor-related protein 1 (*LRP1*), a plasma membrane receptor involved in VSMC and macrophage endocytosis; (4) low density lipoprotein receptor (*LRPR*); and (5) contactin-3 (*CNTN3*) (Boucher et al., 2003; Elmore et al., 2009; Gretarsdottir et al., 2010; Bown et al., 2011).

Candidate gene studies represent complementary approaches to analyze genetics behind AA. The most significant associations with AAA discovered in candidate gene studies are: (1) *SORT1* (lipid metabolism) (Jones et al., 2013); (2) *IL6R* (inflammation) (Harrison et al., 2013); (3) *LPA* (lipid metabolism); (4) *AGTR1* (Renin angistenin system) (Jones et al., 2008); (5) *TGFBR2* (TGFB signaling) (Biros et al., 2011); (6) *MMP3* (Degradation of ECM) (Yoon et al., 1999); (7) *MTHFD1* (methionine metabolism) (Hinterseher et al., 2011); (8) *LRP5* (lipid metabolism) (Boucher et al., 2003). To date, genetic studies targeting ncRNAs are lacking.

Summary and Future Research

Today, there are no clinical tests to detect an aneurysm and the management of aneurismal diseases is unsatisfactory. Surgical intervention is the only valuable treatment option and based only on size. Predictors of biological behavior of an aneurysm wall would enhance the management of aneurysm disease from a one dimensional surgical strategy. This strong clinical need has prompted the research of new biomarker and therapeutic targets to be to translate into clinical care. The explosion on miRNA research in the last year delivered some candidates to be further studied. Basic science still needs to fill several gaps. Firstly, studies in ncRNA biology have focused on the effects of miRNAs or lncRNAs on VSMC and ECs. This research needs to be extended to include miRNAs or lncRNAs in other cellular components of blood vessels, such as fibroblasts and macrophage, which remain to be investigated. NcRNA research focused on their function in blood vessels is currently limited. In addition to miRNAs and lncRNAs, other ncRNAs, such as rasiRNAs and piRNAs, may also be involved in the development of cardiovascular pathology, and their function requires evaluation. Additional work on miRNA drug chemistry and delivery approaches enabling local and systemic but safe delivery of ncRNA-based therapeutics is required, this further necessitates the use of clinical relevant animal models and *in vivo* imaging systems. lncRNAs are vastly unexplored by cardiovascular researchers, but given the enthusiasm of many academic and industry-based scientists who specialize in ncRNAs research, we foresee this will be reversed by a plethora of exciting studies.

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References

Amaral, P. P., Clark, M. B., Gascoigne, D. K., Dinger, M. E., and Mattick, J. S. (2011). lncRNAdb: a reference database for long noncoding RNAs. *Nucleic Acids Res.* 39, D146–D151. doi: 10.1093/nar/gkq1138

Andrews, E., White, W, and Bullock, L. (1975). Spontaneous aortic aneurysms in blotchy mice. *Am. J. Pathol.* 78, 199.

Anidjar, S., Salzmann, J.-L., Gentric, D., Lagneau, P., Camilleri, J.-P., and Michel, J.-B. (1990). Elastase-induced experimental aneurysms in rats. *Circulation* 82, 973–981. doi: 10.1161/01.CIR.82.3.973

Attenhofer Jost, C. H., Greutmann, M., Connolly, H. M., Weber, R., Rohrbach, M., Oxenius, A., et al. (2014). Medical treatment of aortic aneurysms in Marfan syndrome and other heritable conditions. *Curr. Cardiol. Rev.* 10, 161–171. doi: 10.2174/1573403X1002140506124902

Azuma, J., Asagami, T., Dalman, R, and Tsao, P. S. (2009). Creation of murine experimental abdominal aortic aneurysms with elastase. *J. Vis. Exp.* 29. doi: 10.3791/1280

Bell, R. D., Long, X., Lin, M., Bergmann, J. H., Nanda, V., Cowan, S. L., et al. (2014). Identification and initial functional characterization of a human vascular cell–enriched long noncoding RNA. *Arterioscler. Thromb. Vasc. Biol.* 34, 1249–1259. doi: 10.1161/ATVBAHA.114.303240

Biros, E., Norman, P. E., Jones, G. T., van Rij, A. M., Yu, G., Moxon, J. V., et al. (2011). Meta-analysis of the association between single nucleotide polymorphisms in TGF-β receptor genes and abdominal aortic aneurysm. *Atherosclerosis* 219, 218–223. doi: 10.1016/j.atherosclerosis.2011.07.105

Boettger, T., Beetz, N., Kostin, S., Schneider, J., Krüger, M., Hein, L, et al. (2009). Acquisition of the contractile phenotype by murine arterial smooth muscle cells depends on the mir143/145 gene cluster. *J. Clin. Invest.* 119, 2634–2647. doi: 10.1172/JCI38864

Booher, A. M., and Eagle, K. A. (2011). Diagnosis and management issues in thoracic aortic aneurysm. *Am. Heart J.* 162, 38–46.e1. doi: 10.1016/j.ahj.2011.04.010

Boon, R. A., Seeger, T., Heydt, S., Fischer, A., Hergenreider, E., Horrevoets, A. J., et al. (2011). MicroRNA-29 in aortic dilation: implications for aneurysm formation. *Circ. Res.* 109, 1115–1119. doi: 10.1161/CIRCRESAHA.111.255737

Boucher, P., Gotthardt, M., Li, W.-P., Anderson, R. G., and Herz, J. (2003). LRP: role in vascular wall integrity and protection from atherosclerosis. *Science* 300, 329–332. doi: 10.1126/science.1082095

Bown, M. J., Jones, G. T., Harrison, S. C., Wright, B. J., Bumpstead, S., Baas, A. F., et al. (2011). Abdominal aortic aneurysm is associated with a variant in low-density lipoprotein receptor-related protein 1. *Am. J. Hum. Genet.* 89, 619–627. doi: 10.1016/j.ajhg.2011.10.002

Broadbent, H. M., Peden, J. F., Lorkowski, S., Goel, A., Ongen, H., Green, F., et al. (2008). Susceptibility to coronary artery disease and diabetes is encoded by distinct, tightly linked SNPs in the ANRIL locus on chromosome 9p. *Hum. Mol. Genet.* 17, 806–814. doi: 10.1093/hmg/ddm352

Chen, K.-C., Liao, Y.-C., Hsieh, I., Wang, Y.-S., Hu, C.-Y., and Juo, S.-H. H. (2012). OxLDL causes both epigenetic modification and signaling regulation on the microRNA-29b gene: novel mechanisms for cardiovascular diseases. *J. Mol. Cell. Cardiol.* 52, 587–595. doi: 10.1016/j.yjmcc.2011.12.005

Clouse, W. D., Hallett, J. W. Jr., Schaff, H. V., Gayari, M. M., Ilstrup, D. M., and Melton, L. J. III. (1998). Improved prognosis of thoracic aortic aneurysms: a population-based study. *JAMA* 280, 1926–1929. doi: 10.1001/jama.280.22.1926

Coady, M. A., Davies, R. R., Roberts, M., Goldstein, L. J., Rogalski, M. J., Rizzo, J. A., et al. (1999). Familial patterns of thoracic aortic aneurysms. *Arch. Surg.* 134, 361–367. doi: 10.1001/archsurg.134.4.361

Congrains, A., Kamide, K., Katsuya, T., Yasuda, O., Oguro, R., Yamamoto, K., et al. (2012). CVD-associated non-coding RNA, ANRIL, modulates expression of atherogenic pathways in VSMC. *Biochem. Biophys. Res. Commun.* 419, 612–616. doi: 10.1016/j.bbrc.2012.02.050

Cordes, K. R., Sheehy, N. T., White, M. P., Berry, E. C., Morton, S. U., Muth, A. N., et al. (2009). miR-145 and miR-143 regulate smooth muscle cell fate and plasticity. *Nature* 460, 705–710. doi: 10.1038/nature08195

Dai, J., Michineau, S., Franck, G., Desgranges, P., Becquemin, J.-P, Gervais, M, et al. (2011). Long term stabilization of expanding aortic aneurysms by a short course of cyclosporine A through transforming growth factor-beta induction. *PLoS ONE* 6:e28903. doi: 10.1371/journal.pone.0028903

Daugherty, A, and Cassis, L. A. (2004). Mouse models of abdominal aortic aneurysms. *Arterioscler. Thromb. Vasc. Biol.* 24, 429–434. doi: 10.1161/01.ATV.0000118013.72016.ea

Daugherty, A., Rateri, D., Charo, I., Owens, A. III, Howatt, D, and Cassis, L. (2010). Angiotensin II infusion promotes ascending aortic aneurysms: attenuation by CCR2 deficiency in apoEMinus Sign/Minus Sign mice. *Clin. Sci.* 118, 681–689. doi: 10.1042/CS20090372

Davis, B. N., Hilyard, A. C., Nguyen, P. H., Lagna, G, and Hata, A. (2009). Induction of microRNA-221 by platelet-derived growth factor signaling is critical for modulation of vascular smooth muscle phenotype. *J. Biol. Chem.* 284, 3728–3738. doi: 10.1074/jbc.M808788200

Demers, P., Miller, C., Mitchell, R. S., Kee, S. T., Chagonjian, R. L., and Dake, M. D. (2004). Chronic traumatic aneurysms of the descending thoracic aorta: mid-term results of endovascular repair using first and second-generation stent-grafts. *Eur. J. Cardiothorac. Surg.* 25, 394–400. doi: 10.1016/j.ejcts.2003.11.035

Eckhouse, S. R., Logdon, C. B., Oelsen, J. M., Patel, R. K., Rice, A. D., Stroud, R. E., et al. (2013). Reproducible porcine model of thoracic aortic aneurysm. *Circulation* 128:S186–S193. doi: 10.1161/CIRCULATIONAHA.112.000363

El-Hamamsy, I, and Yacoub, M. H. (2009). Cellular and molecular mechanisms of thoracic aortic aneurysms. *Nat. Rev. Cardiol.* 6, 771–786. doi: 10.1038/nrcardio.2009.191

Elia, L., Quintavalle, M., Zhang, J., Contu, R., Cossu, L., Latronico, M. V., et al. (2009). The knockout of miR-143 and-145 alters smooth muscle cell maintenance and vascular homeostasis in mice: correlates with human disease. *Cell Death Differ.* 16, 1590–1598. doi: 10.1038/cdd.2009.153

Elmore, J. R., Obmann, M. A., Kuivaniemi, H., Tromp, G., Gerhard, G. S., Franklin, D. P., et al. (2009). Identification of a genetic variant associated with abdominal aortic aneurysms on chromosome 3p12. 3 by genome wide association. *J. Vasc. Surg.* 49, 1525–1531. doi: 10.1016/j.jvs.2009.01.041

Gregory, R. I., Chendrimada, T. P., and Shiekhattar, R. (2006). "MicroRNA biogenesis: isolation and characterization of the microprocessor complex," in *MicroRNA Protocols*, ed S. Ying (Totowa, NJ: Humana Press), 33–47.

Gretarsdottir, S., Baas, A. F., Thorleifsson, G., Holm, H., den Heijer, M., de Vries, J.-P. P., et al. (2010). Genome-wide association study identifies a sequence variant within the DAB2IP gene conferring susceptibility to abdominal aortic aneurysm. *Nat. Genet.* 42, 692–697. doi: 10.1038/ng.622

Guo, D., Hasham, S., Kuang, S.-Q, Vaughan, C. J., Boerwinkle, E., Chen, H., et al. (2001). Familial thoracic aortic aneurysms and dissections genetic heterogeneity with a major locus mapping to 5q13-14. *Circulation* 103, 2461–2468. doi: 10.1161/01.CIR.103.20.2461

Guo, D. C., Papke, C. L., He, R, and Milewicz, D. M. (2006). Pathogenesis of thoracic and abdominal aortic aneurysms. *Ann. N.Y. Acad. Sci.* 1085, 339–352. doi: 10.1196/annals.1383.013

Hanada, K., Vermeij, M., Garinis, G. A., de Waard, M. C., Kunen, M. G., Myers, L., et al. (2007). Perturbations of vascular homeostasis and aortic valve abnormalities in fibulin-4 deficient mice. *Circ. Res.* 100, 738–746. doi: 10.1161/01.RES.0000260181.19449.95

Harrison, S. C., Smith, A. J., Jones, G. T., Swerdlow, D. I., Rampuri, R., Bown, M. J., et al. (2013). Interleukin-6 receptor pathways in abdominal aortic aneurysm. *Eur. Heart J.* 34, 3707–3716. doi: 10.1093/eurheartj/ehs354

Hinterseher, I., Tromp, G, and Kuivaniemi, H. (2011). Genes and abdominal aortic aneurysm. *Ann. Vasc. Surg.* 25, 388–412. doi: 10.1016/j.avsg.2010.09.004

Ishibashi, M., Egashira, K., Zhao, Q., Hiasa, K., Ohtani, K., Ihara, Y., et al. (2004). Bone marrow–derived monocyte chemoattractant protein-1 receptor CCR2 is critical in angiotensin II–induced acceleration of atherosclerosis and aneurysm formation in hypercholesterolemic mice. *Arterioscler. Thromb. Vasc. Biol.* 24:e174–e178. doi: 10.1161/01.ATV.0000143384.69170.2d

Ji, R., Cheng, Y., Yue, J., Yang, J., Liu, X., Chen, H., et al. (2007). MicroRNA expression signature and antisense-mediated depletion reveal an essential role of MicroRNA in vascular neointimal lesion formation. *Circ. Res.* 100, 1579–1588. doi: 10.1161/CIRCRESAHA.106.141986

Jones, G. T., Bown, M. J., Gretarsdottir, S., Romaine, S. P., Helgadottir, A., Yu, G., et al. (2013). A sequence variant associated with sortilin-1 (SORT1) on 1p13. 3 is independently associated with abdominal aortic aneurysm. *Hum. Mol. Genet.* 22, 2941–2947. doi: 10.1093/hmg/ddt141

Jones, G. T., Thompson, A. R., van Bockxmeer, F. M., Hafez, H., Cooper, J. A., Golledge, J., et al. (2008). Angiotensin II type 1 receptor 1166C polymorphism is associated with abdominal aortic aneurysm in three

independent cohorts. *Arterioscler. Thromb. Vasc. Biol.* 28, 764–770. doi: 10.1161/ATVBAHA.107.155564

Jones, J. A., Stroud, R. E., O'Quinn, E. C., Black, L. E., Barth, J. L., Elefteriades, J. A., et al. (2011). Selective MicroRNA suppression in human thoracic aneurysms relationship of miR-29a to aortic size and proteolytic induction. *Circ. Cardiovasc. Genet.* 4, 605–613. doi: 10.1161/CIRCGENETICS.111.960419

Kapranov, P., Willingham, A. T., and Gingeras, T. R. (2007). Genome-wide transcription and the implications for genomic organization. *Nat. Rev. Genet.* 8, 413–423. doi: 10.1038/nrg2083

Kim, C. W., Kumar, S., Son, D. J., Jang, I.-H., Griendling, K. K., and Jo, H. (2014). Prevention of abdominal aortic aneurysm by Anti–MicroRNA-712 or Anti–MicroRNA-205 in angiotensin II–infused mice. *Arterioscler. Thromb. Vasc. Biol.* 34, 1412–1421. doi: 10.1161/ATVBAHA.113.303134

Kornienko, A. E., Guenzl, P. M., Barlow, D. P., and Pauler, F. M. (2013). Gene regulation by the act of long non-coding RNA transcription. *BMC Biol.* 11:59. doi: 10.1186/1741-7007-11-59

Lederle, F. A., Johnson, G. R., Wilson, S. E., Chute, E. P., Littooy, F. N., Bandyk, D., et al. (1997). Prevalence and associations of abdominal aortic aneurysm detected through screening. *Ann. Intern. Med.* 126, 441–449. doi: 10.7326/0003-4819-126-6-199703150-00004

Lee, C. G., Da Silva, C. A., Cruz, C. S. D., Ahangari, F., Ma, B., Kang, M.-J., et al. (2011). Role of chitin and chitinase/chitinase-like proteins in inflammation, tissue remodeling, and injury. *Ann. Rev. Physiol.* 73, 479–501. doi: 10.1146/annurev-physiol-012110-142250

Leeper, N. J., Raiesdana, A., Kojima, Y., Chun, H. J., Azuma, J., Maegdefessel, L., et al. (2011). MicroRNA−26a is a novel regulator of vascular smooth muscle cell function. *J. Cell. Physiol.* 226, 1035–1043. doi: 10.1002/jcp.22422

Leung, A., Trac, C., Jin, W., Lanting, L., Akbany, A., Sætrom, P., et al. (2013). Novel long noncoding RNAs are regulated by angiotensin II in vascular smooth muscle cells. *Circ. Res.* 113, 266–278. doi: 10.1161/CIRCRESAHA.112.300849

Liao, M., Zou, S., Weng, J., Hou, L., Yang, L., Zhao, Z., et al. (2011). A microRNA profile comparison between thoracic aortic dissection and normal thoracic aorta indicates the potential role of microRNAs in contributing to thoracic aortic dissection pathogenesis. *J. Vas. Surg.* 53, 1341–1349. e3. doi: 10.1016/j.jvs.2010.11.113

Liu, G., Huang, Y., Lu, X., Lu, M., Huang, X., Li, W, et al. (2010). Identification and characteristics of microRNAs with altered expression patterns in a rat model of abdominal aortic aneurysms. *Tohoku J. Exp. Med.* 222, 187–193. doi: 10.1620/tjem.222.187

Liu, X., Cheng, Y., Yang, J., Xu, L, and Zhang, C. (2012). Cell-specific effects of miR-221/222 in vessels: molecular mechanism and therapeutic application. *J. Mol. Cell. Cardiol.* 52, 245–255. doi: 10.1016/j.yjmcc.2011.11.008

Maegdefessel, L., Azuma, J., Toh, R., Merk, D. R., Deng, A., Chin, J. T., et al. (2012). Inhibition of microRNA-29b reduces murine abdominal aortic aneurysm development. *J. Clin. Invest.* 122, 497–506. doi: 10.1172/JCI61598

Maegdefessel, L., Dalman, R. L., and Tsao, P. S. (2014a). Pathogenesis of abdominal aortic aneurysms: microRNAs, proteases, genetic associations. *Ann. Rev. Med.* 65, 49–62. doi: 10.1146/annurev-med-101712-174206

Maegdefessel, L., Spin, J. M., Raaz, U., Eken, S. M., Toh, R., Azuma, J., et al. (2014b). miR-24 limits aortic vascular inflammation and murine abdominal aneurysm development. *Nat. Commun.* 5:5214. doi: 10.1038/ncomms6214

McKellar, S. H., Tester, D. J., Yagubyan, M., Majumdar, R., Ackerman, M. J., and Sundt, T. M. III. (2007). Novel *NOTCH*1 mutations in patients with bicuspid aortic valve disease and thoracic aortic aneurysms. *J. Thorac. Cardiovasc. Surg.* 134, 290–296. doi: 10.1016/j.jtcvs.2007.02.041

Mohammad, F., Pandey, R. R., Nagano, T., Chakalova, L., Mondal, T., Fraser, P, et al. (2008). Kcnq1ot1/Lit1 noncoding RNA mediates transcriptional silencing by targeting to the perinucleolar region. *Mol. Cell. Biol.* 28, 3713–3728. doi: 10.1128/MCB.02263-07

Nuenninghoff, D. M., Hunder, G. G., Christianson, T. J., McClelland, R. L., and Matteson, E. L. (2003). Incidence and predictors of large-artery complication (aortic aneurysm, aortic dissection, and/or large-artery stenosis) in patients with giant cell arteritis: a population-based study over 50 years. *Arthritis. Rheum.* 48, 3522–3531. doi: 10.1002/art.11353

Premakumari, V., Chukkapalli, S., Rivera, M., Velsko, I., Phillippi, J., Gleason, T., et al. (2014). Microrna expression signature in human thoracic and abdominal aortic aneurysms. *Atherosclerosis* 235:e130. doi: 10.1016/j.atherosclerosis.2014.05.363

Riches, K., Angelini, T. G., Mudhar, G. S., Kaye, J., Clark, E., Bailey, M. A., et al. (2013). Exploring smooth muscle phenotype and function in a bioreactor model of abdominal aortic aneurysm. *J. Transl. Med.* 11, 1–13. doi: 10.1186/1479-5876-11-208

Shimizu, K., Mitchell, R. N., and Libby, P. (2006). Inflammation and cellular immune responses in abdominal aortic aneurysms. *Arterioscler. Thromb. Vasc. Biol.* 26, 987–994. doi: 10.1161/01.ATV.0000214999.12921.4f

Skroblin, P, and Mayr, M. (2014). "Going long": long non-coding RNAs as biomarkers. *Circ. Res.* 115, 607–609. doi: 10.1161/CIRCRESAHA.114.304839

Song, J. T., Hu, B., Qu, H. Y., Bi, C. I., Huang, X. Z., and Zhang, M. (2012). Mechanical stretch modulates microRNA 21 expression, participating in proliferation and apoptosis in cultured human aortic smooth muscle cells. *PLoS ONE* 7:e47657. doi: 10.1371/journal.pone.0047657

Sun, J., Sukhova, G. K., Yang, M., Wolters, P. J., MacFarlane, L. A., Libby, P., et al. (2007). Mast cells modulate the pathogenesis of elastase-induced abdominal aortic aneurysms in mice. *J. Clin. Invest.* 117, 3359–3368. doi: 10.1172/JCI31311

Szekanecz, Z., Shah, M. R., Pearce, W. H., and Koch, A. E. (1994). Human atherosclerotic abdominal aortic aneurysms produce interleukin (IL)-6 and interferon-gamma but not IL-2 and IL-4: the possible role for IL-6 and interferon-gamma in vascular inflammation. *Agents Actions* 42, 159–162. doi: 10.1007/BF01983484

Tanaka, A., Hasegawa, T., Chen, Z., Okita, Y, and Okada, K. (2009). A novel rat model of abdominal aortic aneurysm using a combination of intraluminal elastase infusion and extraluminal calcium chloride exposure. *J. Vasc. Surg.* 50, 1423–1432. doi: 10.1016/j.jvs.2009.08.062

Torella, D., Iaconetti, C., Catalucci, D., Ellison, G. M., Leone, A., Waring, C. D., et al. (2011). MicroRNA-133 controls vascular smooth muscle cell phenotypic switch *in vitro* and vascular remodeling *in vivo*. *Circ. Res.* 109, 880–893. doi: 10.1161/CIRCRESAHA.111.240150

Wahlgren, C. M., Larsson, E., Magnusson, P. K., Hultgren, R, and Swedenborg, J. (2010). Genetic and environmental contributions to abdominal aortic aneurysm development in a twin population. *J. Vasc. Surg.* 51, 3–7. discussion: 7. doi: 10.1016/j.jvs.2009.08.036

Wang, S., Zhang, X., Yuan, Y., Tan, M., Zhang, L., Xue, X., et al. (2014). BRG1 expression is increased in thoracic aortic aneurysms and regulates proliferation and apoptosis of vascular smooth muscle cells through the long noncoding RNA HIF1A-AS1 *in vitro*. *Eur. J. Cardiothorac. Surg.* 47, 439–446. doi: 10.1093/ejcts/ezu215

Wang, Y., Ait-Oufella, H., Herbin, O., Bonnin, P., Ramkhelawon, B., Taleb, S., et al. (2010). TGF-β activity protects against inflammatory aortic aneurysm progression and complications in angiotensin II–infused mice. *J. Clin. Invest.* 120, 422–432. doi: 10.1172/JCI38136

Yoon, S., Tromp, G., Vongpunsawad, S., Ronkainen, A., Juvonen, T, and Kuivaniemi, H. (1999). Genetic analysis of MMP3, MMP9, and PAI-1 in Finnish patients with abdominal aortic or intracranial aneurysms. *Biochem. Biophys. Res. Commun.* 265, 563–568. doi: 10.1006/bbrc.1999.1721

Yoshimura, K., Aoki, H., Ikeda, Y., Fujii, K., Akiyama, N., Furutani, A., et al. (2005). Regression of abdominal aortic aneurysm by inhibition of c-Jun N-terminal kinase. *Nat. Med.* 11, 1330–1338. doi: 10.1038/nm1335

Zampetaki, A., Attia, R., Mayr, U., Gomes, R. S., Phinikaridou, A., Yin, X., et al. (2014). Role of miR-195 in aortic aneurysmal disease. *Circ. Res.* 115, 857–866. doi: 10.1161/CIRCRESAHA.115.304361

Zhan, Y., Kim, S., Izumi, Y., Izumiya, Y., Nakao, T., Miyazaki, H, et al. (2003). Role of JNK, p38, and ERK in platelet-derived growth factor–induced vascular proliferation, migration, and gene expression. *Arterioscler. Thromb. Vasc. Biol.* 23, 795–801. doi: 10.1161/01.ATV.0000066132.32063.F2

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REVIEW
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Is Vascular Amyloidosis Intertwined with Arterial Aging, Hypertension and Atherosclerosis?

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Vascular amyloidosis (VA) is a component of aging, but both VA and aging move forward together. Although, not all age-related molecules are involved with VA, some molecules are involved in a crosstalk between both of them. However, the cellular mechanism by which, vascular cells are phenotypically shifted to arterial remodeling, is not only involved in aging but also linked to VA. Additionally, patients with hypertension and atherosclerosis are susceptible to VA, while amyloidosis alone may provide fertile soil for the initiation and progression of subsequent hypertension and atherosclerosis. It is known that hypertension, atherosclerosis and amyloidosis can be viewed as accelerated aging. This review summarizes the available experimental and clinical evidence to help the reader to understand the advance and underlying mechanisms for VA involvement in and interaction with aging. Taken together, it is clear that VA, hypertension and atherosclerosis are closely intertwined with arterial aging as equal partners.

Keywords: vascular amyloidosis, intertwine, vascular aging, hypertension, atherosclerosis

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INTRODUCTION

Amyloid is found in the aortic walls of almost 100% of the population above 50 years of age (Mucchiano et al., 1992), and also aged people are susceptible to hypertension and atherosclerosis, which indicates that vascular amyloidosis (VA), hypertension and atherosclerosis are highly associated with aging. However, few studies have focused on the relationship between amyloidosis and arterial diseases. We reviewed the molecular and cellular mechanisms of VA and give evidence how strongly it is fundamentally intertwined together with hypertension, atherosclerosis, and aging at the cellular and molecular levels (Wang et al., 2010a).

DISTRIBUTION OF AMYLOID PROTEINS

Amyloidosis is a disorder of protein metabolism characterized by extracellular accumulation of abnormal insoluble amyloid fibrils. About 30 proteins are known to form pathogenic amyloid or amyloid-like fibrillary networks in a wide range of human tissues which are associated with diseases having high morbidity and mortality rates (Galant et al., 2017). However, there are only four kinds of amyloid proteins which are mainly associated with VA. Here we review the four main amyloid proteins that are susceptible to deposit in different arteries and interact with different cells (**Table 1**).

In general, these four amyloid proteins TTR (Transthyretin), Apo1 (Apolipoprotein A-1), immunoglobin γ, and medin are susceptible to deposit, respectively at cerebral artery, coronary artery and aorta. If amyloid proteins deposit within the walls of the cerebral vasculature with subsequent aggressive vascular inflammation, it will lead to recurrent hemorrhagic strokes

TABLE 1 | The relationships among the four kinds of amyloid proteins, the arteries likely deposited, the cells likely involved and references.

Amyloid protein	Arterial tree	Cellular role
TTR	Coronary Booth et al., 1995, Cerebral Sekijima, 2015	EC Nunes et al., 2013
Apo1	Coronary Stewart et al., 2007, Aorta Mucchiano et al., 2001	Matrix Ramella et al., 2011
Immunoglobin γ	Cerebra Audemard et al., 2012	EC Truran et al., 2014, Matrix Berghoff et al., 2003
Medin	Aorta Davies et al., 2015a, Cerebral Peng et al., 2005	EC Davies et al., 2015b, VSMC Hagggvist et al., 1999

(Agyare et al., 2014); If they deposit within the walls of the coronary artery, they will lead to angina pectoris, even ischemia cardiomyopathy; If they deposit within the wall of aorta, they will lead to hypertension, atherosclerosis, and even dissecting aneurysm eventually (Wang et al., 2010a).

HYPERTENSION AND VA

What's the relationship between VA and hypertension? Let's review it. In 1997, Rotterdam Study showed that hypertension, as an indicator of atherosclerosis, was not only related to vascular dementia, but also Alzheimer's disease (AD) (Forette et al., 1998). Hypertension may initiate vascular damage before the onset of AD so as to make symptoms more pronounced and progress more rapidly (Liu et al., 2014). Furthermore, compromised vessels may be more vulnerable to the deleterious effects of amyloid β-protein (Aβ). For example, since blood brain barrier (BBB) integrity is damaged by hypertension, blood components may enter the brain prior to the large-scale accumulation of Aβ. These blood components may serve as a seed for Aβ deposition and promote vascular inflammation, resulting in cellular damage and the release of toxic molecules. It is suggested that hypertension has a significant effect on the onset and progression of AD. Epidemiological studies have shown that AD rapidly progressed in elderly hypertensive people. It is also suggested by numerous epidemiological studies that treating hypertension in midlife may be an effective strategy for reducing the likelihood of AD onset later in life (Kruyer et al., 2015).

Conversely, VA, as a main part of vascular aging, is involved with age-associated vascular remodeling. During the whole process, every step is linked closely to the initiation and progression of hypertension. Endothelial dysfunction leads to hypertension directly with the decrease of nitric oxide since endothelium-dependent vasodilation is impaired (Puddu et al., 2000). VSMCs, as the most important cells of vascular wall, shift the phenotypes during VA, which eventually lead to increase vascular wall stiffening, decrease elasticity of vascular wall and hypertension occurs subsequently (Wang et al., 2014a). Fibroblasts, as the main cells in the adventitia of vascular wall, once activated during amyloidosis, affect both intima and adventitia thickening, which make hypertension accompany with it (Peng et al., 2002). The vascular extracellular matrix (ECM) also is involved in amyloidosis and hypertension due to the deposition of amyloid proteins in the ECM, and arterial elasticity can be injured so that hypertension ensues (Larsson et al., 2006). Collectively, all hypertensive patients are vulnerable to VA (Kruyer et al., 2015), while VA accelerates the initiation and progression of hypertension (Larsson et al., 2006).

ATHEROSCLEROSIS AND VA

Similarly, it is certain that the relation between atherosclerosis and VA exists. Roher et al. (2003) found that cerebrovascular atherosclerosis is significantly more frequent and severe in those with AD compared with normal aging and those with other neurodegenerative diseases. Also, Beach et al. (2007) found there was an association between circle of Willis atherosclerosis and AD. Furthermore, they found that this association is relatively specific to AD rather than other major neurodegenerative diseases. Alternatively, the finding that amyloid-β is present in advanced human atherosclerotic lesions suggests that atherosclerosis may be promoted directly by AD pathology, potentially through altering the metabolism of oxidized lipoproteins (Yarchoan et al., 2012). Moreover, the micro-environment produced by MFG-E8 and medin who are intimately involved with VA through promoting the secretion of many inflammatory molecules and the phenotypic shifts of vascular cells render the arterial wall a fertile soil for atherosclerosis to flourish.

MOLECULAR MECHANISMS OF VASCULAR AMYLOIDOSIS

It has been demonstrated that MFG-E8 and medin are rich in the "amyloid-like" aortic media. Simultaneously, MFG-E8, as a novel marker of vascular aging, induces proinflammatory molecules which are involved within the Ang II signal pathway, including Medin, MMP-2/9, MCP-1, as outlined in **Figure 1**.

MFG-E8

Milk fat globule-epidermal growth factor-8 (MFG-E8), is also known as lactadherin. Growing evidence has indicated that MFG-E8 is a secreted inflammatory mediator that orchestrates diverse cellular interactions involved in the pathogenesis of various diseases, including vascular aging and amyloidosis. During aging, as the downstream molecule of Ang II signal pathway, both MFG-E8 transcription and translation increase within the arterial walls of various species (Fu et al., 2009). Moreover, many downstream inflammatory molecules within the Ang II signal pathway are induced by MFG-E8. During amyloidosis, as the origin of amyloid protein, MFG-E8 cleaves into medin which increases the stiffness of vascular wall through the binding to tropoelastin. Furthermore, MFG-E8 leads to the phenotypic shifts of all kinds of vascular cells which supply a fertile soil for arterial diseases. Therefore, MFG-E8 makes VA, aging, hypertension and atherosclerosis intertwine together.

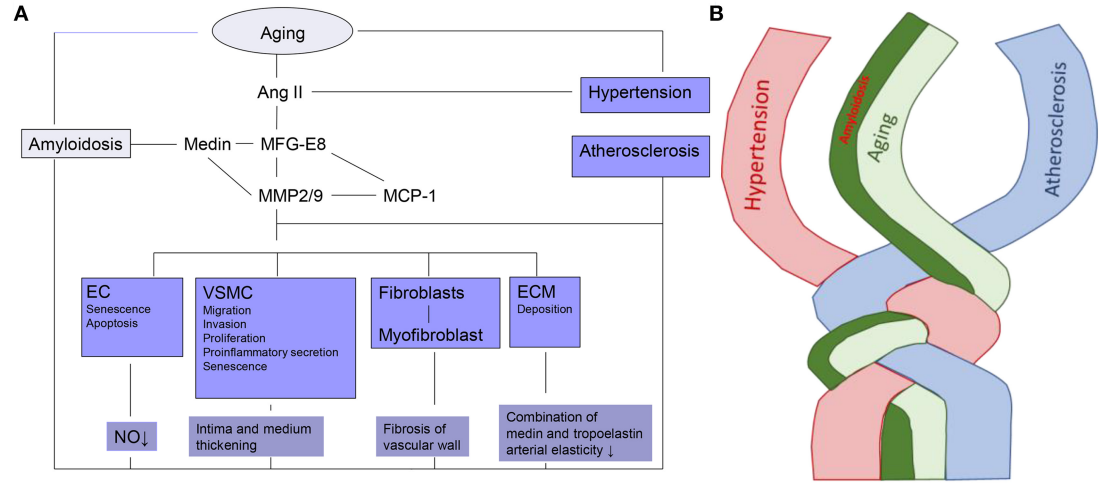

FIGURE 1 | The linkage and twist of VA with aging. **(A)** Cellular and molecular mechanisms of VA. Ec, endothelial cell; VSMC, vascular smooth muscle cell; ECM, extracellular matrix; Ang II, Angiotensin II; MFG-E8, Milk fat globule-epidermal growth factor-8; MMP2/9-zinc, containing endopeptidases2/9; MCP-1, Monocyte chemoattractant protein-1; NO, Nitric oxide. **(B)** Amyloidosis is intertwined with arterial aging, hypertension and atherosclerosis.

Medin

Vascular amyloidosis (VA) is markedly increased with aging (Wang et al., 2013). As mentioned, medin is the most common amyloid protein which deposits in the wall of aorta. Prior studies have shown that a 50 amino acid polypepetide called medin, cleaved from MFG-E8's C2-like domain (Haggqvist et al., 1999), not only tightly binds to tropoelastin but also eventually incorporates into arterial amyloids (Larsson et al., 2006). These medin amyloids have been observed within arterial walls, including that of both aorta and temporal artery (Peng et al., 2005), in Caucasian populations over 50 years old (Davies et al., 2014, 2015b). Recent studies have shown that aortic medin amyloids serve as a trigger for amyloid A-derived amyloidosis (Larsson et al., 2011).

Ang II

Ang II, as a leading proinflammatory vasoactive stressor, can induce the inflammatory molecules in the signaling cascade within the aged arterial wall, in which VA may be progressing. Augmentation of these Ang II biosignals appear to be the foundation of the molecular mechanisms of age-associated adverse arterial structural remodeling (Wang et al., 2010b). It is known that "local" Ang II regulated independently is over 1,000-fold more abundant than "circulating" Ang II and plays an important role in vascular pathophysiology in elderly people (Diz, 2008). Some studies have shown that expression of Ang II protein increases not only in the aged aortic wall of rats but also in the thickened intima of older monkey aortae (Wang et al., 2003; Jiang et al., 2008). Moreover, the immunofluorescence of Ang II increases within the "grossly normal" aortic wall of older people (Wang et al., 2007). Simultaneously, it has been found that MFG-E8 can be induced by Ang II in VSMCs isolated from rat aortae, which indicates that MFG-E8 is required for Ang II to increase some downstream inflammatory molecules. Consequently, MFG-E8, which plays a crucial role in arterial aging and amyloidosis, is a novel link between them.

MMP-2/9

The zinc-containing endopeptidases, MMP-2/-9, are involved in the breakdown of ECM occurred in amyloidosis, aging, hypertension and atherosclerosis (Wang et al., 2014b, 2015). The activity of MMP-2/9 increases with amyloidosis (Peng et al., 2007) and advancing age within the aortic wall which is linked to increased Ang II/MFG-E8/medin signaling (Wang et al., 2003, 2005, 2007). It has been shown that MMP-2/9 activity *in situ* is enhanced both in the "grossly normal" aortic segments of old people (Wang et al., 2010b) and in thoracic aortic aneurysms and dissections (Peng et al., 2007). The proinflammatory microenvironment MMPs create through modifying ECM and the introduction of inflammatory molecules shifts the phenotypes of vascular cells, including endothelial cells (ECs), vascular smooth muscle cells (VSMCs), and fibroblasts, which are so-called arterial remodeling occurred in various arterial diseases. Therefore, MMP2/9, is another group of key factors that intertwines amyloidosis, aging, hypertension and atherosclerosis.

MCP-1

MCP-1, which belongs to the G-protein coupled receptor 1 family, is a notorious inflammatory cytokine downstream of Ang II signaling in the cardiovascular system (Wang and Shah, 2015) and originally functions by recruiting immune cells to sites of inflammation. Vascular amyloidosis (VA), aging, hypertension and atherosclerosis are all a chronic, low-grade inflammation (Wang et al., 2014b, 2015), therefore, MCP-1 is involved intimately in the inflammatory process of arterial diseases. Additionally, it has been demonstrated that MFG-E8 induced by Ang II promotes the expression of MCP-1 in VSMCs

within the old rat aortic wall, which leads MCP-1 within the Ang II/MFG-E8/VSMC invasion signaling cascade (Wang et al., 2014a). The relationship between MCP-1 and MFG-E8 can be viewed as an important signal relationship in both VA and aging.

CELLULAR MECHANISMS OF AMYLOIDOSIS

In arterial diseases, such as aging, amyloidosis, hypertension, and atherosclerosis, all the arterial cells including ECs, VSMC, fibroblasts, and matrix are ultimately the downstream targets of the signal molecules. Adverse arterial restructuring which occurs in all arterial diseases is the result of phenotypic shifts of those arterial cells. The amyloidosis-related molecule MFG-E8 and medin have already been proven to have a close relationship with the arterial cells and matrix, as illustrated in **Figure 1**.

Endothelial Cells (ECs)

Endothelial integrity is important to vascular health, with ECs building the frontline cells of the arterial wall (Wang et al., 2014b). It is suggested that the amyloidosis associated protein medin is toxic to aortic ECs *in vitro* (Madine and Middleton, 2010) and may underlie the pathogenesis of aortic aneurysm *in vivo* through a weakening of the aortic wall (Peng et al., 2007). In addition, the increased inflammatory load, such as elevated MFG-E8 in the old endothelia may damage endothelial mitochondrial DNA and interfere with the mitochondria life cycle via enhanced ROS generation, which consequently initiates and promotes EC senescence and apoptosis (Wang et al., 2005, 2007). These cellular events and micro-environments lead to endothelia dysfunction which renders the arterial wall a fertile soil in which amyloidosis and atherosclerosis may flourish (Najjar et al., 2005; Wang et al., 2014b). Interestingly, endothelial dysfunction also occurs with aging even in healthy adults (Sepulveda et al., 2017). additionally, due to decreased bioavailability of nitric oxide, endothelial dysfunction which impairs endothelium-dependent vasodilation in hypertension (Puddu et al., 2000) may precede the development of clinical hypertension (Najjar et al., 2005). Collectively, endothelial dysfunction can be viewed as a prelude for arterial disease.

Vascular Smooth Muscle Cells (VSMCs)

The phenotypic shifts of VSMC, including enhanced migration, invasion, proliferation, proinflammatory secretion, and senescence, are the most important characters of vascular aging and amyloidosis. They also supply a fertile stage for the initiation and progression of the pathogenesis of hypertension and atherosclerosis in the elderly (Wang et al., 2014a,b). However, those phenotypic shifts are associated directly with the inflammatory molecules Ang II, MFG-E8, and MCP-1. Young VSMCs with the treatment of Ang II secrete MFG-E8 to levels similar to old untreated cells (Gao et al., 2008; Fu et al., 2009). MFG-E8 is induced by Ang II in aging, while MFG-E8 in amyloidosis induces the expression of MCP-1 in VSMCs within the rat aortic wall (Fu et al., 2009), leading to invasion of VSMC. Additionally, it is well-known that MFG-E8, the precursor of medin, is abundantly expressed by VSMCs (Hagggvist et al., 1999; Peng et al., 2002, 2005). Moreover, it has been shown that aortic amyloidosis is also involved with a proinflammatory VSMC phenotypic shift due to the accumulation of MFG-E8 and medin in the aorta (Wang et al., 2014b). Chronic exposure of VSMC to intact MFG-E8 markedly increases proliferation and invasion (Fu et al., 2009; Wang et al., 2012), while it is shown that chronic exposure of VSMC to medin fragments significantly increase secreted MMP-2 levels, which promote phenotypic shifts of vascular cells through the proinflammatory microenvironment (Peng et al., 2007; Lakatta, 2013).

Fibroblasts

Fibroblasts compose the major vascular cells in the adventitia which serves as the artery's final line of defense (Michel et al., 2007). A growing body of evidence indicates that adventitial remodeling intertwines with the VSMC phenotypic shift (Sierevogel et al., 2002; Moos et al., 2005; Passman et al., 2008; Grabner et al., 2009). Variations in the adventitia could be a signal of impending vascular disorders (Wang et al., 2010b). With advancing age or amyloidosis, fibroblasts become activated, synthesize smooth muscle actin and transit into myofibroblasts which contribute to both intimal and adventitial thickening (Najjar et al., 2005; Sepulveda et al., 2017). Additionally, the adventitia and adjacent perivascular white adipose tissue, similar to the intima, is a major source of MMPs (Najjar et al., 2005; Gao et al., 2008; Wang et al., 2012; Sepulveda et al., 2017). The transition of fibroblasts to myofibroblasts points to the remodeling of adventitia and the activation of MMPs (Sierevogel et al., 2002). Therefore, the inhibition of the activation of MMPs may help to retard the progress of arterial diseases.

ECM

The ECM is not only essential for the structural integrity of arterial walls but also serves as a platform for the binding of vascular cell molecules that are crucial for intercellular communication (Wang et al., 2013). Immunostaining and proteomics analyses have also demonstrated that MFG-E8 is a secreted extracellular adhesive molecule within the aortic extracellular space (Hagggvist et al., 1999), while medin, as a unique amyloid protein, is deposited exactly in the ECM (Wang et al., 2013). Furthermore, the MFG-E8-derived medin is not only co-localized with elastic fibers of older arteries in the ECM but also binds to tropo-elastin in a concentration-dependent fashion, forming amyloid-like fibrils within the extracellular space of arterial walls (Larsson et al., 2006; Lakatta, 2013). The related arteries can't stretch and recoil freely as they are subjected to decreased arterial elasticity (Larsson et al., 2006), which leads to hypertension through arterial stiffening which is a hallmark of aging (Zieman et al., 2005). From this view point, amyloidosis is closely intertwined with aging and hypertension.

FUNCTIONAL VARIATIONS IN AMYLOIDOSIS TISSUES

It is known that about 80% of Alzheimer's disease (AD) patients show some degree of Cerebral amyloid angiopathy (CAA) (Michel et al., 2007). CAA is characterized by the deposition

of amyloid beta (Aβ) proteins within the walls of small to medium-sized blood vessels of the brain (Agyare et al., 2014). It has been demonstrated that AD patients with CAA manifest a declining cognitive test performance compared to their early life time. It is believed that several mechanisms linking CAA to cognition include cerebrovascular inflammation, vascular dysfunction (Viswanathan et al., 2008; Arvanitakis et al., 2011; Urbach, 2011; Agyare et al., 2014), oxidative stress, tissue microstructural changes, etc. Additional studies are needed to help elucidate the role of these factors in the relationship between CAA and cognition decline (Greenberg, 2002; Arvanitakis et al., 2011).

If coronary blood flow decreases due to the deposition of amyloid, it will lead to angina pectoris, or even ischemia cardiomyopathy (Bulut et al., 2016). It has been suggested that several mechanisms are involved in the coronary microvascular dysfunction, including wall thickening, luminal narrowing of the vessels due to intramural amyloid deposition in the vessel wall, perivascular and interstitial amyloid deposits causing extrinsic compression of the microvasculature, and endothelial and autonomic dysfunction (Kawata et al., 2013; Bulut et al., 2016).

If amyloid protein, especially medin, deposits within the wall of the aorta, it will lead to hypertension, atherosclerosis and dissecting aneurysm. As we mentioned earlier, it is found that medin has a close association with the internal elastic lamina, which is not only involved with stiffening of aortic wall but also underlies the pathogenesis of sporadic thoracic aortic aneurysm via weakening of the aortic wall (Madine and Middleton, 2010). Annika Larsson, etc. found that aortae from patients suffering from thoracic aortic aneurysm and dissection contain higher levels of medin than normal aortae (Larsson et al., 2007). Westermark and colleagues proved that widely spread medin amyloid deposits in the thoracic aorta are associated with aortic wall weakening. It is notable that other forms of amyloidosis involving arteries can lead to an increased risk for rupture (Peng et al., 2007). In addition, VSMCs incubated together with medin increase the production of matrix metalloproteinase-2 which degrades elastin and collagen and eventually weakens the vessel wall (Peng et al., 2007).

CONCLUSIONS

Collectively, the prevalence of amyloid diseases in the aging population has become a major health concern and consequently many resources are being invested into developing therapies (Merlini and Westermark, 2004). Indeed, it appears that VA may be strongly intertwined with arterial aging, hypertension and atherosclerosis. Since they all have a close relationship with inflammation, targeting proinflammatory signaling molecules, including Ang II, MFG-E8, medin, and MMPs, etc. would be promising approaches to prevent the pathogenesis of clinical cardiovascular events.

AUTHOR CONTRIBUTIONS

YW: Writing the paper. XF: Research of literatures. BS: Research of literatures. JM: Final editing. WZ: Concept of the review.

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REFERENCES

Agyare, E. K., Jaruszewski, K. M., Curran, G. L., Rosenberg, J. T., Grant, S. C., Lowe, V. J., et al. (2014). Engineering theranostic nanovehicles capable of targeting cerebrovascular amyloid deposits. *J. Control. Release* 185, 121–129. doi: 10.1016/j.jconrel.2014.04.010

Arvanitakis, Z., Leurqans, S. E., Wang, Z., Wilson, R. S., Bennett, D. A., and Schneider, A. J. (2011). Cerebral amyloid angiopathy pathology and cognitive domains in older persons. *Ann. Neurol.* 69, 320–327. doi: 10.1002/ana.22112

Audemard, A., Boutemy, J., Galateau-Salle, F., Macro, M., and Bienvenu, B. (2012). AL amyloidosis with temporal artery involvement simulates giant-cell arteritis. *Joint Bone Spine* 79, 195–197. doi: 10.1016/j.jbspin.2011.09.007

Beach, T. G., Wilson, J. R., Sue, L. I., Newell, A., Poston, M., Cisneros, R., et al. (2007). Circle of Willis atherosclerosis: association with Alzheimer's disease, neuritic plaques and neurofibrillary tangles. *Acta Neuropathol.* 113, 13–21. doi: 10.1007/s00401-006-0136-y

Berghoff, M., Kathpal, M., Khan, F., Skinner, M., Falk, R., and Freeman, R. (2003). Endothelial dysfunction precedes C-fiber abnormalities in primary (AL) amyloidosis. *Ann. Neurol.* 53, 725–730. doi: 10.1002/ana.10552

Booth, D. R., Tan, S. Y., Hawkins, P. N., Pepys, M. B., and Frustaci, A. (1995). A novel variant of transthyretin, 59Thr–>Lys, associated with autosomal dominant cardiac amyloidosis in an Italian family. *Circulation* 91, 962–967. doi: 10.1161/01.CIR.91.4.962

Bulut, M., Keles, N., Caliskan, Z., Kostek, O., Aksu, F., Ozdil, K., et al. (2016). Is the presence of AA amyloidosis associated with impaired coronary flow reserve? *Atherosclerosis* 251, 389–395. doi: 10.1016/j.atherosclerosis.2016.05.041

Davies, H. A., Madine, J., and Middleton, D. A. (2015a). Comparisons with amyloid-beta reveal an aspartate residue that stabilizes fibrils of the aortic amyloid peptide medin. *J. Biol. Chem.* 290, 7791–7803. doi: 10.1074/jbc.M114.602177

Davies, H. A., Phelan, M. M., Wilkinson, M. C., Miqrino, R. Q., Truran, S., Franco, D. A., et al. (2015b). Oxidative stress alters the morphology and toxicity of aortic medial amyloid. *Biophys. J.* 109, 2363–2370. doi: 10.1016/j.bpj.2015.10.034

Davies, H. A., Wilkinson, M. C., Gibson, R. P., and Middleton, A. D. (2014). Expression and purification of the aortic amyloid polypeptide medin. *Protein Expr. Purif.* 98, 32–37. doi: 10.1016/j.pep.2014.02.009

Diz, I. D. (2008). Lewis K Dahl memorial lecture: the renin-angiotensin system and aging. *Hypertension* 52, 37–43. doi: 10.1161/HYPERTENSIONAHA.107.108985

Forette, F., Seux, M. L., Staessen, J. A., Thijs, L., Birkenhager, W. H., Babarskiene, M. R., et al. (1998). Prevention of dementia in randomised double-blind placebo-controlled Systolic Hypertension in Europe (Syst-Eur) trial. *Lancet* 352, 1347–1351. doi: 10.1016/S0140-6736(98)03086-4

Fu, Z., Wang, M., Gucek, M., Zhang, J., Wu, J., Jiang, L., et al. (2009). Milk fat globule protein epidermal growth factor-8: a pivotal relay element within the angiotensin II and monocyte chemoattractant protein-1 signaling cascade mediating vascular smooth muscle cells invasion. *Circ. Res.* 104, 1337–1346. doi: 10.1161/CIRCRESAHA.108.187088

Galant, N. J., Westermark, P., Hiqaki, J. N., and Chakrabartty, A. (2017). Transthyretin amyloidosis: an under-recognized neuropathy and cardiomyopathy. *Clin. Sci.* (*Lond)* 131, 395–409. doi: 10.1042/CS20160413

Gao, B. B., Stuart, L., and Feener, P. E. (2008). Label-free quantitative analysis of one-dimensional PAGE LC/MS/MS proteome: application on angiotensin II-stimulated smooth muscle cells secretome. *Mol. Cell. Proteomics* 7, 2399–2409. doi: 10.1074/mcp.M800104-MCP200

Grabner, R., Lotzer, K., Dopping, S., Hildner, M., Radke, D., Beer, M., et al. (2009). Lymphotoxin β receptor signaling promotes tertiary lymphoid organogenesis in the aorta adventitia of aged ApoE$^{-/-}$ mice. *J. Exp. Med.* 206, 233–248. doi: 10.1084/jem.20080752

Greenberg, M. S. (2002). Cerebral amyloid angiopathy and vessel dysfunction. *Cerebrovasc. Dis.* 13(Suppl. 2), 42–47. doi: 10.1159/000049149

Hagggvist, B., Naslund, J., Sletten, K., Westermark, G. T., Mucchiano, G., Tjemberg, L. O., et al. (1999). Medin: an integral fragment of aortic smooth muscle cell-produced lactadherin forms the most common human amyloid. *Proc. Natl. Acad. Sci. U.S.A.* 96, 8669–8674. doi: 10.1073/pnas.96.15.8669

Jiang, L., Wang, M., Zhang, J., Monticone, R. E., Telljohann, R., Spinetti, G., et al. (2008). Increased aortic calpain-1 activity mediates age-associated angiotensin II signaling of vascular smooth muscle cells. *PLoS ONE* 3:e2231. doi: 10.1371/journal.pone.0002231

Kawata, T., Daimon, M., Hasegawa, R., Toyoda, T., Sekine, T., Himi, T., et al. (2013). Prognostic value of coronary flow reserve assessed by transthoracic Doppler echocardiography on long-term outcome in asymptomatic patients with type 2 diabetes without overt coronary artery disease. *Cardiovasc. Diabetol.* 12:121. doi: 10.1186/1475-2840-12-121

Kruyer, A., Soplop, N., Strickland, S., and Norris, H. E. (2015). Chronic hypertension leads to neurodegeneration in the TgSwDI mouse model of Alzheimer's disease. *Hypertension* 66, 175–182. doi: 10.1161/HYPERTENSIONAHA.115.05524

Lakatta, G. E. (2013). The reality of aging viewed from the arterial wall. *Artery Res.* 7, 73–80. doi: 10.1016/j.artres.2013.01.003

Larsson, A., Malmstrom, S., and Westermark, P. (2011). Signs of cross-seeding: aortic medin amyloid as a trigger for protein AA deposition. *Amyloid* 18, 229–234. doi: 10.3109/13506129.2011.630761

Larsson, A., Peng, S., Persson, H., Rosenbloom, J., Abrams, W. R., and Wassberg, E. (2006). Lactadherin binds to elastin–a starting point for medin amyloid formation? *Amyloid* 13, 78–85. doi: 10.1080/13506120600722530

Larsson, A., Soderberg, L., Westermark, G. T., Sletten, K., Engstrom, U., Tjemberg, L. O., et al. (2007). Unwinding fibril formation of medin, the peptide of the most common form of human amyloid. *Biochem. Biophys. Res. Commun.* 361, 822–828. doi: 10.1016/j.bbrc.2007.06.187

Liu, J., Liu, S., Tanabe, C., Maeda, T., Zou, K., Komano, H., et al. (2014). Differential effects of angiotensin II receptor blockers on Abeta generation. *Neurosci. Lett.* 567, 51–56. doi: 10.1016/j.neulet.2014.03.030

Madine, J., and Middleton, A. D. (2010). Comparison of aggregation enhancement and inhibition as strategies for reducing the cytotoxicity of the aortic amyloid polypeptide medin. *Eur. Biophys. J.* 39, 1281–1288. doi: 10.1007/s00249-010-0581-3

Merlini, G., and Westermark, P. (2004). The systemic amyloidoses: clearer understanding of the molecular mechanisms offers hope for more effective therapies. *J. Intern. Med.* 255, 159–178. doi: 10.1046/j.1365-2796.2003.01262.x

Michel, J. B., Thaunat, O., Houard, X., Meilhac, O., Caliqiuri, G., and Nicoletti, A. (2007). Topological determinants and consequences of adventitial responses to arterial wall injury. *Arterioscler. Thromb. Vasc. Biol.* 27, 1259–1268. doi: 10.1161/ATVBAHA.106.137851

Moos, M. P., John, N., Grabner, R., Nossmann, S., Gunther, B., Vollandt, R., et al. (2005). The lamina adventitia is the major site of immune cell accumulation in standard chow-fed apolipoprotein E-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* 25, 2386–2391. doi: 10.1161/01.ATV.0000187470.31662.fe

Mucchiano, G., Cornwell, G. G. III., and Westermark, P. (1992). Senile aortic amyloid. Evidence for two distinct forms of localized deposits. *Am. J. Pathol.* 140, 871–877.

Mucchiano, G. I., Hagggvist, B., Sletten, K., and Westermark, P. (2001). Apolipoprotein A-1-derived amyloid in atherosclerotic plaques of the human aorta. *J. Pathol.* 193, 270–275. doi: 10.1002/1096-9896(2000)9999:9999<::AID-PATH753>3.0.CO;2-S

Najjar, S. S., Scuteri, A., and Lakatta, G. E. (2005). Arterial aging: is it an immutable cardiovascular risk factor? *Hypertension* 46, 454–462. doi: 10.1161/01.HYP.0000177474.06749.98

Nunes, R. J., de Oliveira, P., Lages, A., Becker, J. D., Marcelino, P., Barroso, E., et al. (2013). Transthyretin proteins regulate angiogenesis by conferring different molecular identities to endothelial cells. *J. Biol. Chem.* 288, 31752–31760. doi: 10.1074/jbc.M113.469858

Passman, J. N., Dong, X. R., Wu, S. P., Maquire, C. T., Hoqan, K. A., Bautch, V. L., et al. (2008). A sonic hedgehog signaling domain in the arterial adventitia supports resident Sca1^{+} smooth muscle progenitor cells. *Proc. Natl. Acad. Sci. U.S.A.* 105, 9349–9354. doi: 10.1073/pnas.0711382105

Peng, S., Glennert, J., and Westermark, P. (2005). Medin-amyloid: a recently characterized age-associated arterial amyloid form affects mainly arteries in the upper part of the body. *Amyloid* 12, 96–102. doi: 10.1080/13506120500107006

Peng, S., Larsson, A., Wassberg, E., Gerwins, P., Thelin, S., Fu, X., et al. (2007). Role of aggregated medin in the pathogenesis of thoracic aortic aneurysm and dissection. *Lab. Invest.* 87, 1195–1205. doi: 10.1038/labinvest.3700679

Peng, S., Westermark, G. T., Naslund, J., Haqqqvist, B., Glennert, J., and Westermark, P. (2002). Medin and medin-amyloid in ageing inflamed and non-inflamed temporal arteries. *J. Pathol.* 196, 91–96. doi: 10.1002/path.1014

Puddu, P., Puddu, G. M., Zaca, F., and Muscari, A. (2000). Endothelial dysfunction in hypertension. *Acta Cardiol.* 55, 221–232. doi: 10.2143/AC.55.4.2005744

Ramella, N. A., Rimoldi, O. J., Prieto, E. D., Schinella, G. R., Sanchez, S. A., Jaureguiberry, M. S., et al. (2011). Human apolipoprotein A-I-derived amyloid: its association with atherosclerosis. *PLoS ONE* 6:e22532. doi: 10.1371/journal.pone.0022532

Roher, A. E., Esh, C., Kokjohn, T. A., Kalback, W., Luehrs, D. C., Seward, J. D., et al. (2003). Circle of willis atherosclerosis is a risk factor for sporadic Alzheimer's disease. *Arterioscler. Thromb. Vasc. Biol.* 23, 2055–2062. doi: 10.1161/01.ATV.0000095973.42032.44

Sekijima, Y. (2015). Transthyretin-type cerebral amyloid angiopathy: a serious complication in post-transplant patients with familial amyloid polyneuropathy. *J. Neurol. Neurosurg. Psychiatry* 86:124. doi: 10.1136/jnnp-2014-308576

Sepulveda, C., Palomo, I., and Fuentes, E. (2017). Mechanisms of endothelial dysfunction during aging: predisposition to thrombosis. *Mech. Ageing Dev.* 164, 91–99. doi: 10.1016/j.mad.2017.04.011

Sierevogel, M. J., Velema, E., van der Meer, F. J., Nijhuis, M. O., Smeets, M., de Kleijn, D. P., et al. (2002). Matrix metalloproteinase inhibition reduces adventitial thickening and collagen accumulation following balloon dilation. *Cardiovasc. Res.* 55, 864–869. doi: 10.1016/S0008-6363(02)00467-4

Stewart, C. R., Haw, A. III., Lopez, R., McDonald, T. O., Callaghan, J. M., McConville, M. J., et al. (2007). Serum amyloid P colocalizes with apolipoproteins in human atheroma: functional implications. *J. Lipid Res.* 48, 2162–2171. doi: 10.1194/jlr.M700098-JLR200

Truran, S., Weissiq, V., Ramirez-Alvarado, M., Franco, D. A., Burciu, C., Georges, J., et al. (2014). Nanoliposomes protect against AL amyloid light chain protein-induced endothelial injury. *J. Liposome Res.* 24, 69–73. doi: 10.3109/08982104.2013.838258

Urbach, H. (2011). Comment on: brain microbleeds and Alzheimer's disease: innocent observation or key player?: Cordonnier, C., van der Flier WM Brain;134:335-44. *Clin. Neuroradiol.* 21, 43–44. doi: 10.1007/s00062-011-0063-8

Viswanathan, A., Patel, P., Rahman, R., Nandigam, R. N., Kinnecom, C., Bracoud, L., et al. (2008). Tissue microstructural changes are independently associated with cognitive impairment in cerebral amyloid angiopathy. *Stroke* 39, 1988–1992. doi: 10.1161/STROKEAHA.107.509091

Wang, M., Fu, Z., Wu, J., Zhang, J., Jiang, L., Khazan, B., et al. (2012). MFG-E8 activates proliferation of vascular smooth muscle cells via integrin signaling. *Aging Cell* 11, 500–508. doi: 10.1111/j.1474-9726.2012.00813.x

Wang, M., Monticone, R. E., and Lakatta, E. G. (2010a). Arterial aging: a journey into subclinical arterial disease. *Curr. Opin. Nephrol. Hypertens.* 19, 201–207. doi: 10.1097/MNH.0b013e3283361c0b

Wang, M., Monticone, R. E., and Lakatta, G. E. (2014a). Proinflammation of aging central arteries: a mini-review. *Gerontology* 60, 519–529. doi: 10.1159/000362548

Wang, M., Jiang, L., Monticone, R. E., and Lakatta, G. E. (2014b). Proinflammation: the key to arterial aging. *Trends Endocrinol. Metab.* 25, 72–79. doi: 10.1016/j.tem.2013.10.002

Wang, M., Khazan, B., and Lakatta, G. E. (2010b). Central arterial aging and angiotensin II signaling. *Curr. Hypertens. Rev.* 6, 266–281. doi: 10.2174/157340210793611668

Wang, M., Kim, S. H., Monticone, R. E., and Lakatta, G. E. (2015). Matrix metalloproteinases promote arterial remodeling in aging, hypertension, and atherosclerosis. *Hypertension* 65, 698–703. doi: 10.1161/HYPERTENSIONAHA.114.03618

Wang, M., and Shah, M. A. (2015). Age-associated pro-inflammatory remodeling and functional phenotype in the heart and large arteries. *J. Mol. Cell Cardiol.* 83, 101–111. doi: 10.1016/j.yjmcc.2015.02.004

Wang, M., Takaqi, G., Asai, K., Resuello, R. G., Natividad, F. F., Vatner, D. E., et al. (2003). Aging increases aortic MMP-2 activity and angiotensin II in nonhuman primates. *Hypertension* 41, 1308–1316. doi: 10.1161/01.HYP.0000073843.56046.45

Wang, M., Wang, H. H., and Lakatta, G. E. (2013). Milk fat globule epidermal growth factor VIII signaling in arterial wall remodeling. *Curr. Vasc. Pharmacol.* 11, 768–776. doi: 10.2174/1570161111311050014

Wang, M., Zhang, J., Jiang, L. Q., Spinetti, G., Pintus, G., Monticone, R., et al. (2007). Proinflammatory profile within the grossly normal aged human aortic wall. *Hypertension* 50, 219–227. doi: 10.1161/HYPERTENSIONAHA.107.089409

Wang, M., Zhang, J., Spinetti, G., Jiang, L. Q., Monticone, R., Zhao, D., et al. (2005). Angiotensin II activates matrix metalloproteinase type II and mimics age-associated carotid arterial remodeling in young rats. *Am. J. Pathol.* 167, 1429–1442. doi: 10.1016/S0002-9440(10)61229-1

Yarchoan, M., Xie, S. X., Kling, M. A., Toledo, J. B., Wolk, D. A., Lee, E. B., et al. (2012). Cerebrovascular atherosclerosis correlates with Alzheimer pathology in neurodegenerative dementias. *Brain* 135(Pt 12), 3749–3756. doi: 10.1093/brain/aws271

Zieman, S. J., Melenovsky, V., and Kass, A. D. (2005). Mechanisms, pathophysiology, and therapy of arterial stiffness. *Arterioscler. Thromb. Vasc. Biol.* 25, 932–943. doi: 10.1161/01.ATV.0000160548.78317.29

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Incidence of Peripheral Arterial Disease and Its Association with Pulse Pressure: A Prospective Cohort Study

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Background: The association of pulse pressure and peripheral arterial disease (PAD) has seldom been examined using a prospective design. This study aimed to investigate the association of pulse pressure with PAD incidence in an elderly general population.

Methods: We utilized data from a cohort conducted in Beijing with additionally 2-year follow-up time. PAD was defined as an ankle brachial index value <0.9 in either leg. Cox proportional hazard regression model was used to quantify the magnitude of pulse pressure on PAD incidence.

Results: During a 2-year follow-up time, 357 of 4,201 (8.5%) participants developed PAD with 105 (6.9%) men and 252 (9.4%) women, respectively. After adjusting for baseline age, sex, body mass index, hypertension, diabetes, total cholesterol, and high-density lipoprotein cholesterol, and smoking, the hazard ratio and 95% confidence interval for people with pulse pressure greater than 60 mmHg was 2.20 (1.53, 3.15) compared with those whose pulse pressure was less than 40 mmHg. A linear trend was observed for the association of pulse pressure with PAD.

Conclusion: Higher pulse pressure was associated with higher PAD incidence.

Keywords: pulse pressure, peripheral arterial disease, ankle brachial index, blood pressure, cohort study

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INTRODUCTION

Peripheral arterial disease (PAD) is a common circulatory problem in which narrowed arteries reduce blood flow to limbs (1, 2). It is also a strong predictor for cardiovascular diseases, mortality, and stroke, independent of traditional cardiovascular risk factors (3, 4). Identification of those with PAD has profound public health and clinical implications for earlier disease prevention and treatment (5).

A number of risk factors have been identified for PAD risk assessment, such as smoking, diabetes, height, and dyslipidemia (6–9). Previous investigations suggested higher pulse pressure may be an independent predictor for PAD and this relationship was observed in several studies (10–12).

However, most of these studies were based on cross-sectional design or case–control setting in which the temporal order was difficult to establish due to the weakness of study design. A prospective investigation of the association between pulse pressure and PAD remains scarce.

The present study aimed to examine the incidence of PAD, and to assess its association with pulse pressure in a general senior population.

PATIENTS AND METHODS

Study Design and Participants

This study was a prospective cohort with participants originally recruited for chronic diseases and risk factors assessment in Beijing in 2007 with additional 2-year follow-up until 2009. A more detailed study design was described previously (13, 14). The present study included participants who were 50 years and over. In brief, 5,885 participants from 38 communities were recruited. During the 2-year follow-up duration, 1,095 participants emigrated or relocated, 402 refused to attend the follow-up examination, and 19 died. Of the remaining participants, 164 had baseline PAD and 4 had missing data on core covariates; the final analysis included 4,201 people who were free of PAD at baseline. All participants provided written informed consent to attend this study and subsequent follow-up examination, and the study protocol and ethical approval was obtained from the Ethic Committee of Beijing Municipal Science and Technology Commission.

Measurements

The health interview was performed by trained medical staff at community clinics using a well-established questionnaire to determine demographic and behavioral characteristics of the study population. Information regarding birthday, gender, and smoking status were collected. Physical examination included anthropometric measurements, blood pressure, and medical history. Height and weight were measured to the nearest 0.1 cm and 0.1 kg, respectively, with the subject standing barefoot in light clothes. Waist circumference was measured to the nearest 0.1 cm at the mid-point between the 12th rib and right anterior superior iliac spine. Body mass index (BMI) was calculated as weight (kilogram) to be divided by square of height (meter). Blood pressure was measured using standard mercury sphygmomanometer on the right arm in sitting position after the participants rested for 5 min. Phase 1 and phase 5 Korotkoff sound was used as systolic blood pressure (SBP) and diastolic blood pressure (DBP), respectively. Blood pressure was measured twice with the average results for the data analysis. Medical history was obtained from medical record and confirmed by local general practitioners.

Blood samples were collected from all the participants after an overnight fasting. All the biochemical measurements were conducted in the central laboratory of Peking University People's Hospital. Concentrations of fasting glucose, total cholesterol (TC), high-density lipoprotein cholesterol were measured using an auto analyzer (Hitachi 717, Hitachi Instruments, Inc., Tokyo, Japan). Hypertension was defined as SBP ≥140 mmHg, DBP ≥90 mmHg, or current medication for hypertension, and diabetes mellitus was defined as fasting glucose ≥7.0 mmol/L or current medication for diabetes.

Determination of Pulse Pressure and PAD

Pulse pressure was calculated as the SBP minus the DBP. The ankle brachial index (ABI) was determined complying with a standard protocol. After 5 min of rest, a standard mercury sphygmomanometer and a Doppler stethoscope with 5 mHz probe (Nicolet, Elite 100 R, 5 mHz probe, USA) were used to determine the bilateral brachial, tibial, and dorsal arteries with participants in supine position. Measurements were carried out twice and averaged for analysis. ABI was calculated as the ratio of the highest SBP in the leg to the highest SBP in the arm. The lower value of ABIs was used as the patient-specific ABI for analysis. ABI <0.9 was considered as PAD (15).

Statistical Analysis

Continuous variables were presented as mean ± SD and categorical variables were presented as frequencies and proportions. In the descriptive analysis, we present the basic characteristics of study subjects in men and women separately. Then in the exploratory analysis, we examined the association between pulse pressure and PAD using Cox proportional hazard regression models. Before treating pulse pressure as a continuous variable, pulse pressure was categorized into quartiles with the lowest quartiles as the reference. Three models were used for the analysis. The first model only included pulse pressure followed by the second model adjusted for age and gender as confounders. The third model was further adjusted for BMI, high-density lipoprotein cholesterol, TC, hypertension, diabetes, and smoking status. Hazard ratios (HRs) with 95% confidence interval (CI) were also presented. Kaplan–Meier method was used to estimate the PAD-free probability for different pulse pressure groups. All the analyses were two-tailed and $P < 0.05$ was considered to be statistically significant. All the statistics were obtained using R 3.1.

RESULTS

Basic Characteristics

Among the 4,201 study participants, 1,527 (36.3%) were men and 2,674 (63.7%) were women. As shown in **Table 1**, the mean age was 61.1 (SD, 7.7) for men and 60.4 (SD, 7.8) for women. The mean ABI was 1.09 (SD, 0.08) with 1.11 (SD, 0.09) for men and 1.07 (SD, 0.08) for women. **Tables 2** and **3** described the characteristics by PAD status. During a median 2-year (range: 1.8–2.3 years) follow-up time, 357 (8.5%) participants developed PAD with 105 (6.9%) men and 252 (9.4%) women corresponding to 33.9 per 1,000 person-year in men and 45.5 per 1,000 person-year in women, respectively.

Association between Pulse Pressure and PAD Incidence

Table 4 shows the association between pulse pressure and PAD. Model 1 only included pulse pressure, while Model 2 was adjusted for age and gender followed by Model 3 adjusted for age, gender, BMI, hypertension, diabetes, TC, high-density lipoprotein

TABLE 1 | Baseline characteristics of study participants.

Variables	Men (n = 1,527)	Women (n = 2,674)
Age (years)	61.1 ± 7.7	60.4 ± 7.8
SBP (mmHg)	132.7 ± 18.7	132.4 ± 19.6
DBP (mmHg)	81.7 ± 10.7	80.5 ± 10.1
ABI	1.11 ± 0.09	1.07 ± 0.08
BMI (kg/m^2)	24.9 ± 3.5	26.0 ± 4.0
Glucose (mmol/L)	5.21 ± 1.75	5.29 ± 1.71
TC (mmol/L)	4.75 ± 0.92	5.21 ± 0.93
Hypertension, *n* (%)	657 (43.0)	1,092 (40.8)
Diabetes, *n* (%)	133 (8.7)	251 (9.4)
Smoking, *n* (%)	847 (55.5)	267 (10.0)
Medication (excluding anti-thrombotic), *n* (%)	216 (14.1)	405 (15.1)
Anti-thrombotic, *n* (%)	288 (18.9)	459 (17.2)

SBP, systolic blood pressure; DBP, diastolic blood pressure; ABI, ankle brachial index; BMI, body mass index; TC, total cholesterol.

TABLE 2 | Characteristics of study participants at follow-up.

Variables	Men (n = 1,527)	Women (n = 2,674)
Age (years)	63.16 ± 7.73	62.46 ± 7.77
SBP (mmHg)	134.35 ± 20.05	133.54 ± 19.88
DBP (mmHg)	82.71 ± 11.44	80.17 ± 10.46
ABI	1.08 ± 0.12	1.04 ± 0.11
BMI (kg/m^2)	27.68 ± 58.23	26.95 ± 16.01
Waist (cm)	89.44 ± 10.34	87.48 ± 10.34
Hip (cm)	96.83 ± 6.85	98.11 ± 8.32
Glucose (mmol/L)	5.47 ± 2.01	5.5 ± 1.89
TC (mmol/L)	4.51 ± 0.84	4.97 ± 0.85
Hypertension, *n* (%)	788 (51.6)	1,273 (47.6)
Diabetes, *n* (%)	163 (10.7)	275 (10.3)
Smoking, *n* (%)	774 (50.7)	240 (9.0)
Medication (excluding anti-thrombotic), *n* (%)	198 (13.0)	376 (14.1)
Anti-thrombotic, *n* (%)	192 (12.6)	360 (13.5)

SBP, systolic blood pressure; DBP, diastolic blood pressure; ABI, ankle brachial index; BMI, body mass index; TC, total cholesterol.

TABLE 3 | Characteristics of study participants who developed peripheral arterial disease at follow-up.

Variables	Men (n = 105)	Women (n = 252)
Age (years)	66.45 ± 8.07	67.1 ± 9.35
SBP (mmHg)	138.39 ± 19.53	138.08 ± 21.65
DBP (mmHg)	82.19 ± 10.15	79.79 ± 10.54
ABI	0.81 ± 0.08	0.81 ± 0.1
BMI (kg/m^2)	25.19 ± 4.15	26.33 ± 4.21
Waist (cm)	89.45 ± 11.31	88.57 ± 10.18
Hip (cm)	96.83 ± 7.76	98.35 ± 10.59
W/H ratio	0.92 ± 0.06	0.9 ± 0.08
Glucose (mmol/L)	5.38 ± 1.61	5.59 ± 2.01
TC (mmol/L)	4.71 ± 0.92	5.11 ± 1.07
Hypertension, *n* (%)	57 (54.3)	145 (57.5)
Diabetes, *n* (%)	12 (11.4)	23 (9.1)
Smoking, *n* (%)	54 (51.4)	29 (11.5)
Medication (excluding anti-thrombotic), *n* (%)	16 (15.2)	41 (16.2)
Anti-thrombotic, *n* (%)	15 (14.3)	38 (15.1)

SBP, systolic blood pressure; DBP, diastolic blood pressure; ABI, ankle brachial index; BMI, body mass index; TC, total cholesterol.

TABLE 4 | Association between pulse pressure and peripheral arterial disease incidence, hazard ratio (95% confidence interval).

Pulse pressure	Model 1	Model 2	Model 3	Model 4
0–40 mmHg	1.0	1.0	1.0	1.0
41–50 mmHg	1.38 (0.99, 1.90)	1.22 (0.88, 1.69)	1.25 (0.90, 1.73)	1.25 (0.90, 1.73)
51–60 mmHg	1.99 (1.45, 2.74)	1.59 (1.15, 2.20)	1.63 (1.17, 2.27)	1.63 (1.17, 2.27)
>60 mmHg	3.25 (2.42, 4.38)	2.10 (1.53, 2.87)	2.20 (1.53, 3.15)	2.19 (1.53, 3.14)
Continuous	1.30 (1.23, 1.38)	1.17 (1.10, 1.25)	1.18 (1.10, 1.28)	1.18 (1.10, 1.28)

Model 1 included only pulse pressure; Model 2 was further adjusted for age and sex; Model 3 additionally adjusted for body mass index, hypertension, diabetes, total cholesterol, high-density lipoprotein cholesterol, and smoking. Model 4 was additionally adjusted for medication use.

cholesterol, and smoking. In Model 3, each 10 mmHg increase in pulse pressure was associated with 19% higher risk of PAD when treating pulse pressure as a continuous variable. The HR (95% CI) for people with pulse pressure greater than 60 mmHg was 2.20 (1.53, 3.15) compared with those whose pulse pressure was less than 40 mmHg. Similar result was shown from the Kaplan–Meier plot in **Figure 1**.

DISCUSSION

In the present study, we examined the incidence of PAD and its association with pulse pressure in prospective cohort. We found that higher pulse pressure was associated with high risk of PAD and this association was independent of several risk factors including age, gender, high-density lipoprotein cholesterol, TC, hypertension, diabetes, and smoking status.

There are few studies of PAD incidence based on ABI. The Limburg study reported PAD incidence rate was 17.8 in men and 22.9 in women per 1,000 person-year at the age of 65 and over (16) which is lower than our study. The difference may be due to the different study population, follow-up time, or socioeconomic status. Several observational studies have been conducted to examine whether pulse pressure is associated with PAD (17, 18). One study from Finland reported one 1 mmHg increase of pulse pressure to be associated with 1.03 odds ratio of PAD which was approximately 1.34 odds ratio for 10 mmHg increase of pulse pressure (11). Our previous study also found 5-mmHg increase in pulse pressure was associated with a 17 and 15% increase of PAD risk in men and women (10). A more recent study from Japan observed an odds ratio for PAD was 1.30 for increasing pulse pressure (12). However, all of these studies are based on cross-sectional design or in a case–control setting. It might be difficult to determine if it is pulse pressure affect PAD or the opposite. Our current study, by using the prospectively collected data, found 10 mmHg to be associated with 19% higher risk for 2-year PAD incidence risk. This finding is consistent with published studies while has more strength in terms of its longitudinal cohort design and avoid reverse causation.

In this study, we observed women had higher incidence of PAD compared with men, which was also observed in The

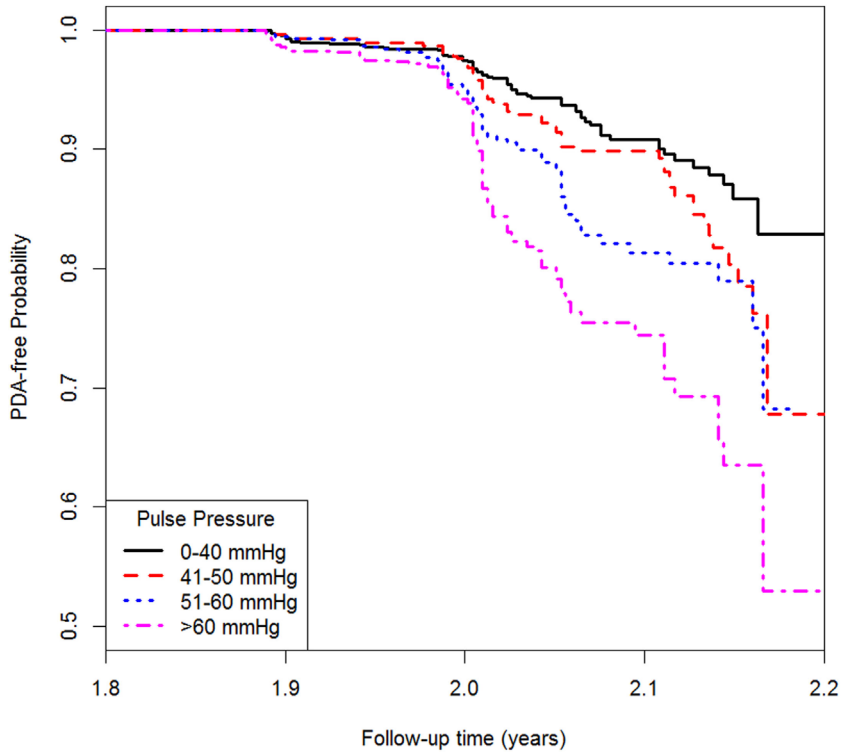

FIGURE 1 | Kaplan–Meier plot for peripheral arterial disease by pulse pressure.

Limburg Study (15). Several factors might contribute to the sex difference in PAD incidence. Firstly, women were less physically active on than men nowadays in China, especially for the elderly (19). Physical activity was a protective factor for PAD; less physical activity could contribute to higher PAD incidence. Secondly, previous studies also revealed sex hormone and menopausal status were associated with lower risk of cardiovascular diseases for women. However, this phenomenon was not observed for PAD. In the Framingham Heart Study, sex hormone concentrations were not associated PAD risk in neither pre-menopausal nor post-menopausal women (20). Thirdly, heart rate was reversely correlated with ABI (21) and women tended to have higher heart rate and heart rate variability than men (22). However, more research is needed to clarify this sex inequality and more attention should be paid to women with higher risks of PAD.

Several potential molecular mechanisms might explain the observed association between pulse pressure and PAD. Pulse pressure was found to impair vascular endothelial function (23), and persistence of endothelial dysfunction could lead to arterial stiffness, atherosclerosis, and PAD (24, 25). Additionally, the *ENPP1* Q121 variant could increase pulse pressure and reduced insulin signaling (26). Insulin resistance was strongly and independently associated with PAD (27). Moreover, pulse pressure promotes the apoptosis of vascular smooth muscle cell and this effect is independent of endothelial and mitogen-activated protein kinase (28). The synergistic effects of all these processes and lipid accumulation (29) accelerate the narrowing of the arteries in the lower extremities and aggravate intermittent claudication.

In order to examine if the observed association was independent of traditional risk factors of PAD, we additionally adjusted for several variables in our analyses. In the multivariable adjusted models, the magnitude of pulse press on PAD incidence was attenuated compared with the unadjusted models. Two reasons might explain this effect size decrease. These adjusted factors, such as age and gender, might be the confounders for both PAD and pulse pressure; thus, the HR will decrease after removing the confounding effects by model adjustment. Other risk factors, such as hypertension, diabetes, or lipid fractions, might mediate the effect from pulse pressure to PAD. However, these explanations need to be further tested in future studies. A few studies examined the relationship between ABI and flow-mediated vasodilation and found they were strongly correlated (30). However, we did not measure flow-mediated vasodilation in our current study.

Limitations

Although the data used in our study was collected prospectively, the median follow-up time was only 2 years. In this study, we only considered several important confounders in the analyses; a residual confounding could still bias the observed association. Additional analysis by collecting more variables, such as genetic variants (31) and other biomarkers (32–34), should be useful. The present study was conducted in an Asia population; the generalization of the results to other ethnic groups may be limited.

CONCLUSION

Higher pulse pressure was associated with PAD incidence, and this association might be mediated by several mechanisms.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Ethic Committee of Beijing Municipal Science and Technology Commission with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Ethic Committee of Beijing Municipal Science and Technology Commission'.

AUTHOR CONTRIBUTIONS

YM, YZ, HY, and JY designed this study. YZ and GY analyzed these data. All authors drafted this manuscript.

REFERENCES

1. Criqui MH, Aboyans V. Epidemiology of peripheral artery disease. *Circ Res* (2015) 116(9):1509–26. doi:10.1161/CIRCRESAHA.116.303849
2. Kullo IJ, Rooke TW. Clinical practice. Peripheral artery disease. *N Engl J Med* (2016) 374(9):861–71. doi:10.1056/NEJMcp1507631
3. Freisinger E, Malyar NM, Reinecke H. Peripheral artery disease is associated with high in-hospital mortality particularly in males with acute myocardial infarction in a nationwide real-world setting. *Vasa* (2016) 45(2):169–74. doi:10.1024/0301-1526/a000512
4. Tziomalos K, Giampatzis V, Bouziana S, Pavlidis A, Spanou M, Papadopoulou M, et al. Predictive value of the ankle brachial index in patients with acute ischemic stroke. *Vasa* (2014) 43(1):55–61. doi:10.1024/0301-1526/a000328
5. Zhan Y, Zhuang J, Dong Y, Xu H, Hu D, Yu J. Predicting the prevalence of peripheral arterial diseases: modelling and validation in different cohorts. *Vasa* (2016) 45(1):31–6. doi:10.1024/0301-1526/a000492
6. Zhan Y, Yu J, Ding R, Sun Y, Hu D. Triglyceride to high density lipoprotein cholesterol ratio, total cholesterol to high density lipoprotein cholesterol ratio and low ankle brachial index in an elderly population. *Vasa* (2014) 43(3):189–97. doi:10.1024/0301-1526/a000348
7. Husmann M, Jacomella V, Thalhammer C, Amann-Vesti BR. Markers of arterial stiffness in peripheral arterial disease. *Vasa* (2015) 44(5):341–8. doi:10.1024/0301-1526/a000452
8. Gavier B, Vazquez F, Gandara E. Antiphospholipid antibodies and lower extremity peripheral arterial disease – a systematic review and meta-analysis. *Vasa* (2016) 45(4):325–30. doi:10.1024/0301-1526/a000545
9. Wang Y, Li J, Zhao D, Wei Y, Hou L, Hu D, et al. Prevalence and characteristics of atherosclerosis and peripheral arterial disease in a Chinese population of Inner Mongolia. *Vasa* (2011) 40(1):49–56. doi:10.1024/0301-1526/a000069
10. Zhan Y, Yu J, Chen R, Sun Y, Fu Y, Zhang L, et al. Prevalence of low ankle brachial index and its association with pulse pressure in an elderly Chinese population: a cross-sectional study. *J Epidemiol* (2012) 22(5):454–61. doi:10.2188/jea.JE20110140
11. Korhonen P, Kautiainen H, Aarnio P. Pulse pressure and subclinical peripheral artery disease. *J Hum Hypertens* (2014) 28(4):242–5. doi:10.1038/jhh.2013.99
12. Kiuchi S, Hisatake S, Watanabe I, Toda M, Kabuki T, Oka T, et al. Pulse pressure and upstroke time are useful parameters for the diagnosis of peripheral artery disease in patients with normal ankle brachial index. *Cardiol Res* (2016) 7(5):161–6. doi:10.14740/cr508e
13. Zhan Y, Yu J, Chen R, Gao J, Ding R, Fu Y, et al. Socioeconomic status and metabolic syndrome in the general population of China: a cross-sectional study. *BMC Public Health* (2012) 12:921. doi:10.1186/1471-2458-12-921
14. Zhan Y, Zhang F, Lu L, Wang J, Sun Y, Ding R, et al. Prevalence of dyslipidemia and its association with insomnia in a community based population in China. *BMC Public Health* (2014) 14:1050. doi:10.1186/1471-2458-14-1050
15. Aboyans V, Criqui MH, Abraham P, Allison MA, Creager MA, Diehm C, et al. Measurement and interpretation of the ankle-brachial index: a scientific statement from the American Heart Association. *Circulation* (2012) 126(24):2890–909. doi:10.1161/CIR.0b013e318276fbcb
16. Hooi JD, Kester AD, Stoffers HE, Overdijk MM, van Ree JW, Knottnerus JA. Incidence of and risk factors for asymptomatic peripheral arterial occlusive disease: a longitudinal study. *Am J Epidemiol* (2001) 153(7):666–72. doi:10.1093/aje/153.7.666
17. Tseng CH. Pulse pressure as a risk factor for peripheral vascular disease in type 2 diabetic patients. *Clin Exp Hypertens* (2003) 25(8):475–85. doi:10.1081/CEH-120025331
18. Subramaniam T, Khaing Nang EE, Lim SC, Wu Y, Khoo CM, Lee J, et al. Distribution of ankle—brachial index and the risk factors of peripheral artery disease in a multi-ethnic Asian population. *Vasc Med* (2011) 16(2):87–95. doi:10.1177/1358863X11400781
19. Zang J, Ng SW. Age, period and cohort effects on adult physical activity levels from 1991 to 2011 in China. *Int J Behav Nutr Phys Act* (2016) 13:40. doi:10.1186/s12966-016-0364-z
20. Haring R, Travison TG, Bhasin S, Vasan RS, Wallaschofski H, Davda MN, et al. Relation between sex hormone concentrations, peripheral arterial disease, and change in ankle-brachial index: findings from the Framingham Heart Study. *J Clin Endocrinol Metab* (2011) 96(12):3724–32. doi:10.1210/jc.2011-1068
21. Su HM, Lee KT, Chu CS, Lee MY, Lin TH, Voon WC, et al. Effects of heart rate on brachial-ankle pulse wave velocity and ankle-brachial pressure index in patients without significant organic heart disease. *Angiology* (2007) 58(1):67–74. doi:10.1177/0003319706295481
22. Jandackova VK, Scholes S, Britton A, Steptoe A. Are changes in heart rate variability in middle-aged and older people normative or caused by pathological conditions? Findings from a large population-based longitudinal cohort study. *J Am Heart Assoc* (2016) 5(2): e002365. doi:10.1161/JAHA.115.002365
23. Beigel R, Dvir D, Arbel Y, Shechter A, Feinberg MS, Shechter M. Pulse pressure is a predictor of vascular endothelial function in middle-aged subjects with no apparent heart disease. *Vasc Med* (2010) 15(4):299–305. doi:10.1177/1358863X10373300
24. Sitia S, Tomasoni L, Atzeni F, Ambrosio G, Cordiano C, Catapano A, et al. From endothelial dysfunction to atherosclerosis. *Autoimmun Rev* (2010) 9(12):830–4. doi:10.1016/j.autrev.2010.07.016
25. Brevetti G, Silvestro A, Di Giacomo S, Bucur R, Di Donato A, Schiano V, et al. Endothelial dysfunction in peripheral arterial disease is related to increase in plasma markers of inflammation and severity of peripheral circulatory impairment but not to classic risk factors and atherosclerotic burden. *J Vasc Surg* (2003) 38(2):374–9. doi:10.1016/S0741-5214(03)00124-1
26. Bacci S, Di Paola R, Menzaghi C, Di Fulvio P, Di Silvestre S, Pellegrini F, et al. ENPP1 Q121 variant, increased pulse pressure and reduced insulin signaling, and nitric oxide synthase activity in endothelial cells. *Arterioscler Thromb Vasc Biol* (2009) 29(10):1678–83. doi:10.1161/ATVBAHA.109.189191
27. Pande RL, Perlstein TS, Beckman JA, Creager MA. Association of insulin resistance and inflammation with peripheral arterial disease: the National Health and Nutrition Examination Survey, 1999 to 2004. *Circulation* (2008) 118(1):33–41. doi:10.1161/CIRCULATIONAHA.107.721878
28. Birney YA, Sweeney CH, Cappadona CR, Sitzmann JV, Cummins PM, Redmond EM, et al. Pulse pressure-induced transmural fluid flux increases bovine aortic smooth muscle cell apoptosis in a mitogen activated protein kinase dependent manner. *J Vasc Res* (2004) 41(4):364–74. doi:10.1159/000080700
29. Kiefer CR, McKenney JB, Trainor JF, Snyder LM. Pulse-pressure-driven neutral lipid accumulation and correlative proinflammatory markers of accelerated atherogenesis. *Atherosclerosis* (2005) 183(1):17–24. doi:10.1016/j.atherosclerosis.2005.02.023
30. Kajikawa M, Maruhashi T, Iwamoto Y, Iwamoto A, Matsumoto T, Hidaka T, et al. Borderline ankle-brachial index value of 0.91-0.99 is associated with

endothelial dysfunction. *Circ J* (2014) 78(7):1740–5. doi:10.1253/circj.CJ-14-0165

31. Kullo IJ, Leeper NJ. The genetic basis of peripheral arterial disease: current knowledge, challenges, and future directions. *Circ Res* (2015) 116(9):1551–60. doi:10.1161/CIRCRESAHA.116.303518
32. Zhan Y, Dong Y, Tang Z, Zhang F, Hu D, Yu J. Serum uric acid, gender, and low ankle brachial index in adults with high cardiovascular risk. *Angiology* (2015) 66(7):687–91. doi:10.1177/0003319714566228
33. Gardner AW, Parker DE, Montgomery PS, Sosnowska D, Casanegra AI, Esponda OL, et al. Impaired vascular endothelial growth factor A and inflammation in patients with peripheral artery disease. *Angiology* (2014) 65(8):683–90. doi:10.1177/0003319713501376
34. Fowkes FGR, Aboyans V, Fowkes FJI, McDermott MM, Sampson UKA, Criqui MH, et al. Peripheral artery disease: epidemiology and global perspectives. *Nat Rev Cardiol* (2017) 14:156–70. doi:10.1038/nrcardio.2016.179

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REVIEW
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Inositol 1,4,5-Trisphosphate Receptors in Hypertension

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Chronic hypertension remains a major cause of global mortality and morbidity. It is a complex disease that is the clinical manifestation of multiple genetic, environmental, nutritional, hormonal, and aging-related disorders. Evidence supports a role for vascular aging in the development of hypertension involving an impairment in endothelial function together with an alteration in vascular smooth muscle cells (VSMCs) calcium homeostasis leading to increased myogenic tone. Changes in free intracellular calcium levels ($[Ca^{2+}]_i$) are mediated either by the influx of Ca^{2+} from the extracellular space or release of Ca^{2+} from intracellular stores, mainly the sarcoplasmic reticulum (SR). The influx of extracellular Ca^{2+} occurs primarily through voltage-gated Ca^{2+} channels (VGCCs), store-operated Ca^{2+} channels (SOC), and Ca^{2+} release-activated channels (CRAC), whereas SR-Ca^{2+} release occurs through inositol trisphosphate receptor (IP_3R) and ryanodine receptors (RyRs). IP_3R-mediated SR-Ca^{2+} release, in the form of Ca^{2+} waves, not only contributes to VSMC contraction and regulates VGCC function but is also intimately involved in structural remodeling of resistance arteries in hypertension. This involves a phenotypic switch of VSMCs as well as an alteration of cytoplasmic Ca^{2+} signaling machinery, a phenomena tightly related to the aging process. Several lines of evidence implicate changes in expression/function levels of IP_3R isoforms in the development of hypertension, VSMC phenotypic switch, and vascular aging. The present review discusses the current knowledge of these mechanisms in an integrative approach and further suggests potential new targets for hypertension management and treatment.

Keywords: aging, hypertension, Ca^{2+}, IP_3R, VSMC

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INTRODUCTION

Cardiovascular diseases (CVD) remain the leading cause of death worldwide, with hypertension being the number one cause of this high mortality (Forouzanfar et al., 2017). Nearly one-third of the yearly global mortality is due to CVD (Chen et al., 2013). At least half or more of ischemic stroke, hemorrhagic stroke, ischemic heart disease and other CVD such as cardiomyopathy, aortic aneurysms, or peripheral vascular disease are intimately attributed to elevated blood pressure (BP), or hypertension (Forouzanfar et al., 2017). This burden is on the rise, despite all therapeutic advances made in recent years, especially in elderly people (Gates et al., 2009; GBD 2013 Risk Factors Collaborators et al., 2015; Harvey et al., 2015; GBD 2015 Risk Factors Collaborators, 2016; Thijssen et al., 2016).

Hypertension is defined as a chronic and persistent elevation of systemic arterial pressure beyond normal values. Etiologically, hypertension is classified as primary and secondary. Primary hypertension, also known as essential hypertension, is the most prevalent form of high BP and constitutes around 90–95% of the cases with unknown etiology (Carretero and Oparil, 2000; Rossier et al., 2017). Secondary hypertension, on the other hand, constitutes around 5–10% of hypertensive cases and arises from known and identifiable causes such as kidney diseases, pregnancy, endocrine disorders, neurological diseases, and others (Chiong et al., 2008).

Chronic hypertension predisposes nearly 1.5 billion individuals in the world to CVD, including ventricular hypertrophy and heart failure, stroke, and renal damage (Chockalingam, 2008). A number of factors are known to increase the risk of high BP development including obesity, sedentary lifestyle, insulin resistance, high alcohol intake, high salt intake, smoking, and aging (Carretero and Oparil, 2000; Gates et al., 2009; Green et al., 2010). The development of essential hypertension involves multiple physiological mechanisms including cardiac output, peripheral resistance, renin–angiotensin–aldosterone system, autonomic nervous system, and vasoactive substances such as endothelin, bradykinin, natriuretic peptides, and others (Beevers et al., 2001; Cain and Khalil, 2002).

The etiology of hypertension is complex and results from the interaction of multiple genetic, neuronal, hormonal, environmental factors, and aging-associated diseases (Garbers and Dubois, 1999; Oparil et al., 2003; Chockalingam, 2008). In fact, with over 50 genes implicated in BP regulation, and other risk factors contributing to the pathogenesis of hypertension, it is rarely possible to determine the etiology of the disease. However, strong evidences support the role of "vascular aging" in the development of hypertension (Green et al., 2010; Fritze et al., 2012; van den Munckhof et al., 2012). In fact, progressive aging implies endothelial dysfunction, loss of nitric oxide (NO) bioavailability, impaired vasodilation, vascular remodeling, and increased arterial stiffness. In addition, the molecular and cellular mechanisms underlying vascular alterations are common and include impaired Ca^{2+} signaling, oxidative stress, and production of pro-inflammatory cytokines and pro-fibrotic growth factors.

Regardless of its etiology, a hallmark of all cases of hypertension is an increased vascular resistance that leads to elevated BP. Resistance arteries, with an internal diameter of less than 350 μm, are key elements in the control of peripheral vascular resistance. The major drop in hydrostatic pressure in the vascular tree occurs at the level of resistance arteries. As described by Poiseuille's law, resistance to blood flow is inversely proportional to the vessel radius to the fourth power; therefore, small variations in the lumen of resistance arteries result in significant effects on peripheral resistance with a pronounced impact on BP. Hence, peripheral resistance is typically a function of the diameter of resistance arteries which, in turn, is intricately linked to the contractility state (vasomotor tone) of vascular smooth muscle cells (VSMCs) (Bosnjak, 1993; Hill et al., 2001). Indeed, it is these VSMCs in resistance arteries and arterioles that act as the main effectors in the continuous regulation of vascular resistance. By stretching VSMCs, BP activates a myriad of signaling events that eventually produces myogenic tone, a distinguishing feature of resistance arteries and arterioles (D'Angelo et al., 1997; Davis, 2012; Mufti et al., 2015; Kroetsch et al., 2017). Furthermore, this tone represents the baseline on which various primary messengers such as neurotransmitters, endothelium-derived vasoactive molecules, local metabolites, or hormones converge and act to modulate constriction and dilatation. Many membrane channels and receptors play a pivotal role in vasotone regulation. VSMCs of resistance arteries express several plasma membrane (PM) ion channels including K^+ channels (Taguchi et al., 1994; Sobey et al., 1998; Tajada et al., 2012), Ca^{2+} channels, Cl^- channels (Bulley et al., 2012; Dam et al., 2014; Heinze et al., 2014), transient receptor potential (TRP) family of ion channels, voltage-gated Ca^{2+} channels (VGCCs) (Hondeghem et al., 1986; Inoue et al., 2001; Liao et al., 2007), epithelial Na^+/acid-sensing channel (ENaC) (Jernigan and Drummond, 2005; Drummond, 2009; Grifoni et al., 2010), and stretch-activated channel, also known as PIEZO1 (Allison, 2017). In addition to these channels, IP_3R and ryanodine receptor (RyR), which are localized SR membrane play an important role in VSMC contractility and the development of hypertension (Long et al., 2007; Mufti et al., 2010; Lin et al., 2016).

It is important to note that with sustained hypertension, vessels undergo progressive alteration characterized by inflammatory responses, VSMC growth and migration, extracellular matrix synthesis and degradation, endothelial dysfunction that increases vascular stiffness and resistance, and decreases vascular elasticity (Shyu, 2009; Dharmashankar and Widlansky, 2010; Renna et al., 2013). Remodeled vessels heavily contribute to the pathophysiology of vascular diseases such as atherosclerosis, and are subsequently at high risk of blockage or rupture that could damage and fail the supplied organ (Renna et al., 2013). This review will highlight the role of alterations in inositol trisphosphate receptors (IP_3R) expression/function in changes in vascular remodeling and vascular tone, and VSM contractility in response to chronic hypertension. A summary of the proposed model is presented in **Figure 1**.

IP_3R IN VSMCs: EXPRESSION, STRUCTURE, AND LOCALIZATION

The IP_3R of VSMCs plays important roles in gene expression, cellular proliferation, and migration, as well as contractility (Wilkerson et al., 2006; Xi et al., 2008; Adebiyi et al., 2010). IP_3R is a tetramer, with each subunit encompassing an amino terminus, six transmembrane domains, and a carboxy terminal tail (Michikawa et al., 1994; Yoshikawa et al., 1996). The amino terminus contains an IP_3-binding domain, a suppressor domain that inhibits IP_3 binding, and a regulatory domain (Yoshikawa et al., 1999). This regulatory domain contains binding sites for Ca^{2+} and ATP as well as consensus phosphorylation sites (Michikawa et al., 1994; Patel et al., 1999; Foskett et al., 2007). Within this regulatory domain, there is also a coupling motif that is important for physical interactions between IP_3R and transient

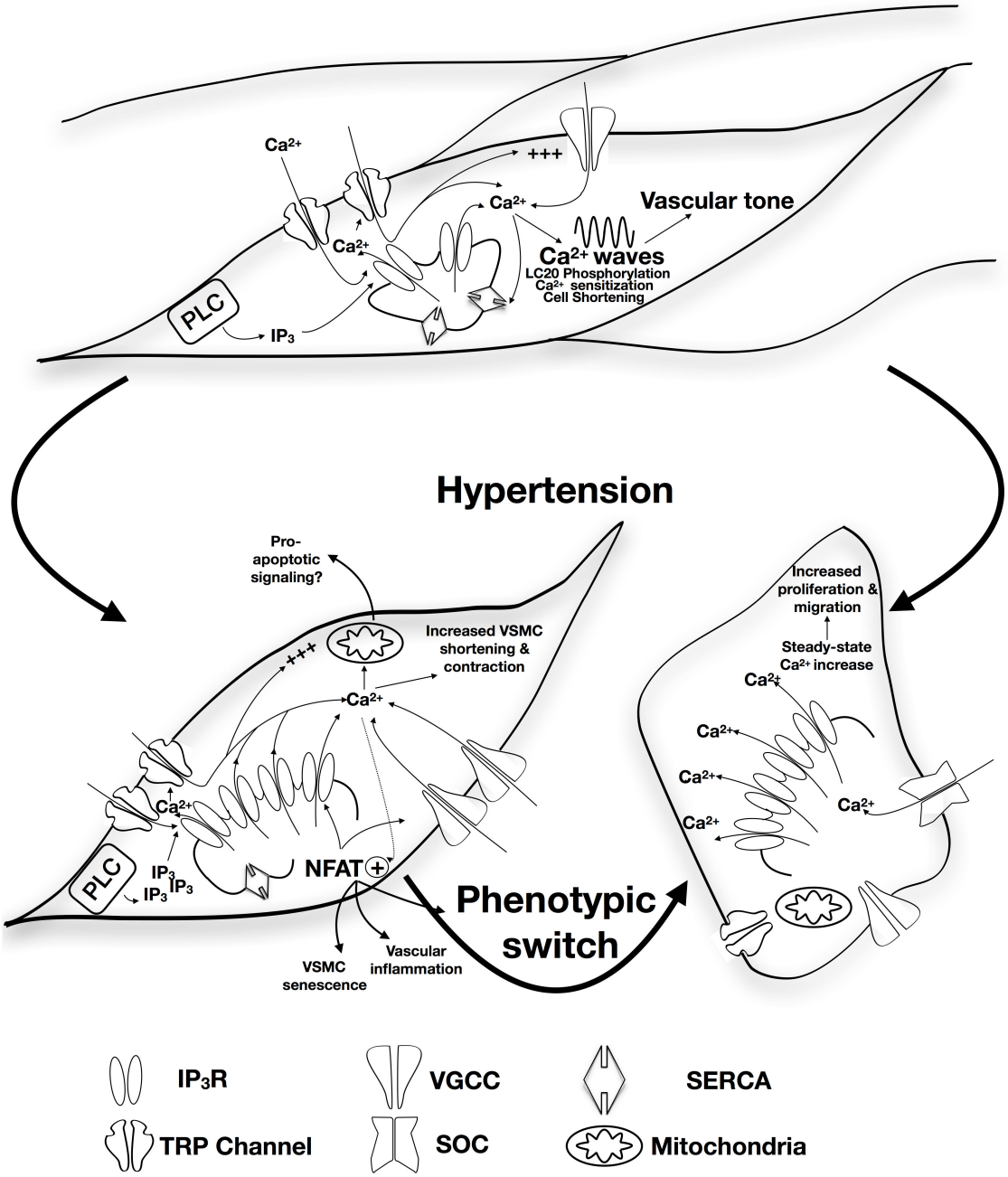

FIGURE 1 | Proposed role of alteration in IP_3R expression/function in the development of hypertension. Apart from contribution to vascular tone and VSM contractility via SR-Ca^{2+} release, calcium wave production, and induction of calcium sensitization, IP3R forms a mechanosensing complex with TRP channels that is proposed to initiate contraction in response to increased intraluminal pressure. Physiological patterns of IP_3R-mediated Ca^{2+} release are affected by the expression of other SR membrane proteins including SERCA. Under circumstances of sustained increased blood pressure, IP_3R expression increases together with increased sensitivity to IP_3 and increased coupling with the TRP mechanosensing machinery resulting in increased intracellular Ca^{2+} release and increased VSM contraction. Interplay with NFAT-mediated signaling pathways might contribute pressure-induced changes in VSMC phenotype, vascular inflammation, and VSMC senescence. Increased VSMC proliferation and migration were reported to involve increased IP_3R-mediated Ca^{2+} release with subsequent intracellular store depletion and increased store-operated Ca^{2+} entry. IP_3R-mediated Ca^{2+} release can potentially relay apoptotic signaling to the mitochondria contributing to vascular aging.

receptor potential canonical (TRPC) channels (Tang et al., 2001; Adebiyi et al., 2010; Zhao et al., 2017). The transmembrane and carboxy terminal domains are essential for tetramerization of IP_3Rs (Mignery and Sudhof, 1990; Sayers et al., 1997).

The IP_3R family comprises three subtypes (IP_3R1, IP_3R2, and IP_3R3) that are encoded by Itpr1, Itpr2, and Itpr3, respectively. Almost all animal cells express IP3Rs (Prole and Taylor, 2016). The human isoforms share approximately 75% amino

acid homology; however, their sensitivity toward IP3 or other regulatory factors is variable, thus adding a layer of complexity for their functions (Foskett et al., 2007; Mikoshiba, 2007). This complexity is further compounded by the existence of many splice variants as well as the possibility of tetramerization (Foskett et al., 2007; Mikoshiba, 2007).

Although most cells express more than one IP3R subtype, the different subtypes exhibit some tissue-specific pattern of expression, with one subtype being expressed at a higher level than the others (Vermassen et al., 2004; Ivanova et al., 2014). Moreover, the different subtypes exhibit marked difference in their affinity for their ligand IP3 (Ivanova et al., 2014; Vervloessem et al., 2015). They also differ in their regulation by Ca^{2+} and ATP as well as their phosphorylation by various kinases (Ivanova et al., 2014; Vervloessem et al., 2015). For instance, although all three isoforms exhibit a biphasic mode of IP3-induced Ca^{2+} release, isoform-specific characteristics of this response are observed (Miyakawa et al., 1999; Mak et al., 2001; Tu et al., 2005). Likewise, ATP regulates all three isoforms but with a clear differential effect on each. For example, IP_3R2 is 10 times more sensitive to ATP than IP_3R3, at least in pancreatic acinar cells (Park et al., 2008). Moreover, while all three isoforms are targets for many kinases such as Akt, PKA, and MAP kinases, isoform-specific regulation by these kinases are markedly noticed. For instance, ERK1/2 can recognize three phosphorylation resides (S436, T945, and S1765) in IP_3R1 but not in IP_3R2 or IP_3R3 (Bosanac et al., 2004).

Vascular smooth muscle cells expresses all three subtypes, with IP_3R1 being the predominant one in these cells (Islam et al., 1996; Wang et al., 2001; Grayson et al., 2004; Zhou et al., 2008). Levels of these proteins are determined by a well-regulated balance between transcription and degradation. For instance, while c-Myb stimulates its expression, retinoic acid and TGF-beta inhibit expression of IP_3R1 (Sharma et al., 1997; Deelman et al., 1998; Afroze et al., 2007). Hydrogen peroxide Jak2 kinase, Herpud1, and vasopressin regulate levels of IP_3R1 by modulating its degradation (Sipma et al., 1998; Wallace et al., 2005; Martin-Garrido et al., 2009; Torrealba et al., 2017).

The main location of IP_3Rs in VSMCs is the SR, both central (perinuclear), and peripheral (beneath the PM) compartments (Nixon et al., 1994; Gordienko et al., 2008; Narayanan et al., 2012). Importantly, this localization may impart a functional effect. For instance, IP_3Rs located around the nucleus are thought to regulate Ca^{2+}-dependent gene expression without affecting the global intracellular pool of Ca^{2+}. On the other hand, peripherally located SR allows their IP_3Rs to be close enough to the PM for localized signaling to membrane proteins to be efficiently elicited (Adebiyi et al., 2010; Zhao et al., 2010). It is also important to note that in addition to their role in Ca^{2+} release, IP3Rs have other significant functions. For example, upon binding, IP3 causes IP_3R—binding protein released with IP_3 (IRBIT) to be released from the IP3-binding site. The now released IRBIT can then modulate other targets such as transporters, channels as well as ribonucleotide reductase (Ando et al., 2003; Arnaoutov and Dasso, 2014). Moreover, IP3Rs, independent of their Ca^{2+} release ability, may also regulate others proteins such as the opening of TRPC (Zhang et al., 2001). As such, cellular distribution of IP_3Rs and their Ca^{2+}-independent roles dictate the functions of these receptors, under both physiologic and pathophysiologic conditions as will be discussed below.

FACTORS AFFECTING VASCULAR TONE: ALTERATION IN HYPERTENSION

Under physiological conditions, individual components of the vascular system maintain a certain degree of spontaneous constriction constituting the vascular tone. This vascular property determines the dilatory capacity of the vascular bed and hence the organ, whereby a higher tone allows for a higher dilatory capacity as in the heart and skeletal muscles, and a lower tone leads to a limited dilatory capacity as in case of cerebral circulation (Klabunde, 2012). Indeed, vascular tone results from the integration of several competing stimuli that modulate the contractile state of VSMC. In isolated vessels, the myogenic response constitutes the fundamental form of vascular reactivity in response to increased intraluminal pressure (Uchida and Bohr, 1969). Extrinsic influences converge to modulate this intrinsic contractility. The overall vascular tone is set as a net outcome of the interaction of endothelial inputs activated by sheer stress (Koller et al., 1993), neuronal regulation (Fleming et al., 1987), humoral mediators (Waldemar and Paulson, 1989), tissue metabolic demand (Chovanes and Richards, 2012), and tubuloglomerular feedback (characteristic to the renal vascular bed) (Burke et al., 2014). The resultant level of constriction determines the extent of systemic vascular resistance and hence contributes to regulating BP, making the examination of alterations in vascular tone an attractive target in the study of hypertension.

Significantly, studies showed substantial alterations in vascular tone in hypertension. Whether it is a causative factor or adaptive consequence of hypertension, enhanced myogenic response was reported in humans and animal models of the disease (Henriksen et al., 1981; Sonoyama et al., 2007). Early studies on spontaneously hypertensive rats showed a reduced ability of cerebral arterioles to dilate increasing the cerebral blood flow in response to intraluminal pressure reduction (Waldemar and Paulson, 1989). Subsequent multiple reports on these animals described an enhanced myogenic constriction in response to intra-luminal pressure in different vascular beds including skeletal muscle arterioles (Falcone et al., 1993; Shibuya et al., 1998), mesenteric arteries (Matrougui et al., 2000), cerebral arterioles (Jarajapu and Knot, 2005), and renal afferent arterioles (Ren et al., 2010).

IP_3R-MEDIATED CALCIUM REGULATION AND VASCULAR TONE GENERATION: ALTERATION IN HYPERTENSION

Among other factors, intracellular Ca^{2+} is known to play a pivotal role in the development and maintenance of vascular

myogenic tone. The increased intraluminal pressure was shown to elicit an increased intracellular Ca^{2+} in a number of vessel preparations that develop myogenic response (Schubert and Mulvany, 1999). Knot and Nelson (1998) reported a strong correlation between vessel constriction in isolated pressurized rat cerebral arteries and intraluminal pressure increase, membrane depolarization, and increased intracellular Ca^{2+}. Early studies using Ca^{2+}-sensitive dyes and two-dimensional electrophoresis showed that the increase in intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) in these vessels was associated with an increase in myosin light chain (LC20) phosphorylation (Zou et al., 1995). Further investigation of the temporal association between increased intraluminal pressure, increased $[Ca^{2+}]_i$, and LC20 phosphorylation showed close coincidence of the three events in vessels examined in pressure myography experiments (Zou et al., 2000). Upon activation by Ca^{2+}/calmodulin, the myosin light-chain kinase (MLCK) specifically phosphorylates LC20 at serine-19 (Kamm and Stull, 2001), an event that is sufficient to activate the ATPase activity of actomyosin, cross-bridge cycling, and cell shortening and contraction (Walsh et al., 1982).

Several receptors and transporters contribute to $[Ca^{2+}]_i$ dynamics in VSM, but the two primary pathways for Ca^{2+} influx are the PM L-type VGCC and the ER membrane IP_3R (Hill et al., 2001). On the one hand, depolarization of the PM activates α_{1C}, the pore-forming subunit of the VGCC, causing a rapid Ca^{2+} entry from extracellular space and thus leading to VSMC contraction. On the other hand, triggering of IP_3R by IP_3 induces Ca^{2+} release from the ER Ca^{2+} stores. The fundamental role of these two Ca^{2+} signaling pathways in the clinical management of hypertension is demonstrated by the fact that pharmacological blockers of the L-type VGCC or α-adrenergic receptors are effective in lowering BP (Oparil et al., 2003). In contractile VSMC, VGCCs are the major determinants of $[Ca^{2+}]_i$ and vascular tone. Indeed, it is mainly via through these channels that Ca^{2+} enters the cell. Nevertheless, studies also implicated RyR-mediated SR Ca^{2+} release not only as a potential contributor to the generation of myogenic tone (Mufti et al., 2010), but also via feedback regulation of VSMC depolarization through activation of large conductance Ca^{2+}-dependent potassium channels (Krishnamoorthy et al., 2014).

The idea of Ca^{2+} influx through VGCC contributing to the development of myogenic response stemmed from early results demonstrating a complete loss of myogenic response following extracellular Ca^{2+} removal in a variety of vessel preparations (Schubert and Mulvany, 1999) and later corroborated by the close association between membrane potential, intracellular Ca^{2+} level, and myogenic contractility (Knot and Nelson, 1998). Voltage-associated Ca^{2+} currents were shown to occur following membrane stretching in cerebral artery (McCarron et al., 1997) and blockade of VGCC, while not affecting the depolarization produced by the increase in intraluminal pressure, inhibited the increase in vessel wall Ca^{2+} and the myogenic response (Knot and Nelson, 1998). Models proposed for this mechanotransduction process spanned the involvement of membrane integrins activating downstream Ca^{2+}-sensitive and insensitive contractile pathways to a role for stretch sensitive channels (Colinas et al., 2015; Mufti et al., 2015).

Out of the several members of the VGCC family, the L-type Ca^{2+} channels received the most and earliest attention as the mediator of the extracellular Ca^{2+} influx in myogenic response. Certainly, L-type Ca^{2+} channels are broadly expressed in VSMC (Abd El-Rahman et al., 2013), and interference with Ca^{2+} influx through these channels with selective blockers was shown to preclude the myogenic response, at least partially, in many vessel preparations in early studies (McCarron et al., 1997; Knot and Nelson, 1998). On the other hand, interventions that increased L-type Ca^{2+} channel expression were associated with an increased myogenic tone (Narayanan et al., 2010). Interestingly, earlier studies of spontaneously hypertensive rats implicated increased Ca^{2+} influx via VGCC in the observed augmentation of myogenic contractility (Ren et al., 2010). It is now widely accepted that an upregulation of VGCC expression and/or function occurs in the context of hypertension (Joseph et al., 2013; Tajada et al., 2013). Several signaling proteins are implicated in this process including protein kinase C (PKC) (Joseph et al., 2013) and PI3K (Carnevale and Lembo, 2012), providing a mechanistic context for the contribution of humoral mediators such as angiotensin in increased vascular resistance.

Of interest, a model was proposed implicating a role for IP_3R in regulating extracellular Ca^{2+} influx in VSMCs. IP_3R activation synergistically enhanced TRP channels mediated stretch-induced depolarization (Gonzales et al., 2014). IP_3R organizes in a signaling complex with TRPC and TRPM channels whereby stretch activates a phospholipase C isoform in addition to Ca^{2+} influx through TRPC channels. The resultant IP_3 sensitizes IP_3R to Ca^{2+} entering through TRPC leading to an increased SR Ca^{2+} release activating TRPM currents establishing VSMC depolarization. Significantly, the physical coupling between IP_3R and TRP channels increased in resistance arteriole myocytes from animal models of genetic hypertension leading to an enhanced IP_3-dependent cationic current and depolarization (Adebiyi et al., 2012).

In addition to the extracellular Ca^{2+} influx, it is well documented that Ca^{2+} release from the SR in the form of Ca^{2+} waves is involved in arterial constriction (Boittin et al., 1999; Jaggar and Nelson, 2000; Lee et al., 2005). Specifically, during the myogenic response, both the number of active cells that display Ca^{2+} waves and the frequency of these waves in a given VSMC dramatically increased upon raising the intraluminal pressure from 20 to 40 mmHg (Mufti et al., 2010). The incidence of Ca^{2+} waves at high pressure was not affected by L-type Ca^{2+} channel blockade but was rather sensitive to interference with SR Ca^{2+} release. SR Ca^{2+} depletion precluded Ca^{2+} wave production, LC20 phosphorylation, and myogenic response generation. Specifically, direct inhibition of IP_3R was associated with impaired Ca^{2+} wave generation and interference with the myogenic tone production (Mufti et al., 2015). Similar effects of IP_3R inhibition on micro-vessel contractility and Ca^{2+} wave production were recently observed in human tissues (Navarro-Dorado et al., 2014). Importantly, the expression of several SR and PM-associated Ca^{2+} handling proteins, including IP_3R, sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA), and Na^+/Ca^{2+} exchanger, was upregulated in different hypertensive animal models. Together with an increased SR Ca^{2+} release,

the upregulation of these proteins leads to enhanced basal and evoked vascular constriction (Linde et al., 2012; Abou-Saleh et al., 2013). Specifically, IP_3R expression was shown to be higher in VSMC from spontaneously hypertensive rats compared to non-hypertensive controls (Bernier and Guillemette, 1993). Moreover, in rat models of genetic hypertension, both IP_3 production and IP_3/IP_3R-binding affinity are increased (Wu and de Champlain, 1996) together with an increased global $[Ca^{2+}]_i$ (Jarajapu and Knot, 2005).

Yet, Osol et al. (2002) showed that increased force production in a myogenic vessel preparation in the pressure range that is associated with myogenic contractility (60–140 mmHg) was not associated with appreciable increases in membrane depolarization or intracellular Ca^{2+} concentration. As well, a number of early studies raised the possibility of the involvement of Ca^{2+}-independent force generation mechanisms in myogenic contractility. Calcium–tone relationships were shown to be fivefold more sensitive during pressure-induced activation (VanBavel et al., 1998), and myogenic contractility persisted in situations with reduced extracellular Ca^{2+} levels (McCarron et al., 1997) or where membrane potential was clamped in a depolarized state with high extracellular potassium precluding further Ca^{2+} entry (McCarron et al., 1997; Lagaud et al., 2002). Multiple lines of evidence implicated signaling pathways involving activation of G proteins, PKC, and Rho-associated protein kinase (ROK) in the generation of Ca^{2+}-independent contraction within the context of the vascular tone development (McCarron et al., 1997; VanBavel et al., 2001; Wesselman et al., 2001; Lagaud et al., 2002). Interestingly, a growing body of evidence suggests that enhanced Ca^{2+} sensitization contributes to augmented vascular tone in models of hypertension (Uehata et al., 1997; Jarajapu and Knot, 2005; Zicha et al., 2014; Behuliak et al., 2017). However, despite the direct observations that a sustained arteriolar constriction could be obtained via enhanced actin cytoskeleton reorganization triggered by a seemingly Ca^{2+}-independent signaling pathways (ROK- and PKC-mediated pathways) (Moreno-Domínguez et al., 2013, 2014; Colinas et al., 2015; El-Yazbi et al., 2015). These studies clearly demonstrated the obligate dependence of the generation of arteriolar vascular tone on Ca^{2+}. Not only interferences with extracellular Ca^{2+} levels affected the myogenic response and the mechanisms of all force generation including the Ca^{2+}-independent pathways; specific inhibition of IP_3R precluded a pressure-dependent increase in Ca^{2+} sensitization (Mufti et al., 2015).

ALTERATIONS IN IP_3R EXPRESSION/ACTIVITY ASSOCIATED WITH VASCULAR REMODELING

Apart from mechanisms contributing to vascular tone through regulation of the contractile machinery, an additional interesting factor is the alteration in structural properties of the vessel wall, referred to as vascular remodeling, a phenomenon strongly associated with age (Baek and Kim, 2011). Initially, vascular remodeling constituted an adaptive response of VSMC to hemodynamic changes that can be sensed by vascular cells, both endothelial and SMCs and translated into structural alteration within the vessel wall. On the long run, however, these adaptations lead to increased media thickness, reduced luminal diameter, and extracellular matrix reorganization (Mulvany et al., 1996; Touyz, 2005; Lemarie et al., 2010; Rizzoni and Agabiti-Rosei, 2012). Furthermore, vascular injury induced by disruption of atheromatous plaque or balloon angioplasty triggers a reparative response that includes inflammation, migration and proliferation of VSMC, and intimal hyperplasia. Ultimately, due to changes in vessel architecture and geometry, this leads to a negative constrictive remodeling of the arterial wall (Gibbons and Dzau, 1994; Faxon et al., 1997).

It is now accepted that structural remodeling in resistance arteries is closely related to the development of hypertension (Lemarie et al., 2010; Rizzoni and Agabiti-Rosei, 2012). In this perspective, smooth muscle cells display a significant degree of phenotypic plasticity and, unlike most other differentiated cells, can change their phenotype even at the differentiated state (Yoshida and Owens, 2005; Matchkov et al., 2012). This involves a phenotypic switch from a contractile to a proliferative, migrating, and or/synthetic phenotype and is associated with gene regulation and alteration of cytoplasmic Ca^{2+} signaling machinery (House et al., 2008; Matchkov et al., 2012). While vascular remodeling in aging has been partially investigated (Wang et al., 2005, 2006, 2007), the molecular mechanisms involved in the remodeling of Ca^{2+} signaling pathways observed in hypertension is still poorly understood.

In VSMCs, resting $[Ca^{2+}]_i$ is slightly higher than in other cells, allowing the vessel to be in a constant state of partial contraction. In the synthetic phenotype, however, this turns to be less important or even voltage-independent. In contrast to the role proposed for VGCC and IP_3R in VSM contraction, it has been suggested that regulation of $[Ca^{2+}]_i$ in synthetic VSMC occurs via alternative pathways including store-operated channels (SOCs) and receptor-operated channels (ROCs) (Berra-Romani et al., 2008; Baryshnikov et al., 2009). SOCs are activated by depletion of internal Ca^{2+} stores mainly through IP_3-mediated Ca^{2+} release (Trebak, 2012), whereas ROCs activation involves different components of the PLC signaling cascade including IP_3 (House et al., 2008). It is beyond the scope of this review to discuss these two pathways in more detail. Of note, however, the expression level of all three IP_3R isoforms increase during VSMC switch from contractile to synthetic phenotype (Berra-Romani et al., 2008). Additionally, IP_3R-mediated Ca^{2+} release increases in proliferating VSMC offering a possible explanation for the observed increased in SOC Ca^{2+} entry (Moses et al., 2001; Wilkerson et al., 2006). Selective inhibition of IP_3R not only reduced VSMC proliferation (Wang et al., 2001; Wilkerson et al., 2006) but also inhibited *in vitro* pressure-induced increase in VSMC migration (Tada et al., 2008). Evidence in synthetic human VSMCs point to an altered mode of Ca^{2+} release via IP_3R (Bobe et al., 2011). IP_3-mediated release in these cells occurs in a steady state followed by store-operated calcium entry. This pattern was restored to the oscillatory Ca^{2+} release pattern characteristic to contractile VSMCs upon upregulation of SERCA pump expression. This switch reduced nuclear factor of activated T cells (NFAT) signaling.

In the context of hypertension, we have previously shown that L-type Ca^{2+} channels and IP_3R are specifically and concomitantly upregulated in an angiotensin-induced hypertension model through a NFAT-dependent pathway (Abou-Saleh et al., 2013). Functionally, this was associated with enhancement and sensitization of IP_3-dependent Ca^{2+} release, thereby resulting in higher basal Ca^{2+} levels and increased VSM contraction. In addition to hypertension, upregulated NFAT signaling in the vasculature was implicated in a number of age-related disorders including post-injury restenosis (Bonnet et al., 2009), vascular inflammation, and aggravation of atherosclerosis in diabetes (Nilsson-Berglund et al., 2010; Zetterqvist et al., 2014), as well as vascular smooth muscle senescence (Min et al., 2009). This latter observation taken together with the evidence regarding the association of increased IP_3R expression/activity, NFAT signaling, and VSMC phenotypic switch may add novel insights into the role of IP_3R in VSMC molecular remodeling as a part of the aging process. Specifically, studies in several cell types demonstrated that different IP_3R isoforms occur in close proximity to the mitochondria and transmit pro-apoptotic Ca^{2+} signals (Simpson et al., 1998; Szalai et al., 1999; Mendes et al., 2005). Yet, it is worth mentioning that the role of IP_3R in aging is far from being clear. Whereas IP_3 content was shown to increase in rat brain (Igwe and Ning, 1993), IP_3R expression and IP_3 binding were shown to be decreased (Igwe and Filla, 1997).

In addition to NFAT, other Ca^{2+} sensitive transcription factors such as serum response factor (SRF), c-response element binding (CREB) seem to play an important role in switching VSMC from a contractile to a synthetic phenotype (Matchkov et al., 2012). Future studies on the role of IP_3R in this process need to be conducted in IP_3R-deficient mice. In this regard, the role of IP_3R in VSMC contractility *in vivo* was recently highlighted in a conditional triple knockout mouse, where the agonist-mediated vascular constriction was attenuated together with a lack of development of hypertension in response to chronic angiotensin infusion (Lin et al., 2016). However, the effect of the conditional knockout on VSMC phenotypic switch in response to hypertension has not been addressed so far.

CONCLUSIONS AND FUTURE PERSPECTIVES

Hypertension is a complex disease that arises from the interaction of multiple genetic, environmental, nutritional, hormonal, and age-related pathological conditions. The etiology of "essential hypertension," which accounts for more than 90% of clinical hypertension, comprises an increased vascular resistance and is associated with structural alterations in the wall of resistance arteries. Modulation of $[Ca^{2+}]_i$ in VSMC allows small arteries and arterioles to establish vasomotor tone and regulate blood flow, and determine peripheral vascular resistance and BP. These changes require a phenotypic switch of VSMC from a contractile quiescent to a versatile proliferative phenotype, a phenomenon widely observed in age-associated vascular remodeling. As described above, IP_3R activity was shown to be essential in almost every cellular mechanism involved in setting vascular tone level. Additionally, modulation of IP_3-dependent Ca^{2+} signaling may represent an essential stimulus for VSMC shift from quiescent to the proliferative state. KT-362 was an investigational drug targeting IP_3R-mediated Ca^{2+} release that showed a clinically relevant antihypertensive action (Hester and Shibata, 1990). However, clinical trials were discontinued at phase II. In addition to known targets for antihypertensive therapy, novel interventions within the PLC–IP_3R pathway constitute attractive therapeutic targets for future research given their ubiquitous involvement in cellular processes leading to hypertension.

AUTHOR CONTRIBUTIONS

AE and HA-S designed and wrote the first draft of the manuscript. AE-Y designed the graphical abstract and reviewed the manuscript. FZ, AA, AO, and MR reviewed the manuscript. HZ, GP, and HA-S proofread and revised the manuscript.

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REFERENCES

Abd El-Rahman, R. R., Harraz, O. F., Brett, S. E., Anfinogenova, Y., Mufti, R. E., Goldman, D., et al. (2013). Identification of L- and T-type Ca^{2+} channels in rat cerebral arteries: role in myogenic tone development. *Am. J. Physiol. Heart Circ. Physiol.* 304, H58–H71. doi: 10.1152/ajpheart.00476.2012

Abou-Saleh, H., Pathan, A. R., Daalis, A., Hubrack, S., Abou-Jassoum, H., Al-Naeimi, H., et al. (2013). Inositol 1,4,5-trisphosphate (IP3) receptor up-regulation in hypertension is associated with sensitization of Ca^{2+} release and vascular smooth muscle contractility. *J. Biol. Chem.* 288, 32941–32951. doi: 10.1074/jbc.M113.496802

Adebiyi, A., Thomas-Gatewood, C. M., Leo, M. D., Kidd, M. W., Neeb, Z. P., and Jaggar, J. H. (2012). An elevation in physical coupling of type 1 inositol 1,4,5-trisphosphate (IP3) receptors to transient receptor potential 3 (TRPC3) channels constricts mesenteric arteries in genetic hypertension. *Hypertension* 60, 1213–1219. doi: 10.1161/hypertensionaha.112.198820

Adebiyi, A., Zhao, G., Narayanan, D., Thomas-Gatewood, C. M., Bannister, J. P., and Jaggar, J. H. (2010). Isoform-selective physical coupling of TRPC3 channels to IP3 receptors in smooth muscle cells regulates arterial contractility. *Circ. Res.* 106, 1603–1612. doi: 10.1161/CIRCRESAHA.110.216804

Afroze, T., Sadi, A. M., Momen, M. A., Gu, S., Heximer, S., and Husain, M. (2007). c-Myb-dependent inositol 1,4,5-trisphosphate receptor type-1 expression in vascular smooth muscle cells. *Arterioscler. Thromb. Vasc. Biol.* 27, 1305–1311. doi: 10.1161/ATVBAHA.107.142059

Allison, S. J. (2017). Hypertension: mechanosensation by PIEZO1 in blood pressure control. *Nat. Rev. Nephrol.* 13:3. doi: 10.1038/nrneph.2016.165

Ando, H., Mizutani, A., Matsu-ura, T., and Mikoshiba, K. (2003). IRBIT, a novel inositol 1,4,5-trisphosphate (IP3) receptor-binding protein, is released from the IP3 receptor upon IP3 binding to the receptor. *J. Biol. Chem.* 278, 10602–10612. doi: 10.1074/jbc.M210119200

Arnaoutov, A., and Dasso, M. (2014). Enzyme regulation. IRBIT is a novel regulator of ribonucleotide reductase in higher eukaryotes. *Science* 345, 1512–1515. doi: 10.1126/science.1251550

Baek, E. B., and Kim, S. J. (2011). Mechanisms of myogenic response: Ca^{2+}-dependent and -independent signaling. *J. Smooth Muscle Res.* 47, 55–65. doi: 10.1540/jsmr.47.55

Baryshnikov, S. G., Pulina, M. V., Zulian, A., Linde, C. I., and Golovina, V. A. (2009). Orai1, a critical component of store-operated Ca^{2+} entry, is functionally associated with Na^{+}/Ca^{2+} exchanger and plasma membrane Ca^{2+} pump in proliferating human arterial myocytes. *Am. J. Physiol. Cell Physiol.* 297, C1103–C1112. doi: 10.1152/ajpcell.00283.2009

Beevers, G., Lip, G. Y., and O'Brien, E. (2001). ABC of hypertension: the pathophysiology of hypertension. *BMJ* 322, 912–916. doi: 10.1136/bmj.322.7291.912

Behuliak, M., Bencze, M., Vaneckova, I., Kunes, J., and Zicha, J. (2017). Basal and activated calcium sensitization mediated by RhoA/Rho kinase pathway in rats with genetic and salt hypertension. *Biomed Res. Int.* 2017:8029728. doi: 10.1155/2017/8029728

Bernier, S., and Guillemette, G. (1993). Increased inositol 1,4,5-trisphosphate binding capacity in vascular smooth muscle of spontaneously hypertensive rats. *Am. J. Hypertens.* 6(3 Pt 1), 217–225.

Berra-Romani, R., Mazzocco-Spezzia, A., Pulina, M. V., and Golovina, V. A. (2008). Ca^{2+} handling is altered when arterial myocytes progress from a contractile to a proliferative phenotype in culture. *Am. J. Physiol. Cell Physiol.* 295, C779–C790. doi: 10.1152/ajpcell.00173.2008

Bobe, R., Hadri, L., Lopez, J. J., Sassi, Y., Atassi, F., Karakikes, I., et al. (2011). SERCA2a controls the mode of agonist-induced intracellular Ca^{2+} signal, transcription factor NFAT and proliferation in human vascular smooth muscle cells. *J. Mol. Cell Cardiol.* 50, 621–633. doi: 10.1016/j.yjmcc.2010.12.016

Boittin, F. X., Macrez, N., Halet, G., and Mironneau, J. (1999). Norepinephrine-induced Ca^{2+} waves depend on InsP(3) and ryanodine receptor activation in vascular myocytes. *Am. J. Physiol.* 277(1 Pt 1), C139–C151. doi: 10.1152/ajpcell.1999.277.1.C139

Bonnet, S., Paulin, R., Sutendra, G., Dromparis, P., Roy, M., Watson, K. O., et al. (2009). Dehydroepiandrosterone reverses systemic vascular remodeling through the inhibition of the Akt/GSK3-beta/NFAT Axis. *Circulation* 120, 1231–1240. doi: 10.1161/Circulationaha.109.848911

Bosanac, I., Michikawa, T., Mikoshiba, K., and Ikura, M. (2004). Structural insights into the regulatory mechanism of IP3 receptor. *Biochim. Biophys. Acta* 1742, 89–102. doi: 10.1016/j.bbamcr.2004.09.016

Bosnjak, Z. J. (1993). Ion channels in vascular smooth muscle. Physiology and pharmacology. *Anesthesiology* 79, 1392–1401. doi: 10.1097/00000542-199312000-00031

Bulley, S., Neeb, Z. P., Burris, S. K., Bannister, J. P., Thomas-Gatewood, C. M., Jangsangthong, W., et al. (2012). TMEM16A/ANO1 channels contribute to the myogenic response in cerebral arteries. *Circ. Res.* 111, 1027–1036. doi: 10.1161/CIRCRESAHA.112.277145

Burke, M., Pabbidi, M. R., Farley, J., and Roman, R. J. (2014). Molecular mechanisms of renal blood flow autoregulation. *Curr. Vasc. Pharmacol.* 12, 845–858. doi: 10.2174/15701611113116660149

Cain, A. E., and Khalil, R. A. (2002). Pathophysiology of essential hypertension: role of the pump, the vessel, and the kidney. *Semin. Nephrol.* 22, 3–16.

Carnevale, D., and Lembo, G. (2012). PI3Kγ in hypertension: a novel therapeutic target controlling vascular myogenic tone and target organ damage. *Cardiovasc. Res.* 95, 403–408. doi: 10.1093/cvr/cvs166

Carretero, O. A., and Oparil, S. (2000). Essential hypertension. Part I: definition and etiology. *Circulation* 101, 329–335. doi: 10.1161/01.CIR.101.3.329

Chen, R., Dharmarajan, K., Kulkarni, V. T., Punnanithinont, N., Gupta, A., Bikdeli, B., et al. (2013). Most important outcomes research papers on hypertension. *Circ. Cardiovasc. Qual. Outcomes* 6, e26–e35. doi: 10.1161/CIRCOUTCOMES.113.000424

Chiong, J. R., Aronow, W. S., Khan, I. A., Nair, C. K., Vijayaraghavan, K., Dart, R. A., et al. (2008). Secondary hypertension: current diagnosis and treatment. *Int. J. Cardiol.* 124, 6–21. doi: 10.1016/j.ijcard.2007.01.119

Chockalingam, A. (2008). World Hypertension Day and global awareness. *Can. J. Cardiol.* 24, 441–444. doi: 10.1016/S0828-282X(08)70617-2

Chovanes, G. I., and Richards, R. M. (2012). The predominance of metabolic regulation of cerebral blood flow and the lack of "Classic" autoregulation curves in the viable brain. *Surg. Neurol. Int.* 3:12. doi: 10.4103/2152-7806.92185

Colinas, O., Moreno-Dominguez, A., Zhu, H. L., Walsh, E. J., Perez-Garcia, M. T., Walsh, M. P., et al. (2015). alpha5-Integrin-mediated cellular signaling contributes to the myogenic response of cerebral resistance arteries. *Biochem. Pharmacol.* 97, 281–291. doi: 10.1016/j.bcp.2015.08.088

Dam, V. S., Boedtkjer, D. M., Nyvad, J., Aalkjaer, C., and Matchkov, V. (2014). TMEM16A knockdown abrogates two different Ca^{2+}-activated Cl^{-} currents and contractility of smooth muscle in rat mesenteric small arteries. *Pflugers Arch.* 466, 1391–1409. doi: 10.1007/s00424-013-1382-1

D'Angelo, G., Davis, M. J., and Meininger, G. A. (1997). Calcium and mechanotransduction of the myogenic response. *Am. J. Physiol.* 273(1 Pt 2), H175–H182. doi: 10.1152/ajpheart.1997.273.1.H175

Davis, M. J. (2012). Perspective: physiological role(s) of the vascular myogenic response. *Microcirculation* 19, 99–114. doi: 10.1111/j.1549-8719.2011.00131.x

Deelman, L. E., Jonk, L. J., and Henning, R. H. (1998). The isolation and characterization of the promoter of the human type 1 inositol 1,4,5-trisphosphate receptor. *Gene* 207, 219–225. doi: 10.1016/S0378-1119(97)00630-6

Dharmashankar, K., and Widlansky, M. E. (2010). Vascular endothelial function and hypertension: insights and directions. *Curr. Hypertens. Rep.* 12, 448–455. doi: 10.1007/s11906-010-0150-2

Drummond, H. A. (2009). Yes, no, maybe so ENaC proteins as mediators of renal myogenic constriction. *Hypertension* 54, 962–963. doi: 10.1161/Hypertensionaha.109.139014

El-Yazbi, A. F., Abd-Elrahman, K. S., and Moreno-Dominguez, A. (2015). PKC-mediated cerebral vasoconstriction: Role of myosin light chain phosphorylation versus actin cytoskeleton reorganization. *Biochem. Pharmacol.* 95, 263–278. doi: 10.1016/j.bcp.2015.04.011

Falcone, J. C., Granger, H. J., and Meininger, G. A. (1993). Enhanced myogenic activation in skeletal muscle arterioles from spontaneously hypertensive rats. *Am. J. Physiol.* 265(6 Pt 2), H1847–H1855. doi: 10.1152/ajpheart.1993.265.6.H1847

Faxon, D. P., Coats, W., and Currier, J. (1997). Remodeling of the coronary artery after vascular injury. *Prog. Cardiovasc. Dis.* 40, 129–140. doi: 10.1016/S0033-0620(97)80005-9

Fleming, B. P., Barron, K. W., Howes, T. W., and Smith, J. K. (1987). Response of the microcirculation in rat cremaster muscle to peripheral and central sympathetic stimulation. *Circ. Res.* 61(5 Pt 2), Ii26–Ii31.

Forouzanfar, M. H., Liu, P., Roth, G. A., Ng, M., Biryukov, S., Marczak, L., et al. (2017). global burden of hypertension and systolic blood pressure of at least 110 to 115 mm Hg, 1990-2015. *JAMA* 317, 165–182. doi: 10.1001/jama.2016.19043

Foskett, J. K., White, C., Cheung, K. H., and Mak, D. O. (2007). Inositol trisphosphate receptor Ca^{2+} release channels. *Physiol. Rev.* 87, 593–658. doi: 10.1152/physrev.00035.2006

Fritze, O., Romero, B., Schleicher, M., Jacob, M. P., Oh, D. Y., Starcher, B., et al. (2012). Age-related changes in the elastic tissue of the human aorta. *J. Vasc. Res.* 49, 77–86. doi: 10.1159/000331278

Garbers, D. L., and Dubois, S. K. (1999). The molecular basis of hypertension. *Annu. Rev. Biochem.* 68, 127–155. doi: 10.1146/annurev.biochem.68.1.127

Gates, P. E., Strain, W. D., and Shore, A. C. (2009). Human endothelial function and microvascular ageing. *Exp. Physiol.* 94, 311–316. doi: 10.1113/expphysiol.2008.043349

GBD 2013 Risk Factors Collaborators, Forouzanfar, M. H., Alexander, L., Anderson, H. R., Bachman, V. F., Biryukov, S., et al. (2015). Global, regional, and national comparative risk assessment of 79 behavioural, environmental and occupational, and metabolic risks or clusters of risks in 188 countries, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet* 386, 2287–2323. doi: 10.1016/S0140-6736(15)00128-2

GBD 2015 Risk Factors Collaborators (2016). Global, regional, and national comparative risk assessment of 79 behavioural, environmental and occupational, and metabolic risks or clusters of risks, 1990-2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet* 388, 1659–1724.

Gibbons, G. H., and Dzau, V. J. (1994). The emerging concept of vascular remodeling. *N. Engl. J. Med.* 330, 1431–1438. doi: 10.1056/NEJM199405193302008

Gonzales, A. L., Yang, Y., Sullivan, M. N., Sanders, L., Dabertrand, F., Hill-Eubanks, D. C., et al. (2014). A PLCgamma1-dependent, force-sensitive signaling network in the myogenic constriction of cerebral arteries. *Sci. Signal.* 7:ra49. doi: 10.1126/scisignal.2004732

Gordienko, D. V., Harhun, M. I., Kustov, M. V., Pucovsky, V., and Bolton, T. B. (2008). Sub-plasmalemmal $[Ca^{2+}]$i upstroke in myocytes of the guinea-pig small intestine evoked by muscarinic stimulation: IP3R-mediated Ca^{2+} release induced by voltage-gated Ca^{2+} entry. *Cell Calcium* 43, 122–141. doi: 10.1016/j.ceca.2007.04.012

Grayson, T. H., Haddock, R. E., Murray, T. P., Wojcikiewicz, R. J. H., and Hill, C. E. (2004). Inositol 1,4,5-trisphosphate receptor subtypes are differentially distributed between smooth muscle and endothelial layers of rat arteries. *Cell Calcium* 36, 447–458. doi: 10.1016/j.ceca.2004.04.005

Green, D. J., Swart, A., Exterkate, A., Naylor, L. H., Black, M. A., Cable, N. T., et al. (2010). Impact of age, sex and exercise on brachial and popliteal artery remodelling in humans. *Atherosclerosis* 210, 525–530. doi: 10.1016/j.atherosclerosis.2010.01.048

Grifoni, S. C., Chiposi, R., McKey, S. E., Ryan, M. J., and Drummond, H. A. (2010). Altered whole kidney blood flow autoregulation in a mouse model of reduced beta-ENaC. *Am. J. Physiol. Renal Physiol.* 298, F285–F292. doi: 10.1152/ajprenal.00496.2009

Harvey, A., Montezano, A. C., and Touyz, R. M. (2015). Vascular biology of ageing—Implications in hypertension. *J. Mol. Cell Cardiol.* 83, 112–121. doi: 10.1016/j.yjmcc.2015.04.011

Heinze, C., Seniuk, A., Sokolov, M. V., Huebner, A. K., Klementowicz, A. E., Szijarto, I. A., et al. (2014). Disruption of vascular Ca^{2+}-activated chloride currents lowers blood pressure. *J. Clin. Invest.* 124, 675–686. doi: 10.1172/JCI70025

Henriksen, O., Skagen, K., Amtorp, O., and Hartling, O. (1981). Augmented vasoconstrictor response to changes in vascular transmural pressure in patients with essential arterial hypertension. *Acta Physiol. Scand.* 112, 323–329. doi: 10.1111/j.1748-1716.1981.tb06823.x

Hester, R. K., and Shibata, S. (1990). KT-362 related effects on intracellular calcium release and associated clinical potential: arrhythmias, myocardial ischemia, and hypertension. *Cardiovasc. Drugs Ther.* 4, 1345–1354. doi: 10.1007/BF02018262

Hill, M. A., Zou, H., Potocnik, S. J., Meininger, G. A., and Davis, M. J. (2001). Arteriolar smooth muscle mechanotransduction: Ca^{2+} signaling pathways underlying myogenic reactivity. *J. Appl. Physiol.* 91, 973–983. doi: 10.1152/jappl.2001.91.2.973

Hondeghem, L. M., Ayad, M. J., and Robertson, R. M. (1986). Verapamil, diltiazem and nifedipine block the depolarization-induced potentiation of norepinephrine contractions in rabbit aorta and porcine coronary arteries. *J. Pharmacol. Exp. Ther.* 239, 808–813.

House, S. J., Potier, M., Bisaillon, J., Singer, H. A., and Trebak, M. (2008). The non-excitable smooth muscle: calcium signaling and phenotypic switching during vascular disease. *Pflugers Arch.* 456, 769–785. doi: 10.1007/s00424-008-0491-8

Igwe, O. J., and Filla, M. B. (1997). Aging-related regulation of myo-inositol 1,4,5-trisphosphate signal transduction pathway in the rat striatum. *Brain Res. Mol. Brain Res.* 46, 39–53. doi: 10.1016/S0169-328X(96)00269-0

Igwe, O. J., and Ning, L. (1993). Inositol 1,4,5-trisphosphate arm of the phosphatidylinositide signal transduction pathway in the rat cerebellum during aging. *Neurosci. Lett.* 164, 167–170. doi: 10.1016/0304-3940(93)90883-M

Inoue, R., Okada, T., Onoue, H., Hara, Y., Shimizu, S., Naitoh, S., et al. (2001). The transient receptor potential protein homologue TRP6 is the essential component of vascular alpha(1)-adrenoceptor-activated Ca^{2+}-permeable cation channel. *Circ. Res.* 88, 325–332. doi: 10.1161/01.RES.88.3.325

Islam, M. O., Yoshida, Y., Koga, T., Kojima, M., Kangawa, K., and Imai, S. (1996). Isolation and characterization of vascular smooth muscle inositol 1,4,5-trisphosphate receptor. *Biochem. J.* 316 (Pt 1), 295–302. doi: 10.1042/bj3160295

Ivanova, H., Vervliet, T., Missiaen, L., Parys, J. B., De Smedt, H., and Bultynck, G. (2014). Inositol 1,4,5-trisphosphate receptor-isoform diversity in cell death and survival. *Biochim. Biophys. Acta* 1843, 2164–2183. doi: 10.1016/j.bbamcr.2014.03.007

Jaggar, J. H., and Nelson, M. T. (2000). Differential regulation of Ca^{2+} sparks and Ca^{2+} waves by UTP in rat cerebral artery smooth muscle cells. *Am. J. Physiol. Cell Physiol.* 279, C1528–C1539. doi: 10.1152/ajpcell.2000.279.5.C1528

Jarajapu, Y. P., and Knot, H. J. (2005). Relative contribution of Rho kinase and protein kinase C to myogenic tone in rat cerebral arteries in hypertension. *Am. J. Physiol. Heart Circ. Physiol.* 289, H1917–1922. doi: 10.1152/ajpheart.01012.2004

Jernigan, N. L., and Drummond, H. A. (2005). Vascular ENaC proteins are required for renal myogenic constriction. *Am. J. Physiol. Renal Physiol.* 289, F891–F901. doi: 10.1152/ajprenal.00019.2005

Joseph, B. K., Thakali, K. M., Moore, C. L., and Rhee, S. W. (2013). Ion channel remodeling in vascular smooth muscle during hypertension: implications for novel therapeutic approaches. *Pharmacol. Res.* 70, 126–138. doi: 10.1016/j.phrs.2013.01.008

Kamm, K. E., and Stull, J. T. (2001). Dedicated myosin light chain kinases with diverse cellular functions. *J. Biol. Chem.* 276, 4527–4530. doi: 10.1074/jbc.R000028200

Klabunde, R. E. (2012). *Cardiovascular Physiology Concepts*. Philadelphia, PA: Lippincott Williams & Wilkins.

Knot, H. J., and Nelson, M. T. (1998). Regulation of arterial diameter and wall $[Ca^{2+}]$ in cerebral arteries of rat by membrane potential and intravascular pressure. *J. Physiol.* 508 (Pt 1), 199–209. doi: 10.1111/j.1469-7793.1998.199br.x

Koller, A., Sun, D., and Kaley, G. (1993). Role of shear stress and endothelial prostaglandins in flow- and viscosity-induced dilation of arterioles in vitro. *Circ. Res.* 72, 1276–1284. doi: 10.1161/01.RES.72.6.1276

Krishnamoorthy, G., Sonkusare, S. K., Heppner, T. J., and Nelson, M. T. (2014). Opposing roles of smooth muscle BK channels and ryanodine receptors in the regulation of nerve-evoked constriction of mesenteric resistance arteries. *Am. J. Physiol. Heart Circ. Physiol.* 306, H981–H988. doi: 10.1152/ajpheart.00866.2013

Kroetsch, J. T., Levy, A. S., Zhang, H., Aschar-Sobbi, R., Lidington, D., Offermanns, S., et al. (2017). Constitutive smooth muscle tumour necrosis factor regulates microvascular myogenic responsiveness and systemic blood pressure. *Nat. Commun.* 8:14805. doi: 10.1038/ncomms14805

Lagaud, G., Gaudreault, N., Moore, E. D., Van Breemen, C., and Laher, I. (2002). Pressure-dependent myogenic constriction of cerebral arteries occurs independently of voltage-dependent activation. *Am. J. Physiol. Heart Circ. Physiol.* 283, H2187–H2195. doi: 10.1152/ajpheart.00554.2002

Lee, C.-H., Kuo, K.-H., Dai, J., and van Breemen, C. (2005). Asynchronous calcium waves in smooth muscle cells. *Can. J. Physiol. Pharmacol.* 83, 733–741. doi: 10.1139/y05-083

Lemarie, C. A., Tharaux, P. L., and Lehoux, S. (2010). Extracellular matrix alterations in hypertensive vascular remodeling. *J. Mol. Cell. Cardiol.* 48, 433–439. doi: 10.1016/j.yjmcc.2009.09.018

Liao, P., Yu, D., Li, G., Yong, T. F., Soon, J. L., Chua, Y. L., et al. (2007). A smooth muscle Cav1.2 calcium channel splice variant underlies hyperpolarized window current and enhanced state-dependent inhibition by nifedipine. *J. Biol. Chem.* 282, 35133–35142. doi: 10.1074/jbc.M705478200

Lin, Q., Zhao, G., Fang, X., Peng, X., Tang, H., Wang, H., et al. (2016). IP3 receptors regulate vascular smooth muscle contractility and hypertension. *JCI Insight* 1:e89402. doi: 10.1172/jci.insight.89402

Linde, C. I., Karashima, E., Raina, H., Zulian, A., Wier, W. G., Hamlyn, J. M., et al. (2012). Increased arterial smooth muscle Ca^{2+} signaling, vasoconstriction, and myogenic reactivity in Milan hypertensive rats. *Am. J. Physiol. Heart Circ. Physiol.* 302, H611–H620. doi: 10.1152/ajpheart.00950.2011

Long, C., Cook, L. G., Hamilton, S. L., Wu, G. Y., and Mitchell, B. M. (2007). FK506 binding protein 12/12.6 depletion increases endothelial nitric oxide synthase threonine 495 phosphorylation and blood pressure. *Hypertension* 49, 569–576. doi: 10.1161/01.hyp.0000257914.80918.72

Mak, D. O., McBride, S., and Foskett, J. K. (2001). Regulation by Ca^{2+} and inositol 1,4,5-trisphosphate (InsP3) of single recombinant type 3 InsP3 receptor channels. Ca^{2+} activation uniquely distinguishes types 1 and 3 insp3 receptors. *J. Gen. Physiol.* 117, 435–446. doi: 10.1085/jgp.117.5.435

Martin-Garrido, A., Boyano-Adanez, M. C., Alique, M., Calleros, L., Serrano, I., Griera, M., et al. (2009). Hydrogen peroxide down-regulates inositol 1,4,5-trisphosphate receptor content through proteasome activation. *Free Radic. Biol. Med.* 47, 1362–1370. doi: 10.1016/j.freeradbiomed.2009.07.006

Matchkov, V. V., Kudryavtseva, O., and Aalkjaer, C. (2012). Intracellular Ca^{2+} signalling and phenotype of vascular smooth muscle cells. *Basic Clin. Pharmacol. Toxicol.* 110, 42–48. doi: 10.1111/j.1742-7843.2011.00818.x

Matrougui, K., Levy, B. I., and Henrion, D. (2000). Tissue angiotensin II and endothelin-1 modulate differently the response to flow in mesenteric resistance arteries of normotensive and spontaneously hypertensive rats. *Br. J. Pharmacol.* 130, 521–526. doi: 10.1038/sj.bjp.0703371

McCarron, J. G., Crichton, C. A., Langton, P. D., MacKenzie, A., and Smith, G. L. (1997). Myogenic contraction by modulation of voltage-dependent calcium currents in isolated rat cerebral arteries. *J. Physiol.* 498 (Pt 2), 371–379. doi: 10.1113/jphysiol.1997.sp021864

Mendes, C. C. P., Gomes, D. A., Thompson, M., Souto, N. C., Goes, T. S., Goes, A. M., et al. (2005). The type III inositol 1,4,5-trisphosphate receptor preferentially transmits apoptotic Ca^{2+} signals into mitochondria. *J. Biol. Chem.* 280, 40892–40900. doi: 10.1074/jbc.M506623200

Michikawa, T., Hamanaka, H., Otsu, H., Yamamoto, A., Miyawaki, A., Furuichi, T., et al. (1994). Transmembrane topology and sites of N-glycosylation of inositol 1,4,5-trisphosphate receptor. *J. Biol. Chem.* 269, 9184–9189.

Mignery, G. A., and Sudhof, T. C. (1990). The ligand binding site and transduction mechanism in the inositol-1,4,5-triphosphate receptor. *EMBO J.* 9, 3893–3898.

Mikoshiba, K. (2007). IP3 receptor/Ca^{2+} channel: from discovery to new signaling concepts. *J. Neurochem.* 102, 1426–1446. doi: 10.1111/j.1471-4159.2007.04825.x

Min, L. J., Mogi, M., Tamura, K., Iwanami, J., Sakata, A., Fujita, T., et al. (2009). Angiotensin II type 1 receptor-associated protein prevents vascular smooth muscle cell senescence via inactivation of calcineurin/nuclear factor of activated T cells pathway. *J. Mol. Cell. Cardiol.* 47, 798–809. doi: 10.1016/j.yjmcc.2009.09.006

Miyakawa, T., Maeda, A., Yamazawa, T., Hirose, K., Kurosaki, T., and Iino, M. (1999). Encoding of Ca^{2+} signals by differential expression of IP3 receptor subtypes. *EMBO J.* 18, 1303–1308. doi: 10.1093/emboj/18.5.1303

Moreno-Domínguez, A., Colinas, O., El-Yazbi, A., Walsh, E. J., Hill, M. A., Walsh, M. P., et al. (2013). Ca^{2+} sensitization due to myosin light chain phosphatase inhibition and cytoskeletal reorganization in the myogenic response of skeletal muscle resistance arteries. *J. Physiol.* 591, 1235–1250. doi: 10.1113/jphysiol.2012.243576

Moreno-Domínguez, A., El-Yazbi, A. F., Zhu, H. L., Colinas, O., Zhong, X. Z., Walsh, E. J., et al. (2014). Cytoskeletal reorganization evoked by Rho-associated kinase- and protein kinase C-catalyzed phosphorylation of cofilin and heat shock protein 27, respectively, contributes to myogenic constriction of rat cerebral arteries. *J. Biol. Chem.* 289, 20939–20952. doi: 10.1074/jbc.M114.553743

Moses, S., Dreja, K., Lindqvist, A., Lovdahl, C., Hellstrand, P., and Hultgardh-Nilsson, A. (2001). Smooth muscle cell response to mechanical injury involves intracellular calcium release and ERK1/ERK2 phosphorylation. *Exp. Cell Res.* 269, 88–96. doi: 10.1006/excr.2001.5308

Mufti, R. E., Brett, S. E., Tran, C. H. T., Abd El-Rahman, R., Anfinogenova, Y., El-Yazbi, A., et al. (2010). Intravascular pressure augments cerebral arterial constriction by inducing voltage-insensitive Ca^{2+} waves. *J. Physiol.* 588(Pt 20), 3983–4005. doi: 10.1113/jphysiol.2010.193300

Mufti, R. E., Zechariah, A., Sancho, M., Mazumdar, N., Brett, S. E., and Welsh, D. G. (2015). Implications of alphavbeta3 Integrin Signaling in the Regulation of Ca^{2+} Waves and Myogenic Tone in Cerebral Arteries. *Arterioscler. Thromb. Vasc. Biol.* 35, 2571–2578. doi: 10.1161/atvbaha.115.305619

Mulvany, M. J., Baumbach, G. L., Aalkjaer, C., Heagerty, A. M., Korsgaard, N., Schiffrin, E. L., et al. (1996). Vascular remodeling. *Hypertension* 28, 505–506.

Narayanan, D., Adebiyi, A., and Jaggar, J. H. (2012). Inositol trisphosphate receptors in smooth muscle cells. *Am. J. Physiol. Heart Circ. Physiol.* 302, H2190–H2210. doi: 10.1152/ajpheart.01146.2011

Narayanan, D., Xi, Q., Pfeffer, L. M., and Jaggar, J. H. (2010). Mitochondria control functional CaV1.2 expression in smooth muscle cells of cerebral arteries. *Circ. Res.* 107, 631–641. doi: 10.1161/circresaha.110.224345

Navarro-Dorado, J., Garcia-Alonso, M., van Breemen, C., Tejerina, T., and Fameli, N. (2014). Calcium oscillations in human mesenteric vascular smooth muscle. *Biochem. Biophys. Res. Commun.* 445, 84–88. doi: 10.1016/j.bbrc.2014.01.150

Nilsson-Berglund, L. M., Zetterqvist, A. V., Nilsson-Ohman, J., Sigvardsson, M., Gonzalez Bosc, L. V., Smith, M. L., et al. (2010). Nuclear factor of activated T cells regulates osteopontin expression in arterial smooth muscle in response to diabetes-induced hyperglycemia. *Arterioscler. Thromb. Vasc. Biol.* 30, 218–224. doi: 10.1161/ATVBAHA.109.199299

Nixon, G. F., Mignery, G. A., and Somlyo, A. V. (1994). Immunogold localization of inositol 1,4,5-trisphosphate receptors and characterization of ultrastructural features of the sarcoplasmic reticulum in phasic and tonic smooth muscle. *J. Muscle Res. Cell Motil.* 15, 682–700. doi: 10.1007/BF00121075

Oparil, S., Zaman, M. A., and Calhoun, D. A. (2003). Pathogenesis of hypertension. *Ann. Intern. Med.* 139, 761–776. doi: 10.7326/0003-4819-139-9-200311040-00011

Osol, G., Brekke, J. F., McElroy-Yaggy, K., and Gokina, N. I. (2002). Myogenic tone, reactivity, and forced dilatation: a three-phase model of in vitro arterial myogenic behavior. *Am. J. Physiol. Heart Circ. Physiol.* 283, H2260–H2267. doi: 10.1152/ajpheart.00634.2002

Park, H. S., Betzenhauser, M. J., Won, J. H., Chen, J., and Yule, D. I. (2008). The type 2 inositol (1,4,5)-trisphosphate (InsP3) receptor determines the sensitivity of InsP3-induced Ca^{2+} release to ATP in pancreatic acinar cells. *J. Biol. Chem.* 283, 26081–26088. doi: 10.1074/jbc.M804184200

Patel, S., Joseph, S. K., and Thomas, A. P. (1999). Molecular properties of inositol 1,4,5-trisphosphate receptors. *Cell Calcium* 25, 247–264. doi: 10.1054/ceca.1999.0021

Prole, D. L., and Taylor, C. W. (2016). Inositol 1,4,5-trisphosphate receptors and their protein partners as signalling hubs. *J. Physiol.* 594, 2849–2866. doi: 10.1113/JP271139

Ren, Y., D'Ambrosio, M. A., Liu, R., Pagano, P. J., Garvin, J. L., and Carretero, O. A. (2010). Enhanced myogenic response in the afferent arteriole of spontaneously hypertensive rats. *Am. J. Physiol. Heart Circ. Physiol.* 298, H1769–H1775. doi: 10.1152/ajpheart.00537.2009

Renna, N. F., de Las Heras, N., and Miatello, R. M. (2013). Pathophysiology of vascular remodeling in hypertension. *Int. J. Hypertens.* 2013:808353. doi: 10.1155/2013/808353

Rizzoni, D., and Agabiti-Rosei, E. (2012). Structural abnormalities of small resistance arteries in essential hypertension. *Intern. Emerg. Med.* 7, 205–212. doi: 10.1007/s11739-011-0548-0

Rossier, B. C., Bochud, M., and Devuyst, O. (2017). The hypertension pandemic: an evolutionary perspective. *Physiology* 32, 112–125. doi: 10.1152/physiol.00026.2016

Sayers, L. G., Miyawaki, A., Muto, A., Takeshita, H., Yamamoto, A., Michikawa, T., et al. (1997). Intracellular targeting and homotetramer formation of a truncated inositol 1,4,5-trisphosphate receptor-green fluorescent protein chimera in Xenopus laevis oocytes: evidence for the involvement of the transmembrane spanning domain in endoplasmic reticulum targeting and homotetramer complex formation. *Biochem. J.* 323 (Pt 1), 273–280. doi: 10.1042/bj3230273

Schubert, R., and Mulvany, M. J. (1999). The myogenic response: established facts and attractive hypotheses. *Clin. Sci.* 96, 313–326. doi: 10.1042/cs0960313

Sharma, K., Wang, L., Zhu, Y., Bokkala, S., and Joseph, S. K. (1997). Transforming growth factor-beta1 inhibits type I inositol 1,4,5-trisphosphate receptor expression and enhances its phosphorylation in mesangial cells. *J. Biol. Chem.* 272, 14617–14623. doi: 10.1074/jbc.272.23.14617

Shibuya, J., Ohyanagi, M., and Iwasaki, T. (1998). Enhanced myogenic response in resistance small arteries from spontaneously hypertensive rats: relationship to the voltage-dependent calcium channel. *Am. J. Hypertens.* 11, 767–773. doi: 10.1016/S0895-7061(98)00055-7

Shyu, K. G. (2009). Cellular and molecular effects of mechanical stretch on vascular cells and cardiac myocytes. *Clin. Sci.* 116, 377–389. doi: 10.1042/CS20080163

Simpson, P. B., Mehotra, S., Langley, D., Sheppard, C. A., and Russell, J. T. (1998). Specialized distributions of mitochondria and endoplasmic reticulum proteins define Ca^{2+} wave amplification sites in cultured astrocytes. *J. Neurosci. Res.* 52, 672–683. doi: 10.1002/(SICI)1097-4547(19980615)52:6<672::AID-JNR6>3.0.CO;2-5

Sipma, H., Deelman, L., Smedt, H. D., Missiaen, L., Parys, J. B., Vanlingen, S., et al. (1998). Agonist-induced down-regulation of type 1 and type 3 inositol 1,4,5-trisphosphate receptors in A7r5 and DDT1 MF-2 smooth muscle cells. *Cell Calcium* 23, 11–21. doi: 10.1016/S0143-4160(98)90070-7

Sobey, C. G., Heistad, D. D., and Faraci, F. M. (1998). Potassium channels mediate dilatation of cerebral arterioles in response to arachidonate. *Am. J. Physiol.* 275(5 Pt 2), H1606–H1612. doi: 10.1152/ajpheart.1998.275.5.H1606

Sonoyama, K., Greenstein, A., Price, A., Khavandi, K., and Heagerty, T. (2007). Vascular remodeling: implications for small artery function and target organ damage. *Ther. Adv. Cardiovasc. Dis.* 1, 129–137. doi: 10.1177/1753944707086358

Szalai, G., Krishnamurthy, R., and Hajnoczky, G. (1999). Apoptosis driven by IP(3)-linked mitochondrial calcium signals. *EMBO J.* 18, 6349–6361. doi: 10.1093/emboj/18.22.6349

Tada, T., Nawata, J., Wang, H., Onoue, N., Zhulanqiqige, D., Ito, K., et al. (2008). Enhanced pulsatile pressure accelerates vascular smooth muscle migration: implications for atherogenesis of hypertension. *Cardiovasc. Res.* 80, 346–353. doi: 10.1093/cvr/cvn211

Taguchi, H., Heistad, D. D., Kitazono, T., and Faraci, F. M. (1994). ATP-sensitive K^+ channels mediate dilatation of cerebral arterioles during hypoxia. *Circ. Res.* 74, 1005–1008. doi: 10.1161/01.RES.74.5.1005

Tajada, S., Cidad, P., Colinas, O., Santana, L. F., Lopez-Lopez, J. R., and Perez-Garcia, M. T. (2013). Down-regulation of CaV1.2 channels during hypertension: how fewer CaV1.2 channels allow more Ca^{2+} into hypertensive arterial smooth muscle. *J. Physiol.* 591, 6175–6191. doi: 10.1113/jphysiol.2013.265751

Tajada, S., Cidad, P., Moreno-Dominguez, A., Perez-Garcia, M. T., and Lopez-Lopez, J. R. (2012). High blood pressure associates with the remodelling of inward rectifier K^+ channels in mice mesenteric vascular smooth muscle cells. *J. Physiol.* 590, 6075–6091. doi: 10.1113/jphysiol.2012.236190

Tang, J., Lin, Y., Zhang, Z., Tikunova, S., Birnbaumer, L., and Zhu, M. X. (2001). Identification of common binding sites for calmodulin and inositol 1,4,5-trisphosphate receptors on the carboxyl termini of trp channels. *J. Biol. Chem.* 276, 21303–21310. doi: 10.1074/jbc.M102316200

Thijssen, D. H., Carter, S. E., and Green, D. J. (2016). Arterial structure and function in vascular ageing: are you as old as your arteries? *J. Physiol.* 594, 2275–2284. doi: 10.1113/JP270597

Torrealba, N., Navarro-Marquez, M., Garrido, V., Pedrozo, Z., Romero, D., Eura, Y., et al. (2017). Herpud1 negatively regulates pathological cardiac hypertrophy by inducing IP3 receptor degradation. *Sci. Rep.* 7:13402. doi: 10.1038/s41598-017-13797-z

Touyz, R. M. (2005). Intracellular mechanisms involved in vascular remodelling of resistance arteries in hypertension: role of angiotensin II. *Exp. Physiol.* 90, 449–455. doi: 10.1113/expphysiol.2005.030080

Trebak, M. (2012). STIM/Orai signalling complexes in vascular smooth muscle. *J. Physiol.* 590, 4201–4208. doi: 10.1113/jphysiol.2012.233353

Tu, H., Wang, Z., and Bezprozvanny, I. (2005). Modulation of mammalian inositol 1,4,5-trisphosphate receptor isoforms by calcium: a role of calcium sensor region. *Biophys. J.* 88, 1056–1069. doi: 10.1529/biophysj.104.049601

Uchida, E., and Bohr, D. F. (1969). Myogenic tone in isolated perfused vessels. Occurrence among vascular beds and along vascular trees. *Circ. Res.* 25, 549–555. doi: 10.1161/01.RES.25.5.549

Uehata, M., Ishizaki, T., Satoh, H., Ono, T., Kawahara, T., Morishita, T., et al. (1997). Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature* 389, 990–994. doi: 10.1038/40187

van den Munckhof, I., Scholten, R., Cable, N. T., Hopman, M. T., Green, D. J., and Thijssen, D. H. (2012). Impact of age and sex on carotid and peripheral arterial wall thickness in humans. *Acta Physiol.* 206, 220–228. doi: 10.1111/j.1748-1716.2012.02457.x

VanBavel, E., van der Meulen, E. T., and Spaan, J. A. (2001). Role of Rho-associated protein kinase in tone and calcium sensitivity of cannulated rat mesenteric small arteries. *Exp. Physiol.* 86, 585–592. doi: 10.1113/eph8602217

VanBavel, E., Wesselman, J. P., and Spaan, J. A. (1998). Myogenic activation and calcium sensitivity of cannulated rat mesenteric small arteries. *Circ. Res.* 82, 210–220. doi: 10.1161/01.RES.82.2.210

Vermassen, E., Parys, J. B., and Mauger, J. P. (2004). Subcellular distribution of the inositol 1,4,5-trisphosphate receptors: functional relevance and molecular determinants. *Biol. Cell* 96, 3–17. doi: 10.1016/j.biolcel.2003.11.004

Vervloessem, T., Yule, D. I., Bultynck, G., and Parys, J. B. (2015). The type 2 inositol 1,4,5-trisphosphate receptor, emerging functions for an intriguing Ca^{2+}-release channel. *Biochim. Biophys. Acta* 1853, 1992–2005. doi: 10.1016/j.bbamcr.2014.12.006

Waldemar, G., and Paulson, O. B. (1989). Angiotensin converting enzyme inhibition and cerebral circulation–a review. *Br. J. Clin. Pharmacol.* 28(Suppl. 2), 177s–182s. doi: 10.1111/j.1365-2125.1989.tb03593.x

Wallace, T. A., Xia, S. L., and Sayeski, P. P. (2005). Jak2 tyrosine kinase prevents angiotensin II-mediated inositol 1,4,5 trisphosphate receptor degradation. *Vascul. Pharmacol.* 43, 336–345. doi: 10.1016/j.vph.2005.08.022

Walsh, M. P., Bridenbaugh, R., Hartshorne, D. J., and Kerrick, W. G. (1982). Phosphorylation-dependent activated tension in skinned gizzard muscle fibers in the absence of Ca^{2+}. *J. Biol. Chem.* 257, 5987–5990.

Wang, M., Zhang, J., Jiang, L.-Q., Spinetti, G., Pintus, G., Monticone, R., et al. (2007). Proinflammatory profile within the grossly normal aged human aortic wall. *Hypertension* 50, 219–227. doi: 10.1161/HYPERTENSIONAHA.107.089409

Wang, M., Zhang, J., Spinetti, G., Jiang, L.-Q., Monticone, R., Zhao, D., et al. (2005). Angiotensin II activates matrix metalloproteinase type II and mimics age-associated carotid arterial remodeling in young rats. *Am. J. Pathol.* 167, 1429–1442. doi: 10.1016/S0002-9440(10)61229-1

Wang, M., Zhao, D., Spinetti, G., Zhang, J., Jiang, L.-Q., Pintus, G., et al. (2006). Matrix metalloproteinase 2 activation of transforming growth factor-β1 (TGF-β1) and TGF-β1–Type II receptor signaling within the aged arterial wall. *Arterioscler. Thromb. Vasc. Biol.* 26, 1503–1509. doi: 10.1161/01.ATV.0000225777.58488.f2

Wang, Y., Chen, J., Wang, Y., Taylor, C. W., Hirata, Y., Hagiwara, H., et al. (2001). Crucial role of type 1, but not type 3, inositol 1,4,5-trisphosphate (IP(3)) receptors in IP(3)-induced Ca^{2+} release, capacitative Ca^{2+} entry, and proliferation of A7r5 vascular smooth muscle cells. *Circ. Res.* 88, 202–209. doi: 10.1161/01.RES.88.2.202

Wesselman, J. P., Spaan, J. A., van der Meulen, E. T., and VanBavel, E. (2001). Role of protein kinase C in myogenic calcium-contraction coupling of rat cannulated mesenteric small arteries. *Clin. Exp. Pharmacol. Physiol.* 28, 848–855. doi: 10.1046/j.1440-1681.2001.03534.x

Wilkerson, M. K., Heppner, T. J., Bonev, A. D., and Nelson, M. T. (2006). Inositol trisphosphate receptor calcium release is required for cerebral artery smooth muscle cell proliferation. *Am. J. Physiol. Heart Circ. Physiol.* 290, H240–H247. doi: 10.1152/ajpheart.01191.2004

Wu, L., and de Champlain, J. (1996). Inhibition by cyclic AMP of basal and induced inositol phosphate production in cultured aortic smooth muscle cells from Wistar-Kyoto and spontaneously hypertensive rats. *J. Hypertens.* 14, 593–599. doi: 10.1097/00004872-199605000-00008

Xi, Q., Adebiyi, A., Zhao, G., Chapman, K. E., Waters, C. M., Hassid, A., et al. (2008). IP3 constricts cerebral arteries via IP3 receptor-mediated TRPC3 channel activation and independently of sarcoplasmic reticulum Ca^{2+} release. *Circ. Res.* 102, 1118–1126. doi: 10.1161/CIRCRESAHA.108.173948

Yoshida, T., and Owens, G. K. (2005). Molecular determinants of vascular smooth muscle cell diversity. *Circ. Res.* 96, 280–291. doi: 10.1161/01.RES.0000155951.62152.2e

Yoshikawa, F., Iwasaki, H., Michikawa, T., Furuichi, T., and Mikoshiba, K. (1999). Cooperative formation of the ligand-binding site of the inositol 1,4, 5-trisphosphate receptor by two separable domains. *J. Biol. Chem.* 274, 328–334. doi: 10.1074/jbc.274.1.328

Yoshikawa, F., Morita, M., Monkawa, T., Michikawa, T., Furuichi, T., and Mikoshiba, K. (1996). Mutational analysis of the ligand binding site of the inositol 1,4,5-trisphosphate receptor. *J. Biol. Chem.* 271, 18277–18284. doi: 10.1074/jbc.271.30.18277

Zetterqvist, A. V., Berglund, L. M., Blanco, F., Garcia-Vaz, E., Wigren, M., Duner, P., et al. (2014). Inhibition of nuclear factor of activated T-cells (NFAT) suppresses accelerated atherosclerosis in diabetic mice. *PLoS One* 8, e65020. doi: 10.1371/journal.pone.0065020

Zhang, Z., Tang, J., Tikunova, S., Johnson, J. D., Chen, Z., Qin, N., et al. (2001). Activation of Trp3 by inositol 1,4,5-trisphosphate receptors through displacement of inhibitory calmodulin from a common binding domain. *Proc. Natl. Acad. Sci. U.S.A.* 98, 3168–3173. doi: 10.1073/pnas.051632698

Zhao, G., Neeb, Z. P., Leo, M. D., Pachuau, J., Adebiyi, A., Ouyang, K., et al. (2010). Type 1 IP3 receptors activate BKCa channels via local molecular coupling in arterial smooth muscle cells. *J. Gen. Physiol.* 136, 283–291. doi: 10.1085/jgp.201010453

Zhao, M., Jia, H. H., Liu, L. Z., Bi, X. Y., Xu, M., Yu, X. J., et al. (2017). Acetylcholine attenuated TNF-alpha-induced intracellular Ca^{2+} overload by inhibiting the formation of the NCX1-TRPC3-IP3R1 complex in human umbilical vein endothelial cells. *J. Mol. Cell. Cardiol.* 107, 1–12. doi: 10.1016/j.yjmcc.2017.04.001

Zhou, H., Nakamura, T., Matsumoto, N., Hisatsune, C., Mizutani, A., Iesaki, T., et al. (2008). Predominant role of type 1 IP3 receptor in aortic vascular muscle contraction. *Biochem. Biophys. Res. Commun.* 369, 213–219. doi: 10.1016/j.bbrc.2007.12.194

Zicha, J., Behuliak, M., Pinterova, M., Bencze, M., Kunes, J., and Vaneckova, I. (2014). The interaction of calcium entry and calcium sensitization in the control of vascular tone and blood pressure of normotensive and hypertensive rats. *Physiol. Res.* 63(Suppl. 1), S19–S27.

Zou, H., Ratz, P. H., and Hill, M. A. (1995). Role of myosin phosphorylation and $[Ca^{2+}]$i in myogenic reactivity and arteriolar tone. *Am. J. Physiol.* 269(5 Pt 2), H1590–H1596. doi: 10.1152/ajpheart.1995.269.5.H1590

Zou, H., Ratz, P. H., and Hill, M. A. (2000). Temporal aspects of Ca^{2+} and myosin phosphorylation during myogenic and norepinephrine-induced arteriolar constriction. *J. Vasc. Res.* 37, 556–567. doi: 10.1159/000054089

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Hydrogen Sulfide Attenuates Atherosclerosis in a Partially Ligated Carotid Artery Mouse model via Regulating Angiotensin Converting Enzyme 2 Expression

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Hydrogen sulfide has been suggested to play an essential role in atherogenesis. There is a paucity of information about the association between H_2S and angiotensin converting enzyme 2 (ACE2), a novel homolog of ACE. Therefore, the aim of the study was to explore the role of H_2S in atherosclerosis with respect to ACE2 both *in vitro* and *in vivo*. Here, a murine model of acutely disturbed flow-induced atherosclerosis by left common carotid artery (LCA) partial ligation was utilized. We found that carotid partial ligation in high-fat fed apoE$^{-/-}$ mice significantly inhibited endogenous H_2S synthesis in LCA. Application of NaHS, an H_2S donor considerably attenuated the severity of atherosclerosis with upregulating carotid expression of ACE2, thus converting pro-atherosclerotic angiotensin II (Ang II) to anti-atherosclerotic angiotensin 1-7 (Ang-(1-7)). The anti-atherosclerotic effect of NaHS was dramatically abolished by treatment with MLN-4760, an ACE2 inhibitor. In contrast, blockage of H_2S formation by DL-propargylglycine exacerbated the burden of atherosclerotic plaques accompanied by inhibiting carotid expression of ACE2. At the cellular level, NaHS dose-dependently promoted the expression of ACE2 and conversion from Ang II to Ang-(1-7) in unstimulated or LPS-stimulated endothelial cells, thus exerting anti-inflammatory properties. The anti-inflammatory effect of NaHS was abrogated by pretreatment with DX600, a selective ACE2 inhibitor. In conclusion, these data provide direct evidences that endogenous H_2S insufficiency exists in acute flow disturbance-induced atherosclerosis and that application of H_2S may protect against atherosclerosis via upregulating ACE2 expression in endothelial cells.

Keywords: hydrogen sulfide, atherosclerosis, ACE2, Ang-(1-7), Ang II

INTRODUCTION

Hydrogen sulfide (H_2S) is recently considered to be a novel gaseous mediator, which is endogenously produced during cysteine metabolism mainly by two pyridoxal phosphate-dependent enzymes, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) (Renga, 2011). It has become clear that H_2S exerts various effects in mammalian cardiovascular tissues.

Endogenous H_2S has been suggested to be involved in the pathogenesis of atherosclerosis (Lefer, 2007; Elsey et al., 2010). Wang et al. found for the first time the association between H_2S and atherosclerosis in apoE$^{-/-}$mice (Wang et al., 2009). The causative effect of H_2S in atherosclerosis was further confirmed in CSE gene-deficient mice (Mani et al., 2013). Deletion of CSE gene in mice led to decreased H_2S formation, elevated blood pressure and impaired endothelium-dependent vasorelaxation (Yang et al., 2008). As a result, CSE knockout mice fed a high cholesterol diet predisposed to develop early atherosclerotic lesions (Mani et al., 2013). Mice with both CSE and apoE gene knockout had more extensive atherosclerosis burden than those with either apoE or CSE knockout (Mani et al., 2013). Furthermore, various studies have investigated the precise mechanisms by which H_2S hinders the development of atherosclerosis. H_2S might protect vascular tissue from atherogenic damage by inhibiting vascular intimal proliferation, reducing adhesion molecules expression, suppressing oxidative stress and limiting foam cell formation (Laggner et al., 2007; Meng et al., 2007; Zhao et al., 2011). However, due to the complexity of the atherogenic process, the anti-atherogenic mechanism of H_2S is still far from clear.

It is well-known that renin-angiotensin system (RAS) is a key modulator of cardiovascular function and plays essential roles in endothelial dysfunction and atherosclerosis (Dzau, 2005; Savoia and Schiffrin, 2006). Angiotensin-converting enzyme (ACE)/angiotensin II (Ang II)/angiotensin II type 1 receptor (AT1R) axis is the main pathway of RAS and contributes to the pathogenesis of atherosclerosis (Dzau, 2005; Savoia and Schiffrin, 2006). Recently, the understanding of RAS has been greatly expanded after a homolog of ACE, namely ACE2 was identified. Unlike ACE, ACE2 stimulates the degradation of Ang II into Ang-(1-7), an anti-inflammatory vasodilator and anti-trophic heptapeptide (Donoghue et al., 2000; Oudit et al., 2003; Danilczyk and Penninger, 2006), therefore exerting protective effect in atherosclerosis. Moreover, the anti-atherosclerotic effect of ACE2 has been proposed by overexpression of ACE2 in mice or rabbits, as characterized by an increase in tissue Ang-(1-7) and a decrease in tissue Ang II (Dong et al., 2008; Lovren et al., 2008). Overexpression of ACE2 promoted the stability of atherosclerotic lesions by suppressing macrophage infiltration, decreasing lipid deposition, increasing collagen content and lowering matrix metalloproteinase activity in plaques (Dong et al., 2008). However, ACE2-deficicency in LDLR$^{-/-}$ or apoE$^{-/-}$ mice exacerbated the development of atherosclerosis (Thomas et al., 2010; Thatcher et al., 2011). Thus, ACE2 may provide a therapeutic target in the treatment of atherosclerotic cardiovascular diseases.

In light of the importance of RAS in atherosclerosis, some studies explored the relationship between H_2S metabolism and RAS. It was found that H_2S improved endothelial function and myocardial remodeling via downregulating Ang II/AT1R pathway in renovascular hypertensive rats (Xue et al., 2015; Liu et al., 2017). Application of NaHS, an H_2S donor, inhibited hyperglycemia-induced ACE-Ang II-AT1R activation in cultured renal mesangial cells and kidneys from diabetic rats (Xue et al., 2013). Previous studies have investigated the role of H_2S in ACE-Ang II- AT1R axis, the classical pathway of RAS. However, there is little information about the link between H_2S and ACE2 in cardiovascular system. Given this background, this study was designed to probe the possible effect of H_2S on ACE2-Ang-(1-7) *in vitro* and *in vivo*. Our data indicate that deficiency of endogenous H_2S formation accompanies the development and progression of disturbed flow-induced atherosclerosis. Supplement of H_2S upregulated ACE2 expression and production of Ang-(1-7) in endothelial cells, resulting in attenuation of atherosclerosis.

MATERIALS AND METHODS

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were obtained from American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, USA), 100 units/ml penicillin and 100 mg/ml streptomycin. Cells were plated in 6-well plates or 100-mm tissue culture dishes 1 day before experiments. Near-confluent cultures were starved overnight in medium containing 0.5% FBS before NaHS treatment or stimulation with LPS.

Cell Treatment

All treatments were performed in serum-free culture medium with penicillin and streptomycin. Cells were washed twice with serum-free culture medium and pre-incubated with saline or NaHS (50, 100, and 200 μM) for 24 h. Some cells were then stimulated with LPS (100 ng/ml) for 24 h in the continuous presence of NaHS. In the time course experiment, cells were pre-incubated with saline or NaHS (100 μM) for 0, 6, 24, or 48 h. For experiments using inhibitors, cells were pre-treated with DX600 (1 μM, an ACE2 inhibitor, Phoenix Pharmaceuticals; Pedersen et al., 2011) for 2 h before pre-incubation with NaHS (100 μM) for 24 h and subsequent stimulation with LPS (100 ng/ml) for 24 h.

Animal Model

C57BL/6J male apoE$^{-/-}$ mice were purchased from the Animal Center of the Beijing University, Beijing, China. The study was carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Shanghai JiaoTong University School of Medicine. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Shanghai JiaoTong University School of Medicine (Permit Number: [2015]-117). At age of 8 weeks, mice were randomly assigned to partial ligation of left carotid artery (LCA) or sham operation, followed by being fed a high-fat diet for 4 weeks that contained 10% fat from lard and was supplemented with 2% (w/w) cholesterol. LCA was partially ligated as previously described (Sullivan, 2002; Nam et al., 2009) with slight modifications. In brief, mice were intrapeitoneally anesthetized by the mixture of xylazine (10 mg/kg) and ketamine (80 mg/kg). The neck was epilated and then disinfected with iodophor. LCA was exposed by a ventral midline incision (4–5 mm) in the neck. Except that the superior thyroid artery remained intact, left external carotid, internal carotid and occipital artery were ligated with

6–0 silk (**Figure 1A**). The skin was sutured and mice were then kept in a warm chamber until recovery. After LCA partial ligation or sham operation, mice were randomly given saline, NaHS (1 mg/kg/day, i.p.) or DL-propargylglycine (PAG, 10 mg/kg/day, i.p.). Some NaHS-treated mice were intraperitoneally given MLN-4760, a selective ACE2 inhibitor (0.5 mg/kg, daily, Millennium Pharmaceuticals) 2 weeks after initiation of NaHS treatment (Ye et al., 2012). Four weeks postligation, all the mice were sacrificed. Samples of carotid arteries and blood were collected and stored at −80°C. At the beginning and the end of the study, systolic blood pressure (SBP) was monitored by a tail cuff system (Blood Pressure Analysis System BP- 98AW monitor, Japan).

Histological Examination and Masson Staining

Anesthetized mice underwent left ventricle perfusion with buffered saline and 10% neutralized formalin at 100 mmHg. LCA was collected en bloc with the trachea and esophagus. LCA were embedded in paraffin. Fifteen serial sections (5 μm) were taken 500–1,000 μm proximal to the location of ligation. Five sections from each mouse were stained with hematoxylin and eosin. The staining were then examined by light microscopy ($n = 6$ mice in each group, objective lens magnification of ×20; eyepiece magnification of ×10).The lesions were quantified by Image J software (National Institutes of Health, USA) as described in the literatures (Lessner et al., 2002; Sullivan, 2002; Nam et al., 2009; Zhang et al., 2012, 2015). Measurement of intima and media of carotid arteries was acquired by tracing the border of the lumen and the internal and external elastic laminae (**Figure S1**). The mean area of the intima and media was measured by tracing multiple sections, and the ratio of the area of intima to media was calculated. Sections were also stained with Masson's trichrome to display collagen components in a given plaque. To calculate proportions of collagen content to neointima, we measured intimal lesion areas and Masson's trichrome positive blue areas in at least three sequential sections (Tasaki et al., 2013). Every morphological parameter was quantified by one investigator blinded for the treatment.

Immunohistochemistry of ACE2

LCA were deparaffinized in xylene and rehydrated in aqueous solutions with decreasing alcohol content, followed by a wash in PBST (1 × PBS with 0.5% Tween 20, pH 7.4). Antigen retrieval was achieved by heating the slides in 10 mM sodium citrate (PH 6.0) at 95°C for 20 min. Slides were gradually cooled down at room temperature and washed in H_2O and PBST. To inactivate endogenous peroxidase, sections were treated with 3% H_2O_2 for 15 min, followed by incubation with 10% normal goat serum for 20 min to block nonspecific staining. After incubation overnight at 4°C with primary antibody (rabbit anti-mouse ACE2 in a dilution of 1:75, Abcam, USA), slides were incubated with secondary antibody (HRP-conjugated, goat anti-rabbit IgG in a dilution of 1:200, Santa Cruz Biotechnology, USA) for 2 h at room temperature. Specific staining for ACE2 was developed by the reaction with 3, 3′-diaminobenzidine and counterstaining was applied with hematoxylin. The immunohistochemical staining was viewed by light microscopy (objective lens magnification of ×40; eyepiece magnification of ×10).

Real-Time RT-PCR

Total RNA from cells was extracted using Trizol ® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The concentration of isolated RNA was assayed by measuring the absorbance at 260 nm and the integrity of RNA was visualized by ethidium bromide staining of 18S and 28S on a denaturing agarose gel. One microgram RNA was reversely transcribed using iScript™ cDNA Synthesis Kit (Biorad, USA) at 25°C for 5 min, 42°C for 30 min, followed by 85°C for 5 min. The resulting cDNA was then used as a template for real time PCR amplification. The forward and reverse primers of ACE2, ACE, Mas, and β-actin gene were shown in **Table 1**. Real-time PCR was performed using Lightcycler 2.0 system (Roche Applied Science, USA). Relative expression of gene mRNA was analyzed using a comparative method described in the user bulletin. The data were calculated with $2^{-\Delta\Delta CT}$ method and normalized to β-actin expression.

Western Immunoblot

Cells (3×10^6) were collected and lysated at 4°C using radioimmunoprecipitation assay lysis buffer. Tissues of LCA were homogenized on ice in radioimmunoprecipitation assay lysis buffer. Cell lysates or tissue homogenates were centrifuged at 14,000 g for 10 min at 4°C. Protein concentration in the soluble fraction was determined by Bradford method. Proteins (20 μg) were size-fractionated by 8% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked for 2–3 h with 5% nonfat milk and then probed overnight at 4°C with rabbit polyclonal anti-ACE2 (1:500, molecular weight 97 kDa, Abcam, USA), β-actin antibodies (1:1,000, Santa Cruz Biotechnology, USA) and mouse monoclonal anti-CSE antibody (1:1,000, molecular weight 45 kDa, Abnova, Taiwan) respectively, followed by secondary antibody for 2 hs with a 1:2,000 dilution of HRP-conjugated, goat anti-rabbit IgG or goat anti-mouse IgG (Santa Cruz Biotechnology, USA). The blots were visualized using a standard enhanced chemiluminescence system.

ELISA

MCP-1, TNF- α and IL-6 (Quantikine, R&D systems), as well as Ang-(1-7) and Ang II (Cloud-Clone Corp., USA) were assayed using ELISA kits according to the manufacturers' instructions. Results for the levels of Ang-(1-7) and Ang II in carotid arteries were expressed as pg/mg protein after correction for the protein concentration in tissue homogenates (determined using the Bradford assay).

Measurement of Plasma H_2S

Ten percent Trichloroacetic acid (120 μl), 1% zinc acetate (60 μl), 20 μM N, N-dimethyl-p-phenylenediamine sulfate (40 μl) in 7.2 M hydrochloride acid and 30 μM $FeCl_3$ (40 μl) in 1.2 M hydrochloride acid were mixed with plasma (120 μl) and distilled water (100 μl). After 10 min, the absorbance of the mixture was assayed by spectrophotometry at 670 nm (Tecan Systems Inc.).

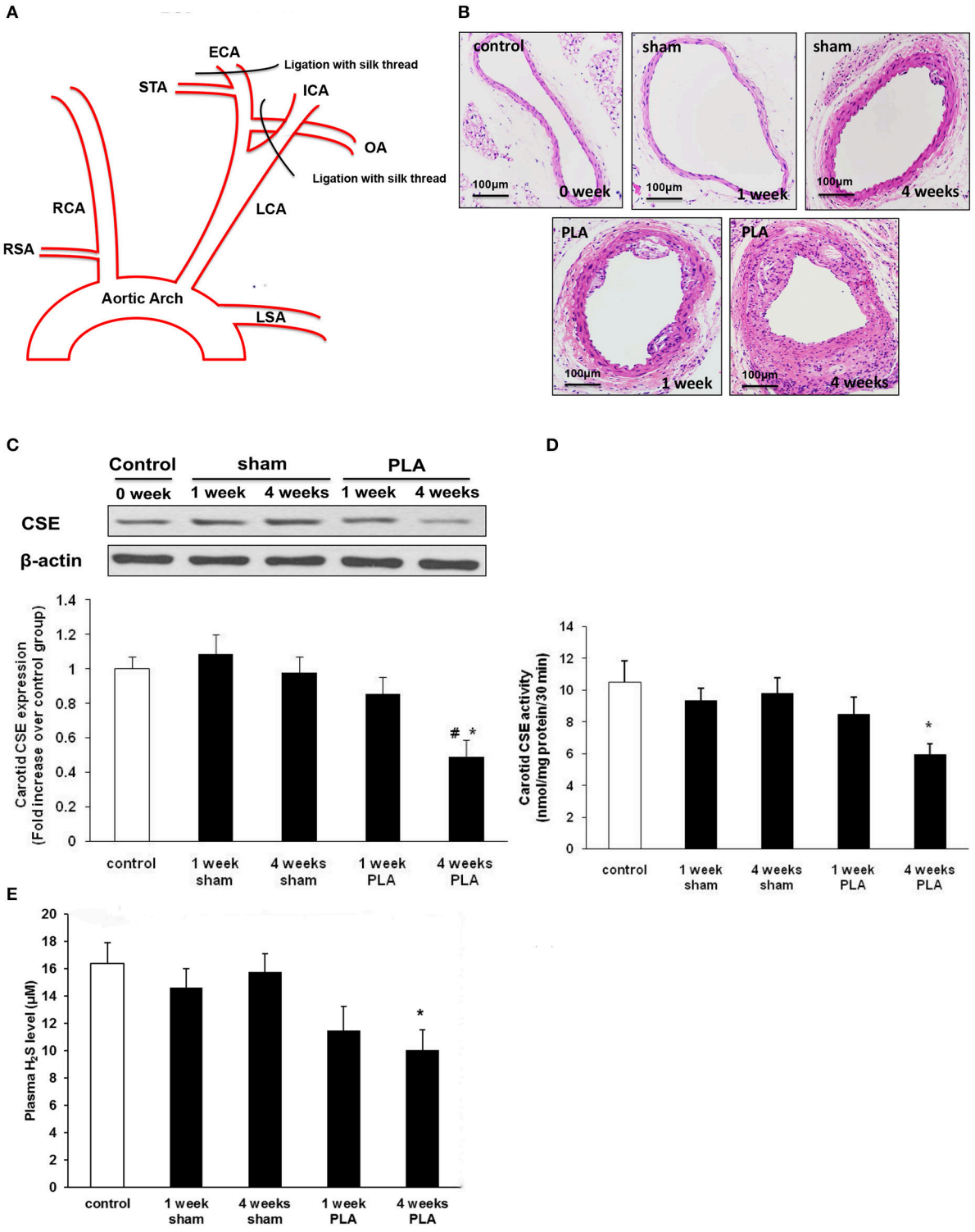

FIGURE 1 | (A) Schematic diagram of low and oscillatory flow-induced atherosclerosis by partial ligation of LCA (PLA). Three branches of LCA, external carotid artery (ECA), internal carotid artery (ICA), and occipital artery (OA) were ligated with 6-0 silk suture, while leaving the superior thyroid artery (STA) intact. **(B)** Representative light microscopy images of hematoxylin and eosin-stained LCA cross sections were taken from high-fat fed apoE$^{-/-}$ mice 1 or 4 weeks after PLA or sham operation. Scale bar for histological images = 100 μm. **(C–E)** Alterations in H_2S biosynthesis during PLA induced atherosclerosis in high-fat fed apoE$^{-/-}$ mice. CSE expression **(C)** and activity **(D)** in LCA, and plasma H_2S level **(E)** were assayed at indicated time points (1 or 4 weeks after PLA or sham operation). Results shown are the mean ± SEM ($n = 6$ animals in each group). *$P < 0.05$ for the comparison between mice 4 weeks after sham operation and mice 4 weeks after PLA. $^{\#}P < 0.05$ for the comparison between mice 1 week after PLA and mice 4 weeks after PLA.

TABLE 1 | Real time PCR primers.

Genes	Primers
ACE2	F: 5′-ACCCTTCTTACATCAGCCCTACTG-3′ R: 5′-TGTCCAAAACCTACCCCACATAT-3′
ACE	F: 5′-CAGCTTCATCAT-CCAGTTCC-3′ R: 5'-CCAGGAAGAG-CAGCAGCCAC-3′
Mas	F: 5′-ACAACACGGGCCTCTATCTG-3′ R: 5′-CTCATGGGCATAGCGAAGAT-3′
β-actin	F: 5′-GGATGCAGAAGG AGATCACTG-3′ R: 5′-CGATCCACACGGA GTACTTG-3′

Plasma level of H_2S was calculated using a standard curve of NaHS with a concentration from 3.125 to 100 μM.

H_2S Synthesizing Activity Assay

H_2S synthesizing activity in LCA was measured as described previously (Zhang et al., 2006). In brief, 4.5% w/v tissue homogenate (430 μl) in 100 mM potassium phosphate buffer (pH 7.4) was mixed with 20 mM L-cysteine (20 μl), 2 mM pyridoxyal 5′-phosphate (20 μl) and saline (30 μl). The reaction started in tightly sealed tubes after tubes were transferred from ice to water bath at 37°C. After incubation for half an hour, 1% zinc acetate (250 μl) was added and trapped the evolved H_2S, followed by 10% trichloroacetic acid (250 μl) to cease the reaction. Afterward, 20 μM N, N-dimethyl-p-phenylenediamine sulfate (133 μl) in 7.2 M hydrochloride acid was added, immediately followed by 30 μM FeCl3 (133 μl) in 1.2 M hydrochloride acid. The absorbance of the resulting mixture was assayed by spectrophotometry at 670 nm (Tecan Systems Inc). H_2S concentration was calculated using a standard curve of NaHS with a range from 3.125 to 100 μM. Results were expressed as nmoles H_2S produced per mg protein in tissue homogenates (determined using the Bradford assay).

Statistics

The data were expressed as mean ± SEM. The significance of differences among groups was evaluated by analysis of variance (ANOVA) with post-hoc Tukey's test when comparing three or more groups. The significance of differences between two groups was evaluated by *T*-test. A $P < 0.05$ was regarded as statistically significant.

RESULTS

Alterations of Endogenous H_2S Synthesis during Disturbed Flow-Induced Atherosclerosis

Atherosclerosis is known to be closely associated with disturbed flow characterized by low and oscillatory shear stress (Sullivan, 2002; Pedersen et al., 2011). However, studies directly linking H_2S to disturbed flow condition in atherogenesis are lacking. Here, we investigated the alterations of H_2S metabolism in a murine model of acutely disturbed flow-induced atherosclerosis by partial carotid ligation (**Figure 1A**). ApoE$^{-/-}$ mice underwent either LCA partial ligation or sham operation and then were fed a high-fat diet for 1 or 4 weeks. By the first week, LCA showed slight evidences of atherosclerotic lesions as determined by H&E staining (**Figure 1B**). By 4 weeks, LCA developed accelerated atherosclerosis (**Figure 1B**). However, only minor or no lesions were observed in carotid arteries isolated from mice fed a high-fat diet for 1 or 4 weeks after sham operation (**Figure 1B**).

Then, we examined the time-dependent alterations of carotid H_2S bio-synthesis in disturbed flow-induced atherosclerosis. As shown in **Figures 1C,D**, H_2S synthesizing activity and CSE expression in LCA decreased in a time-dependent fashion, with a significant reduction 4 weeks after ligation. Furthermore, plasma H_2S concentration gradually declined in a time-dependent manner (**Figure 1E**). There was a statistically significant reduction in plasma H_2S level by 4 weeks after ligation. These data suggest both local and systemic H_2S insufficiency in LCA partial ligation induced atherosclerosis. In another word, acute flow disturbance induced by partial ligation inhibited carotid CSE expression and CSE activity, thus resulting in an evident decline in production of endogenous H_2S.

Alterations of ACE2 in Carotid Arteries during Disturbed Flow-Induced Atherosclerosis

Next, we explored the alterations of carotid ACE2 over time in disturbed flow-induced atherosclerosis (**Figure 2A**). Immunostaining showed that ACE2 was finely expressed in vascular endothelial cells from normal mice. By 1 week postligation, expression of ACE2 in LCA endothelim was significantly upregulated. However, by 4 weeks, the endothelial expression of ACE2 dramatically decreased with the progression of atherosclerosis (**Figures 2A,B**). Four weeks after ligation, LCA endothelium exhibited less intense staining with ACE2 than 1 week after ligation. As a result, the level of Ang-(1-7) in LCA was high at the beginning of ligation and then gradually reduced in a time dependent manner (**Figure 2C**). The level of Ang II in LCA changed oppositely (**Figure 2D**). These data indicate that disturbed flow in carotid arteries initially induced the endothelial expression of ACE2 in an attempt to inhibit the initiation of atherosclerosis. However, with the development and progression of atherosclerosis, endothelial ACE2 expression was inhibited, therefore shifting a balance from anti-atherosclerotic Ang-(1-7) to pro-atherosclerotic Ang II.

Effect of H_2S on Disturbed Flow-Induced Atherosclerosis

In the time course study, we found that the biosynthesis of H_2S significantly decreased 4 weeks after PLA whereas the carotid ACE2 expression started rising 1 week after PLA (**Figures 1**, **2**). This finding suggested that H_2S seems to play a part in regulating ACE2 expression in the advanced stage of atherosclerosis (4

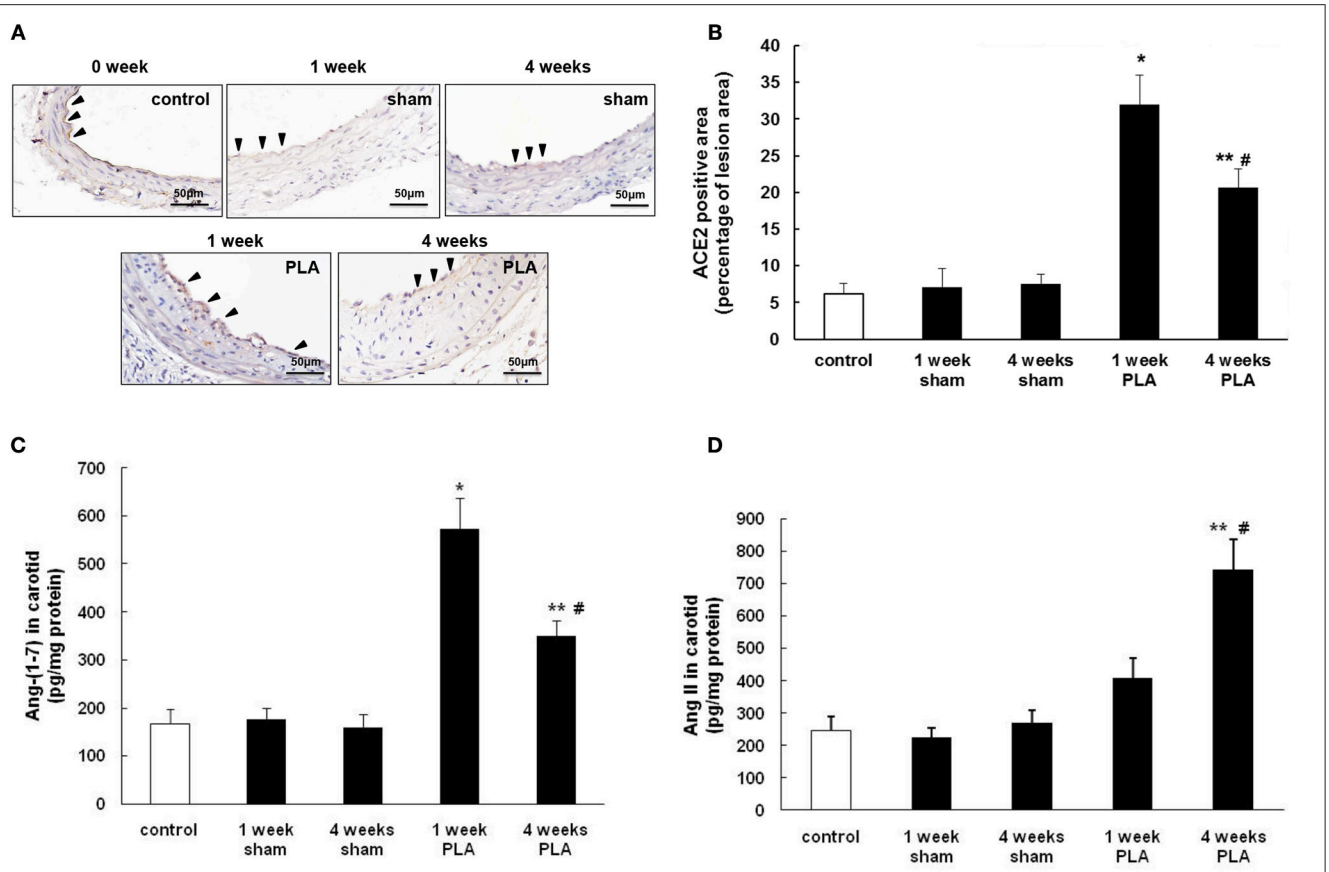

FIGURE 2 | Alterations in ACE2 expression during PLA induced atherosclerosis in high-fat fed apoE$^{-/-}$ mice. **(A)** Representative immunohistochemical images of LCA cross sections were taken from high-fat fed apoE$^{-/-}$ mice 1 or 4 weeks after PLA or sham operation. Scale bar for histological images = 50 μm. **(B)** LCA sections from mice 1 or 4 weeks after PLA or sham operation were quantified immunohistochemically for ACE2 positive staining. Levels of Ang-(1-7) **(C)** and Ang II **(D)** in LCA were assayed by ELISA at indicated time points (1 or 4 weeks after PLA or sham operation). Results shown are the mean ± SEM ($n = 6$ animals in each group). Arrowheads represented positive staining for ACE2 in endothelial cells. *$P < 0.05$ for the comparison between mice 1 week after sham operation and mice 1 week after PLA. **$P < 0.05$ for the comparison between mice 4 weeks after sham operation and mice 4 weeks after PLA. $^{\#}P < 0.05$ for the comparison between mice 1 week after PLA and mice 4 weeks after PLA.

weeks after PLA) but not in the early phase of atherosclerosis (1 week after PLA). In addition to H_2S, there are some other factors contributing to ACE2 expression in different stages of atherosclerosis. Here, we investigated the effect of H_2S on carotid ACE2 expression 4 weeks after PLA.

NaHS (1 mg/kg/day, i.p.), DL-propargylglycine (PAG, 10 mg/kg/day, i.p.) or saline was randomly given to mice after partial ligation. After 4 weeks, the extent of atherosclerotic lesions was assessed by histological analysis (**Figure 3A**). Histological analysis showed that NaHS treatment significantly impeded the plaque development, as characterized by alleviated neointimal hyperplasia and less atherosclerotic lesions in LCA (**Figures 3B,C**). On the other hand, inhibition of H_2S formation by PAG induced advanced atherosclerosis, as evidenced by more severe neointimal hyperplasia and more obvious narrowness in LCA (**Figures 3B,C**). Masson's staining revealed that NaHS reduced the amount of collagen deposits whereas PAG enhanced hyperplasia of collagen fibers in neointimal lesions (**Figures 3D,E**). In addition, NaHS or PAG had negligible effect on body weight, plasma lipid profiles, and blood pressure (**Table 2**).

Effect of H_2S on ACE2-Ang-(1-7) Expression in Atherosclerosis

Immunohistochemistry revealed that supplement with NaHS enhanced the expression of ACE2 in endothelial cells whereas PAG inhibited it in atherosclerotic lesions (**Figures 4A,B**). Then, we used Western blot to quantify the ACE2 expression level in LCA. Consistent with the findings obtained by immunostaining, NaHS significantly upregulated carotid ACE2 expression while PAG downregulated it (**Figure 4C**). As a result, NaHS notably promoted the local production of Ang (1-7) in LCA but PAG inhibited it (**Figure 4D**). The carotid level of Ang II was blunted by NaHS while it was raised by PAG (**Figure 4E**).

In order to reinforce the influence of H_2S on ACE2-Ang-(1-7), MLN-4760, a selective and potent inhibitor against mouse

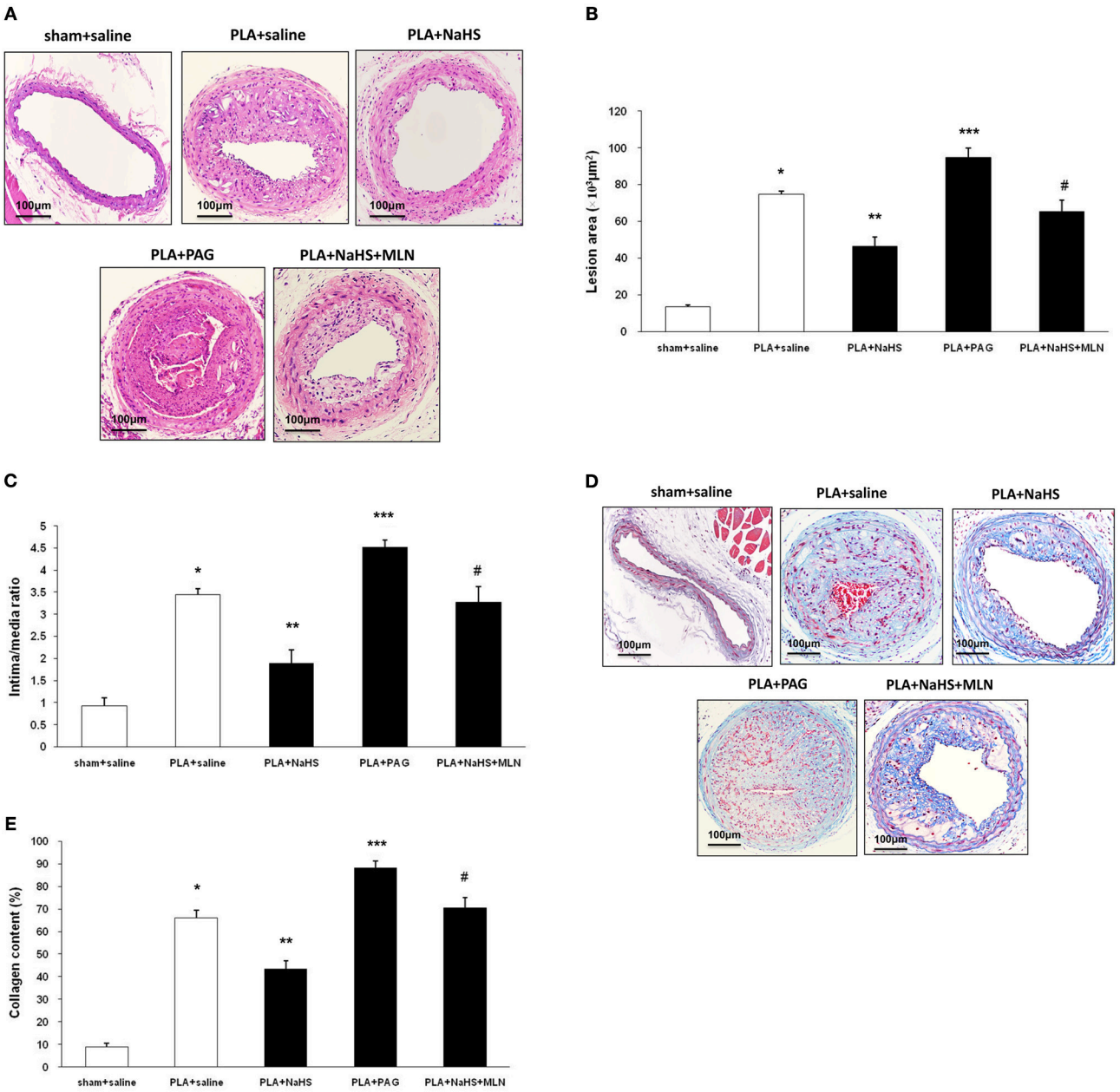

FIGURE 3 | Effect of NaHS or PAG on PLA-induced atherosclerosis in LCA. Representative light microscopy images of hematoxylin and eosin-stained **(A)** or Masson trichrome-stained **(D)** LCA cross sections were taken from sham-operated mice with saline, PLA mice with NaHS, PLA mice with PAG, or PLA mice with NaHS and MLN (intervention with MLN-4760 for 14 days). Scale bar for histological images = 100 μm. Lesion area of intima **(B)**, intima/media ratio **(C)** and collagen deposits **(E)** in the neointimal hyperplasia in LCA were measured as described in Materials and Methods. Results shown are the mean ± SEM ($n = 6$ animals in each group). $^{*}P < 0.05$ for the comparison between sham+saline and PLA+saline. $^{**}P < 0.05$ for the comparison between PLA+saline and PLA+NaHS. $^{***}P < 0.05$ for the comparison between PLA+saline and PLA+PAG. $^{\#}P < 0.05$ for the comparison between PLA+NaHS and PLA+NaHS+MLN.

ACE2 was applied (Ye et al., 2012). As shown in **Figures 4D,E**, blockage of ACE2 activity by MLN-4760 significantly reversed the elevation of carotid Ang-(1-7) level and the decline of carotid Ang II level induced by NaHS. The anti-atherosclerotic property of NaHS was also significantly abolished by treatment with MLN-4760 (**Figures 3A,D**).

Effect of H_2S on ACE2 in Endothelial Cells

Since ACE2 is highly expressed in carotid endothelium, we sought to examine the effect of H_2S on ACE2-Ang-(1-7) in HUVECs. We found that NaHS time-dependently (**Figures 5A–C**) and dose-dependently (**Figures 5D–F**) increased the mRNA and protein expression of ACE2 in HUVECs.

TABLE 2 | Effects of treatment with NaHS on blood pressure and plasma lipids.

	Body Weight (g)	Systolic Blood Pressure (mmHg)	Total Cholesterol (mmol/L)	Triglycerides (mmol/L)	High-density Lipoprotein (mmol/L)	Low-density Lipoprotein (mmol/L)
sham +saline	33.11 ± 5.61	128.74 ± 17.27	10.21 ± 2.51	1.87 ± 0.66	1.97 ± 0.71	7.21 ± 2.05
PLA+saline	31.65 ± 3.27	125.86 ± 15.26	11.43 ± 3.45	1.90 ± 0.67	2.02 ± 0.63	7.32 ± 3.68
PLA+NaHS	30.56 ± 3.36	109.23 ± 25.26	10.78 ± 2.96	2.01 ± 0.85	1.84 ± 0.77	8.10 ± 3.18
PLA+PAG	29.06 ± 4.85	134.45 ± 21.88	11.19 ± 3.09	2.14 ± 0.99	2.16 ± 0.63	7.65 ± 2.77
PLA+NaHS+MLN	28.75 ± 5.86	112.06 ± 23.32	11.52 ± 3.18	2.34 ± 1.04	2.08 ± 0.69	8.19 ± 2.74

Subsequently, NaHS enhanced the production of Ang-(1-7) (**Figures 5G,I**) but decreased the level of Ang II (**Figures 5H,J**) in a time-dependent and dose-dependent manner. Moreover, similar effect of NaHS was observed in LPS-stimulated HUVECs (**Figure 6**). LPS at a concentration of 100 ng/ml significantly downregulated the expression of ACE2 and Ang-(1-7) but increased the level of Ang II in HUVECs. The inhibition of endothelial ACE2-Ang-(1-7) expression induced by LPS was reversed by NaHS in a dose dependent manner (**Figure 6**). NaHS also dose-dependently reduced the level of Ang II in LPS-stimulated HUVECs (**Figure 6E**). As Mas receptor is a functional receptor for Ang-(1-7) (Santos et al., 2003), we evaluated the effect of NaHS on the mRNA expression of Mas in HUVECs. NaHS did not alter the mRNA expression of Mas in unstimulated or LPS-stimulated HUVECs (**Figure S2A**). In addition, we examined the effect of H_2S on ACE, the classical component of RAS in HUVECs. After treatment with NaHS, the mRNA expression level of ACE remained unchangeable in unstimulated or LPS-stimulated HUVECs (**Figure S2B**).

H_2S Inhibits the Production of Cytokines and Chemokine in Endothelial Cells by an ACE2 Dependent Mechanism

As shown in **Figure 7**, the production of TNF-α, IL-6, and MCP-1 was dose-dependently repressed by NaHS in either unstimulated or LPS-stimulated HUVECs. The inhibitory effect of NaHS was significantly blunted by DX600, a selective ACE2 inhibitor (Nam et al., 2009), suggesting that H_2S may inhibit endothelial activation through an ACE2-dependent mechanism (**Figure 8**).

In addition to endothelial cells, positive immunostaining for ACE2 in atherosclerotic plaques was also present in foam cells and macrophages (**Figure 4A**). H_2S has been shown to be a potent regulator of monocyte/ macrophage activation (Zhi et al., 2007; Zhang et al., 2012), which is central to the pathogenesis of atherosclerosis. Therefore, we explored whether H_2S would affect the activation of macrophages via ACE2-Ang-(1-7). As shown in **Figure S3**, NaHS significantly inhibited the production of TNF-α and MCP-1 in unstimulated or LPS-stimulated RAW264.7 cells. The anti-inflammatory property of NaHS was not reversed by pretreatment with DX 600, suggesting that H_2S likely modulates macrophage functions through other pathways but not ACE2.

DISCUSSION

It is well established that atherosclerosis develops preferentially at particular sites in branched or curved arteries, which expose endothelial cells to disturbed flow characterized by low and oscillatory wall shear stress, even if there are various risk factors such as smoking, hyperlipidemia, diabetes and hypertension (Ku et al., 1985; VanderLaan et al., 2004). The present study utilized partial carotid ligation in high-fat fed apoE$^{-/-}$ mice as an animal model of acutely disturbed flow-induced atherosclerosis and then investigated the role of H_2S in it. To the best of our knowledge, this is the first study to explore the alterations of H_2S biosynthesis in disturbed flow-induced atherosclerosis. We found that partial carotid ligation inhibited local CSE expression and CSE activity, thus resulting in an evident decline in endogenous production of H_2S. Consistent with our findings, deficit of H_2S synthesis was also obtained in fat-fed apoE$^{-/-}$ mice or balloon injury induced neointimal hyperplasis (Meng et al., 2007; Wang et al., 2009; Zhang et al., 2012; Mani et al., 2013).

The biological importance of H_2S in disturbed flow-induced atherosclerosis is further underlined by application of NaHS to manipulate endogenous H_2S deficiency. NaHS treatment significantly reduced LCA atherosclerotic burden and impeded the progression of atherosclerosis. Furthermore, abolishing systemic H_2S by PAG aggravated the extent of atherosclerosis in carotid arteries. These interesting findings highlight the potential role of H_2S in the pathogenesis of atherosclerosis induced by low and oscillatory shear stress.

It is well documented that ACE2 plays a permissive role in reducing atherosclerosis. ACE2 is thought to counterbalance ACE by degrading pro-atherosclerotic Ang II to the putative protective peptide, Ang-(1-7) (Oudit et al., 2003; Dong et al., 2008; Lovren et al., 2008; Thomas et al., 2010; Thatcher et al., 2011). The anti-atherosclerotic properties of ACE2 were also evaluated in disturbed flow induced-atherosclerosis. We found that ACE2 was predominantly expressed in carotid endothelial cells in normal mice and that the expression of ACE2 significantly increased 1 week after partial ligation. With the progression of atherosclerosis, the expression level of ACE2 dramatically decreased. As a result, the level of Ang-(1-7) in LCA gradually reduced in a time dependent manner although it was high at the beginning of ligation. The level of Ang II in LCA changed oppositely. These data suggest that disturbed flow in carotid arteries initially upregulated the expression of ACE2 in an attempt to inhibit the initiation of atherosclerosis. However,

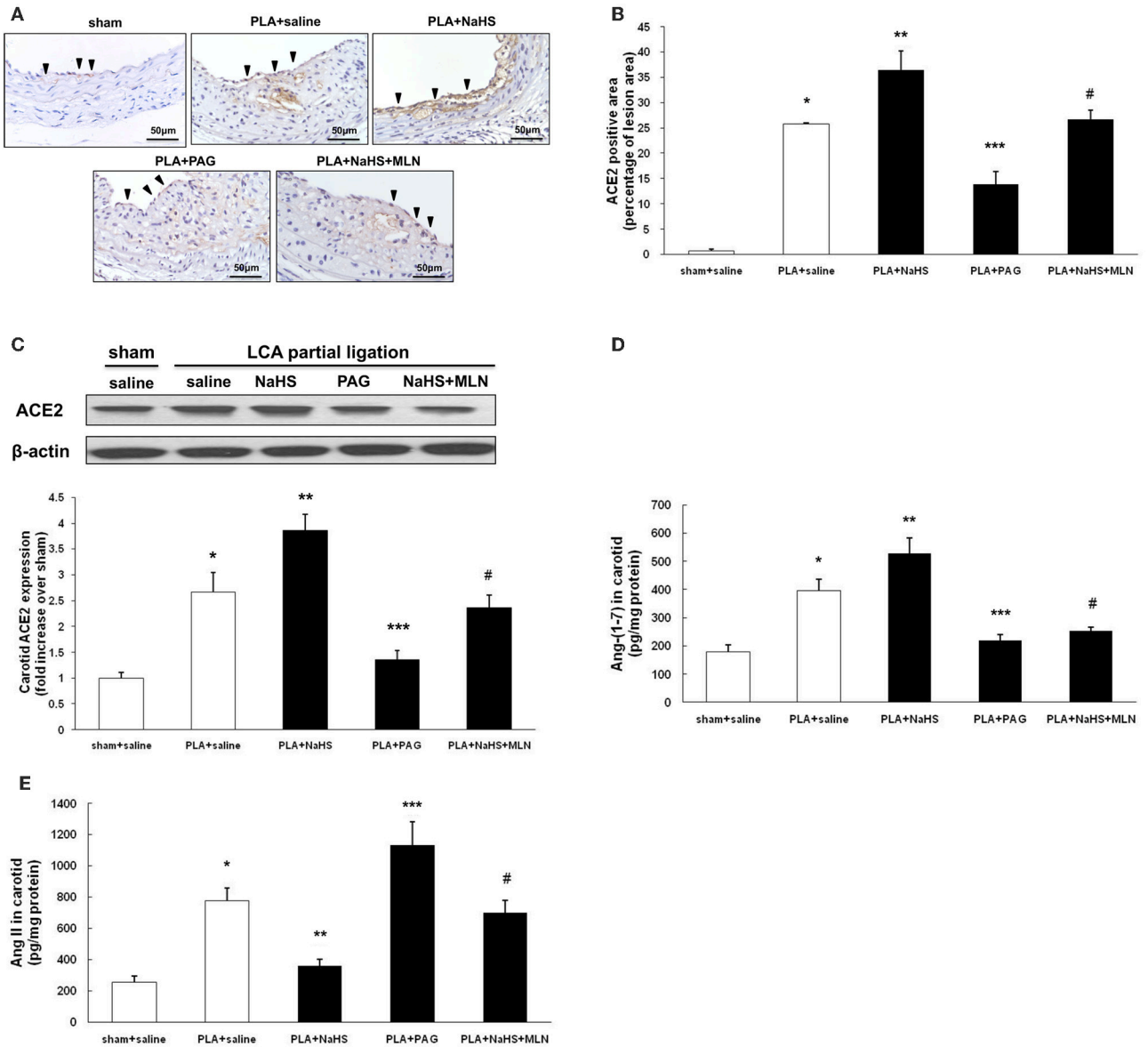

FIGURE 4 | Effect of NaHS or PAG on ACE2 expression in LCA from mice with PLA-induced atherosclerosis. **(A)** Representative immunohistochemical images of LCA cross sections were taken from sham-operated mice with saline, PLA mice with NaHS, PLA mice with PAG, or PLA mice with NaHS and MLN (intervention with MLN-4760 for 14 days). Scale bar for histological images = 50 μm. **(B)** LCA sections were quantified immunohistochemically for ACE2 positive staining. **(C)** ACE2 expression in LCA was measured by Western blot as described in Materials and Methods. Levels of Ang (1-7) **(D)** and Ang II **(E)** in LCA were assayed by ELISA. Results shown are the mean ± SEM ($n = 6$ animals in each group). Arrowheads represented positive staining for ACE2 in endothelial cells. $^{*}P < 0.05$ for the comparison between sham+saline and PLA+saline. $^{**}P < 0.05$ for the comparison between PLA+saline and PLA+NaHS. $^{***}P < 0.05$ for the comparison between PLA+saline and PLA+PAG. $^{\#}P < 0.05$ for the comparison between PLA+NaHS and PLA+NaHS+MLN.

with the development and progression of atherosclerosis, ACE2 expression decreased, thus shifting the balance from anti-atherosclerotic Ang-(1-7) to pro-atherosclerotic Ang II microenvironment. Consistent with our findings, expression of ACE2 mRNA and protein was observed in early and advanced human carotid atherosclerotic lesions (Sluimer et al., 2008). Overexpression of ACE2 by gene transfer attenuated the progression of atherosclerotic lesions in a rabbit model of atherosclerosis or mouse studies (Dong et al., 2008; Lovren et al., 2008). Knockout ACE2 gene in $LDLR^{-/-}$ or $apoE^{-/-}$ mice increased the development of atherosclerosis in aortic arch and sinus (Thomas et al., 2010; Thatcher et al., 2011). A meta-analysis involving 11,051 subjects suggests that genetic variants in ACE2 gene might have a potential effect on ACE2 activity, ACE2 level or Ang-(1-7) production and that ACE2 gene polymorphism may be a genetic risk factor for essential hypertension (Lu et al., 2012).

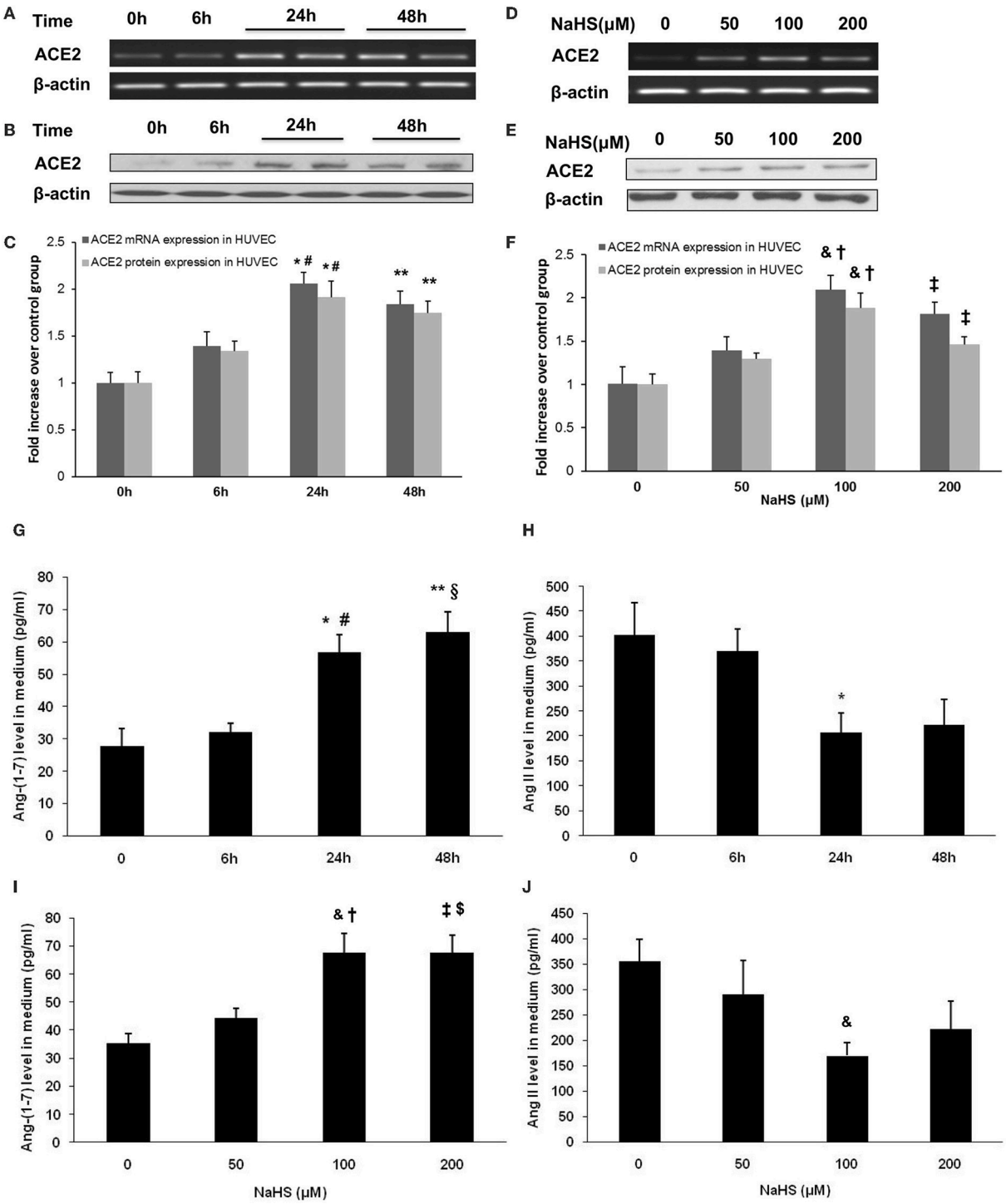

FIGURE 5 | Effect of NaHS on ACE2 and Ang-(1-7) expression in HUVECs. In the time course study, cells were pre-incubated with saline or NaHS (100 μM) for 0, 6, 24 or 48 h **(A–C,G,H)**. In the dose ranging study, cells were pre-incubated with saline or NaHS (50, 100, and 200 μM) for 24 h **(D–F,I,J)**. ACE2 mRNA **(A,C,D,F)** and protein expression **(B,C,E,F)** were analyzed by real time PCR and Western blot respectively. Levels of Ang (1-7) **(G,I)** and Ang II **(H,J)** in culture medium were assayed by ELISA. The data are means ± SEM of at least three independent experiments. $^{*}P < 0.05$ for the comparison between control at baseline and HUVECs treated with NaHS for 24 h. $^{**}P < 0.05$ for the comparison between control at baseline and HUVECs treated with NaHS for 48 h. $^{\#}P < 0.05$ for the comparison between HUVECs treated with NaHS for 6 h and HUVECs treated with NaHS for 24 h. $^{\S}P < 0.05$ for the comparison between HUVECs treated with NaHS for 6 h and HUVECs treated with NaHS for 48 h. $^{\&}P < 0.05$ for the comparison between control at baseline and HUVECs treated with NaHS at a concentration of 100 μM. $^{\dagger}P < 0.05$ for the

(Continued)

FIGURE 5 | Continued
comparison between HUVECs treated with NaHS at a concentration of 50 μM and HUVECs treated with NaHS at a concentration of 100 μM. $^{‡}P < 0.05$ for the comparison between control at baseline and HUVECs treated with NaHS at a concentration of 200 μM. $^{\$}P < 0.05$ for the comparison between HUVECs treated with NaHS at a concentration of 50 μM and HUVECs treated with NaHS at a concentration of 200 μM.

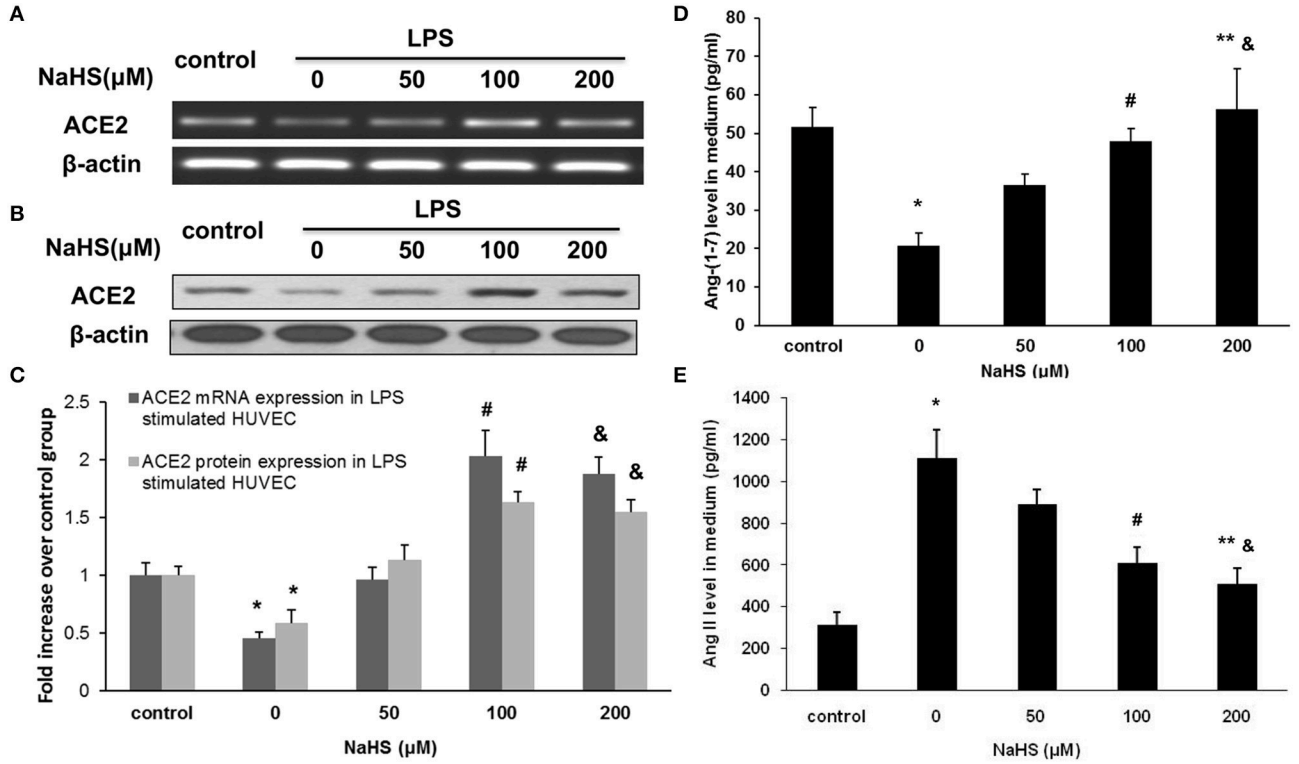

FIGURE 6 | Effect of NaHS on ACE2 and Ang-(1-7) expression in HUVECs stimulated with LPS (100 ng/ml). HUVECs were pre-incubated with saline or NaHS (50, 100, and 200 μM) for 24 h and subsequently stimulated with saline or LPS (100 ng/ml) for 24 h. ACE2 mRNA **(A,C)** and protein **(B,C)** expression were analyzed by real time PCR and Western blot respectively. Levels of Ang (1-7) **(D)** and Ang II **(E)** in culture medium were assayed by ELISA. The data are means ± SEM of at least three independent experiments. $^{*}P < 0.05$ for the comparison between control at baseline and LPS-stimulated HUVECs treated with saline. $^{\#}P < 0.05$ for the comparison between LPS-stimulated HUVECs treated with saline and LPS-stimulated HUVECs treated with NaHS at a concentration of 100 μM. $^{\&}P < 0.05$ for the comparison between LPS-stimulated HUVECs treated with saline and LPS-stimulated HUVECs treated with NaHS at a concentration of 200 μM. $^{**}P < 0.05$ for the comparison between LPS-stimulated HUVECs treated with NaHS at a concentration of 50 μM and LPS-stimulated HUVECs treated with NaHS at a concentration of 200 μM.

Furthermore, the association between H_2S and ACE2 in atherosclerosis was explored in the present study. We found that application of exogenous H_2S reversed partial ligation induced downregulation of ACE2 and Ang-(1-7) in LCA while blockage of H_2S synthesis by PAG significantly aggravated it. The local level of Ang II was reduced by NaHS, but raised by PAG. Similar observations were obtained in cellular experiments. NaHS dose-dependently and time-dependently increased the expression of ACE2 in unstimulated or LPS-stimulated HUVECs. As a result, NaHS enhanced the production of Ang-(1-7) but decreased the level of Ang II. On the other hand, NaHS significantly inhibited the production of TNF-α, IL-6 and MCP-1 in unstimulated or LPS-stimulated HUVECs. The anti-inflammatory effect of NaHS in HUVECs was blunted by DX600. The anti-atherosclerotic benefit of NaHS was also abrogated by treatment with MLN-4760. Taken together, our findings provide solid evidences proposing that H_2S plays a critical role in modulating endothelial ACE2 expression and promoting the cleavage of pro-atherosclerotic Ang II to anti-atherosclerotic Ang-(1-7), thereby impeding the development and progression of atherosclerosis. However, the precise mechanism by which H_2S regulates the expression of ACE2 in endothelia cells during the initiation and progression of atherosclerosis remains elusive.

Peroxisome proliferator-activated receptors (PPAR) are nuclear receptors and function as transcription factors. PPARα has been suggested to affect the signaling pathway of RAS (Banks and Oyekan, 2008; Ibarra-Lara et al., 2010). Stimulation of PPARα with clofibrate favored ACE2/Ang-(1–7)/ATR2 axis in aortic coarctation-induced hypertensive rats as evidenced by enhancing Ang-(1-7) and ACE2 expression in the heart (Ibarra-Lara et al., 2016). Recent studies found that H_2S

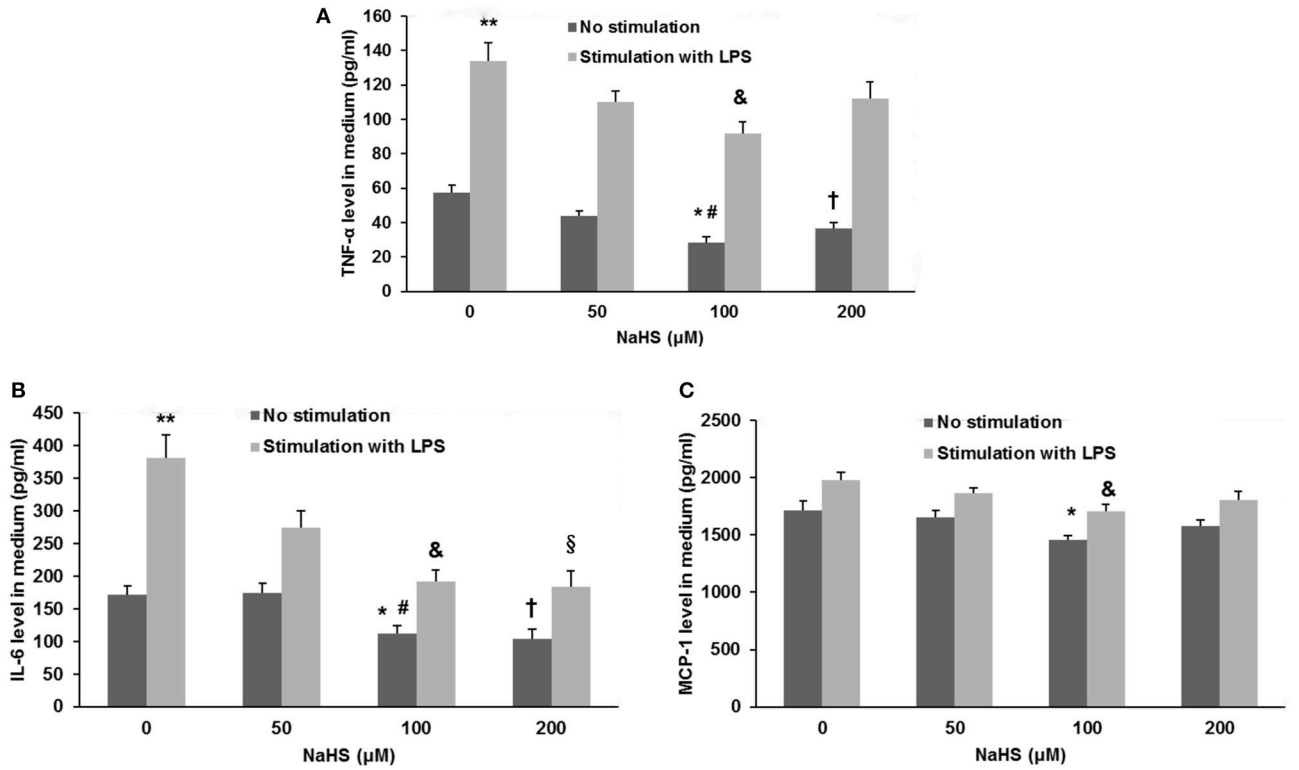

FIGURE 7 | Effect of NaHS on cytokine and chemokine production in unstimulated or LPS-stimulated HUVECs. HUVECs were pre-incubated with saline or NaHS (50, 100, and 200 μM) for 24 h and subsequently stimulated with saline or LPS (100 ng/ml) for 24 h. Production of cytokines (TNF-α, IL-6) **(A,B)** and chemokine (MCP-1) **(C)** was assayed by ELISA. The data are means ± SEM of at least three independent experiments. $^{*}P < 0.05$ for the comparison between unstimulated HUVECs treated with saline and unstimulated HUVECs treated with NaHS at a concentration of 100 μM. $^{\#}P < 0.05$ for the comparison between unstimulated HUVECs treated with NaHS at a concentration of 50 μM and unstimulated HUVECs treated with NaHS at a concentration of 100 μM. $^{\dagger}P < 0.05$ for the comparison between unstimulated HUVECs treated with saline and unstimulated HUVECs treated with NaHS at a concentration of 200 μM. $^{**}P < 0.05$ for the comparison between unstimulated HUVECs treated with saline and LPS-stimulated HUVECs treated with saline. $^{\&}P < 0.05$ for the comparison between LPS-stimulated HUVECs treated with saline and LPS-stimulated HUVECs treated with NaHS at a concentration of 100 μM. $^{\S}P < 0.05$ for the comparison between LPS-stimulated HUVECs treated with saline and LPS-stimulated HUVECs treated with NaHS at a concentration of 200 μM.

promoted the activation of PPARγ in macrophages (Zhang et al., 2012) and facilitated the nuclear translocation of PPARα in fat-fed apoE$^{-/-}$ mice, thus exerting beneficial effect on atherogenesis (Li et al., 2016). Therefore, it offers an original possibility that H_2S might induce the expression of ACE2 in endothelial cells via modulating the activation of PPARα. Moreover, latest studies reported that H_2S participated in regulating the expression of microRNAs (miR-129, miR-299b, and miR-369) in Ang II-induced hypertensive kidney (Weber et al., 2017). Na_2S, an H_2S donor alleviated ischemic and inflammatory injury in cardiomyocytes through upregulation of miR-21 (Toldo et al., 2014). Epigenetic modulation of miRNAs raises another possible way that H_2S may post-transcriptionally regulate ACE2 expression through miRNAs in atherosclerosis. To further prove these assumptions and elucidate the precise mechanism for the induction of ACE2 expression by H_2S, more research is warranted.

In addition to endothelial cells, macrophages are the main source of proinflammatory mediators and contribute to the initiation and development of atherosclerosis. Previous studies have implied the regulatory role of H_2S in monocyte/macrophage activation (Zhi et al., 2007; Zhang et al., 2012). Here, we further investigated whether H_2S affected the function of macrophages involving the pathway of ACE2-Ang-(1-7). It was found that NaHS significantly suppressed the production of TNF-α and MCP-1 in RAW264.7 cells. The anti-inflammatory property of NaHS was not reversed by pretreatment with DX 600. These findings indicate that H_2S possibly inhibited the activation of macrophages through other pathways, but not ACE2 dependent pathway. Clearly, further studies are needed to elucidate the correlation between H_2S and RAS in macrophages. In contrast to the anti-inflammatory effect of H_2S observed in the present study, some researchers demonstrate that H_2S acts as a pro-inflammatory mediator in sepsis and acute pancreatitis (Bhatia et al., 2005; Zhang et al., 2006). H_2S stimulated the synthesis of pro-inflammatory cytokines in human monocyte cell line (Zhi et al., 2007). The discrepancy suggests that H_2S plays various roles in different inflammatory conditions. Different cell types and animal models used in these studies may contribute to this divergence.

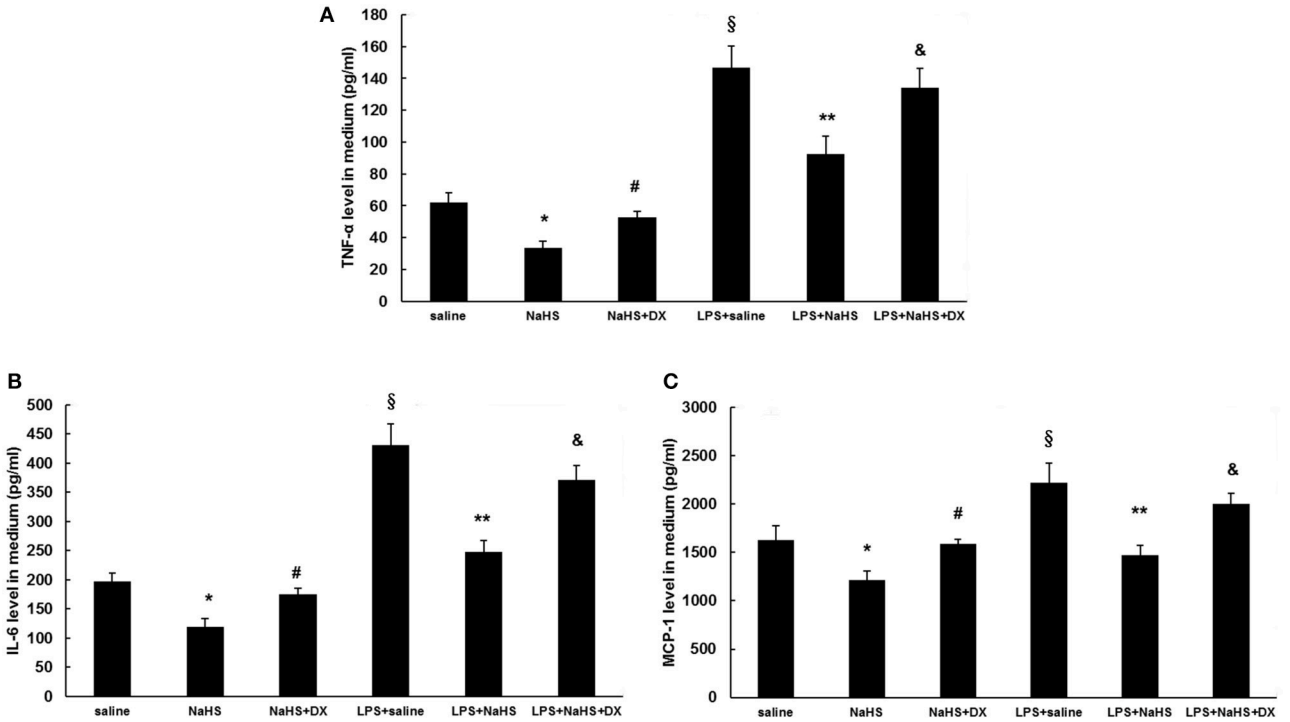

FIGURE 8 | Effect of pretreatment with DX600, a selective ACE2 antagonist on suppressing the production of cytokines and chemokine induced by NaHS in unstimulated or LPS-stimulated HUVECs. Before pre-incubation with NaHS (100 μM) for 24 h, HUVECs were pre-treated with DX600 (1 μM) for 2 h. Then the cells were stimulated with saline or LPS (100 ng/ml) for 24 h. Production of cytokines (TNF-α, IL-6) **(A,B)** and chemokine (MCP-1) **(C)** was assayed by ELISA. The data are means ± SEM of at least three independent experiments. $^{*}P < 0.05$ for the comparison between control at baseline and unstimulated HUVECs treated with NaHS. $^{\#}P < 0.05$ for the comparison between unstimulated HUVECs treated with NaHS and unstimulated HUVECs treated with NaHS and DX600. $^{\S}P < 0.05$ for the comparison between control at baseline and LPS-stimulated HUVECs treated with saline. $^{**}P < 0.05$ for the comparison between LPS-stimulated HUVECs treated with saline and LPS-stimulated HUVECs treated with NaHS. $^{\&}P < 0.05$ for the comparison between LPS-stimulated HUVECs treated with NaHS and LPS-stimulated HUVECs treated with NaHS and DX600.

ACE is known to assist the conversion from Ang I to Ang II while ACE2 is a major enzyme to degrade Ang II to Ang-(1-7). Here, H_2S was found to have negligible effect on the mRNA expression of ACE in endothelial cells. Thus, H_2S regulated the carotid level of Ang II mainly by local ACE2. However, some studies proposed the role of H_2S in regulating ACE-Ang II-AT1R axis. H_2S inhibited Ang II/AT1R pathway and improved endothelial function and myocardial remodeling in renovascular hypertensive rats (Xue et al., 2015; Liu et al., 2017). NaHS also inhibited hyperglycemia-induced ACE-Ang II-AT1R activation in cultured renal mesangial cells and kidneys from diabetic rats (Xue et al., 2013). The discrepancy about the role of H_2S in regulating ACE may be due to different animal models and cell lines utilized in experiments. Future work is needed to clarify this divergence and expand the understanding of the effect of H_2S on RAS.

In conclusion, our findings propose deficiency of endogenous H_2S formation as well as downregulation of ACE2 in carotid atherosclerosis induced by disturbed flow and high fat diet. Supplement of H_2S promotes ACE2 expression and production of Ang-(1-7) in endothelial cells, resulting in attenuation of atherosclerosis.

AUTHOR CONTRIBUTIONS

HZh and CW supervised the whole project. YL, HZe, and LG performed the major experiments. TG provided the technical support.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2017.00782/full#supplementary-material

Figure S1 | Representative sections used for morphometric analysis. Sections were measured by image analysis stained with hematoxylin and eosin (H&E) and Masson's trichrome. Arrowheads indicated external elastic lamina (EEL). Small arrows indicated internal elastic lamina (IEL).

Figure S2 | Effect of NaHS on Mas, a functional receptor for Ang-1-7 and ACE expression in unstimulated HUVECs or HUVECs stimulated with LPS (100 ng/ml). HUVECs were pre-incubated with saline or NaHS (100 μM) for 24 and then stimulated with saline or LPS (100 ng/ml) for 24 h. Mas **(A)** and ACE **(B)** mRNA expression were analyzed by RT-PCR.

Figure S3 | Effect of pretreatment with DX600, a selective ACE2 antagonist on suppressing the production of TNF-α nd MCP-1 induced by NaHS in unstimulated or LPS-stimulated murine macrophage cell line (RAW264.7). Before pre-incubation with NaHS (100 μM) for 24 h, RAW264.7 cells were pre-treated with DX600 (1 μM) for 2 h. Then the cells were stimulated with saline or LPS (100 ng/ml) for 6 h. Production of TNF-α **(A)** and MCP-1 **(B)** was assayed by ELISA. The data are means ± SEM of at least three independent experiments. $^{*}P < 0.05$ for the comparison between control at baseline and unstimulated HUVECs treated with NaHS. $^{\#}P < 0.05$ for the comparison between control at baseline and LPS-stimulated HUVECs treated with saline. $^{**}P < 0.05$ for the comparison between LPS-stimulated HUVECs treated with saline and LPS-stimulated HUVECs treated with NaHS.

REFERENCES

Banks, T., and Oyekan, A. (2008). Peroxisome proliferator-activated receptor alpha activationattenuated angiotensin type 1-mediated but enhanced angiotensin type 2-mediated hemodynamic effects to angiotensin II in the rat. *J. Hypertens.* 26, 468–477. doi: 10.1097/HJH.0b013e3282f2f0f3

Bhatia, M., Wong, F. L., Fu, D., Lau, H. Y., Moochhala, S. M., and Moore, P. K. (2005). Role of hydrogen sulfide in acute pancreatitis and associated lung injury. *FASEB J.* 19, 623–625. doi: 10.1096/fj.04-3023fje

Danilczyk, U., and Penninger, J. M. (2006). Angiotensin-converting enzyme II in the heart and the kidney. *Circ. Res.* 98, 463–471. doi: 10.1161/01.RES.0000205761.22353.5f

Dong, B., Zhang, C., Feng, J. B., Zhao, Y. X., Li, S. Y., Yang, Y. P., et al. (2008). Overexpression of ACE2 enhances plaque stability in a rabbit model of atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* 28, 1270–1276. doi: 10.1161/ATVBAHA.108.164715

Donoghue, M., Hsieh, F., Baronas, E., Godbout, K., Gosselin, M., Stagliano, N., et al. (2000). A novel angiotensin-converting enzyme–related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. *Cir. Res.* 87, e1–e9. doi: 10.1161/01.RES.87.5.e1

Dzau, V. (2005). The cardiovscular continuum and renin-angiotensin-aldosterone system blockade. *J. Hypertens.* 13, S9–S17. doi: 10.1097/01.hjh.0000165623.72310.dd

Elsey, D. J., Fowkes, R. C., and Baxter, G. F. (2010). Regulation of cardiovascular cell function by hydrogen sulfide (H(2)S). *Cell Biochem. Funct.* 28, 95–106. doi: 10.1002/cbf.1618

Ibarra-Lara, L., Cervantes-Perez, L. G., Perez-Severiano, F., Del Valle, L., Rubio-Ruiz, E., Soria-Castro, E., et al. (2010). PPARα stimulation exerts a blood pressure lowering effect through different mechanisms in a time-dependent manner. *Eur. J. Pharmacol.* 627, 185–193. doi: 10.1016/j.ejphar.2009.10.039

Ibarra-Lara, L., Del Valle-Mondragón, L., Soria-Castro, E., Torres-Narváez, J. C., Pérez-Severiano, F., Sánchez-Aguilar, M., et al. (2016). Peroxisome proliferator-activated receptor-α stimulation by clofibrate favors an antioxidant and vasodilator environment in a stressed left ventricle. *Pharmacol. Rep.* 68, 692–702. doi: 10.1016/j.pharep.2016.03.002

Ku, D. N., Giddens, D. P., Zarins, C. K., and Glagov, S. (1985). Pulsatile flow and atherosclerosis in the human carotid bifurcation. Positive correlation between plaque location and low oscillating shear stress. *Arterioscler. Thromb. Vasc. Biol.* 5, 293–302. doi: 10.1161/01.ATV.5.3.293

Laggner, H., Muellner, M. K., Schreier, S., Sturm, B., Hermann, M., Exner, M., et al. (2007). Hydrogen sulphide: a novel physiological inhibitor of LDL atherogenic modification by HOCl. *Free Radic. Res.* 41, 741–747. doi: 10.1080/10715760701263265

Lefer, D. J. (2007). A new gaseous signaling molecule emerges: cardioprotective role of hydrogen sulfide. *Proc. Natl. Acad. Sci. U.S.A.* 104, 17907–17908. doi: 10.1073/pnas.0709010104

Lessner, S. M., Prado, H. L., Waller, E. K., and Galis, Z. S. (2002). Atherosclerotic lesions grow through recruitment and proliferation of circulating monocytes in a murine model. *Am. J. Pathol.* 160, 2145–2155. doi: 10.1016/S0002-9440(10)61163-7

Li, D., Xiong, Q., Peng, J., Hu, B., Li, W., Zhu, Y., et al. (2016). Hydrogen sulfide up-regulates the expression of ATP-Binding cassette transporter A1 via promoting nuclear translocation of PPARα. *Int. J. Mol. Sci.* 17:635. doi: 10.3390/ijms17050635

Liu, S. Y., Duan, X. C., Jin, S., Teng, X., Xiao, L., Xue, H. M., et al. (2017). Hydrogen sulfide improves myocardial remodeling via downregulated angiotensin /AT1R pathway in renovascular hypertensive rats. *Am. J. Hypertens.* 30, 67–74. doi: 10.1093/ajh/hpw104

Lovren, F., Pan, Y., Quan, A., Teoh, H., Wang, G., Shukla, P. C., et al. (2008). Angiotensin converting enzyme-2 confers endothelial protection and attenuates atherosclerosis. *Am. J. Physiol. Heart Circ. Physiol.* 295, H1377–H1384. doi: 10.1152/ajpheart.00331.2008

Lu, N., Yang, Y., Wang, Y., Liu, Y., Fu, G., Chen, D., et al. (2012). ACE2 gene polymorphism and essential hypertension: an updated meta-analysis involving 11,051 subjects. *Mol. Biol. Rep.* 39, 6581–6589. doi: 10.1007/s11033-012-1487-1

Mani, S., Li, H., Untereiner, A., Wu, L., Yang, G., Austin, R. C., et al. (2013). Decreased endogenous production of hydrogen sulfide accelerates atherosclerosis. *Circulation* 127, 2523–2534. doi: 10.1161/CIRCULATIONAHA.113.002208

Meng, Q. H., Yang, G., Yang, W., Jiang, B., Wu, L., and Wang, R. (2007). Protective effect of hydrogen sulfide on balloon injury-induced neointima hyperplasia in rat carotid arteries. *Am. J. Pathol.* 170, 1406–1414. doi: 10.2353/ajpath.2007.060939

Nam, D., Ni, C. W., Rezvan, A., Suo, J., Budzyn, K., Llanos, A., et al. (2009). Partial carotid ligation is a model of acutely induced disturbed flow, leading to rapid endothelial dysfunction and atherosclerosis. *Am. J. Physiol. Heart Circ. Physiol.* 297, H1535–H1543. doi: 10.1152/ajpheart.00510.2009

Oudit, G. Y., Crackower, M. A., Backx, P. H., and Penninger, J. M. (2003). The role of ACE2 in cardiovascular physiology *Trends Cardiovas. Med.* 13, 93–101. doi: 10.1016/S1050-1738(02)00233-5

Pedersen, K. B., Sriramula, S., Chhabra, K. H., Xia, H., and Lazartigues, E. (2011). Species-specific inhibitor sensitivity of angiotensin-converting enzyme 2 (ACE2) and its implication for ACE2 activity assays. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 301, R1293–1299. doi: 10.1152/ajpregu.00339.2011

Renga, B. (2011). Hydrogen sulfide generation in mammals: the molecular biology of cystathionine-β-synthase (CBS) and cytathionine-γ-Lyase (CSE). *Inflamm. Allergy Drug Targets* 10, 85–91. doi: 10.2174/187152811794776286

Santos, R. A. S., Simoes e Silva, A. C., Maric, C., Silva, D. M., Machado, R. P., de Buhr, I., et al. (2003). Angiotensin-(1–7) is an endogenous ligand for the G protein-coupled receptor Mas. *Proc. Natl. Acad. Sci. U.S.A.* 100, 8258–8263. doi: 10.1073/pnas.1432869100

Savoia, C., and Schiffrin, E. L. (2006). Inhibition of the renin angiotensin system: implications for the endothelium. *Curr. Diab. Rep.* 6, 274–278. doi: 10.1007/s11892-006-0060-5

Sluimer, J. C., Gasc, J. M., Hamming, I., van Goor, H., Michaud, A., van den Akker, L. H., et al. (2008). Angiotensin-converting enzyme 2 (ACE2) expression and activity in human carotid atherosclerotic lesions. *J. Pathol.* 215, 273–279. doi: 10.1002/path.2357

Sullivan, C. J. (2002). Flow-dependent remodeling in the carotid artery of fibroblast growth factor-2 knockout mice. *Arterioscler. Thromb. Vasc. Biol.* 22, 1100–1105. doi: 10.1161/01.ATV.0000023230.17493.E3

Tasaki, T., Yamada, S., Guo, X., Tanimoto, A., Wang, K. Y., Nabeshima, A., et al. (2013). Apoptosis signal-regulating kinase 1 deficiency attenuates vascular injury-induced neointimal hyperplasia by suppressing apoptosis in smooth muscle cells. *Am. J. Pathol.* 182, 597–609. doi: 10.1016/j.ajpath.2012.10.008

Thatcher, S. E., Zhang, X., Howatt, D. A., Lu, H., Gurley, S. B., Daugherty, A., et al. (2011). Angiotensin-converting enzyme 2 deficiency in whole body or bone marrow-derived cells increases atherosclerosis in low-density lipoprotein receptor–/– mice. *Arterioscler. Thromb. Vasc. Biol.* 31, 758–765. doi: 10.1161/ATVBAHA.110.221614

Thomas, M. C., Pickering, R. J., Tsorotes, D., Koitka, A., Sheehy, K., Bernardi, S., et al. (2010). Genetic Ace2 deficiency accentuates vascular inflammation

and atherosclerosis in the ApoE knockout mouse. *Circ. Res.* 107, 888–897. doi: 10.1161/CIRCRESAHA.110.219279

Toldo, S., Das, A., Mezzaroma, E., Chau, V. Q., Marchetti, C., Durrant, D., et al. (2014). Induction of microRNA-21 with exogenous hydrogen sulfide attenuates myocardial ischemic and inflammatory injury in mice. *Circ. Cardiovasc. Genet.* 7, 311–320. doi: 10.1161/CIRCGENETICS.113.000381

VanderLaan, P. A., Reardon, C. A., and Getz, G. S. (2004). Site specificity of atherosclerosis: site-selective responses to atherosclerotic modulators. *Arterioscler. Thromb. Vasc. Biol.* 24, 12–22. doi: 10.1161/01.ATV.0000105054.43931.f0

Wang, Y., Zhao, X., Jin, H., Wei, H., Li, W., Bu, D., et al. (2009). Role of hydrogen sulfide in the development of atherosclerotic lesions in apolipoprotein E knockout mice. *Arterioscler. Thromb. Vasc. Biol.* 29, 173–179. doi: 10.1161/ATVBAHA.108.179333

Weber, G. J., Pushpakumar, S. B., and Sen, U. (2017). Hydrogen sulfide alleviates hypertensive kidney dysfunction through an epigenetic mechanism. *Am. J. Physiol. Heart Circ. Physiol.* 312, H874–H885. doi: 10.1152/ajpheart.00637.2016

Xue, H., Yuan, P., Ni, J., Li, C., Shao, D., Liu, J., et al. (2013). H_2S inhibits hyperglycemia-induced intrarenal renin-angiotensin system activation via attenuation of reactive oxygen species generation. *PLoS ONE* 8:e74366. doi: 10.1371/journal.pone.0074366

Xue, H., Zhou, S., Xiao, L., Guo, Q., Liu, S., and Wu, Y. (2015). Hydrogen sulfide improves the endothelial dysfunction in renovascular hypertensive rats. *Physiol. Res.* 64, 663–672.

Yang, G., Wu, L., Jiang, B., Yang, W., Qi, J., Cao, K., et al. (2008). H_2S as a physiologic vasorelaxant: hypertension in mice with deletion of cystathionine gamma-lyase. *Science* 322, 587–590. doi: 10.1126/science.1162667

Ye, M., Wysocki, J., Gonzalez-Pacheco, F. R., Salem, M., Evora, K., Garcia-Halpin, L., et al. (2012). Murine recombinant angiotensin-converting enzyme 2: effect on angiotensin II-dependent hypertension and distinctive angiotensin-converting enzyme 2 inhibitor characteristics on rodent and human angiotensin-converting enzyme 2. *Hypertension* 60, 730–740. doi: 10.1161/HYPERTENSIONAHA.112.198622

Zhang, H., Guo, C., Wu, D., Zhang, A., Gu, T., Wang, L., et al. (2012). Hydrogen sulfide inhibits the development of atherosclerosis with suppressing CX3CR1 and CX3CL1 expression. *PLoS ONE* 7:e41147. doi: 10.1371/journal.pone.0041147

Zhang, H., Zhi, L., Moore, P. K., and Bhatia, M. (2006). Role of hydrogen sulfide in cecal ligation and puncture-induced sepsis in the mouse. *Am. J. Physiol. Lung Cell Mol. Physiol.* 290, L1193–L1201. doi: 10.1152/ajplung.00489.2005

Zhang, X., Zhang, T., Gao, F., Li, Q., Shen, C., Li, Y., et al. (2015). Fasudil, a Rhokinase inhibitor, prevents intimamedia thickening in a partially ligated carotid artery mouse model: effects of fasudil in flowinduced vascular remodeling. *Mol. Med. Rep.* 12, 7317–7325. doi: 10.3892/mmr.2015.4409

Zhao, Z. Z., Wang, Z., Li, G. H., Wang, R., Tan, J. M., Cao, X., et al. (2011). Hydrogen sulfide inhibits macrophage-derived foam cell formation. *Exp. Biol. Med. (Maywood)* 236, 169–176. doi: 10.1258/ebm.2010.010308

Zhi, L., Ang, A. D., Zhang, H., Moore, P. K., and Bhatia, M. (2007). Hydrogen sulfide induces the synthesis of proinflammatory cytokines in human monocyte cell line U937 via the ERK-NF-kappaB pathway. *J. Leukoc. Biol.* 81, 1322–1332. doi: 10.1189/jlb.1006599

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REVIEW
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Effects of Apelin on Cardiovascular Aging

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Apelin is the endogenous ligand of APJ, the orphan G protein-coupled receptor. The apelin–APJ signal transduction pathway is widely expressed in the cardiovascular system and is an important factor in cardiovascular homeostasis. This signal transduction pathway has long been related to diseases with high morbidity in the elderly, such as atherosclerosis, coronary atherosclerotic heart disease, hypertension, calcific aortic valve disease, heart failure and atrial fibrillation. In this review, we discuss the apelin–APJ signal transduction pathway related to age-associated cardiovascular diseases.

Keywords: Apelin, cardiovascular diseases, aging, RAAS, Atherosclerosis/CAD

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INTRODUCTION

Apelin was discovered in 1998 as the endogenous ligand of APJ, the orphan G protein-coupled receptor (O'Dowd et al., 1993; Tatemoto et al., 1998). The gene for the APJ receptor has high sequence homology to the angiotensin receptor ATR (O'Dowd et al., 1993; Tatemoto et al., 1998). The preprotein of apelin is a 77-amino acid that is sequentially decomposed by an angiotensin-converting enzyme into four active peptides, i.e., apelin-13, apelin-12, apelin-17, and apelin-36 (Tatemoto et al., 1998; Habata et al., 1999; Hosoya et al., 2000; Lee et al., 2000); among these, the most potent peptide that has the primary active biological function is apelin-13 (Kawamata et al., 2001; Tatemoto et al., 2001).

Aging is one of the primary risk factors in cardiovascular diseases (CVDs) (Dai et al., 2012). Studies have found that the renin-angiotensin system was related to cardiovascular aging. The renin-angiotensin system is one of the major signaling pathways related to the progress of the chronic proinflammatory profile within aged arteries (Wang et al., 2014). Ang II increased markedly in the thickened intima of rats, nonhuman and human primates (Wang et al., 2003, 2005, 2007; Fu et al., 2009). The Ang II receptor, AT1, is upregulated in aged arterial walls (Wang et al., 2005, 2007, 2010).

Ang II was also found to be related to structural, functional, and molecular changes that were found in the hearts of aged animals (Groban et al., 2006; Dai et al., 2009). Ang II levels increased significantly with age in myocardial tissue. Inhibition of Ang II signaling by either angiotensin-converting enzyme inhibitor or angiotensin receptor type II inhibitor was found to slow the progress of age-related cardiovascular changes, providing evidence for the role of Ang II and the effect of RAAS inhibitor in cardiovascular disease in aged people (Basso et al., 2007). Angiotensin-converting enzyme inhibitor and angiotensin receptor type II inhibitor have been shown to inhibit myocardial fibrosis and fibrosis-related arrhythmias in aged mice (Stein et al., 2010).

Because the gene for the APJ receptor has high sequence homology to the angiotensin receptor ATR, many studies concerning apelin–APJ in age-related cardiovascular diseases have

been performed. Diseases that are prominent in the elderly, such as atherosclerosis, hypertension, coronary atherosclerotic heart diseases, heart failure, atrial fibrillation and calcific aortic valve disease (CAVD), have been associated with the apelin–APJ signaling system. This review will focus on the apelin–APJ signaling system related to age-associated cardiovascular diseases.

APELIN/APJ CELLULAR SIGNALING PATHWAYS IN THE CARDIOVASCULAR SYSTEM

A number of studies have indicated that the apelin–APJ system is a powerful factor in the cardiovascular system in addition to Angiotension II and ATR. In the cardiovascular system, apelin binds to the APJ receptor on endothelial cells, vascular smooth muscle cells, and cardiac myocytes. As a result, vasodilatation (Reaux et al., 2001) and cardiac inotropic effect are performed (Dai et al., 2006; Yu et al., 2014). Previous studies showed that apelin could inhibit cardiac fibrosis via the prevention of cardiac fibroblast activation and collagen production (Pchejetski et al., 2012).

ENDOTHELIAL CELLS

It was found that apelin could act as a vasodilator in the presence of NO and endothelium (Tatemoto et al., 2001). *In vitro* studies showed that apelin caused NO-dependent vasodilation in human mesenteric arteries (Jia et al., 2007). However, apelin-13 may conduct vasoconstriction and deteriorate hypertension in rats after harming the vascular endothelium (Han et al., 2013).

VASCULAR SMOOTH MUSCLE CELLS

Recent studies showed that apelin-13 could induce vascular smooth muscle cell (VSMC) proliferation by upregulating the expression of Cyclin D1 (Li et al., 2013a). Cui et al. found that apelin prominently reduces apoptosis of human VSMCs; apoptosis was induced by serum deprivation (Cui et al., 2010). Wang et al. determined that apelin promotes VSMC migration through a PI3K/Akt/FoxO3a/MMP-2 pathway (Wang et al., 2015).

CARDIOMYOCYTES

The cardiac inotropic effect of apelin has been found in recent studies. Apelin showed direct effects on the contractility of cardiomyocytes. Apelin significantly improved sarcomere shortening in normal and failing cardiomyocytes. One of the mechanisms may be an increased myofilament sensitivity to Ca(2+), because apelin enhanced the activity of the Na(+)/H(+) exchanger with consequent intracellular alkalinization (Farkasfalvi et al., 2007). Isolated left ventricular cardiomyocytes lacking either apelin or APJ show less sarcomeric shortening and a decreased velocity of contraction (Charo et al., 2009).

APELIN AND AGING-RELATED CARDIOVASCULAR DISEASES

Apelin and Atherosclerosis

The most important part in atherosclerotic progress is atherosclerotic plaque formation. Angiotensin had been proved to be an atherosclerosis inducer, so it is hypothesized that apelin is also a critical factor in the progress of atherosclerosis (Li et al., 2010). Pitkin SL et al. found that apelin was upregulated in human atherosclerotic coronary arteries and is also localized to the plaque, co-localizing with markers for macrophages and smooth muscle cells (Pitkin et al., 2010). Chun et al. (2008) found that apelin downregulated AS formation by inhibiting AngII actions in mice. However, Hashimoto et al. (2007) found that apelin can promote AS by mediating oxidative stress-related AS in vascular tissue. Although it is clear that apelin is an important factor for AS, it is still difficult to define whether apelin/APJ has a beneficial or harmful role in atherosclerosis. The contribution of apelin in the development of AS remains to be determined.

Apelin and Cardiac Atherosclerotic Diseases

Angiogenesis is one of the most important mechanisms of myocardial repair for cardiac atherosclerotic diseases, such as myocardial infarctions (MI) and ischemic heart diseases. The effect of apelin in angiogenesis in animal models of AMI and ischemic heart disease have been demonstrated with positive results (Li et al., 2007; Mao et al., 2011). It was reported that apelin decreased in patients with MI, and a lower apelin level was associated with downregulated myocardial angiogenesis (Li et al., 2010). Injection of apelin into the ischemic myocardium stimulated neovascularization in the peri-infarct area through paracrine activity (Tempel et al., 2012). Li et al. (2008) found that apelin-13 could promote myocardial angiogenesis, inhibit cardiac fibrosis, attenuate cardiac hypertrophy, and improve cardiac function at 14 days after myocardial infarction. Regarding the mechanism for apelin-13 promoting angiogenesis after myocardial infarction, studies explored that apelin could upregulate the expression of SDF-1a/CXCR-4 and the homing of vascular progenitor cells (Wang et al., 2013). To confirm the angiogenesis effect of apelin in the heart, a further study was performed in which murine bone marrow cells were pretreated by apelin and later delivered into myocardium. As a result, myocardial angiogenesis increased and cardiac fibrosis was attenuated (Kidoya et al., 2010).

Because myocardial angiogenesis plays an important role in cardiac function in cardiac atherosclerotic diseases, the positive effect of apelin indicates that it could be used as a myocardial protecting factor after myocardial infarction. Further clinical studies are needed to confirm this effect of apelin.

Apelin and Hypertension

Hypertension is highly related to endothelial dysfunction and arterial stiffness. In healthy individuals, age is an essential factor in arterial structure and function alteration (Azizi et al., 2013). Increases in arterial stiffness are mostly attributed to aging-induced endothelial dysfunction (Arnett et al., 1994; Blacher et al., 1999; Li et al., 2012, 2013b). NO plays an important role

in vasodilation (Laurent et al., 2001). Aging is associated with the impairment of arterial eNOS mRNA and protein expression, which contribute to increased arterial stiffness and elevated blood pressure (Csiszar et al., 2002; LeBlanc et al., 2008; Donato et al., 2009; Novella et al., 2013).

Apelin administration caused a powerful antihypertensive effect in normal and hypertensive animal models (Rowe, 1987; Katugampola et al., 2001; Napoli and Ignarro, 2001). Administration of apelin to patients causes NO-mediated arterial vasodilation with no significant effect on peripheral venous tonus (Japp et al., 2008; Quazi et al., 2009). This antihypertensive effect was blocked in the co-presence of NOS inhibitor, indicating that apelin leads to vasodilation through a mechanism associated with NO (Szokodi et al., 2002). The antihypertensive effect of apelin was inhibited, and at the same time, the eNOS phosphorylation in the endothelial cells was downregulated in APJ-deficient mice (Zhang et al., 2006). Therefore, reductions of NO expression may be associated with reduced plasma apelin levels in the elderly and may result in endothelial dysfunction and arterial stiffness. Moreover, the concentration-dependent vasodilatation effect of apelin was normal in endothelium-intact mammary arteries but disappeared after endothelial removal, indicating that the antihypertensive effect of apelin is endothelium-dependent (Charles et al., 2006; Maguire et al., 2009).

Future research about the effect of apelin in patients with hypertension should focus on the mechanism in addition to the NO pathway in order to find hidden side effects of apelin in patients with hypertension in further clinical studies.

Apelin and Heart Failure

Because it was demonstrated that apelin had a potent inotropic effect in myocardial cells, further *in vivo* studies were performed to find the effect of apelin in heart failure. Both myocardial and plasma apelin levels of heart failure patients decreased simultaneously, suggesting that the heart is a major source of circulating apelin; it plays an essential role in the maintenance of myocardial systolic function (Dalzell et al., 2015). Several studies focused on aged animals and humans with heart failure. Compared with control aged mice, apelin$^{-/-}$ mice have an increased risk of progressive left ventricular systolic dysfunction with age (Lee et al., 2005). Infusion of apelin-13 in aged apelin$^{-/-}$ mice could improve left ventricular systolic dysfunction (Ishida et al., 2004). In humans, plasma apelin levels decreased in advanced heart failure in most studies, but the studies that focused on the early stages of heart failure demonstrated that apelin levels remained normal or even increased in early stages (Chen et al., 2003; Kuba et al., 2007; Miettinen et al., 2007; Japp et al., 2010). A study from Pitkin SL et al. may explain this phenomenon. They found that apelin receptor APJ's density significantly decreased in the left ventricle of patients with dilated cardiomyopathy or ischemic heart disease compared with that in the left ventricle of control patients, but apelin peptide levels remained unchanged. The decrease in receptor density in heart failure may limit the positive inotropic actions of apelin, resulting in an initial compensatory mechanism by increasing apelin to improve myocardial contractility (Pitkin et al., 2010). Serum apelin levels were upregulated after cardiac resynchronization therapy together with an improvement in myocardial systolic function (Földes et al., 2003). The administration of apelin in patients with heart failure led to the improvement of cardiac output and vasodilatation (Chong et al., 2006).

Thus, apelin could be used as a factor that has both a cardiotonic and afterload lowering effect in heart failure patients. It seems that apelin has a similar effect to that of BNP in heart failure, so future clinical studies could be designed to compare these two factors, because Nesiritide's effect has been confirmed.

Apelin and Atrial Fibrillation (AF)

The expression of apelin in normal atrial myocardium of humans is extremely high (Miettinen et al., 2007). Compared with control subjects with sinus rhythm, patients with atrial fibrillation had significantly lower plasma apelin levels (Francia et al., 2007). Another study showed that if patients could remain in sinus rhythm, the circulating apelin level would rise subsequently as a result (Ellinor et al., 2006). Atrial fibrillation will lead to the loss of atrial systolic function and atrial tissue remodeling. It may be deduced that downregulation of atrial apelin synthesis is a result of increased atrial diastolic filling pressures in patients with atrial fibrillation. Moreover, it has been shown that apelin significantly changes atrial electrophysiology with a shortening of action potential duration that may be caused by its effects on multiple ionic currents (Cheng et al., 2013).

The morbidity of atrial fibrillation increases with age in humans. Based on the existing studies concerning apelin and atrial fibrillation, the level of apelin in patients with AF may reflect the systolic function of the atrium. Further studies could focus on the predictive effect of apelin in the morbidity of atrial fibrillation and the possibility of maintaining a sinus rhythm.

Apelin and Calcific Aortic Valve Disease

Aortic stenosis and calcific aortic valve disease (CAVD) are leading valvular heart diseases in the elderly (Kallergis et al., 2010). The prevalence of aortic stenosis is only approximately 0.2% in adults over 50 years of age but increases to 9.8% for adults over 80 years of age (Otto and Prendergast, 2014). In tissues of stenotic aortic valves, the expression levels of both mRNA and protein of apelin increased (Nishimura et al., 2014). The levels of apelin and its receptor APJ are upregulated in patients with calcified aortic valve stenosis (Peltonen et al., 2009). Apelin may be upregulated compensatorily in the development of aortic valve stenosis. APJ receptor antagonists might be beneficial in the treatment of aortic valve stenosis by suppressing angiogenesis, osteoblast activity and collagen synthesis (Peltonen et al., 2009).

CONCLUSION

CVDs are the most common causes of death in most countries of the world, and old age is a risk factor for CVDs. Studies have found that RAAS plays an important role in cardiac aging. As the newest member in the RAAS system, it has been shown that apelin can increase cardiac contractility, lower blood pressure, increase atherosclerotic plaque stability and ameliorate the harmful effects of AT1 receptor activation in the progression of aortic valve stenosis. Further clinical trials are necessary to study the application of apelin in the treatment of cardiac aging, hypertensive cardiomyopathy, and heart failure.

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REFERENCES

Arnett, D. K., Evans, G. W., and Riley, W. A. (1994). Arterial stiffness: a new cardiovascular risk factor? *Am. J. Epidemiol.* 140, 669–682. doi: 10.1093/oxfordjournals.aje.a117315

Azizi, Y., Faghihi, M., Imani, A., Roghani, M., and Nazari, A. (2013). Post-infarct treatment with [Pyr1]-apelin-13 reduces myocardial damage through reduction of oxidative injury and nitric oxide enhancement in the rat model of myocardial infarction. *Peptides* 46, 76–82. doi: 10.1016/j.peptides.2013.05.006

Basso, N., Cini, R., Pietrelli, A., Ferder, L., Terragno, N. A., and Inserra, F. (2007). Protective effect of long-term angiotensin II inhibition. *Am. J. Physiol. Heart Circ. Physiol.* 293, H1351–H1358. doi: 10.1152/ajpheart.00393.2007

Blacher, J., Asmar, R., Djane, S., London, G. M., and Safar, M. E. (1999). Aortic pulse wave velocity as a marker of cardiovascular risk in hypertensive patients. *Hypertension* 33, 1111–1117. doi: 10.1161/01.HYP.33.5.1111

Charles, C. J., Rademaker, M. T., and Richards, A. M. (2006). Apelin-13 induces a biphasic haemodynamic response and hormonal activation in normal conscious sheep. *J. Endocrinol.* 189, 701–710. doi: 10.1677/joe.1.06804

Charo, D. N., Ho, M., Fajardo, G., Kawana, M., Kundu, R. K., Sheikh, A. Y., et al. (2009). Endogenous regulation of cardiovascular function by apelin–APJ. *Am. J. Physiol. Heart Circ. Physiol.* 297, H1904–H1913. doi: 10.1152/ajpheart.00686.2009

Chen, M. M., Ashley, E. A., Deng, D. X., Tsalenko, A., Deng, A., Tabibiazar, R., et al. (2003). Novel role for the potent endogenous inotrope apelin in human cardiac dysfunction. *Circulation* 108, 1432–1439. doi: 10.1161/01.CIR.0000091235.94914.75

Cheng, C. C., Weerateerangkul, P., Lu, Y. Y., Chen, Y. C., Lin, Y. K., Chen, S. A., et al. (2013). Apelin regulates the electrophysiological characteristics of atrial myocytes. *Eur. J. Clin. Invest.* 43, 34–40. doi: 10.1111/eci.12012

Chong, K. S., Gardner, R. S., Morton, J. J., Ashley, E. A., and McDonagh, T. A. (2006). Plasma concentrations of the novel peptide apelin are decreased in patients with chronic heart failure. *Eur. J. Heart Fail.* 8, 355–360. doi: 10.1016/j.ejheart.2005.10.007

Chun, H. J., Ali, Z. A., Kojima, Y., Kundu, R. K., Sheikh, A. Y., Agrawal, R., et al. (2008). Apelin signaling antagonizes Ang II effects in mouse models of atherosclerosis. *J. Clin. Invest.* 118, 3343–3354. doi: 10.1172/JCI34871

Csiszar, A., Ungvari, Z., Edwards, J. G., Kaminski, P., Wolin, M. S., Koller, A., et al. (2002). Aging-induced phenotypic changes and oxidative stress impair coronary arteriolar function. *Circ. Res.* 90, 1159–1166. doi: 10.1161/01.RES.0000020401.61826.EA

Cui, R. R., Mao, D. A., Yi, L., Wang, C., Zhang, X. X., Xie, H., et al. (2010). Apelin suppresses apoptosis of human vascular smooth muscle cells via APJ/PI3-K/Akt signaling pathways. *Amino Acids* 39, 1193–1200. doi: 10.1007/s00726-010-0555-x

Dai, D. F., Chen, T., Johnson, S. C., Szeto, H., and Rabinovitch, P. S. (2012). Cardiac aging: from molecular mechanisms to significance in human health and disease. *Antioxid. Redox Signal.* 16, 1492–1526. doi: 10.1089/ars.2011.4179

Dai, D. F., Santana, L. F., Vermulst, M., Tomazela, D. M., Emond, M. J., MacCoss, M. J., et al. (2009). Overexpression of catalase targeted to mitochondria attenuates murine cardiac aging. *Circulation* 119, 2789–2797. doi: 10.1161/CIRCULATIONAHA.108.822403

Dai, T., Ramirez-Correa, G., and Gao, W. D. (2006). Apelin increases contractility in failing cardiac muscle. *Eur. J. Pharmacol.* 53, 222–228. doi: 10.1016/j.ejphar.2006.09.034

Dalzell, J. R., Rocchiccioli, J. P., Weir, R. A., Jackson, C. E., Padmanabhan, N., Gardner, R. S., et al. (2015). The emerging potential of the Apelin-APJ system in heart failure. *J. Card. Fail.* 21, 489–498. doi: 10.1016/j.cardfail.2015.03.007

Donato, A. J., Gano, L. B., Eskurza, I., Silver, A. E., Gates, P. E., Jablonski, K., et al. (2009). Vascular endothelial dysfunction with aging: endothelin-1 and endothelial nitric oxide synthase. *Am. J. Physiol. Heart Circ. Physiol.* 297, H425–H432. doi: 10.1152/ajpheart.00689.2008

Ellinor, P. T., Low, A. F., and Macrae, C. A. (2006). Reduced apelin levels in lone atrial fibrillation. *Eur. Heart J.* 27, 222e226. doi: 10.1093/eurheartj/ehi648

Farkasfalvi, K., Stagg, M. A., Coppen, S. R., Siedlecka, U., Lee, J., Soppa, G. K., et al. (2007). Direct effects of apelin on cardiomyocyte contractility and electrophysiology. *Biochem. Biophys. Res. Commun.* 357, 889–895. doi: 10.1016/j.bbrc.2007.04.017

Földes, G., Horkay, F., Szokodi, I., Vuolteenaho, O., Ilves, M., Lindstedt, K. A., et al. (2003). Circulating and cardiac levels of apelin, the novel ligand of the orphan receptor APJ, in patients with heart failure. *Biochem. Biophys. Res. Commun.* 308, 480–485. doi: 10.1016/S0006-291X(03)01424-4

Francia, P., Salvati, A., Balla, C., De Paolis, P., Pagannone, E., Borro, M., et al. (2007). Cardiac resynchronization therapy increases plasma levels of the endogenous inotrope apelin. *Eur. J. Heart Fail.* 9, 306–309. doi: 10.1016/j.ejheart.2006.06.005

Fu, Z., Wang, M., Gucek, M., Wu, J., Jiang, L., Monticone, R. E., et al. (2009). Milk fat globule protein epidermal growth factor-8: a pivotal relay element within the angiotensin II and monocyte chemoattractant protein-1 signaling cascade mediating vascular smooth muscle cells invasion. *Circ. Res.* 104, 1337–1346. doi: 10.1161/CIRCRESAHA.108.187088

Groban, L., Pailes, N. A., Bennett, C. D., Carter, C. S., Chappell, M. C., Kitzman, D. W., et al. (2006). Growth hormone replacement attenuates diastolic dysfunction and cardiac angiotensin II expression in senescent rats. *J. Gerontol. A Biol. Sci. Med. Sci.* 61, 28–35. doi: 10.1093/gerona/61.1.28

Habata, Y., Fujii, R., Hosoya, M., Fukusumi, S., Kawamata, Y., Hinuma, S., et al. (1999). Apelin, the natural ligand of the orphan receptor APJ, is abundantly secreted in the colostrum. *Biochim. Biophys. Acta* 1452, 25–35. doi: 10.1016/S0167-4889(99)00114-7

Han, X., Zhang, D. L., Yin, D. X., Zhang, Q. D., and Liu, W. H. (2013). Apelin-13 deteriorates hypertension in rats after damage of the vascular endothelium by ADMA. *Can. J. Physiol Pharmacol.* 91, 708–714. doi: 10.1139/cjpp-2013-0046

Hashimoto, T., Kihara, M., Imai, N., Yoshida, S., Shimoyamada, H., Yasuzaki, H., et al. (2007). Requirement of apelin-apelin receptor system for oxidative stress-linked atherosclerosis. *Am. J. Pathol.* 171, 1705–1712. doi: 10.2353/ajpath.2007.070471

Hosoya, M., Kawamata, Y., Fukusumi, S., Fujii, R., Habata, Y., Hinuma, S., et al. (2000). Molecular and functional characteristics of APJ. Tissue distribution of mRNA and interaction with the endogenous ligand apelin. *J. Biol. Chem.* 275, 21061–21067. doi: 10.1074/jbc.M908417199

Ishida, J., Hashimoto, T., Hashimoto, Y., Nishiwaki, S., Iguchi, T., Harada, S., et al. (2004). Regulatory roles for APJ, a seven-transmembrane receptor related to angiotensin-type 1 receptor in blood pressure *in vivo*. *J. Biol. Chem.* 279, 26274–26279. doi: 10.1074/jbc.M.404149200

Japp, A. G., Cruden, N. L., Amer, D. A., Li, V. K., Goudie, E. B., Johnston, N. R., et al. (2008). Vascular effects of apelin *in vivo* in man. *J. Am. Coll. Cardiol.* 52, 908–913. doi: 10.1016/j.jacc.2008.06.013

Japp, A. G., Cruden, N. L., Barnes, G., van Gemeren, N., Mathews, J., Adamson, J., et al. (2010). Acute cardiovascular effects of apelin in humans: potential role in patients with chronic heart failure. *Circulation* 121, 1818–1827. doi: 10.1161/CIRCULATIONAHA.109.911339

Jia, Y. X., Lu, Z. F., Zhang, J., Pan, C. S., Yang, J. H., Zhao, J., et al. (2007). Apelin activates L-arginine/nitric oxide synthase/nitric oxide pathway in rat aortas. *Peptides* 28, 2023–2029. doi: 10.1016/j.peptides.2007.07.016

Kallergis, E. M., Manios, E. G., Kanoupakis, E. M., Mavrakis, H. E., Goudis, C. A., Maliaraki, N. E., et al. (2010). Effect of sinus rhythm restoration after electrical cardioversion on apelin and brain natriuretic peptide prohormone levels in patients with persistent atrial fibrillation. *Am. J. Cardiol.* 105, 90–94. doi: 10.1016/j.amjcard.2009.08.656

Katugampola, S. D., MacGuire, J. J., Mathewson, S. R., and Davenport, A. P. (2001). [125I]-(Pyr1)Apelin-13 is a novel radioligand for localizing the APJ orphan receptor in human and rat tissues with evidence for a vasoconstrictor role in man. *Br. J. Pharmacol.* 132, 1255–1260. doi: 10.1038/sj.bjp.0703939

Kawamata, Y., Habata, Y., Fukusumi, S., Hosoya, M., Fujii, R., Hinuma, S., et al. (2001). Molecular properties of apelin, tissue distribution and receptor binding. *Biochim. Biophys. Acta* 1538, 162–171. doi: 10.1016/S0167-4889(00)00143-9

Kidoya, H., Naito, H., and Takakura, N. (2010). Apelin induces enlarged and nonleaky blood vessels for functional recovery from ischemia. *Blood* 115, 3166–3174. doi: 10.1182/blood-2009-07-232306

Kuba, K., Zhang, L., Imai, Y., Arab, S., Chen, M., Maekawa, Y., et al. (2007). Impaired heart contractility in apelin gene-deficient mice associated with aging and pressure overload. *Circ. Res.* 101, e32–e42. doi: 10.1161/CIRCRESAHA.107.158659

Laurent, S., Boutouyrie, P., Asmar, R., Gautier, I., Laloux, B., Guize, L., et al. (2001). Aortic stiffness is an independent predictor of all-cause and cardiovascular mortality in hypertensive patients. *Hypertension* 37, 1236–1241. doi: 10.1161/01.HYP.37.5.1236

LeBlanc, A. J., Shipley, R. D., Kang, L. S., and Muller-Delp, J. M. (2008). Age impairs Flk-1 signaling and NO-mediated vasodilation in coronary arterioles. *Am. J. Physiol. Heart Circ. Physiol.* 295, H2280–H2288. doi: 10.1152/ajpheart.00541.2008

Lee, D. K., Cheng, R., Nguyen, T., Fan, T., Kariyawasam, A. P., Liu, Y., et al. (2000). Characterization of apelin, the ligand for the APJ receptor. *J. Neurochem.* 74, 34–41. doi: 10.1046/j.1471-4159.2000.0740034.x

Lee, D. K., Saldivia, V. R., Nguyen, T., Cheng, R., George, S. R.,and O'Dowd, B. F. (2005). Modification of the terminal residue of apelin-13 antagonizes its hypotensive action. *Endocrinology* 146, 231–236. doi: 10.1210/en.2004-0359

Li, F., Li, L. F., Qin, X. P., Pan, W. N., Feng, F., Chen, F., et al. (2007). The study of vascular smooth muscle cells proliferation stimulated by apelin-13. *Chin. Pharmacol. Bull.* 23, 949–953.

Li, F., Li, L., Qin, X., Pan, W., Feng, F., Chen, F., et al. (2008). Apelin-induced vascular smooth muscle cell proliferation: the regulation of cyclin D1. *Front Biosci.* 13, 3786–3792. doi: 10.2741/2967

Li, L., Li, L., Xie, F., Zhang, Z., Guo, Y., Tang, G., et al. (2013a). Jagged-1/Notch3 signaling transduction pathway is involved in apelin-13-induced vascular smooth muscle cells proliferation. *Acta Biochim. Biophys. Sin.* 45, 875–881. doi: 10.1093/abbs/gmt085

Li, L., Zeng, H., and Chen, J. X. (2012). Apelin-13 increases myocardial progenitor cells and improves repair postmyocardial infarction. *Am. J. Physiol. Heart Circ. Physiol.* 303, H605–H618. doi: 10.1152/ajpheart.00366.2012

Li, L., Zeng, H., Hou, X., He, X., and Chen, J. X. (2013b). Myocardial injection of apelinoverexpressing bone marrow cells improves cardiac repair via upregulation of Sirt3 after myocardial infarction. *PLoS ONE* 8:e71041. doi: 10.1371/journal.pone.0071041

Li, X., Zhang, X., Li, F., Chen, L., Li, L., Qin, X., et al. (2010). 14-3-3 mediates apelin-13-induced enhancement of adhesion of monocytes to human umbilical vein endothelial cells. *Acta Biochim. Biophys. Sin.* 42, 403–409. doi: 10.1093/abbs/gmq036

Maguire, J. J., Kleinz, M. J., Pitkin, S. L., and Davenport, A. P. (2009). [Pyr1]apelin-13 identified as the predominant apelin isoform in the human heart: vasoactive mechanisms and inotropic action in disease. *Hypertension* 54, 598–604. doi: 10.1161/HYPERTENSIONAHA.109.134619

Mao, X., Su, T., Zhang, X., Li, F., Qin, X., Liao, D., et al. (2011). Apelin-13 promote monocytes adhesion to HUVECs via PI3K signaling. *Prog. Biochem. Biophys.* 38, 1162–1170. doi: 10.3724/SP.J.1206.2011.00335

Miettinen, K. H., Magga, J., Vuolteenaho, O., Vanninen, E. J., Punnonen, K. R., Ylitalo, K., et al. (2007). Utility of plasma apelin and other indices of cardiac dysfunction in the clinical assessment of patients with dilated cardiomyopathy. *Regul. Pept.* 140, 178–184. doi: 10.1016/j.regpep.2006.12.004

Napoli, C., and Ignarro, L. J. (2001). Nitric oxide and atherosclerosis. *Nitric Oxide* 5, 88–97. doi: 10.1006/niox.2001.0337

Nishimura, R. A., Otto, C. M., Bonow, R. O., Carabello, B. A., Erwin, J. P. III., Guyton, R. A., et al. (2014). 2014 AHA/ACC guideline for the management of patients with valvular heart disease: executive summary: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. *J. Am. Coll. Cardiol.* 6322, 2438–2488. doi: 10.1016/j.jacc.2014.02.537

Novella, S., Dantas, A. P., Segarra, G., Vidal-Gómez, X., Mompeón, A., Garabito, M., et al. (2013). Aging-related endothelial dysfunction in the aorta from female senescence-accelerated mice is associated with decreased nitric oxide synthase expression. *Exp. Gerontol.* 48, 1329–1337. doi: 10.1016/j.exger.2013.08.003

O'Dowd, B. F., Heiber, M., Chan, A., Heng, H. H., Tsui, L. C., Kennedy, J. L., et al. (1993). A human gene that shows identity with the gene encoding the angiotensin receptor is located on chromosome 11. *Gene* 136, 355–360. doi: 10.1016/0378-1119(93)90495-O

Otto, C. M., and Prendergast, B. (2014). Aortic-valve stenosis–from patients at risk to severe valve obstruction. *N. Engl. J. Med.* 371, 744–756. doi: 10.1056/NEJMra1313875

Pchejetski, D., Foussal, C., Alfarano, C., Lairez, O., Calise, D., Guilbeau-Frugier, C. et al. (2012). Apelin prevents cardiac fibroblast activation and collagen production through inhibition of sphingosine kinase 1. *Eur. Heart J.* 33, 2360–2369. doi: 10.1093/eurheartj/ehr389

Peltonen, T., Napankangas, J., Vuolteenaho, O., Ohtonen, P., Soini, Y., Juvonen, T., et al. (2009). Apelin and its receptor APJ in human aortic valve stenosis. *J. Heart Valve Dis.* 18, 644–652.

Pitkin, S. L., Maguire, J. J., Kuc, R. E., and Davenport, A. P. (2010). Modulation of the apelin/APJ system in heart failure and atherosclerosis in man. *Br. J. Pharmacol.* 160, 1785–1795. doi: 10.1111/j.1476-5381.2010.00821.x

Quazi, R., Palaniswamy, C., and Frishman, W. H. (2009). The emerging role of apelin in cardiovascular disease and health. *Cardiol. Rev.* 17, 283–286. doi: 10.1097/CRD.0b013e3181b3fe0d

Reaux, A., De Mota, N., Skultetyova, I., Lenkei, Z., El Messari, S., Gallatz, K., et al. (2001). Physiological role of a novel neuropeptide, apelin, and its receptor in the rat brain. *J. Neurochem.* 77, 1085–1096. doi: 10.1046/j.1471-4159.2001.00320.x

Rowe, J. W. (1987). Clinical consequences of age-related impairments in vascular compliance. *Am. J. Cardiol.* 60, 68G−71G. doi: 10.1016/0002-9149(87)90594-7

Stein, M., Boulaksil, M., Jansen, J. A., Herold, E., Noorman, M., Joles, J. A., et al. (2010). Reduction of fibrosisrelated arrhythmias by chronic renin-angiotensin-aldosterone system inhibitors in an aged mouse model. *Am. J. Physiol. Heart Circ. Physiol.* 299, H310–H321. doi: 10.1152/ajpheart.01137.2009

Szokodi, I., Tavi, P., Foldes, G., Voutilainen-Myllylä, S., Ilves, M., Tokola, H., et al. (2002). Apelin, the novel endogenous ligand of the orphan receptor APJ, regulates cardiac contractility. *Circ. Res.* 91, 434–440. doi: 10.1161/01.RES.0000033522.37861.69

Tatemoto, K., Hosoya, M., Habata, Y., Fujii, R., Kakegawa, T., Zou, M. X., et al. (1998). Isolation and characterization of a novel endogenous peptide ligand for the human APJ receptor. *Biochem. Biophys. Res. Commun.* 251, 471–476. doi: 10.1006/bbrc.1998.9489

Tatemoto, K., Takayama, K., Zou, M. X., Kumaki, I., Zhang, W., Kumano, K., et al. (2001). The novel peptide apelin lowers blood pressure via a nitric oxide-dependent mechanism. *Regul. Pept.* 99, 87–92. doi: 10.1016/S0167-0115(01)00236-1

Tempel, D., de Boer, M., van Deel, E. D., Haasdijk, R. A., Duncker, D. J., Cheng, C., et al. (2012). Apelin enhances cardiac neovascularization after myocardial infarction by recruiting aplnr+ circulating cells. *Circ. Res.* 111, 585–598. doi: 10.1161/CIRCRESAHA.111.262097

Wang, C., Wen, J., Zhou, Y., Li, L., Cui, X., Wang, J., et al. (2015). Apelin induces vascular smooth muscle cells migration via a PI3K/Akt/FoxO3a/MMP-2 pathway. *Int. J. Biochem. Cell Biol.* 69, 173–182. doi: 10.1016/j.biocel.2015.10.015

Wang, M., Jiang, L., Monticone, R. E., and Lakatta, E. G. (2014). Proinflammation: the key to arterial aging. *Trends Endocrinol. Metab.* 25, 72–79. doi: 10.1016/j.tem.2013.10.002

Wang, M., Takagi, G., Asai, K., Resuello, R. G., Natividad, F. F., Vatner, D. E., et al. (2003). Aging increases aortic MMP-2 activity and angiotensin II in nonhuman primates. *Hypertension* 41, 1308–1316. doi: 10.1161/01.HYP.0000073843.56046.45

Wang, M., Zhang, J., Jiang, L. Q., Spinetti, G., Pintus, G., Monticone, R., et al. (2007). Proinflammatory profile within the grossly normal aged human aortic wall. *Hypertension* 50, 219–227. doi: 10.1161/HYPERTENSIONAHA.107.089409

Wang, M., Zhang, J., Spinetti, G., Jiang, L. Q., Monticone, R., Zhao, D., et al. (2005). Angiotensin II activates matrix metalloproteinase type II and mimics age-associated carotid arterial remodeling in young rats. *Am. J. Pathol.* 167, 1429–1442. doi: 10.1016/S0002-9440(10)61229-1

Wang, M., Zhang, J., Walker, S. J., Dworakowski, R., Lakatta, E. G., and Shah, A. M. (2010). Involvement of NADPH oxidase in ageassociated cardiac remodeling. *J. Mol. Cell. Cardiol.* 48, 765–772. doi: 10.1016/j.yjmcc.2010.01.006

Wang, W., McKinnie, S. M., Patel, V. B., Haddad, G., Wang, Z., Zhabyeyev, P., et al. (2013). Loss of Apelin exacerbates myocardial infarction adverse remodeling and ischemia–reperfusion injury: therapeutic potential of synthetic Apelin analogues. *J. Am. Heart Assoc.* 2:e000249. doi: 10.1161/JAHA.113.000249

Yu, X. H., Tang, Z. B., Liu, L. J., Qian, H., Tang, S. L., Zhang, D. W., et al. (2014). Apelin and its receptor APJ in cardiovascular diseases. *Clin. Chim. Acta* 428, 1–8. doi: 10.1016/j.cca.2013.09.001

Zhang, J., Ren, C. X., Qi, Y. F., Lou, L. X., Chen, L., Zhang, L. K., et al. (2006). Exercise training promotes expression of apelin and APJ of cardiovascular tissues in spontaneously hypertensive rats. *Life Sci.* 79, 1153–1159. doi: 10.1016/j.lfs.2006.03.040

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Chronic Exercise Training Improved Aortic Endothelial and Mitochondrial Function via an AMPKα2-Dependent Manner

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Chronic exercise training is known to protect the vasculature; however, the underlying mechanisms remain obscure. The present study hypothesized that exercise may improve aortic endothelial and mitochondrial function through an adenosine monophosphate-activated protein kinase α2 (AMPKα2)-dependent manner. Ten-week-old AMPKα2 knockout (AMPKα2$^{-/-}$) mice and age-matched wild-type (WT) mice were subjected to daily treadmill running for 6 weeks, and the thoracic aorta from these mice were used for further examination. Our results showed that exercise significantly promoted vasodilatation and increased expression and phosphorylation of endothelial nitric oxide synthase (eNOS), concomitant with increased AMPKα2 expression in WT mice. These effects were not observed in AMPKα2$^{-/-}$ mice. Furthermore, exercise training increased thoracic aortic mitochondrial content as indicated by increased Complex I and mitochondrial DNA (mtDNA) in WT mice but not in AMPKα2$^{-/-}$ mice. This may be caused by decreased mitochondrial autophagy since the expression of BH3 domain-containing BCL2 family members BNIP3-like (BNIP3L) and LC3B were decreased in WT mice with exercise. And these changes were absent with AMPKα2 deletion in mice. Importantly, exercise increased the expression of manganous superoxide dismutase (MnSOD) and catalase, suggesting that mitochondrial antioxidative capacity was increased. Notably, the improved antioxidative capacity was lost in AMPKα2$^{-/-}$ mice with exercise. In conclusion, this study illustrated that AMPKα2 plays a critical role in exercise-related vascular protection via increasing endothelial and mitochondrial function in the artery.

Keywords: AMPK, exercise, vascular, endothelial function, mitochondrial function

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INTRODUCTION

It is well documented that exercise training can effectively prevent cardiovascular risk factors such as obesity, hypertension, and diabetes in the long term (Stewart, 2002). For example, it has been shown that exercise improved vascular endothelial function in hypertensive animal models (Kumral et al., 2016) and patients with coronary artery disease (Hambrecht et al., 2000). In vessel

samples from animals with exercise, the expression and Ser^{1177} phosphorylation of endothelial nitric oxide synthase (eNOS) were increased, whereas the oxidative stress was decreased. These changes lead to increased NO availability and improved vascular function (Kojda et al., 2001; Hambrecht et al., 2003; Adams et al., 2005). However, the mechanisms by which exercise exerts these beneficial effects on the vasculature are little known.

Adenosine monophosphate-activated protein kinase (AMPK) is a serine/threonine kinase consisting of α, β, and γ subunits. The β and γ regulatory subunits maintain the stability of the kinase, and the α subunit possesses catalytic activity (Steinberg and Kemp, 2009). In particular, two isoforms of AMPKα, AMPKα1, and AMPKα2 are both expressed in endothelial cells and in smooth muscle cells (Goirand et al., 2007). Furthermore, it has been shown that AMPKα2 is increased during exercise in skeletal muscle cells (Magnoni et al., 2014), and the increased AMPKα2 can regulate gene and protein expression through direct interaction with the nucleus (McGee et al., 2003; Jørgensen et al., 2006). Recent studies suggest that AMPK has a much more important role in the vasculature as it activates and phosphorylates endothelial nitric oxide synthase (eNOS) (Morrow et al., 2003), protects endothelial cells against oxidative stress (Schulz et al., 2008) and prevents vascular smooth muscle proliferation (Nagata et al., 2004). These results may suggest a protective role of AMPK in the vascular system.

Mitochondria are mobile organelles that exist in dynamic networks. To maintain a healthy population of mitochondria, the content of mitochondria is critically regulated by biogenesis, fusion-fission, and autophagy. These regulations ultimately determine the quantity, quality, and function of mitochondria, thereby contributing to cell function (Yan et al., 2012). It has been shown that maintaining mitochondrial content and functional network is crucially important for proper function of both endothelial cells and vascular smooth muscle cells (Salabei and Hill, 2013; Szewczyk et al., 2015). As expected, the abnormal content and network regulation of mitochondria lead to various cardiovascular diseases, such as diabetic vascular dysfunction (Pangare and Makino, 2012) and hypertension (Jin et al., 2011). It has been shown that exercise can increase mitochondrial content and function in skeletal muscle (Russell et al., 2014). In addition, mitochondrial antioxidant enzymes can reduce the damaging effects of reactive oxygen species (ROS, Tang et al., 2014). For example, mitochondrial manganese superoxide dismutase (MnSOD) deficiency aggravated age-dependent vascular dysfunction and increased mitochondrial oxidative stress (Wenzel et al., 2008). Over-expressing mitochondria MnSOD in mice attenuated angiotensin II (Ang-II) induced hypertension (Dikalova et al., 2010). However, whether exercise training could improve mitochondrial function in the vasculature remains unclear. In the present study, we hypothesize that chronic exercise training may improve endothelial function and mitochondrial function in aortas and that AMPKα2 may contribute these protective effects by mediating the expression of the corresponding proteins.

MATERIALS AND METHODS

Animals and Exercise Protocol

All animal treatment complied with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). All animal procedures were approved in accordance with the institutional guidelines established by the *Committee of Ethics on Animal Experiments at the Chinese Academy of Sciences*. Wild type (WT) mice were provided by the Institute of Laboratory Animal Science of Peking Union Medical College. AMPKα2-knockout ($AMPK\alpha2^{-/-}$) mice were kindly provided by Dr. Benoit Viollet (Department of Endocrinology, Metabolism and Cancer, Institute Cochin, University Paris Descartes, Paris, France) and bred in a specific pathogen-free environment. Male $AMPK\alpha2^{-/-}$ and WT mice were both with C57BL/6J genetic background. All mice were 2 months old with a mean body weight of 18 ± 2 g at the start of the experiment.

After allowing acclimatization to their housing and the treadmill, WT mice ($n = 20$), and $AMPK\alpha2^{-/-}$ mice ($n = 20$) were randomly divided into two groups: the control group and the training group, with 10 mice in each group. Mice in the training group ran on the treadmill for 90 min/day at 9.0 meters/min (0% grade), 5 days/week for 6 weeks (Fernando et al., 1993). Body weight, heart rate and systolic/diastolic blood pressure were assessed in all animals. After 12 h of the last training, mice were anesthetized of pentobarbital (5 mg/100 g) with an intraperitoneal injection and sacrificed.

Western Blot Analysis

The thoracic aortas were dissected out and immersed in liquid nitrogen immediately. Then the frozen tissues were lysed in RIPA (Radio Immunoprecipitation Assay) buffer containing 150 mM NaCl, 50 mM Tris (pH 7.4), 1% sodium deoxycholate, 1% Triton X-10, 0.1% SDS, protease inhibitor (sodium fluoride, sodium orthovanadate, leupeptin, EDTA) (Beyotime, Haimen, China). After sonication on ice for 30 min and centrifugation at 12 000 rpm for 20 min at 4°C, the supernatant was collected for Western blotting as previously described (Li et al., 2012). The primary antibodies were as follows: anti-MnSOD (ABclonal, MA, USA), anti-AMPKα2 (Abcam, Cambridge, England), anti-phospho-AMPKα1/α2 (Thr^{172}), anti-BNIP3L (BH3 domain-containing BCL2 family members BNIP3-like) (Bioworld, St. Louis, Park, USA), anti-eNOS, anti-phospho-eNOS (Ser^{1177}) (BD Biosciences, NJ, UK), anti-Complex I, anti-PGC-1α (peroxisome proliferator-activated receptor gamma coactivator 1 alpha), anti-Drp1(dynamin related protein 1), anti-Mfn1 (mitofusin 1), anti-LC3, anti-catalase, anti-GAPDH (Santa Cruz, CA, USA), anti-mTOR (mammalian target of rapamycin), anti-phospho-mTOR (Ser^{2448}) (Sigma-Aldrich, St. Louis, MO, USA). Immunoreactive bands were highlighted by electrochemiluminescence (ECL) technology and quantified by densitometry using imaging software (Image Jversion 1.46, NIH, Maryland, USA). The individual values were originally expressed as a percentage of a target protein and an internal protein standard (GAPDH) (target protein content/GAPDH content) and then expressed as a fold change of the normal

WT control group (target protein content/GAPDH content) value.

Immunofluorescence

The paraffin sections were deparaffinized by dimethylbenzene and rehydrated by graded alcohol. Antigen retrieval was processed by citric acid buffer (pH 6.0) for 5 min at 100°C. Then the slides were incubated in hydrogen peroxide for 10 min and were blocked in TBST (tris-buffered saline and tween) containing 5% Bovine Serum Albumin at room temperature for 30 min. Some sections were subsequently incubated with 300 nM MitoTracker Green (Invitrogen, CA, USA) at room temperature for 30 min. Other sections were incubated at 4°C overnight with antibodies against AMPKα2 (1:100, Abcam, Cambridge, England), fluorescent anti-rabbit secondary antibody at a 1:400 dilution for 30 min, and then nucleus dye 4,6-diamidino-2-phenylindole (DAPI) for 3 min. All images were taken by using a Zeiss Pascal LSM 710 confocal microscope (Germany). Fluorescence intensity was analyzed with Image Pro Plus in three independent samples.

Mitochondrial DNA Copy Number

Genomic DNA of the thoracic aorta tissue was extracted by using UniversalGen DNA Kit(Cwbiotech, Beijing, China). The mitochondrial (mt) copy number was analyzed by real-time PCR (ABI 7900 Real Time PCR System; Foster City, CA) as previously described (Ray Hamidie et al., 2015), through the relative value of mitochondrial and nuclear DNA (mt:nuclear DNA) which reflects the amounts of mitochondria per cell. The mitochondrial DNA (mtDNA) forward primer was CCTAGGGATAACAGCGCAAT (5′-3′) and the reverse primer was ATCGTTGAACAAACGAACCA. The nuclear DNA (nDNA) forward primer was AGAGCTCTGCGGGTACATCT and the reverse primer was CATCAGTGACGGTGCCTTAC. Q-PCR were performed in a real time PCR system: the PCR began with 95°C denaturation for 30 s followed by 40 cycles of 95°C denaturation for 5 s, and annealing and elongation for 34 s at 60°C. Samples were assayed in triplicate. Cycle threshold (CT) was used for data analysis, and CT (nDNA)—CT (mtDNA) or ΔCT was used to reflect the difference in CT values. Results were expressed as the copy number of mtDNA per cell, $2 \times 2^{-\Delta CT}$.

Thoracic Aorta Ring Assay

Mice thoracic aortas were separated, cleared of fat and connective tissues, cut into 2–3 mm rings, and fixed on isometric force transducers (Danish Myo Technology Model 610 M, Denmark) in a 5 ml organ bath, and aerated with 95% O_2 and 5% CO_2 under an initial resting tension of 2.5 mN (Zhou et al., 2014). Force was recorded in a PowerLab/8sp data acquisition system (A.D. Instruments, Castle Hill, Australia). After 1 h of incubation in oxygenated Krebs' medium (containing: KCl 4.7 mmol/L, NaCl 118 mmol/L, $CaCl_2$ 2.5 mmol/L, KH_2PO_4 1.2 mmol/L, $MgSO_4$ 1.2 mmol/L, glucose 11 mmol/L and $NaHCO_3$ 25 mmol/L) at pH 7.4 and 37°C, rings contractility was tested 3 times by high K^+ mediums (60 mM KCI) to stabilize the contraction. Cumulative response curve of phenylephrine (10^{-8} to 10^{-4} mol/L) was performed to assess the vasoconstriction response and cumulative concentration-response curves of acetylcholine (10^{-8} to 10^{-4} mol/L) were constructed with a phenylephrine pre-contraction (3 μmol/L).

Statistical Analysis

All values are reported as means ± SD. Comparison of groups involved Student's unpaired two tailed *t*-test or two-way ANOVA with the Bonferroni test for post-hoc analysis (SigmaPlot Software, San Jose, CA, USA). $P < 0.05$ was considered statistically significant.

RESULTS

The Protein Expression and Phosphorylation of AMPKα2 were Increased in Mice Aorta with Chronic Exercise

Firstly, there was no significant difference of body weight and systolic blood pressure in WT and AMPKα2$^{-/-}$ mice pre and post exercise as shown in Table S1. Table S1 also showed that heart rate was decreased in the exercise group by 17.7%, which was comparable in WT and AMPKα2$^{-/-}$ mice.

Next, we evaluated whether chronic exercise would have any effect on AMPKα2 expression. As shown in **Figure 1A**, AMPKα2 expression was dramatically increased in the aorta after chronic exercise training in immunofluorescence staining, although the overall vascular architecture had no significant difference in the four groups of mice in H&E staining (Figure S1). This was consistent with western blot results showing that exercise training induced a significant increase in aortic AMPKα2 protein expression by 31% in WT mice (**Figure 1B**). Furthermore, it was found that phosphor-AMPKα (p-AMPKα) (T172) was also significantly increased in the aorta of exercised mice by using phosphor-specific antibody against both α1 and α2 isoforms of AMPK. In AMPKα2$^{-/-}$ mice, the protein of AMPKα2 was not detectable due to gene knockout, and p-AMPKα (T172) had similar basal levels to WT mice but showed no increase in response to exercise. These results indicated that exercise induced a significant increase in the expression and phosphorylaton of AMPKα2 in the aorta.

AMPKα2 Deficiency Decreased Vasodilation and eNOS of Aorta in Exercise

We then analyzed whether exercise would have any beneficial effect on vasodilation. As shown in **Figure 2A**, the vascular relaxation to acetylcholine was decreased in aorta rings from AMPKα2$^{-/-}$ mice compared with age-matched wild type mice, indicating that AMPKα2 was involved in NO-dependent vasodilation. Importantly, the improvement of vasodilation was significantly lower in AMPKα2$^{-/-}$ mice compared with WT mice, although exercise increased the vasorelaxation ability of the aorta in both WT and AMPKα2$^{-/-}$ mice (**Figure 2A**). These results indicated that AMPKα2 played an important role in exercise-related vasorelaxation. In contrast, the vasoconstriction of aortas responding to phenylephrine was similar among the

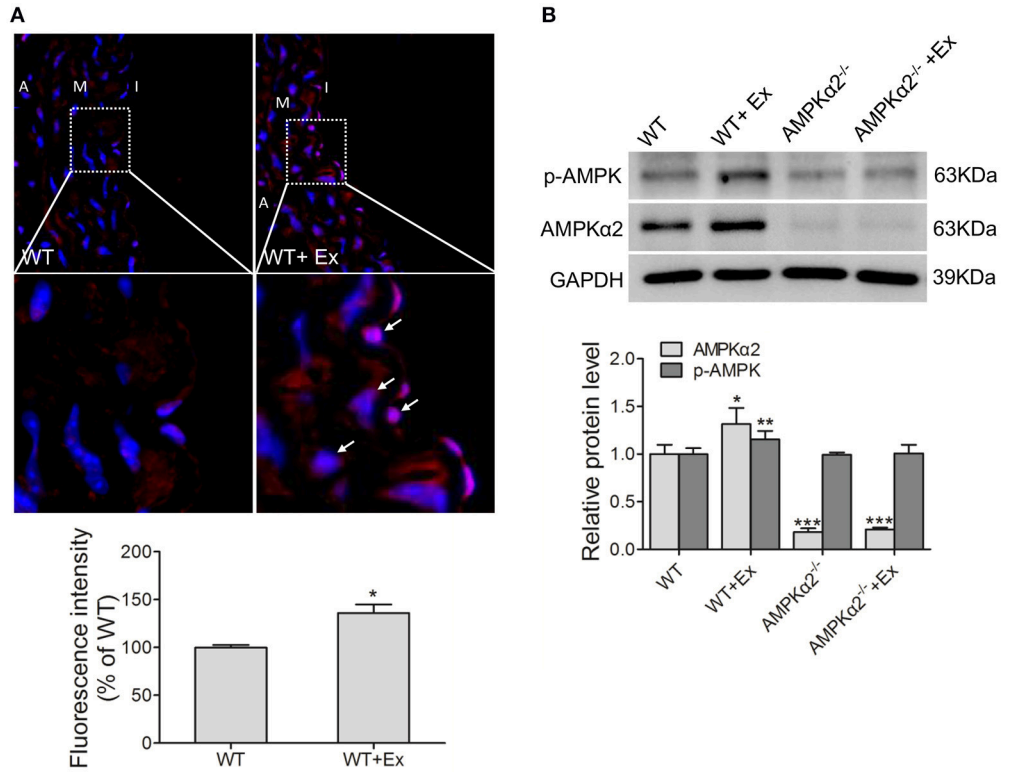

FIGURE 1 | Exercise training increased thoracic aortic AMPKα2 activity. (A) Representative immunofluorescence images and fluorescence intensity analysis showing the expression of AMPKα2 in aorta, in which fluorescence-positive cells were stained in red (arrow) and nuclei were counterstained with DAPI (blue). **(B)** Representative western blot images and summarized data showing the expression of p-AMPKα2 and AMPKα2 of aorta from WT and AMPKα2 knockout mice with or without exercise. The protein levels of p-AMPKα2 and AMPKα2 were normalized to GAPDH. WT, wild type; Ex, exercise. Values are mean ± SD ($n = 6$ in each group). $^{*}p < 0.05$; $^{**}p < 0.01$ versus WT. $^{***}p < 0.001$ vs. WT+Ex. I indicates intima; M, media; A, adventitia.

four groups (**Figure 2B**), suggesting that exercise might have no effect on vasocontraction.

We then further investigated whether the difference in vasodilation was due to changes in eNOS/p-eNOS and the possible involvement of AMPKα2. As expected, WT mice with exercise exhibited increased eNOS protein expression and phosphorylation in aorta compared with WT mice without exercise (**Figure 2C**). In contrast, AMPKα2$^{-/-}$ mice with exercise did not show any increase in total eNOS level or p-eNOS level in aorta compared with AMPKα2$^{-/-}$ mice without exercise. These results indicated that the improved vasodilation of aortas during exercise training in mice might be through an AMPKα2-dependent mechanism.

AMPKα2 Deficiency Results in Loss of Aortic Mitochondrial Content Increase with Exercise

Accumulating studies indicated that mitochondrial content plays a critical role in maintaining vascular function. We thus evaluated whether exercise would have any effect on aortic mitochondrial content and the possible involvement of AMPKα2 by assessing mitochondrial fluorescence intensity, mtDNA copy number and Complex I protein expression.

As shown in **Figure 3A**, MitoTracker Green fluorescence intensity increased significantly in WT mice with exercise compared with WT mice without exercise. Consistently, the mtDNA copy number was upregulated by 34% in WT mice during exercise (**Figure 3B**). Meanwhile, Complex I protein expression also showed an increase of 2-folds in WT exercise mice compared to control mice (**Figure 3C**). In contrast, AMPKα2$^{-/-}$ mice with exercise did not show any increase in mitochondrial fluorescence intensity, mtDNA copy number and Complex I protein compared with AMPKα2$^{-/-}$ mice without exercise (**Figure 3**). These data suggested that exercise increased aortic mitochondrial content, and this effect was dependent on the presence of AMPKα2.

Effect of AMPKα2 Deficiency on Autophagy of Aortic Mitochondria with Exercise

It has been reported that mitochondrial quantity and quality were controlled by biogenesis, fusion-fission, and autophagy. We therefore examined the effect of exercise on the expression of these relative proteins and the possible role of AMPKα2$^{-/-}$. We found that the expression of LC3B, an indicator of autophagy, and BNIP3L, a mitochondria-associated protein, were decreased in WT mice with exercise compared with WT mice without

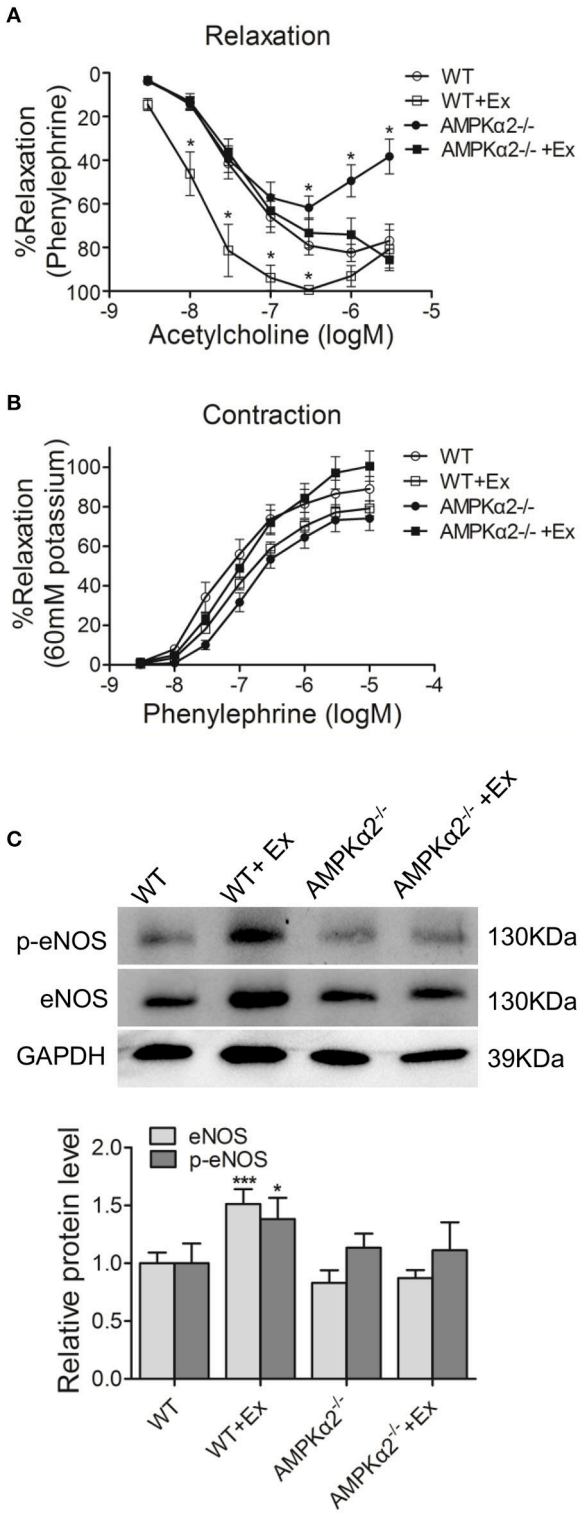

FIGURE 2 | Exercise improved vasodilation and endothelial function in an AMPKα2-dependent manner. (A) Dose-dependent vasorelaxation to acetylcholine of aorta from WT and AMPKα2 knockout mice with or without exercise. **(B)** Dose-dependent vasocontraction to phenylephrine of aorta from WT and AMPKα2 knockout mice with or without exercise. **(C)** Representative *(Continued)*

FIGURE 2 | Continued
western blot images and summarized data showing the expression of p-eNOS and eNOS of aorta from WT and AMPKα2 knockout mice with or without exercise. The protein levels of p-eNOS and eNOS were normalized to GAPDH. WT, wild type; Ex, exercise. Values are mean ± SD ($n = 6$ in each group). $^{*}p < 0.05$; $^{***}p < 0.001$ versus WT.

exercise (**Figure 4B**). Then we detected the protein expression of mTOR, the major autophagy negative regulator, and its phosphorylation at Ser2448. It was shown that WT exercise mice showed increased mTOR protein content compared to WT mice without exercise, but no significant alteration in phosphorylation activity (**Figure 4B**). In contrast, there was no significant difference in either LC3B/BNIP3L or mTOR protein levels in AMPKα2$^{-/-}$ mice with exercise compared with these knockout mice without exercise. These results indicated that decreased autophagy may be responsible for exercise-related increase of mitochondrial content, and this effect was dependent on AMPKα2.

In contrast, there was no significant difference of PGC-1α (peroxisome proliferator-activated receptor gamma coactivator 1 alpha) protein expression, the main regulator of mitochondrial biogenesis, in the WT and AMPKα2$^{-/-}$ mice with exercise compared with matched strain without exercise (Figure S2). Similarly, the protein levels of Drp1 (dynamin related protein 1) and Mfn1 (mitofusin 1), fission and fusion markers, also remained unchanged in the WT and AMPKα2$^{-/-}$ mice with exercise compared with matched stains without exercise. (Figure S2). These results indicated that mitochondrial biogenesis, fission and fusion might not be involved in exercise-related mitochondria content increase in the aorta.

AMPKα2 Deficiency Attenuates Exercise-Mediated Increase in Aortic Mitochondrial Antioxidant Capacity

Finally, we evaluated the effect of exercise on mitochondrial antioxidant capacity and the possible involvement of AMPKα2. MnSOD and catalase are both critical to mitochondrial specific antioxidant defense. **Figure 5A** shows that the expression of catalase protein was significantly increased in the WT mice by 58% following exercise exposure, but not in AMPKα2$^{-/-}$ mice. MnSOD protein content was significantly increased in WT mice after exercise intervention. Conversely, a marked decrease of MnSOD was observed in AMPKα2$^{-/-}$ mice with exercise compared with those without exercise (**Figure 5A**). These results indicated that exercise might increase mitochondrial antioxidant response in the aorta, and this effect was dependent on AMPKα2.

DISCUSSION

In the present study, we provide strong evidence that exercise promoted vasodilation, increased eNOS expression/^{S1177}-phosphorylation, and increased mitochondrial content and mitochondrial antioxidant capacity, resulting in increased vascular function. Importantly, these beneficial effects are dependent on the presence of AMPKα2 (**Figure 5B**).

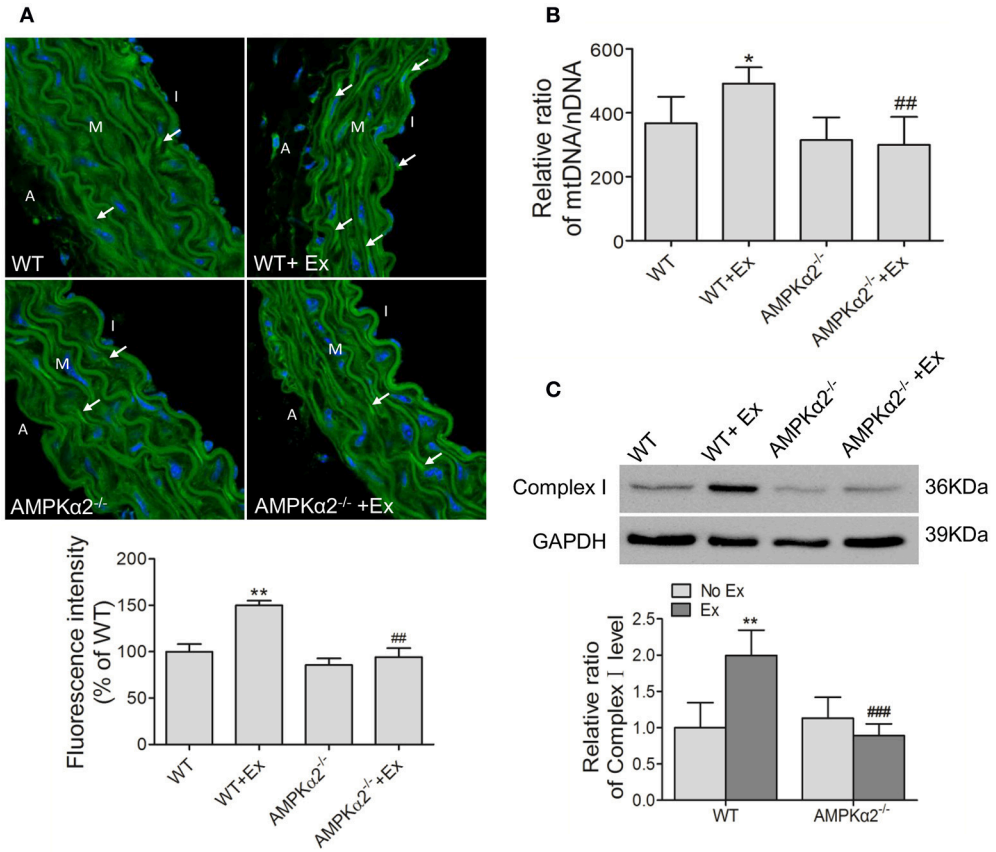

FIGURE 3 | Exercise training increased aortic mitochondrial content connected with AMPKα2. (A) Representative MitoTracker Green immunofluorescent images and fluorescence intensity analysis of aorta from WT and AMPKα2 knockout mice with or without exercise. Mitochondria were stained in green (arrow) and nuclei were counterstained with DAPI (blue). **(B)** Summarized data showing the mitochondria DNA copy number of aorta from WT and AMPKα2 knockout mice with or without exercise. **(C)** Representative western blot images and summarized data showing the expression of Complex I of aorta from WT and AMPKα2 knockout mice with or without exercise. The mitochondria DNA copy numbers were normalized to nuclear DNA, and the protein levels of Complex I were normalized to GAPDH. WT, wild type; Ex, exercise. Values are mean ± SD ($n = 6$ in each group). $^{*}p < 0.05$; $^{**}p < 0.01$ versus WT; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ versus WT+Ex. I indicates intima; M, media; A, adventitia.

Previous studies show that acute and long-term exercise training potently stimulate AMPK activity in skeletal muscles (Richter and Ruderman, 2009). Goirand et al. and Musi et al. have provided evidence that exercise training could activate cardiac and vascular AMPK in mice (Musi et al., 2005; Goirand et al., 2007). Consistent with these studies, the present study shows that exercise training significantly increased AMPKα2 activity in the vasculature. Furthermore, the present study shows that chronic exercise training increased vasodilation, eNOS expression and phosphorylation in aorta from mice, and these effects were lost in AMPKα2 knockout mice. It has been reported in a previous study that the activation of AMPKα1 was associated with increased vasodilatation and eNOS activation in mouse aorta during exercise training (Kröller-Schon et al., 2012). In this present study, we observed that AMPKα2 activation in response to exercise-related protection also occurs in the vasculature. Moreover, acute exercise activated eNOS associated with the presence of AMPK in mouse aorta (Cacicedo et al., 2011), which is consistent with the present study showing that exercise increased eNOS activity through an AMPKα2 dependent manner.

Increasing evidence demonstrated that maintaining mitochondrial content/function and stability is essential to normal vascular systems (Dromparis and Michelakis, 2013; Kröller-Schon et al., 2013), and that exercise can stimulate key stress signals that control mitochondrial content and function in skeletal muscles (Russell et al., 2014). In the current study, we find that chronic exercise training induces an adaptive increase of mitochondrial quantity in the aorta, including the increased mtDNA and Complex I protein content. Further study shows that the expression of autophagy markers LC3B and BNIP3L (Zhu et al., 2013) was decreased, and that the expression of autophagy inhibitor marker mTOR (Kim et al., 2011) was increased in exercise training. These results indicate that autophagy was decreased in exercise and that the decreased autophagy might be responsible for the increased mitochondrial content in exercise. Consistent with the present study, it has been

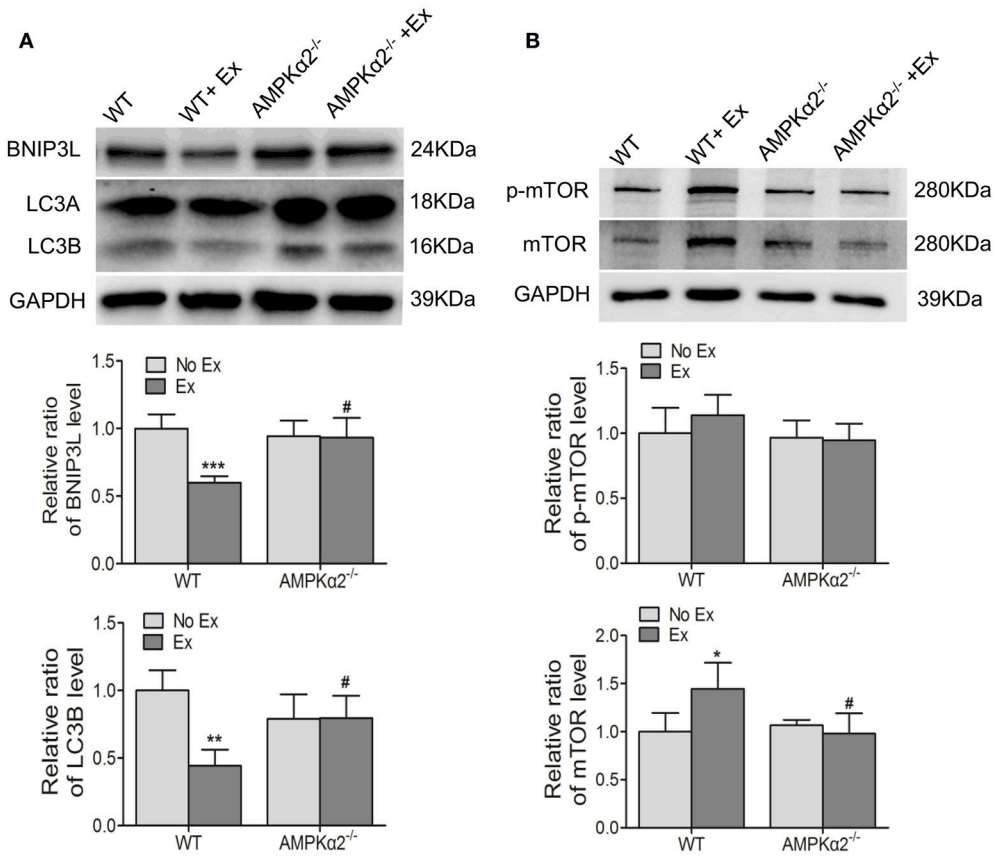

FIGURE 4 | The changes of mitochondria autophagy-related protein LC3B, BNIP3L, and mTOR during exercise training were dependent on AMPKα2. **(A)** Representative western blot images and summarized data showing the expression of LC3B and BNIP3L of aorta from WT and AMPKα2 knockout mice with or without exercise. **(B)** Representative western blot images and summarized data showing the expression of mTOR and p-mTOR of aorta from WT and AMPKα2 knockout mice with or without exercise. The protein levels of LC3B, BNIP3L, p-Mtor, and mTOR were normalized to GAPDH. WT, wild type; Ex, exercise. Values are mean ± SD ($n = 6$ in each group). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus WT. $^{\#}p < 0.05$ versus WT+Ex.

shown that autophagy is decreased in skeletal muscle cells after exercise (Kim et al., 2012). Besides autophagy, mitochondria content can also be regulated by biogenesis, fission, and fusion (Youle and Narendra, 2011; Ding et al., 2013). In our study, mitochondrial biogenesis and mitochondrial fusion and fission may have no effect on the changes in aorta with exercise training, since the expression of PGC-1α, Mfn1, and Drp1 was not changed in exercised mice compared mice without exercise. In contrast, previous studies suggest that AMPK works through PGC-1α to promote mitochondrial biogenesis in acute exercise in skeletal muscles (Kahn et al., 2005; Reznick and Shulman, 2006), and that endurance training increases Mfn1 content to induce mitochondrial fusion in rat liver (Gonçalves et al., 2016). These results suggest that mitochondrial content may be regulated through different signaling pathways in different cells in exercise training.

Furthermore, our study demonstrates that chronic exercise training upregulated the protein expression of MnSOD and catalase, and the increases are depended on the AMPKα2 isoform. Indeed, it has been reported that antioxidant enzymes were activated by exercise training in rat brains (Marosi et al., 2012; Marcelino et al., 2013), and chronic aerobic exercise training increased aortic mitochondrial antioxidant enzyme in aged rats (Gu et al., 2014). Consistent with our results, AMPKα2 has been reported to be involved in the protective effect of swimming training against isoproterenol-induced ROS production and promote the expression of antioxidant enzymes in mouse hearts (Ma et al., 2015). AMPK activity has been reported to be associated with the redox reaction in different tissues in the cardiovascular system (Ma et al., 2015). Activation of AMPK by 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) could significantly decrease ROS which was induced by palmitic acid in human aortic endothelial cells and increased expression of the antioxidant thioredoxin (Li et al., 2009). In addition, AMPKα2 suppressed NADPH oxidase expression and reduced ROS production in endothelial cells (Wang et al., 2010) as well as induced manganese SOD (Kukidome et al., 2006).

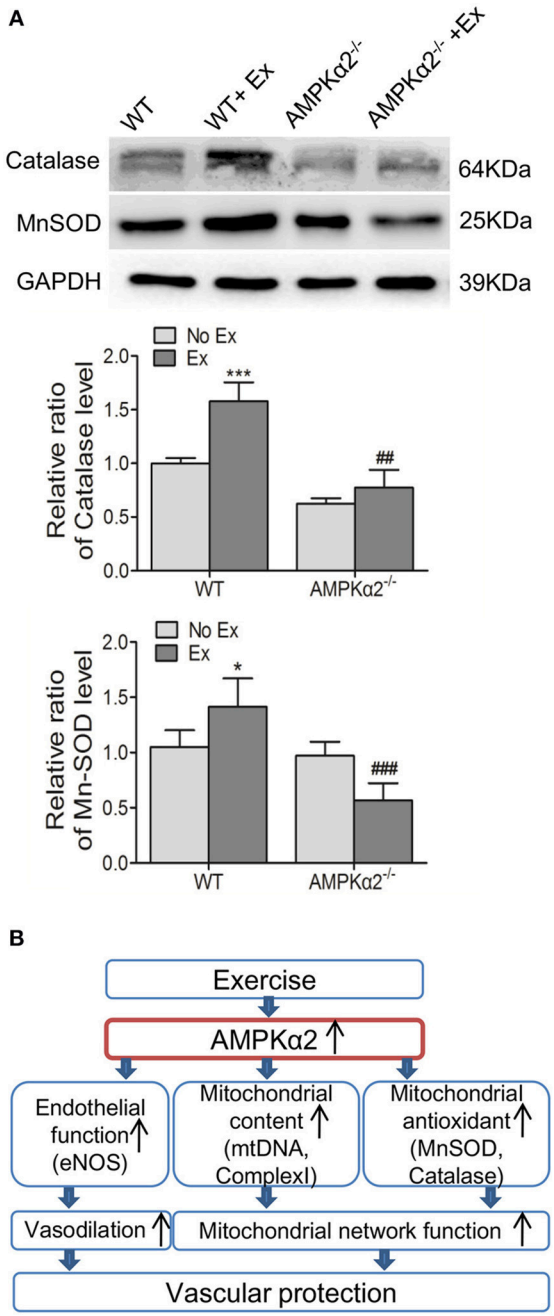

FIGURE 5 | Exercise training enhanced aortic mitochondrial MnSOD and catalase via AMPKα2. (A) Representative western blot images and summarized data showing the expression of MnSOD and catalase of aorta from WT and AMPKα2 knockout mice with or without exercise. **(B)** Scheme illustrates that AMPKα2 plays a critical role in exercise-related vascular protection via increasing endothelial and mitochondrial function in the artery. AMPKα2 is activated after chronic exercise training and in turn, mediates increased eNOS expression and activation, increased mitochondrial content (mtDNA and Complex I) and mitochondrial antioxidant capacity (MnSOD and catalase). These events contribute to the promoted vasodilation and mitochondrial network function, resulting in improved vascular function. The protein levels of MnSOD and catalase were normalized to GAPDH. WT, wild type; Ex, exercise. Values are mean ± SD ($n = 6$ in each group). $^{*}p < 0.05$; $^{***}p < 0.001$ versus WT. $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ versus WT+Ex.

Finally, our results show that exercise-related increases of eNOS expression/phosphorylation, mitochondrial content and MnSOD/catalase expression were lost in AMPKα2 knockout mice. As demonstrated in the present study, the increased vasodilation and eNOS activation during exercise were decreased in AMPKα2 deficient mice. This may be caused by the finding that AMPK is able to phosphorylate eNOS, thereby leading to eNOS activation and increased NO production (Morrow et al., 2003), and that the loss of AMPKα2 may prevent exercise-related eNOS activation. As for the increased expression of eNOS, mitochondria content and increased expression of MnSOD and catalase in aorta with exercise, the increased AMPKα2 may induce the expression of these proteins through direct interaction with the nucleus in WT mice as previously reported (McGee et al., 2003; Jørgensen et al., 2006). In AMPKα2 knockout mice, however, these corresponding proteins were not increased due to the lack of AMPKα2 to promote the expression of these proteins. Indeed, a recent report showed that AMPK signaling is required for the metabolic response to exercise *in vivo*, and AMPK activation was proposed as a regulatory mechanism that underlies exercise-induced glucose uptake in muscles, thereby leading to increased systemic insulin sensitivity (Steinberg and Jørgensen, 2007).

It has been shown that AMPK plays a protective role in diabetes and hypertension. For example, Wang et al. reported that AMPK activation was reduced in diabetic mice, and that metformin, an AMPK activator, normalized the acetylcholine-induced endothelial relaxation (Wang et al., 2009). Sun et al. found that resveratrol, a compound that activates AMPK, induced vasodilation and lowered blood pressure in DOCA-hypertensive mice(Sun et al., 2015). Metformin attenuated cytokine-induced expression of proinflammatory factors via AMPK activation in human umbilical vein endothelial cells (Hattori et al., 2006). The present finding that exercise improved aortic endothelial and mitochondrial function via AMPKα2 activation suggests that AMPKα2 may play a critical role in exercise-related improvement of vascular function in diabetes and hypertension.

In summary, our study shows that chronic exercise training mediates vascular protection through improving aortic endothelial and mitochondrial function, and that vascular AMPK isoform AMPKα2 is a key signaling molecule that mediates the protective effects of exercise in the vasculature. These findings may provide a novel mechanism in exercise-related cardiovascular protection.

AUTHOR CONTRIBUTIONS

XC designed the study, performed the experiments, collected and analyzed the data, and wrote and revised the final version of the manuscript. XA and DC contributed to data collection. WS, YZ, and PG designed the study. MY, WH, and PG critically revised the final version of the manuscript. All authors read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2016.00631/full#supplementary-material

REFERENCES

Adams, V., Linke, A., Kränkel, N., Erbs, S., Gielen, S., Möbius-Winkler, S., et al. (2005). Impact of regular physical activity on the NAD(P)H oxidase and angiotensin receptor system in patients with coronary artery disease. *Circulation* 111, 555–562. doi: 10.1161/01.CIR.0000154560.88933.7E

Cacicedo, J. M., Gauthier, M. S., Lebrasseur, N. K., Jasuja, R., Ruderman, N. B., and Ido, Y. (2011). Acute exercise activates AMPK and eNOS in the mouse aorta. *Am. J. Physiol. Heart Circ. Physiol.* 301, H1255–H1265. doi: 10.1152/ajpheart.01279.2010

Dikalova, A. E., Bikineyeva, A. T., Budzyn, K., Nazarewicz, R. R., McCann, L., Lewis, W., et al. (2010). Therapeutic targeting of mitochondrial superoxide in hypertension. *Circ. Res.* 107, U106–U221. doi: 10.1161/CIRCRESAHA.109.214601

Ding, H., Zhang, Z. Y., Zhang, J. W., and Zhang, Y. (2013). Role of mitochondrial quality control in exercise-induced health adaptation. *Zhongguo Ying Yong Sheng Li Xue Za Zhi* 29, 543–553.

Dromparis, P., and Michelakis, E. D. (2013). Mitochondria in vascular health and disease. *Annu. Rev. Physiol.* 75, 95–126. doi: 10.1146/annurev-physiol-030212-183804

Fernando, P., Bonen, A., and Hoffman-Goetz, L. (1993). Predicting submaximal oxygen consumption during treadmill running in mice. *Can. J. Physiol. Pharmacol.* 71, 854–857. doi: 10.1139/y93-128

Goirand, F., Solar, M., Athea, Y., Viollet, B., Mateo, P., Fortin, D., et al. (2007). Activation of AMP kinase α1 subunit induces aortic vasorelaxation in mice. *J. Physiol.* 581(Pt 3), 1163–1171. doi: 10.1113/jphysiol.2007.132589

Gonçalves, I. O., Passos, E., Diogo, C. V., Rocha-Rodrigues, S., Santos-Alves, E., Oliveira, P. J., et al. (2016). Exercise mitigates mitochondrial permeability transition pore and quality control mechanisms alterations in nonalcoholic steatohepatitis. *Appl. Physiol. Nutr. Metab.* 41, 298–306. doi: 10.1139/apnm-2015-0470

Gu, Q., Wang, B., Zhang, X. F., Ma, Y. P., Liu, J. D., and Wang, X. Z. (2014). Chronic aerobic exercise training attenuates aortic stiffening and endothelial dysfunction through preserving aortic mitochondrial function in aged rats. *Exp. Gerontol.* 56, 37–44. doi: 10.1016/j.exger.2014.02.014

Hambrecht, R., Adams, V., Erbs, S., Linke, A., Kränkel, N., Shu, Y., et al. (2003). Regular physical activity improves endothelial function in patients with coronary artery disease by increasing phosphorylation of endothelial nitric oxide synthase. *Circulation* 107, 3152–3158. doi: 10.1161/01.CIR.0000074229.93804.5C

Hambrecht, R., Wolf, A., Gielen, S., Linke, A., Hofer, J., Erbs, S., et al. (2000). Effect of exercise on coronary endothelial function in patients with coronary artery disease. *N. Engl. J. Med.* 342, 454–460. doi: 10.1056/NEJM200002173420702

Hattori, Y., Suzuki, K., Hattori, S., and Kasai, K. (2006). Metformin inhibits cytokine-induced nuclear factor κB activation via AMP-activated protein kinase activation in vascular endothelial cells. *Hypertension* 47, 1183–1188. doi: 10.1161/01.HYP.0000221429.94591.72

Jin, H. S., Sober, S., Hong, K. W., Org, E., Kim, B. Y., Laan, M., et al. (2011). Age-dependent association of the polymorphisms in the mitochondria-shaping gene, OPA1, with blood pressure and hypertension in Korean population. *Am. J. Hypertens.* 24, 1127–1135. doi: 10.1038/ajh.2011.131

Jørgensen, S. B., Richter, E. A., and Wojtaszewski, J. F. (2006). Role of AMPK in skeletal muscle metabolic regulation and adaptation in relation to exercise. *J. Physiol. Lond.* 574, 17–31. doi: 10.1113/jphysiol.2006.109942

Kahn, B. B., Alquier, T., Carling, D., and Hardie, D. G. (2005). AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab.* 1, 15–25. doi: 10.1016/j.cmet.2004.12.003

Kim, J., Kundu, M., Viollet, B., and Guan, K. L. (2011). AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat. Cell Biol.* 13, U132–U171. doi: 10.1038/ncb2152

Kim, Y. A., Kim, Y. S., and Song, W. (2012). Autophagic response to a single bout of moderate exercise in murine skeletal muscle. *J. Physiol. Biochem.* 68, 229–235. doi: 10.1007/s13105-011-0135-x

Kojda, G., Cheng, Y. C., Burchfield, J., and Harrison, D. G. (2001). Dysfunctional regulation of endothelial nitric oxide synthase (eNOS) expression in response to exercise in mice lacking one eNOS gene. *Circulation* 103, 2839–2844. doi: 10.1161/01.CIR.103.23.2839

Kröller-Schon, S., Jansen, T., Hauptmann, F., Schüler, A., Heeren, T., Hausding, M., et al. (2012). α1AMP-activated protein kinase mediates vascular protective effects of exercise. *Arterioscler. Thromb. Vasc. Biol.* 32, 1632–1641. doi: 10.1161/ATVBAHA.111.243980

Kröller-Schon, S., Jansen, T., Schüler, A., Oelze, M., Wenzel, P., Hausding, M., et al. (2013). Peroxisome proliferator-activated receptor gamma, coactivator 1α deletion induces angiotensin II-associated vascular dysfunction by increasing mitochondrial oxidative stress and vascular inflammation. *Arterioscler. Thromb. Vasc. Biol.* 33, 1928–1935. doi: 10.1161/ATVBAHA.113.301717

Kukidome, D., Nishikawa, T., Sonoda, K., Imoto, K., Fujisawa, K., Yano, M., et al. (2006). Activation of AMP-activated protein kinase reduces hyperglycemia-Induced mitochondrial reactive oxygen species production and promotes mitochondrial biogenesis in human umbilical vein endothelial cells. *Diabetes* 55, 120–127. doi: 10.2337/diabetes.55.01.06.db05-0943

Kumral, Z. N., Sener, G., Ozgur, S., Koc, M., Suleymanoglu, S., Hurdag, C., et al. (2016). Regular exercise alleviates renovascular hypertension-induced cardiac/endothelial dysfunction and oxidative injury in rats. *J. Physiol. Pharmacol.* 67, 45–55.

Li, X. D., Chen, J., Ruan, C. C., Zhu, D. L., and Gao, P. J. (2012). Vascular endothelial growth factor-induced osteopontin expression mediates vascular inflammation and neointima formation via Flt-1 in adventitial fibroblasts. *Arterioscler. Thromb. Vasc. Biol.* 32, 2250–2258. doi: 10.1161/ATVBAHA.112.255216

Li, X. N., Song, J., Zhang, L., LeMaire, S. A., Hou, X. Y., Zhang, C., et al. (2009). Activation of the AMPK-FOXO3 pathway reduces fatty acid-induced increase in intracellular reactive oxygen species by upregulating thioredoxin. *Diabetes* 58, 2246–2257. doi: 10.2337/db08-1512

Ma, X. W., Fu, Y. N., Xiao, H., Song, Y., Chen, R. F., Shen, J., et al. (2015). Cardiac fibrosis alleviated by exercise training is AMPK-dependent. *PLoS ONE* 10:e0129971. doi: 10.1371/journal.pone.0129971

Magnoni, L. J., Palstra, A. P., and Planas, J. V. (2014). Fueling the engine: induction of AMP-activated protein kinase in trout skeletal muscle by swimming. *J. Exp. Biol.* 217(Pt 10), 1649–1652. doi: 10.1242/jeb.099192

Marcelino, T. B., Longoni, A., Kudo, K. Y., Stone, V., Rech, A., de Assis, A. M., et al. (2013). Evidences that maternal swimming exercise improves antioxidant

defenses and induces mitochondrial biogenesis in the brain of young wistar rats. *Neuroscience* 246, 28–39. doi: 10.1016/j.neuroscience.2013.04.043

Marosi, K., Bori, Z., Hart, N., Sárga, L., Koltai, E., Radák, Z., et al. (2012). Long-term exercise treatment reduces oxidative stress in the hippocampus of aging rats. *Neuroscience* 226, 21–28. doi: 10.1016/j.neuroscience.2012.09.001

McGee, S. L., Howlett, K. F., Starkie, R. L., Cameron-Smith, D., Kemp, B. E., and Hargreaves, M. (2003). Exercise increases nuclear AMPK α(2) in human skeletal muscle. *Diabetes* 52, 926–928. doi: 10.2337/diabetes.52.4.926

Morrow, V. A., Foufelle, F., Connell, J. M., Petrie, J. R., Gould, G. W., and Salt, I. P. (2003). Direct activation of AMP-activated protein kinase stimulates nitric-oxide synthesis in human aortic endothelial cells. *J. Biol. Chem.* 278, 31629–31639. doi: 10.1074/jbc.M212831200

Musi, N., Hirshman, M. F., Arad, M., Xing, Y., Fujii, N., Pomerleau, J., et al. (2005). Functional role of AMP-activated protein kinase in the heart during exercise. *FEBS Lett.* 579, 2045–2050. doi: 10.1016/j.febslet.2005.02.052

Nagata, D., Takeda, R., Sata, M., Satonaka, H., Suzuki, E., Nagano, T., et al. (2004). AMP-activated protein kinase inhibits angiotensin II-stimulated vascular smooth muscle cell proliferation. *Circulation* 110, 444–451. doi: 10.1161/01.CIR.0000136025.96811.76

Pangare, M., and Makino, A. (2012). Mitochondrial function in vascular endothelial cell in diabetes. *J. Smooth Muscle Res.* 48, 1–26. doi: 10.1540/jsmr.48.1

Ray Hamidie, R. D., Yamada, T., Ishizawa, R., Saito, Y., and Masuda, K. (2015). Curcumin treatment enhances the effect of exercise on mitochondrial biogenesis in skeletal muscle by increasing cAMP levels. *Metab. Clin. Exp.* 64, 1334–1347. doi: 10.1016/j.metabol.2015.07.010

Reznick, R. M., and Shulman, G. I. (2006). The role of AMP-activated protein kinase in mitochondrial biogenesis. *J. Physiol.* 574(Pt 1), 33–39. doi: 10.1113/jphysiol.2006.109512

Richter, E. A., and Ruderman, N. B. (2009). AMPK and the biochemistry of exercise: implications for human health and disease. *Biochem. J.* 418, 261–275. doi: 10.1042/BJ20082055

Russell, A. P., Foletta, V. C., Snow, R. J., and Wadley, G. D. (2014). Skeletal muscle mitochondria: a major player in exercise, health and disease. *Biochim. Biophys. Acta* 1840, 1276–1284. doi: 10.1016/j.bbagen.2013.11.016

Salabei, J. K., and Hill, B. G. (2013). Mitochondrial fission induced by platelet-derived growth factor regulates vascular smooth muscle cell bioenergetics and cell proliferation. *Redox Biol.* 1, 542–551. doi: 10.1016/j.redox.2013.10.011

Schulz, E., Dopheide, J., Schuhmacher, S., Thomas, S. R., Chen, K., Daiber, A., et al. (2008). Suppression of the JNK pathway by induction of a metabolic stress response prevents vascular injury and dysfunction. *Circulation* 118, 1347–1357. doi: 10.1161/CIRCULATIONAHA.108.784289

Steinberg, G. R., and Jørgensen, S. B. (2007). The AMP-activated protein kinase: role in regulation of skeletal muscle metabolism and insulin sensitivity. *Mini Rev. Med. Chem.* 7, 519–526. doi: 10.2174/138955707780619662

Steinberg, G. R., and Kemp, B. E. (2009). AMPK in health and disease. *Physiol. Rev.* 89, 1025–1078. doi: 10.1152/physrev.00011.2008

Stewart, K. J. (2002). Exercise training and the cardiovascular consequences of type 2 diabetes and hypertension: plausible mechanisms for improving cardiovascular health. *JAMA* 288, 1622–1631. doi: 10.1001/jama.288.13.1622

Sun, G. Q., Li, Y. B., Du, B., and Meng, Y. (2015). Resveratrol via activation of AMPK lowers blood pressure in DOCA-salt hypertensive mice. *Clin. Exp. Hypertens* 37, 616–621. doi: 10.3109/10641963.2015.1036060

Szewczyk, A., Jarmuszkiewicz, W., Koziel, A., Sobieraj, I., Nobik, W., Lukasiak, A., et al. (2015). Mitochondrial mechanisms of endothelial dysfunction. *Pharmacol. Rep.* 67, 704–710. doi: 10.1016/j.pharep.2015.04.009

Tang, X., Luo, Y. X., Chen, H. Z., and Liu, D. P. (2014). Mitochondria, endothelial cell function, and vascular diseases. *Front. Physiol.* 5:175. doi: 10.3389/fphys.2014.00175

Wang, S., Xu, J., Song, P., Viollet, B., and Zou, M. H. (2009). *In vivo* activation of AMP-activated protein kinase attenuates diabetes-enhanced degradation of GTP cyclohydrolase I. *Diabetes* 58, 1893–1901. doi: 10.2337/db09-0267

Wang, S., Zhang, M., Liang, B., Xu, J., Xie, Z., Liu, C., et al. (2010). AMPKα2 deletion causes aberrant expression and activation of NAD(P)H oxidase and consequent endothelial dysfunction *in vivo*: role of 26S proteasomes. *Circ. Res.* 106, 1117–1128. doi: 10.1161/CIRCRESAHA.109.212530

Wenzel, P., Schuhmacher, S., Kienhöfer, J., Müller, J., Hortmann, M., Oelze, M., et al. (2008). Manganese superoxide dismutase and aldehyde dehydrogenase deficiency increase mitochondrial oxidative stress and aggravate age-dependent vascular dysfunction. *Cardiovasc. Res.* 80, 280–289. doi: 10.1093/cvr/cvn182

Yan, Z., Lira, V. A., and Greene, N. P. (2012). Exercise training-induced regulation of mitochondrial quality. *Exerc. Sport Sci. Rev.* 40, 159–164. doi: 10.1097/jes.0b013e3182575599

Youle, R. J., and Narendra, D. P. (2011). Mechanisms of mitophagy. *Nat. Rev. Mol. Cell Biol.* 12, 9–14. doi: 10.1038/nrm3028

Zhou, W., Hong, M., Zhang, K., Chen, D., Han, W., Shen, W., et al. (2014). Mechanisms of improved aortic stiffness by arotinolol in spontaneously hypertensive rats. *PLoS ONE* 9:e88722. doi: 10.1371/journal.pone.0088722

Zhu, Y. Y., Massen, S., Terenzio, M., Lang, V., Chen-Lindner, S., Eils, R., et al. (2013). Modulation of serines 17 and 24 in the LC3-interacting region of Bnip3 determines pro-survival mitophagy versus apoptosis. *J. Biol. Chem.* 288, 1099–1113. doi: 10.1074/jbc.M112.399345

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Greater Progression of Age-Related Aortic Stiffening in Adults with Poor Trunk Flexibility: A 5-Year Longitudinal Study

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Purpose: Having a low level of physical fitness, especially cardiorespiratory fitness, appears to accelerate age-related aortic stiffening. Whereas, some studies have reported that trunk flexibility is a component of physical fitness, it is also negatively associated with arterial stiffening independent of cardiorespiratory fitness in cross-sectional studies. However, no long-term longitudinal study has determined whether poor trunk flexibility accelerates the progression of age-related aortic stiffening. We examined trunk flexibility and aortic stiffness progression in a 5-year longitudinal study.

Methods and Results: A total of 305 apparently healthy men and women participated in this study (49.6 ± 9.5 years of age). Trunk flexibility was measured using a sit-and-reach test. Aortic stiffness was assessed using carotid-femoral pulse wave velocity (cfPWV) at baseline and after 5 years. Analysis of covariance (ANCOVA) was used to assess the association of the annual rate of cfPWV across flexibility levels (low, middle, high). There were no significant differences in baseline cfPWV among the three groups (835 ± 164, 853 ± 140, 855 ± 2.68 cm/s; $P = 0.577$). Annual ΔcfPWV was significantly higher in the low-flexibility group than in the high-flexibility group ($P = 0.009$). ANCOVA revealed an inverse relationship between flexibility level and annual ΔcfPWV (14.41 ± 2.73, 9.79 ± 2.59, 2.62 ± 2.68 cm/s/year; P for trend = 0.011). Multiple regression analysis revealed that baseline sit and reach (β = −0.12, −0.70 to −0.01) was independently correlated with ΔcfPWV following adjustment for baseline peak oxygen uptake, age, sex, body fat, heart rate, and cfPWV. The 5-year change in cfPWV was not significantly correlated with 5-year change in sit-and-reach performance ($P = 0.859$).

Conclusion: Poor trunk flexibility is associated with greater progression of age-related aortic stiffening in healthy adults. However, we failed to confirm a significant association between 5-year change in aortic stiffness and 5-year change in trunk flexibility. The association between increased age-related increase in aortic stiffness and deterioration in flexibility due to age may require observation for more than 5 years.

Keywords: arteriosclerosis, aging, fitness, trunk flexibility, primary prevention

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INTRODUCTION

Aortic stiffness, as indexed based on pulse wave velocity (PWV), increases progressively with advancing age (Lakatta, 2003) and is a major risk factor for cardiovascular disease and all-cause mortality (Vlachopoulos et al., 2010).

Having a high level of physical fitness, especially higher cardiorespiratory fitness, is associated with the suppression of age-related arterial stiffening (Vaitkevicius et al., 1993; Gando et al., 2016). Recently, we and others have reported that flexibility, a component of physical fitness (Cureton, 1941), is also negatively associated with arterial stiffening independent of cardiorespiratory fitness in cross-sectional studies (Yamamoto et al., 2009; Nishiwaki et al., 2014). It was reported that healthy children with stiffer skin and joints have higher blood pressure and higher pulse pressure levels, independent of several confounders (Uiterwaal et al., 2003). Moreover, the loss of trunk flexibility with aging accelerates at the fourth or fifth decade of life, based on 6,000 Flexitest results (Medeiros et al., 2013). The same tendency is observed for age-related arterial stiffening. However, no long-term longitudinal study has determined whether poor trunk flexibility accelerates the progression of age-related arterial stiffening.

Flexibility (body stiffness), which can be measured non-invasively, easily, quickly, and safely, may represent the phenotypic stiffness of various body parts. Flexibility is determined by connective tissue in the tendons, muscles, ligaments, and joint capsules (Alter, 2004). Increased aortic PWV is linked to structural alterations in the arterial wall, including increased connective tissue (Lakatta, 2003). Some studies have reported that benign joint hypermobility syndrome and Ehlers-Danlos syndrome, one of the most common heritable disorders of connective tissue, are characterized by joint laxity and associated with increased aortic compliance (Neil-Dwyer et al., 1983; Handler et al., 1985), distensibility (Boutouyrie et al., 2004), and lower aortic stiffness (Francois et al., 1986; Yazici et al., 2004). It is possible that poor flexibility may accelerate the progression of age-related arterial stiffening. Therefore, we hypothesized that the age-related increases in aortic stiffness were higher in individuals with low flexibility compared to those with high flexibility. The aim of this study was to investigate the association between flexibility and the progression of aortic stiffening in a longitudinal study.

METHODS

Participants

The study population is part of the Nutrition and Exercise Intervention Study (NEXIS) cohort, registered at Clinical Trials.gov, identifier: NCT00926744. For the purpose of this study, a total of 305 Japanese adults (80 men and 225 women; mean age, 49.6 ± 9.5 years; range, 27–66 years) were selected from among 1,125 participants. Participants underwent anthropometric measurements, physical fitness testing (cardiorespiratory fitness and flexibility), physical activity assessments, arterial stiffness measurement, and blood examinations (baseline measurement). They underwent the same tests (except for physical activity) at 5-year follow-up (follow-up measurement). We excluded participants with a history of stroke, cardiac disease, or chronic renal failure, and those undergoing medical treatment for hypertension, dyslipidemia, or diabetes. We also excluded current smokers. Participants in this sample had an ankle-brachial pressure index between 0.9 and 1.3 at both baseline and the follow-up visit (during the observation period). All participants gave their written informed consent before participating in the study. The study was approved by the ethical committees of the National Institutes of Biomedical Innovation, Health and Nutrition, and Okayama Health Foundation, and the study was performed in accordance with the guidelines of the Declaration of Helsinki.

Arterial Stiffness and Blood Pressure

We measured carotid and femoral PWV (cfPWV) as indicators of aortic stiffness and blood pressure with a vascular test device (form PWV/ABI; Omron Colin, Japan) as described previously (Gando et al., 2016). Waveforms were measured by applanation tonometry according to a standardized protocol. The standard deviation of the difference for interobserver reproducibility was 62 cm/s in our laboratory (Gando et al., 2010). Heart rate (HR) was simultaneously determined during the measurement of cfPWV (form PWV/ABI; Omron Colin, Japan). Recordings were made in triplicate, with participants in the supine position, and conformed strictly to American Heart Association guidelines (Pickering et al., 2005). The mean right and left brachial BPs were used for analysis.

Flexibility

We measured sit-and-reach test performance as an indicator of trunk flexibility with a trunk flexion meter (T.K.K.5112; Takeikiki, Japan) as described previously (Yamamoto et al., 2009). Participants sat on the floor with legs stretched out straight in front of the body. They put both hands on the trunk flexion meter and flexed forward slowly. The device then displayed the distance moved. The standard deviation of the difference for interobserver reproducibility was 2.3 cm in our laboratory (Yamamoto et al., 2009).

Participants were classified into low, moderate, or high trunk flexibility categories according to the distribution of sex- and age (20–29, 30–39, 40–49, 50–59, and 60–69 years) -specific sit and reach test results: lowest tertile, poor flexibility; middle tertile, mid-range flexibility; and highest tertile, high flexibility.

Cardiorespiratory Fitness

We measured peak oxygen uptake as an indicator of cardiorespiratory fitness, which was measured according to the protocol of a graded exercise load using a cycle ergometer (Ergomedic 828E Test Cycle, Monark, Sweden, or Excalibur V2.0, The Netherlands), as described previously (Gando et al., 2016).

Abbreviations: ANCOVA, analysis of covariance; ANOVA, analysis of variance; BMI, body mass index; cfPWV, carotid and femoral pulse wave velocity; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein; HR, heart rate; METs, metabolic equivalents; PWV, pulse wave velocity; SBP, systolic blood pressure.

Physical Activity

We assessed physical activity using triaxial accelerometry (Actimarker EW4800; Panasonic Electric Works, Japan), as described previously (Gando et al., 2010). Participants wore a triaxial accelerometer for 28 days with habitual physical activity. They were instructed to wear the accelerometer from the time they woke up until they went to bed. We used the data for at least 14 days (2 weeks) and obtained daily physical activity durations corresponding to 1.1 to 2.9 metabolic equivalents (METs) (light), 3.0 to 5.9 METs (moderate), or ≥6.0 METs (vigorous) (Haskell et al., 2007).

Body Composition

We assessed body composition using dual-energy X-ray absorptiometry (Hologic QDR-4500; Hologic, Waltham, MA). We measured waist circumference using a tape measure. Body mass index (BMI) was calculated as measured weight in kilograms divided by the square of measured height in meters.

Blood Samples

Blood samples were obtained after at least 10 h of overnight fasting. Fasting plasma glucose and glycated hemoglobin (HbA1c) and serum levels of total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides were measured.

Statistical Analyses

Means ± standard deviations were calculated for continuous variables. Analysis of variance (ANOVA) was used to assess the relationship of the continuous variables to categories of flexibility levels. The differences between baseline and follow-up measurements were assessed by paired *t*-test and McNemar's non-parametric test. In the ANOVA, Scheffe's method was used to identify significant differences among mean values.

Pearson's correlation coefficients were used to analyze the relationships between the 5-year changes in cfPWV and baseline values of factors known to influence vascular stiffness and 5-year changes in these variables.

Analysis of covariance (ANCOVA) models were estimated to test differences in the annual rate of cfPWV [annual ΔcfPWV: (follow-up cfPWV—baseline cfPWV)/follow-up years] across flexibility levels. The annual ΔcfPWV was entered as a dependent variable; the tertile flexibility category was entered as a fixed factor; and baseline age, weight, body fat, systolic blood pressure (SBP), HR, cfPWV, peak oxygen uptake, moderate physical activity time, vigorous physical activity time, and sex were entered as covariates for adjustment. Pairwise *post-hoc* comparisons were examined using a Bonferroni test. In ANCOVA, data were expressed as estimated marginal mean ± standard error.

A multiple regression analysis was used to determine the influences of baseline values of factors known to influence vascular stiffness and changes in these variables (annual rate of change) on the annual ΔcfPWV.

P values < 0.05 were considered statistically significant. Data were analyzed using SPSS software (IBM Japan v.20.0, Japan).

RESULTS

Table 1 shows the baseline characteristics and changes in these variables of the participants divided by flexibility levels. There were no significant differences in baseline cfPWV among the three groups ($P = 0.577$). The paired *t*-test demonstrated that cfPWV increased significantly during the follow-up period in low and middle flexibility groups. The body fat, HbA1c, SBP increased significantly during the follow-up period in all flexibility groups.

Pearson's correlation coefficients between the 5-year change in cfPWV and baseline and 5-year changes in other parameters were as follows: the 5-year change in cfPWV was correlated with baseline sit-and-reach ($r = -0.16$, $P = 0.005$); weight ($r = 0.15$, $P = 0.007$); BMI ($r = 0.15$, $P = 0.011$); waist ($r = 0.13$, $P = 0.029$); cfPWV ($r = -0.26$, $P < 0.001$); peak oxygen uptake ($r = -0.12$, $P = 0.030$); and 5-year changes in SBP ($r = 0.24$, $P < 0.001$), diastolic blood pressure (DBP) ($r = 0.24$, $P < 0.001$), and HR ($r = 0.21$, $P < 0.001$). The 5-year change in cfPWV was not significantly correlated with the 5-year-change in sit-and-reach. Moreover, univariate regression analyses were used to assess the relationships between sit-and-reach values and the 5-year changes of cfPWV in different age categories (young, middle, old). The Pearson's correlation coefficient was larger in older subjects than in young and middle-aged subjects (young, $r = -0.13$; middle-aged, $r = -0.15$; older, $r = -0.26$). These results suggest that poor trunk flexibility is associated with greater progression of age-related aortic stiffening, especially in older adults.

Figure 1 shows the crude and adjusted values of the annual ΔcfPWV across flexibility levels. ANCOVA revealed an inverse relationship between flexibility level and the annual ΔcfPWV (14.41 ± 2.73, 9.79 ± 2.59, 2.62 ± 2.68 cm/s/year; *P* for trend = 0.011). The annual ΔcfPWV was significantly higher in the low-flexibility group than in the high-flexibility group ($P = 0.009$). Moreover, we performed ANCOVA analyses by sex. In men, ANCOVA revealed an inverse relationship between flexibility level and the annual ΔcfPWV (21.86 ± 6.65, 15.40 ± 5.46, −0.94 ± 6.00 cm/s/year; *P* for trend = 0.044). In women, ANCOVA revealed an inverse relationship (not statistically significant) between flexibility level and annual ΔcfPWV (11.23 ± 3.00, 7.68 ± 2.95, 4.67 ± 3.01 cm/s/year; *P* for trend = 0.329).

As shown in **Table 2**, multiple regression analysis revealed that baseline sit-and-reach and peak oxygen uptake were independent correlates of annual ΔcfPWV (Model 1). Upon further adjustment including body fat, HR, and cfPWV (Model 2), baseline sit-and-reach and peak oxygen uptake were independent correlates of annual ΔcfPWV. Annual ΔHR was independently correlated with annual ΔcfPWV (Model 3). However, annual Δsit-and-reach, Δpeak oxygen uptake, and Δbody fat were not independent correlates of annual ΔcfPWV.

DISCUSSION

In this 5-year longitudinal study, the age-related increases in aortic stiffness were higher in the low-flexibility groups compared with those in the high-flexibility groups. Moreover,

TABLE 1 | Changes in participants' characteristics during the study period.

Variables	Low		Middle		High	
	Baseline	Follow-up	Baseline	Follow-up	Baseline	Follow-up
N (men/women)	99 (23/76)		104 (30/74)		102 (27/75)	
Follow-up year, years	5.0 ± 0.1		5.0 ± 0.1		5.0 ± 0.1	
Sit-and-reach, cm	30.2 ± 6.0	31.1 ± 7.8	40.0 ± 4.8	38.7 ± 7.7†	49.7 ± 5.5	48.7 ± 7.1†
Premenopausal Women, *n* (%)	31 (31)	19 (19)†	30 (29)	23 (22)	34 (33)	22 (22)†
Age, years	49.8 ± 9.4	54.8 ± 9.4†	49.4 ± 9.7	54.4 ± 9.7†	49.5 ± 9.5	54.6 ± 9.5†
Height, cm	159.7 ± 8.3	159.4 ± 8.3†	160.7 ± 7.5	160.4 ± 7.5†	162.6 ± 8.6*	162.2 ± 8.6*†
Weight, kg	57.3 ± 9.7	56.9 ± 9.4	58.2 ± 9.1	58.4 ± 8.9	58.1 ± 9.2	58.1 ± 9.6
BMI, kg/m^2	22.3 ± 2.8	22.3 ± 2.8	22.4 ± 2.4	22.5 ± 2.3	21.9 ± 2.5	22.0 ± 2.8
Waist circumference, cm	81.0 ± 8.3	81.1 ± 7.7	80.2 ± 7.6	81.6 ± 7.6†	79.1 ± 8.4	79.3 ± 8.5
Body fat, %	27.4 ± 6.2	28.0 ± 6.6†	25.8 ± 6.3	27.0 ± 6.4†	24.1 ± 7.0*	25.9 ± 7.4†
Glucose, mg/dL	89.3 ± 8.1	86.7 ± 9.9†	90.6 ± 13.1	88.6 ± 19.3†	89.7 ± 10.3	85.4 ± 9.9 †
HbA1c, %	5.3 ± 0.3	5.4 ± 0.3†	5.3 ± 0.5	5.5 ± 0.7†	5.3 ± 0.4	5.4 ± 0.3†
Total cholesterol, mg/dL	212 ± 38	221 ± 36 †	213 ± 33	219 ± 35	208 ± 36	217 ± 35†
HDL cholesterol, mg/dL	67 ± 20	71 ± 20 †	65 ± 17	67 ± 20	68 ± 16	71 ± 17†
Triglycerides, mg/dL	88 ± 47	103 ± 182	97 ± 71	100 ± 69	81 ± 54	77 ± 38
SBP, mmHg	117 ± 14	120 ± 16 †	115 ± 12	117 ± 13†	118 ± 13	121 ± 15†
DBP, mmHg	71 ± 11	73 ± 11 †	70 ± 9	71 ± 10†	71 ± 10	72 ± 10
HR, beats per minute	62 ± 10	61 ± 9	62 ± 11	61 ± 9	62 ± 12	60 ± 10
cfPWV, cm/s	835 ± 164	913 ± 212†	853 ± 140	895 ± 167†	855 ± 140	871 ± 156
N (men/women)	99 (23/76)	94 (23/71)	104 (30/74)	95 (28/67)	102 (27/75)	94 (26/68)
Peak oxygen uptake, mL/kg/min	29.7 ± 5.9	30.0 ± 7.2	31.8 ± 7.8	31.5 ± 7.8	33.1 ± 7.3*	34.0 ± 9.0*
N (men/women)	99 (23/76)	–	104 (30/74)	–	102 (27/75)	–
Daily time spent in physical activity						
Light, min/day	586 ± 117	–	571 ± 115	–	587 ± 106	–
Moderate, min/day	55 ± 21	–	63 ± 25	–	64 ± 4*	–
Vigorous, min/day	1.7 ± 6.3	–	2.8 ± 5.9	–	3.8 ± 7.7	–

Values are mean ± SD, or n (%).

**P < 0.05 vs. Low (assessed by 1-way analysis of variance with post-hoc multiple comparisons by Scheffe's test).*

†P < 0.05 vs. Baseline (assessed by paired t-test for continuous variables, and by McNemar's non-parametric test for premenopausal women).

BMI indicates body mass index; HbA1c, hemoglobin A1c; HDL cholesterol, high-density lipoprotein cholesterol; SBP, systolic blood pressure; DBP, diastolic blood pressure; and cfPWV, carotid-femoral pulse wave velocity.

trunk flexibility was inversely associated with the annual rate of change in cfPWV. This association was independent of other confounders. These findings suggest that poor trunk flexibility is associated with greater progression of age-related aortic stiffening in healthy adults.

The current study has several strengths that set it apart from previous studies. Previous cross-sectional studies indicated that poor trunk flexibility was associated with arterial stiffening (Yamamoto et al., 2009; Nishiwaki et al., 2014). However, to our knowledge, there have been no long-term longitudinal studies with repeated measures of cfPWV and assessment of other risk factors regarding the relationships between flexibility and the progression of aortic stiffness. Here, we determined the relationship between flexibility and the 5-year change in cfPWV. It is well-known that cardiorespiratory fitness is inversely related to arterial stiffness (Vaitkevicius et al., 1993; Gando et al., 2016). Similar to previous findings, the present study also confirmed that arterial stiffness was significantly related to cardiorespiratory fitness. More importantly, the present study demonstrated that trunk flexibility, which has been identified as a component of physical fitness (Cureton, 1941), was also inversely related with arterial stiffening, independent of cardiorespiratory fitness and other confounders. The present findings statistically support the hypothesis that flexibility is a predictor of arterial stiffness, independent of cardiorespiratory fitness and other confounders. By contrast, the 5-year change in cfPWV was not significantly correlated with the 5-year-change in sit-and-reach performance. This result was unexpected, because some studies have suggested that stretching (Nishiwaki et al., 2015) and yoga (Hunter et al., 2013) exercise interventions significantly improved sit-and-reach performance and arterial compliance and reduced arterial stiffness. Our data indicate that the 5-year change in sit-and-reach was small (mean 5-year change, −0.45 ± 5.44 cm), which may be hardly detectable. A significant correlation between the changes in cfPWV and sit-and-reach might be detectable over a longer-term follow-up period. Further research is needed to understand this association.

On the other hand, similar to a previous study (Tomiyama et al., 2010), the present study also showed that the 5-year changes in HR was independently correlated with 5-year change

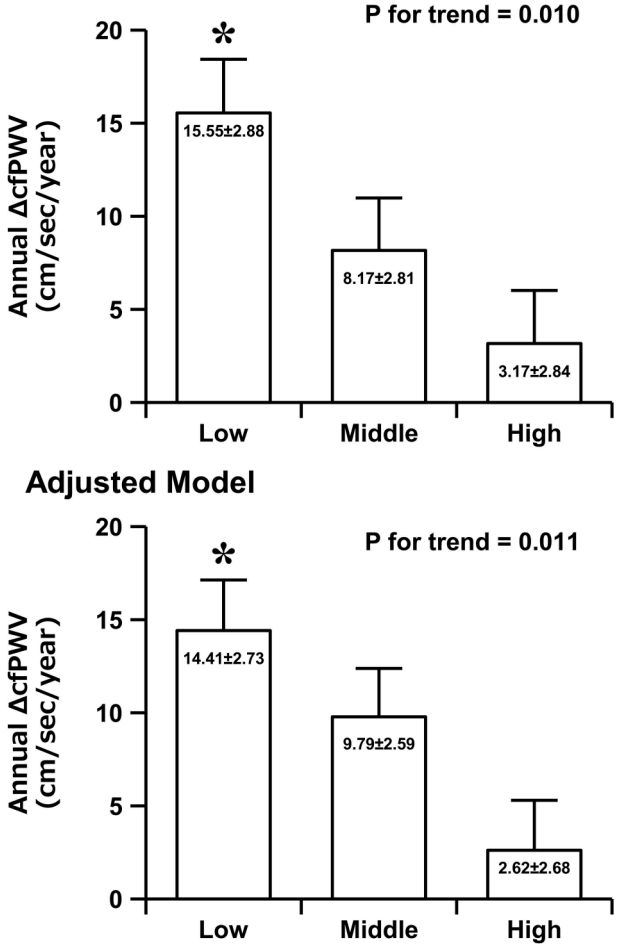

FIGURE 1 | The crude and adjusted values of the annual rate of change in carotid-femoral pulse wave velocity (ΔcfPWV) across the flexibility levels. Data (adjusted model) are adjusted for baseline age, weight, body fat, SBP, HR, cfPWV, peak oxygen uptake, moderate physical activity time, vigorous physical activity time, and sex. Data are presented as mean ± standard error.
*$P < 0.05$ vs. the high-flexibility group.

TABLE 2 | Multiple regression analysis showing the influences of baseline and changes in these variables (annual rate of change) on the annual ΔcfPWV.

	Non-standardized coefficient	95% CI		Standardized coefficient	*P*-value
ΔcfPWV	**Model 1 (R^2 changes = 0.047)**				
Sit-and-reach, cm	−0.37	−0.73	−0.01	−0.12	0.043
Peak oxygen uptake, mL/kg/min	−0.72	−1.3	−0.13	−0.18	0.016
Age, years	−0.12	−0.52	0.27	0.04	0.537
Sex, men = 0; women = 1	−6.73	−15.51	2.06	−0.10	0.133
ΔcfPWV	**Model 2 (R^2 changes = 0.151)**				
Sit-and-reach, cm	−0.36	−0.70	−0.01	−0.12	0.043
Peak oxygen uptake, mL/kg/min	−0.86	−1.52	−0.22	−0.21	0.010
Age, years	0.41	−0.02	0.84	0.13	0.063
Sex, men = 0; women = 1	−12.0	−21.42	−2.50	−0.18	0.013
Body fat, %	−0.35	−1.10	0.40	−0.08	0.361
HR, beats per minute	0.02	−0.29	0.34	0.01	0.877
cfPWV, cm/s	−0.07	−0.10	−0.05	−0.36	0.000
ΔcfPWV	**Model 3 (R^2 changes = 0.083)**				
ΔSit-and-reach, cm	−0.33	−3.96	3.31	−0.01	0.859
ΔPeak oxygen uptake, mL/kg/min	−2.14	−6.93	2.65	−0.07	0.380
ΔBody fat, %	−4.03	−10.67	2.62	−0.10	0.233
ΔHR, beats per minute	3.61	1.42	5.81	0.26	0.001

cfPWV indicates carotid-femoral pulse wave velocity; 95% CI, 95% confidence interval.

in cfPWV. Moreover, flexibility is influenced by age and environmental and genetic factors. Age is associated with both flexibility (Medeiros et al., 2013) and arterial stiffness (Lakatta, 2003). Environmental factors such as exercise habits or physical activity levels are also associated with flexibility (Harvey et al., 2002) and arterial stiffness (Gando et al., 2010). Our findings demonstrate that flexibility is associated greater progression of age-related aortic stiffening, independent of age or physical activity level. Thus, flexibility might be an independent factor of age-related arterial stiffening. Our recent study indicated that ACTN3 R577X genotype could be an independent genetic factor of trunk flexibility (Kikuchi et al., 2017). This study indicated that RR genotype was associated with significantly lower flexibility than XX. Moreover, Deschamps et al. (2015) reported that RR genotype was associated with higher systolic and diastolic blood pressure. Blood pressure is a strong determinant of arterial stiffening. Therefore, this genetic factor might be related to the present findings. However, flexibility levels were not associated with the 5-year progression of SBP (low: 3 ± 1, middle: 2 ± 1, high: 3 ± 1 mmHg, P for trend = 0.495) in the present study. Further research is necessary to determine the relationship between R577X genotype and age-related arterial stiffening. In general, there are gender differences in age-related arterial stiffening (AlGhatrif et al., 2013). We performed ANCOVA analyses by sex. Similar to previous findings (Nishiwaki et al., 2014), our results seem to indicate a stronger correlation in men. It is not clear why men show a stronger relationship between flexibility and arterial stiffness. Estrogen may has a key role in the relationship between body flexibility and arterial stiffness. However, the effect of estrogen on arterial stiffness is not consistent (Crews and Khalil, 1999; Rodriguez-Macias et al., 2002; Tatchum-Talom et al., 2002). Aortic remodeling is related to sex and women display less aortic dilation than men (Lam et al., 2010). Therefore, age-related arterial wall remodeling may be associated with our findings. Further research is necessary to determine the gender differences.

We speculate on several possible reasons for the greater progression of aortic stiffening in the low-flexibility group. First,

flexibility and arterial wall stiffness at least partly interact through connective tissue metabolism (Nichols and O'Rourke, 2005). Age-related structural alterations in the arterial wall may show a consistent link with age-related alterations in body flexibility within the same individual. Second, aortic stiffness is functionally influenced by vasomotor tone (Nichols and O'Rourke, 2005). Passive muscle stretch leads to an increase in sympathetic nerve activity via the central nervous system (Yamamoto et al., 2004). Repetitive sympathoexcitation induced by habitual stretching exercises may be related to the improvements in sympathetic control of vasomotor tone (Wong and Figueroa, 2014). These improvements in sympathetic nerve control may result in a decrease in arterial stiffness (Sugawara et al., 2009). Third, it is possible that physical inactivity also contributes to this relationship. In the present study, we found that daily time spent in moderate and vigorous physical activity was longer in the high-flexibility group than in the low-flexibility group. Previous studies have indicated that moderate-to-vigorous physical activity is associated with age-related arterial stiffening (Seals et al., 2009; Gando et al., 2010). However, the present findings revealed a statistically significant inverse relationship between trunk flexibility and aortic stiffening, independent of moderate and vigorous physical activity. Fourth, PWV is also affected by other physiologic factors, such as endothelial-mediated vasodilation function and structural alterations in the arterial wall. Further research is needed to understand the association between flexibility levels and arterial function and structure.

We believe that our findings have potentially important implications for preventive and preemptive medicine. Trunk flexibility can be non-invasively, easily, quickly, and safely evaluated over all ages and in many settings. Thus, the measurement of flexibility may be useful for the screening and prevention of age-related arterial stiffening. Additional research on the mechanisms underlying the relations between flexibility and the progression of age-related arterial stiffening will help to explain these associations. If research continues to confirm causal links between flexibility and the progression of age-related arterial stiffening, it may have a positive public health impact and aid in the detection of preclinical markers of arterial stiffening. Moreover, high flexibility can be achieved through stretching or yoga, which need not be cardiorespiratory fitness-enhancing exercise (e.g., aerobic exercise), and therefore may be a practical and achievable preventive strategy in older people. Studies have indicated that acute (Yamato et al., 2016) and chronic stretching exercise (Cortez-Cooper et al., 2008; Nishiwaki et al., 2015) and yoga (Patel and North, 1975; Patil et al., 2015) significantly increased arterial compliance and reduced arterial stiffness and blood pressure. Additional research is needed to determine whether these practices may represent a new preventive and/or treatment strategy for age-related arterial stiffening. Nonetheless, we should emphasize that cardiorespiratory fitness-enhancing exercise training is an important approach for preventing aortic stiffening. In addition, the difference in 5-year change in cfPWV data between the low- and high-flexibility groups was 62 cm/s/5 years. A previous meta-analysis (Vlachopoulos et al., 2010) suggested that an increase in aortic PWV by 100 cm/s was associated with increases of 14, 15, and 15% in cardiovascular events, cardiovascular mortality, and all-cause mortality, respectively. Therefore, our findings suggest that poor flexibility exposure may be associated with future cardiovascular health.

There are several limitations to the present study. First, the sit-and-reach test used as an indicator of flexibility is a multifactorial test, which may compromise the interpretation of information. We did not evaluate the flexibility of other body regions such as the elbow, shoulder, knee, or ankle. Further studies are required to refine our understanding of the link between flexibility and arterial stiffness. Second, arterial stiffness is influenced by the phases of the menstrual cycle. We could not control for the menstrual cycle in this study. However, the number of premenopausal women was relatively low (32%), and there were no significant differences among the three groups. Therefore, we think that this factor had a small effect on aortic stiffening. Third, there were no significant differences in baseline cfPWV among the three groups ($P = 0.577$). Because the present study included subjects with a broad age range (27–66 years), our data may be less sensitive to cfPWV compared with previous reports. Our previous study suggested that trunk flexibility was correlated with arterial stiffness in older adults, but not in young adults (Yamamoto et al., 2009). Nishiwaki et al. (2014) observed a relationship between flexibility and arterial stiffness in men and elderly women. Thus, we confirmed the flexibility-arterial stiffness relationship according to age and sex. Pearson's correlation coefficients between baseline sit-and-reach and cfPWV were, in men, $r = -0.05$ (young) and -0.32 (older) and, in women, $r = -0.02$ (young) and -0.13 (older). These results seem to show greater sensitivity to cfPWV in older men. Therefore, there might be no significant differences in baseline cfPWV among the three groups. Fourth, we carried out this study as a sub-analysis of the NEXIS, which is an ongoing prospective study. Therefore, we used an observational study design. More research is needed to determine cause-and-effect relationships between flexibility and arterial stiffness.

CONCLUSIONS

The present longitudinal study suggests that poor flexibility is associated with greater progression of age-related aortic stiffening. This association was independent of known confounders including cardiorespiratory fitness. Therefore, trunk flexibility may be an effective measure for preventing age-related aortic stiffening.

AUTHOR CONTRIBUTIONS

YG designed the work, acquired, analyzed and interpreted the data, and wrote the first draft of manuscript. HM planned, supervised the study, and acquired the data. KY conceived the idea for the study, wrote the first draft of the manuscript, and acquired and interpreted the data. RK, HO, and NM acquired and interpreted the data. SS interpreted the data. MM designed the work, interpreted the data, wrote the first draft of the article, and supervised the study. All authors read and approved the final manuscript.

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REFERENCES

AlGhatrif, M., Strait, J. B., Morrell, C. H., Canepa, M., Wright, J., Elango, P., et al. (2013). Longitudinal trajectories of arterial stiffness and the role of blood pressure: the Baltimore Longitudinal Study of Aging. *Hypertension* 62, 934–941. doi: 10.1161/HYPERTENSIONAHA.113.01445

Alter, M. J. (2004). *Science of Flexibility, 3rd Edn*. Champaign, IL: Human Kinetics.

Boutouyrie, P., Germain, D. P., Fiessinger, J. N., Laloux, B., Perdu, J., and Laurent, S. (2004). Increased carotid wall stress in vascular Ehlers-Danlos syndrome. *Circulation* 109, 1530–1535. doi: 10.1161/01.CIR.0000121741.50315.C2

Cortez-Cooper, M. Y., Anton, M. M., Devan, A. E., Neidre, D. B., Cook, J. N., and Tanaka, H. (2008). The effects of strength training on central arterial compliance in middle-aged and older adults. *Eur. J. Cardiovasc. Prev. Rehabil.* 15, 149–155. doi: 10.1097/HJR.0b013e3282f02fe2

Crews, J. K., and Khalil, R. A. (1999). Antagonistic effects of 17 beta-estradiol, progesterone, and testosterone on Ca^{2+} entry mechanisms of coronary vasoconstriction. *Arterioscler. Thromb. Vasc. Biol.* 19, 1034–1040. doi: 10.1161/01.ATV.19.4.1034

Cureton, T. K. (1941). Flexibility as an aspect of physical fitness. *Res. Quart.* 12, 381–390.

Deschamps, C. L., Connors, K. E., Klein, M. S., Johnsen, V. L., Shearer, J., Vogel, H. J., et al. (2015). The ACTN3 R577X Polymorphism is associated with cardiometabolic fitness in healthy young adults. *PLoS ONE* 10:e0130644. doi: 10.1371/journal.pone.0130644

Francois, B., De Paepe, A., Matton, M. T., and Clement, D. (1986). Pulse wave velocity recordings in a family with ecchymotic Ehlers-Danlos syndrome. *Int. Angiol.* 5, 1–5.

Gando, Y., Murakami, H., Kawakami, R., Yamamoto, K., Kawano, H., Tanaka, N., et al. (2016). Cardiorespiratory fitness suppresses age-related arterial stiffening in healthy adults: a 2-year longitudinal observational study. *J. Clin. Hypertens.*18, 292–298. doi: 10.1111/jch.12753

Gando, Y., Yamamoto, K., Murakami, H., Ohmori, Y., Kawakami, R., Sanada, K., et al. (2010). Longer time spent in light physical activity is associated with reduced arterial stiffness in older adults. *Hypertension* 56, 540–546. doi: 10.1161/HYPERTENSIONAHA.110.156331

Handler, C. E., Child, A., Light, N. D., and Dorrance, D. E. (1985). Mitral valve prolapse, aortic compliance, and skin collagen in joint hypermobility syndrome. *Br. Heart J.* 54, 501–508. doi: 10.1136/hrt.54.5.501

Harvey, L., Herbert, R., and Crosbie, J. (2002). Does stretching induce lasting increases in joint ROM? A systematic review. *Physiother. Res. Int.* 7, 1–13. doi: 10.1002/pri.236

Haskell, W. L., Lee, I. M., Pate, R. R., Powell, K. E., Blair, S. N., Franklin, B. A., et al. (2007). Physical activity and public health: updated recommendation for adults from the American College of Sports Medicine and the American Heart Association. *Circulation* 116, 1081–1093. doi: 10.1161/CIRCULATIONAHA.107.185649

Hunter, S. D., Dhindsa, M. S., Cunningham, E., Tarumi, T., Alkatan, M., Nualnim, N., et al. (2013). The effect of Bikram yoga on arterial stiffness in young and older adults. *J. Altern. Complement. Med.* 19, 930–934. doi: 10.1089/acm.2012.0709

Kikuchi, N., Zempo, H., Fuku, N., Murakami, H., Sakamaki-Sunaga, M., Okamoto, T., et al. (2017). Association between ACTN3 R577X polymorphism and trunk flexibility in 2 different cohorts. *Int. J. Sports Med.* 38, 402–406. doi: 10.1055/s-0042-118649

Lakatta, E. G. (2003). Arterial and Cardiac aging: major shareholders in cardiovascular disease enterprises: part I: aging arteries: a "set up" for vascular disease. *Circulation* 107, 139–146. doi: 10.1161/01.CIR.0000048892.83521.58

Lam, C. S., Xanthakis, V., Sullivan, L. M., Lieb, W., Aragam, J., Redfield, M. M., et al. (2010). Aortic root remodeling over the adult life course: longitudinal data from the Framingham Heart Study. *Circulation* 122, 884–890. doi: 10.1161/CIRCULATIONAHA.110.937839

Medeiros, H. B., de Araujo, D. S., and de Araujo, C. G. (2013). Age-related mobility loss is joint-specific: an analysis from 6,000 Flexitest results. *Age* 35, 2399—2407. doi: 10.1007/s11357-013-9525-z

Neil-Dwyer, G., Child, A. H., Dorrance, D. E., Pope, F. M., and Bartlett, J. (1983). Aortic compliance in patients with ruptured intracranial aneurysms. *Lancet* 1, 939–940. doi: 10.1016/S0140-6736(83)91376-4

Nichols, W., and O'Rourke, M. (2005). *McDonald's Blood Flow in Arteries: Theoretical, Experimental and Clinical Principles 5th Edn*. London: CRC Press.

Nishiwaki, M., Kurobe, K., Kiuchi, A., Nakamura, T., and Matsumoto, N. (2014). Sex differences in flexibility-arterial stiffness relationship and its application for diagnosis of arterial stiffening: a cross-sectional observational study. *PLoS ONE* 9:e113646. doi: 10.1371/journal.pone.0113646

Nishiwaki, M., Yonemura, H., Kurobe, K., and Matsumoto, N. (2015). Four weeks of regular static stretching reduces arterial stiffness in middle-aged men. *Springerplus* 4:555. doi: 10.1186/s40064-015-1337-4

Patel, C., and North, W. R. (1975). Randomised controlled trial of yoga and bio-feedback in management of hypertension. *Lancet* 2, 93–95. doi: 10.1016/S0140-6736(75)90002-1

Patil, S. G., Aithala, M. R., and Das, K. K. (2015). Effect of yoga on arterial stiffness in elderly subjects with increased pulse pressure: a randomized controlled study. *Complement. Ther. Med.* 23, 562–569. doi: 10.1016/j.ctim.2015.06.002

Pickering, T. G., Hall, J. E., Appel, L. J., Falkner, B. E., Graves, J., Hill, M. N., et al. (2005). Recommendations for blood pressure measurement in humans and experimental animals: part 1: blood pressure measurement in humans: a statement for professionals from the Subcommittee of Professional and Public Education of the American Heart Association Council on High Blood Pressure Research. *Circulation* 111, 697–716. doi: 10.1161/01.CIR.0000154900.76284.F6

Rodriguez-Macias, K. A., Naessen, T., Bostrom, A., and Bergqvist, D. (2002). Arterial stiffness is not improved in long-term use of estrogen. *Am. J. Obstet. Gynecol.* 186, 189–194. doi: 10.1067/mob.2002.119808

Seals, D. R., Walker, A. E., Pierce, G. L., and Lesniewski, L. A. (2009). Habitual exercise and vascular ageing. *J. Physiol.* 587(Pt 23), 5541–5549. doi: 10.1113/jphysiol.2009.178822

Sugawara, J., Komine, H., Hayashi, K., Yoshizawa, M., Otsuki, T., Shimojo, N., et al. (2009). Reduction in alpha-adrenergic receptor-mediated vascular tone contributes to improved arterial compliance with endurance training. *Int. J. Cardiol.* 135, 346–352. doi: 10.1016/j.ijcard.2008.04.007

Tatchum-Talom, R., Martel, C., and Marette, A. (2002). Influence of estrogen on aortic stiffness and endothelial function in female rats. *Am. J. Physiol. Heart Circ. Physiol.* 282, H491–H498. doi: 10.1152/ajpheart.00589.2001

Tomiyama, H., Hashimoto, H., Tanaka, H., Matsumoto, C., Odaira, M., Yamada, J., et al. (2010). Synergistic relationship between changes in the pulse wave velocity and changes in the heart rate in middle-aged Japanese adults: a prospective study. *J. Hypertens.* 28, 687–694. doi: 10.1097/HJH.0b013e3283369fe8

Uiterwaal, C. S., Grobbee, D. E., Sakkers, R. J., Helders, P. J., Bank, R. A., and Engelbert, R. H. (2003). A relation between blood pressure and stiffness of joints and skin. *Epidemiology* 14, 223–227. doi: 10.1097/01.EDE.0000040327.31385.9B

Vaitkevicius, P. V., Fleg, J. L., Engel, J. H., O'Connor, F. C., Wright, J. G., Lakatta, L. E., et al. (1993). Effects of age and aerobic capacity on arterial stiffness in healthy adults. *Circulation* 88(4 Pt 1), 1456–1462. doi: 10.1161/01.CIR.88.4.1456

Vlachopoulos, C., Aznaouridis, K., and Stefanadis, C. (2010). Prediction of cardiovascular events and all-cause mortality with arterial stiffness: a

systematic review and meta-analysis. *J. Am. Coll. Cardiol.* 55, 1318–1327. doi: 10.1016/j.jacc.2009.10.061

Wong, A., and Figueroa, A. (2014). Eight weeks of stretching training reduces aortic wave reflection magnitude and blood pressure in obese postmenopausal women. *J. Hum. Hypertens.* 28, 246–250. doi: 10.1038/jhh.2013.98

Yamamoto, K., Kawada, T., Kamiya, A., Takaki, H., Miyamoto, T., Sugimachi, M., et al. (2004). Muscle mechanoreflex induces the pressor response by resetting the arterial baroreflex neural arc. *Am. J. Physiol. Heart Circ. Physiol.* 286, H1382–H1388. doi: 10.1152/ajpheart.00801.2003

Yamamoto, K., Kawano, H., Gando, Y., Iemitsu, M., Murakami, H., Sanada, K., et al. (2009). Poor trunk flexibility is associated with arterial stiffening. *Am. J. Physiol. Heart Circ. Physiol.* 297, H1314–H1318. doi: 10.1152/ajpheart.00061.2009

Yamato, Y., Hasegawa, N., Sato, K., Hamaoka, T., Ogoh, S., and Iemitsu, M. (2016). Acute effect of static stretching exercise on arterial stiffness in healthy young adults. *Am. J. Phys. Med. Rehabil.* 95, 764–770. doi: 10.1097/PHM.0000000000000498

Yazici, M., Ataoglu, S., Makarc, S., Sari, I., Erbilen, E., Albayrak, S., et al. (2004). The relationship between echocardiographic features of mitral valve and elastic properties of aortic wall and Beighton hypermobility score in patients with mitral valve prolapse. *Jpn. Heart J.* 45, 447–460. doi: 10.1536/jhj.45.447

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