Aging of the Nitric Oxide System: Are We as Old as Our NO?

Aaron L. Sverdlov, MBBS, PhD, FRACP;* Doan T.M. Ngo, B.Pharm, B.Health Sci (Hons.), PhD;*
Wai P.A. Chan, MBBS, PhD, FRACP; Yuliy Y. Chirkov, PhD; John D. Horowitz, MBBS, PhD, FRACP, FAHA

Background—Impaired generation and signaling of nitric oxide (NO) contribute substantially to cardiovascular (CV) risk (CVR) associated with hypertension, hyperlipidemia, and diabetes mellitus. In our rapidly aging society, advanced age is, in itself, a consistent and independent CVR factor. Many processes involved in aging are modulated by NO. We therefore postulated that aging might be independently associated with impaired NO signaling.

Methods and Results—In a prospective cohort study of 204 subjects (mean age 63 ± 6 at study entry), we evaluated the effects of 4 years of aging on parameters of NO generation and effect, including platelet aggregability and responsiveness to NO, and plasma concentrations of the NO synthase inhibitor, asymmetric dimethylarginine (ADMA). Clinical history, lipid profile, high-sensitivity C-reactive protein, routine biochemistry, and 25-hydroxyvitamin D levels were obtained at study entry and after 4 years of follow-up. Aging was associated with marked deterioration of responsiveness of platelets to NO ($P<0.0001$) and increases in plasma ADMA concentrations ($P<0.0001$). There was a significant correlation between changes in these parameters over time ($r=0.2$; $P=0.013$). On multivariable analyses, the independent correlates of deterioration of responsiveness of platelets to NO were female gender ($\beta=0.17$; $P=0.034$) and low vitamin D concentrations ($\beta=0.16$; $P=0.04$), whereas increases in ADMA were associated with presence of diabetes ($\beta=0.16$; $P=0.03$) and impaired renal function ($\beta=0.2$; $P=0.004$).

Conclusions—Aging is associated with marked impairment of determinants of NO generation and effect, to an extent which is commensurate with adverse impact on CV outcomes. This deterioration represents a potential target for therapeutic interventions. (*J Am Heart Assoc*. 2014;3:e000973 doi: 10.1161/JAHA.114.000973)

Key Words: aging • endothelium • platelets • nitric oxide

Population aging is one of the most distinctive current demographic events. Increases in the proportions of older persons (60 years or older) are being accompanied by declines in the proportions of the young (under age 15). By 2050, the number of older persons in the world will exceed the number of young for the first time in history.[1] These trends have clear social and economic implications, including healthcare costs. Furthermore, advanced age itself has long been an independent risk factor for a wide variety of disease states.

Epidemiological studies have delineated the status of lipid levels, systolic hypertension (HTN), diabetes, sedentary lifestyle, smoking, obesity, and genetic factors as components of risk for coronary disease, congestive heart failure, and stroke within our society.[2] However, advanced age represents a substantial and independent basis for incremental risk—not only does clinically overt cardiovascular (CV) disease (CVD) increase dramatically with aging, but so do subclinical or occult diseases, such as silent coronary atherosclerosis[3] and aortic sclerosis.[4]

Among age-associated changes are increased intimal thickening and arterial stiffness, aberrations of vascular tone, left ventricular (LV) hypertrophy, reduced threshold for cell calcium overload, reduced CV reserve, reduced heart rate variability, and reduced myocardial contractility.[5,6] While precise mechanisms underlying these changes are not fully elucidated, many of these pathophysiological processes are modulated by the nitric oxide (NO) system, especially arterial stiffness, vascular tone, platelet function, myocardial hypertrophy, and contractility.[7–10]

The NO-signaling system has a number of potential points of vulnerability—biochemical and cellular processes, which may result in impairment of the entire NO cascade, as illustrated in Figure 1. This cascade can be assessed readily in
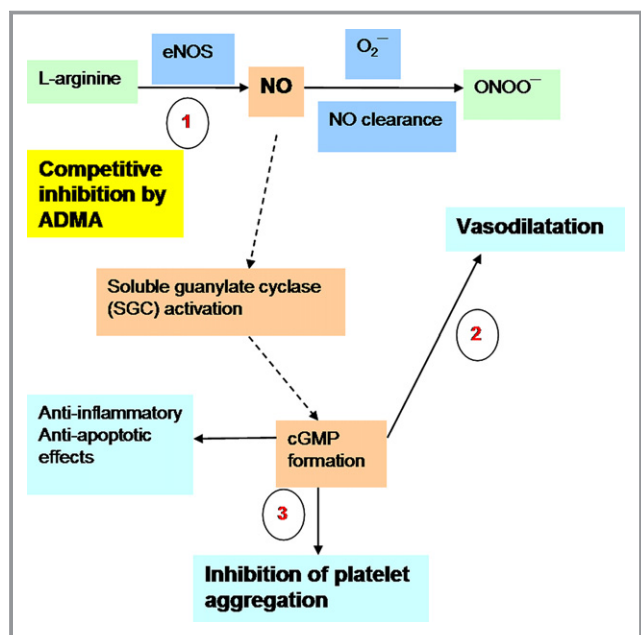
Figure 1. Interactions between NOS, activation of soluble guanylate cyclase (sGC) and physiological actions of NO. Sites evaluated in this study: (1) ADMA concentrations; (2) NO–mediated effect on arterial stiffness; and (3) inhibition of platelet aggregation by NO. ADMA indicates asymmetric dimethylarginine; NOS, nitric oxide synthase.

humans. Plasma concentrations of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase (NOS), reflect modulation of NO production and represent an established marker of cardiovascular risk (CVR).[11] ADMA concentrations vary inversely with those of vitamin D, and directly with those of high-sensitivity C-reactive protein (hs-CRP),[12] indicating complex interactions with propensity to CVR. Furthermore, platelet NO responsiveness, another independent correlate of CVR,[13] can be evaluated by NO-dependant inhibition of platelet aggregation.

Although ADMA levels are thought to increase in older individuals,[14] this has not been extensively studied. In a cross-sectional all-female study, we have found that ADMA levels, platelet NO responsiveness, and augmentation index correlate with age.[15] However, longitudinal effect of aging per se upon vascular and platelet NO responsiveness has not been reported on. Given the critical role of the NO system in cardiac and vascular function, we have now sought to establish the impact of aging on the platelet and vascular NO system in an aging, but otherwise unselected, population.

Methods

Patient Selection

The study was conducted on a cohort of 253 subjects selected randomly from the North Western Adelaide Health

Study population.[16] Criteria for selection were (1) age over 55 years and (2) absence of known terminal illness. Subjects were evaluated regarding NO physiology at baseline and after an interval of 4 years. The study was approved by the Ethics of Human Research Committee of The Queen Elizabeth Hospital (Adelaide, Australia).

Patient Data

All patients' CVR factors were delineated at interview. HTN was defined on the basis of treatment with antihypertensive drugs or blood pressure greater than 140/80 mm Hg. Hypercholesterolemia was defined by current treatment with cholesterol-lowering drugs or total cholesterol greater than 5.5 mmol/L. Diabetes mellitus (DM) was defined as current treatment for diabetes or fasting blood glucose greater than 7.8 mmol/L. Known coronary artery disease (CAD) was defined on the basis of patient history of coronary revascularization, history of myocardial infarction, or known significant coronary disease from previous angiogram, if available. Framingham risk scores were estimated for each subject using an online calculator at http://cvd-risk.nhlbi.nih.gov.

Biochemical and Physiological Assessment

These can be summarized as follows.

Physiological measures

1. Extent of adenosine diphosphate (ADP)–induced platelet aggregation and platelet responsiveness to the inhibitory effect of NO donor sodium nitroprusside (SNP) were assessed using whole-blood aggregometry, with a dual-channel impedence aggregometer (model 560; Chrono-Log, Havertown, PA), as previously described.[17]

2. Resting augmentation index (AIx), a measure of arterial stiffness, was determined by applanation tonometry using a commercially available SphygmoCor system (AtCor Medical, Sydney, Australia), as previously described.[18–20] Briefly, all subjects were asked to lie down in a quiet room for 15 minutes before the procedure. Pulse wave analysis was computed from the radial artery at the wrist and recorded by applanation tonometry using a high-fidelity micromanometer. Three recordings of 10 sequential waveforms were acquired for each subject; a validated, generalized transfer function was used to generate the corresponding central aortic pressure waveform, from which AIx was derived. Only high-quality recordings with in-device quality index ≥90% were used. All augmentation indices

were corrected for a standard heart rate of 75 beats per minute.

Biochemical measures

1. Plasma concentrations of ADMA were determined by high-performance liquid chromatography with the derivatization reagent, AccQ-Fluor (Waters, Milford, MA), after solid-phase extraction, as previously described.[21]
2. hs-CRP concentrations, lipid profile, creatinine, serum calcium levels, and 1,25 dihydroxy-cholecalciferol (vitamin D levels) were measured by a ^{125}I-radioimmunoassay (Immunodiagnostic Systems Ltd, Bolden, UK). C-terminal telopeptide of collagen type 1 and N-terminal peptide of procollagen I (P1NP) concentrations were measured as markers of collagen homeostasis.
3. Creatinine clearance (CrCl) was calculated according to the Cockcroft-Gault equation and indexed for body surface area with the Dubois and Dubois formula.

Statistical Analyses

All data are expressed as mean±SD, unless otherwise stated. Normal distribution was tested for all continuous variables, and skewed data were normalized either by log or square root transformation. Comparisons between groups for normally distributed data were performed with nonpaired t tests and, and comparisons for nonparametric data were made with Mann-Whitney's test. Comparisons between baseline and end-of-study (4-year follow-up) parameters were made with paired t tests or Wilcoxon's matched-pairs signed-rank test for nonparametric data. Correlations between continuous transformed nonparametric data were made with linear regression.

Baseline determinants of platelet ADP-induced aggregation and NO responsiveness, plasma ADMA concentrations, and AIx were evaluated utilizing univariate, and then multivariable, analyses. Variables selected for all multivariable backward regression analyses were on the basis of univariate significance ($P<0.2$).

Changes in platelet ADP-induced aggregation, NO responsiveness, and plasma ADMA concentrations with time were used as dependent variables for the purpose of further linear regression analyses. Variables selected for all multivariable backward regression analyses were on the basis of univariate significance ($P<0.2$).

In uni- and multivariable analyses, β refers to standardized regression coefficients, whereas B and SE represent unstandardized regression coefficients and standard error, respectively. All analyses were performed with SPSS software (version 17; SPSS, Inc., Chicago, IL), and $P<0.05$ was considered to be statistically significant.

Results

Patient Characteristics

A total of 253 subjects were initially recruited and underwent baseline evaluation. Nineteen percent of subjects did not undergo follow-up evaluation—12 were lost to follow-up, 7 were deceased (5 resulting from cancers, and for the remaining 2, the cause of death could not be ascertained), 6 had developed terminal illness or were receiving chemotherapy, and 24 declined to participate in follow-up, citing personal reasons. Table 1 summarizes baseline and end-of-study patient characteristics of the 204 subjects that completed the study. Over the study period, there were significant increases in the proportion of subjects diagnosed with HTN, dyslipidemia, and diabetes, as well as a decrease in that of active smokers. More people were treated with angiotensin-converting enzyme inhibitors/angiotensin II receptor antagonists (ACEIs/ARBs) by the end of the study.

Table 2 summarizes biochemical data at baseline and follow-up. This cohort had well-preserved renal function—no subject had CrCl less than 30 mL/min. An apparent increase in mean CrCl probably resulted from a change in methodology of creatinine assay over the study period from the Olympus AU5400 Chemistry-Immuno Analyzer (Olympus America, Melville, NY) to the Advia 2400 Chemistry System (Siemens Healthcare Diagnostics, Deerfield, IL) and associated reagent kits.

Change in Parameters of NO System With Time

Table 3 summarizes baseline and follow-up data as regards integrity of the NO system. Results can be categorized as follows.

Table 1. Patient Characteristics (n=204)

Parameter	Baseline (%)	End of Study (%)	P Value
Age, y	63±6	67±6	
Gender	42.4% male		
Diabetes	24 (12%)	33 (16%)	0.013
Hypertension	85 (42%)	106 (52%)	<0.001
Dyslipidaemia	118 (58%)	137 (67.5%)	0.004
Smoking	28 (14%)	13 (6.4%)	<0.01
Coronary disease	24 (12%)	28 (14%)	0.26
Statin use	65 (32%)	71 (35%)	0.24
ACEI/ARB use	69 (34%)	83 (41%)	0.008
BMI, kg/m^2	28.2±5.2	28.2±5.2	0.37

ACEI indicates angiotensin-converting enzyme inhibitors; ARB, angiotensin receptor blocker; BMI, body mass index.

Table 2. Biochemical Profile (n=204)

Parameter	Baseline	End of Study	P Value
Total cholesterol, mmol/L	4.9±0.9	5±1.1	0.02
LDL, mmol/L	2.8±0.8	2.9±1	0.75
HDL, mmol/L	1.3±0.3	1.5±0.4	<0.001
Calcium level, mmol/L	2.2±0.1	2.3±0.1	<0.001
Vitamin D level, mmol/L	72±23.1	74.7±26.6	0.29
CrCl, mL/min per 1.73 m^2	92±21.6	98±28.6	<0.001
hs-CRP, mmol/L	3.5±3.7	3.1±3.8	0.14
CTx (median), pg/mL	242±143	283±145	0.001
P1NP (median), μg/L	40.5±19.8	43.4±50.6	0.45

CrCl indicates creatinine clearance; CTx, C-terminal telopeptide of collagen type 1; HDL, high-density lipoprotein; hs-CRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; P1NP, N-terminal peptide of procollagen I.

Platelet data

Given that platelet aggregation in response to ADP increased and that the antiaggregatory response to the NO donor, SNP, decreased markedly (data shown in Figure 2A and 2B, respectively), the possibility of physiological antagonism[22] was investigated. While there was a strong correlation between baseline and follow-up measurements of ADP-induced aggregation and platelet NO responsiveness (*P*<0.001 for both; Figure 3), there was no relationship between changes in ADP-induced aggregation and those in platelet NO responsiveness (*P*=0.42).

Arterial stiffness (AIx)

Mean AIx of 27.2±8.3% (normal range: 15±16%) at baseline was substantially greater than previously reported values for normal young adults.[23] However, there was no significant change in AIx with time (mean 27.2±8.3% vs. 27±6.9%; *P*=not significant [NS]).

ADMA

Mean plasma ADMA concentrations at baseline were within the previously described normal range for the methodology

used.[21,24] However, there was a significant rise in plasma ADMA concentration over the 4-year follow-up period: 0.52±0.08 to 0.60±0.09 μmol/L (*P*<0.0001), consistent with deterioration of endothelial function with time (Figure 2C).

Univariate Correlates

Univariate correlates of baseline (1) ADP-induced aggregation, (2) (impaired) inhibition of aggregation by the NO donor, SNP, (3) higher baseline AIx, and (4) elevated baseline ADMA concentrations are summarized in Table 4. Notably, female gender was a significant direct correlate of all 4 of these measures of impaired vascular and platelet function, whereas age was a significant correlate of ADMA concentrations.

Univariate correlates of changes in the same 4 parameters of vascular and platelet function over 4 years of follow-up are also summarized in Table 4. As regards decreases in platelet NO responsiveness and increases in ADMA concentrations, there was a strong correlation with calcium-phosphate product (Ca$_x$PO$_4$) and plasma cholesterol concentrations. Lack of use of ACEIs/ARBs was strongly predictive of increases in ADMA concentrations.

Furthermore, increasing ADMA concentrations correlated strongly with worsening platelet NO responsiveness (*P*=0.013; Figure 4). But, change in ADP-induced platelet aggregation did not correlate with change in ADMA (*P*=0.9) nor with change in SNP responsiveness (*P*=0.43).

Multivariable Analyses

Baseline

Multivariable predictors of higher *baseline ADP-induced aggregation* were: female gender (*P*<0.001; β=0.223; B=2.36); higher total cholesterol concentrations (*P*=0.002; β=0.254; B=0.63). Thus, females had 2.36 Ohms greater baseline ADP-induced platelet aggregation after adjustment for covariates and confounders.

Table 3. Parameters Relevant to NO Generation/Responsiveness at Baseline and End of Study

Parameter	Baseline	End of Study	P Value
ADP-induced platelet aggregation (median [25% to 75%]), Ohms	7.8 (5.05 to 10.2)	8.8 (6.8 to 10.4)	0.0002*
Platelet NO responsiveness (median [25% to 75%]), %	28.4 (13.8 to 49.5)	15.6 (3.7 to 38.6)	<0.0001*
ADMA concentrations (mean±SD), μmol/L	0.52±0.08	0.60±0.09	<0.0001†
AIx (mean±SD), %	27.2±8.3	27±6.9	NS†

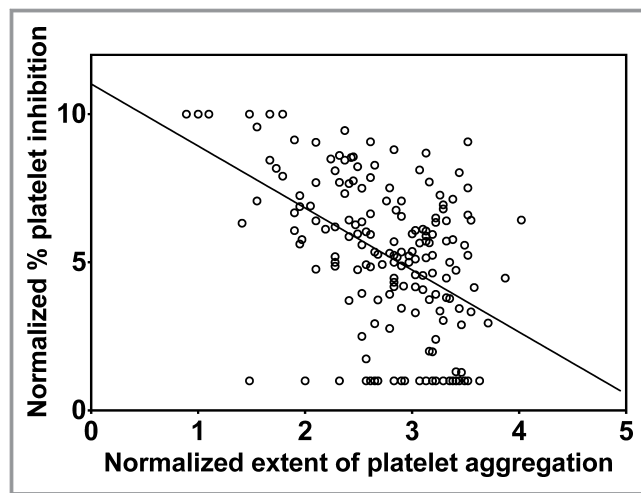ADMA indicates asymmetric dimethylarginine; ADP, adenosine diphosphate; AIx, augmentation index; NO, nitric oxide.
*Wilcoxon's matched-pairs signed-rank test.
†Paired *t* test.
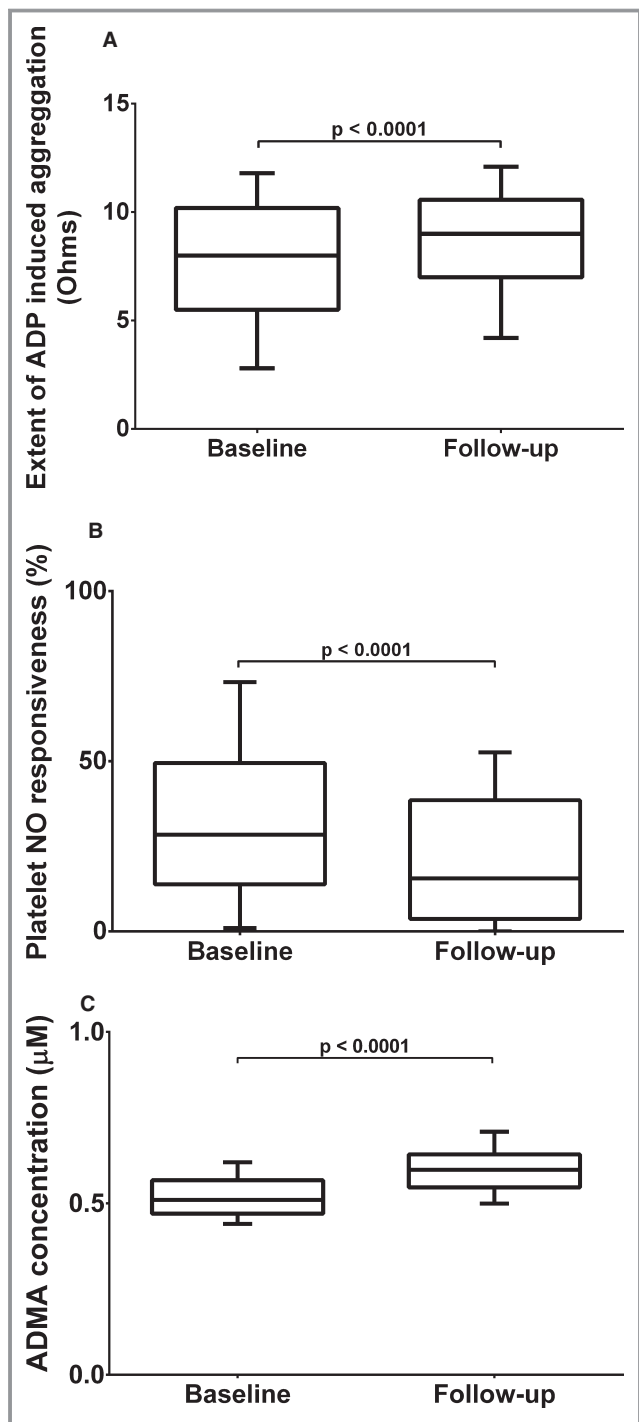
Figure 2. A, Extent of ADP-induced platelet aggregation over the 4-year study period. B, Platelet NO responsiveness over the 4-year study period. C, Plasma ADMA concentrations over the 4-year study period (box and whiskers represent interquartile and 10% to 90% ranges, respectively, whereas line represents medians or mean, as appropriate). ADMA indicates asymmetric dimethylarginine; ADP, adenosine diphosphate; NO, nitric oxide.

Figure 3. Correlation ($r=-0.49$; $P<0.001$) between extent of ADP-induced platelet aggregation and of inhibition of aggregation (platelet NO responsiveness) at baseline. Data were normalized by square root transformation (see *Statistical Analyses* section). ADP indicates adenosine diphosphate; NO, nitric oxide.

$\beta=0.161$; B=0.351). Importantly, females had 3.85% lower platelet NO responsiveness upon adjustments for covariates and confounders.

Multivariable correlates of higher *baseline ADMA concentrations* were: higher concentrations of N-terminal peptide of procollagen I ($P=0.001$; $\beta=0.241$; B=0.001), lower vitamin D concentrations ($P=0.01$; $\beta=0.18$; B=0.001), and age ($P=0.045$; $\beta=0.14$; B=0.002).

Multivariable predictors of high *baseline AIx* were: female gender ($P<0.001$; $\beta=0.5$; B=8.25), higher diastolic blood pressure ($P=0.004$; $\beta=0.184$; B=0.169), lack of use of ACEIs/ARBs use ($P=0.015$; $\beta=0.153$; B=2.64), and age ($P=0.02$; $\beta=0.147$; B=0.2). Thus, females have 8.25% higher AIx, whereas use of ACEIs/ARBs was associated with a 2.64% reduction in AIx.

Changes over time

Multivariable correlates of *deterioration of platelet NO responsiveness* were: female gender ($P=0.034$; $\beta=0.17$; B=9.84) and lower vitamin D concentrations ($P=0.04$; $\beta=0.16$; B=0.21). Specifically, female gender was associated with a 9.8% greater drop in platelet NO responsiveness over 4 years after adjustment for covariates and confounders.

Multivariable correlates of *increasing ADMA concentrations* were: increased Ca_xPO_4 ($P=0.001$; $\beta=0.226$; B=0.004), lower CrCl ($P=0.004$; $\beta=0.202$; B=0.001), and presence of DM ($P=0.03$; $\beta=0.158$; B=0.048). Thus, diabetic subjects had, on average, a 0.048-μmol/L greater rise in ADMA concentrations over time of study after adjustment for covariates and confounders.

Multivariable predictors of impaired *baseline platelet NO responsiveness* were: female gender ($P=0.003$; $\beta=0.226$; B=3.85) and elevated systolic blood pressure ($P=0.035$;

Table 4. Univariate Correlates of Markers of NO Generation/Effect ($P \leq 0.2$ for Inclusion into Multivariable Model)

| Marker of NO Generation/Effect	Baseline			Change Over Time		
	Parameter	β Coefficient (SE)*	P Value	Parameter	β Coefficient (SE)*	P Value
Increased ADP-induced platelet aggregation, Ohms	Female gender	2.6 (0.49)	<0.001	Higher diastolic blood pressure (mm Hg)	0.043 (0.027)	0.11
	Higher total cholesterol concentrations (mmol/L)	0.91 (0.28)	<0.001			
	Higher Ca_xPO_4 (units)	1.77 (0.58)	0.005			
	Higher AIx (%)	0.08 (0.03)	0.017			
	Known CAD	1.9 (0.81)	0.023			
	History of hypertension	1.17 (0.01)	0.034			
	Higher Framingham 10 year CAD risk score	0.08 (0.04)	0.11			
	Lack of use of ACEIs/ARBs	0.62 (0.55)	0.16			
Impaired platelet NO responsiveness, %	Female gender	12.7 (3.98)	0.001	Higher Ca_xPO_4 (units)	14.7 (5.12)	0.005
				Higher total cholesterol concentrations (mmol/L)	5.33 (2.6)	0.042
				Lower vitamin D concentrations (mmol/L)	0.194 (0.1)	0.057
	Lack of use of ACEIs/ARBs	1.5 (0.9)	0.074	Female gender	7.88 (4.75)	0.11
	Higher Framingham 10 year CAD risk score	0.67 (0.27)	0.013	Higher systolic blood pressure (mm Hg)	0.22 (0.14)	0.11
	Higher CTx concentrations (pg/mL)	0.32 (0.16)	0.052	History of hypertension	6.95 (4.87)	0.16
	Higher systolic blood pressure (mm Hg)	0.22 (0.12)	0.09	Higher CTx concentrations (pg/mL)	0.035 (0.016)	0.17
	Higher total cholesterol concentrations (mmol/L)	5.19 (2.24)	0.16			
Elevated ADMA concentrations, µmol/L	Higher P1NP concentrations (µg/L)	0.001 (0.0003)	<0.001	Lack of use of ACEIs/ARBs	0.038 (0.012)	0.002
	Lower vitamin D concentrations (mmol/L)	0.001 (0.0002)	0.002	Higher Ca_xPO_4 (units)	0.042 (0.013)	0.002
				History of hypertension	0.033 (0.012)	0.007
				Lower CrCl (mL/min per 1.73 m^2)	0.001 (0.0003)	0.01
				Higher hs-CRP concentration (mmol/L)	0.003 (0.002)	0.017
				Higher CTx concentrations (pg/mL)	0.0001 (0.00004)	0.018
				Higher total cholesterol concentrations (mmol/L)	0.013 (0.006)	0.045

Continued

Table 4. Continued

Marker of NO Generation/ Effect	Baseline			Change Over Time		
	Parameter	β Coefficient (SE)*	P Value	Parameter	β Coefficient (SE)*	P Value
	Higher CTx concentrations (pg/mL)	0.00007 (0.00004)	0.008	Higher P1NP concentrations (μ/L)	0.001 (0.0003)	0.061
	History of DM	0.042 (0.016)	0.017	History of DM	0.044 (0.018)	0.076
	Lack of use of ACEIs/ARBs	0.023 (0.011)	0.025	Higher BMI (kg/m^2)	0.002 (0.001)	0.15
	Female gender	0.023 (0.011)	0.027	Higher AIx (%)	0.001 (0.001)	0.18
	Age (years)	0.002 (0.001)	0.035			
	Higher total cholesterol concentrations (mmol/L)	0.011 (0.006)	0.069			
	AIx (%)	0.001 (0.001)	0.078			
	Lower CrCl (mL/min per 1.73 m^2)	0.0004 (0.0003)	0.15			
	Higher Ca$_x$PO$_4$ (units)	0.017 (0.012)	0.16			
Greater AIx, (%)	Female gender	8.1 (1.04)	<0.001	None		
	Lower CrCl (mL/min per 1.73 m^2)	0.093 (0.028)	0.001			
	Higher Framingham 10 year CAD score	0.21 (0.08)	0.01			
	Higher diastolic blood pressure (mm Hg)	0.17 (0.064)	0.01			
	Greater ADP induced platelet aggregation (Ohms)	0.42 (0.16)	0.017			
	History of DM	3.93 (1.79)	0.024			
	Lack of use of ACEIs/ARB	2.69 (1.22)	0.027			
	Higher total cholesterol concentrations (mmol/L)	1.32 (0.637)	0.039			
	History of CAD	3.23 (1.83)	0.06			
	Higher ADMA concentrations (μmol/L)	13.5 (7.6)	0.078			
	Age (years)	0.17 (0.1)	0.085			
	Higher Ca$_x$PO$_4$ (units)	2.1 (1.34)	0.12			
	Higher systolic blood pressure (mm Hg)	0.056 (0.035)	0.12			

ACEIs/ARBs indicates angiotensin-converting enzyme inhibitors/angiotensin receptor blockers; ADMA, asymmetric dimethylarginine; ADP, adenosine diphosphate; AIx, augmentation index; BMI, body mass index; CAD, coronary artery disease; Ca$_x$PO$_4$, calcium-phosphate product; CrCl, creatinine clearance; CTx, C-terminal telopeptide of collagen type 1; DM, diabetes mellitus; hs-CRP, high-sensitivity C-reactive protein; MD, mean difference; NO, nitric oxide; P1NP, N-terminal peptide of procollagen I.

*β estimate (standard error) for a change in dependent variable per 1 unit change in predictor for continuous variables and presence of predictor for dichotomous variables.
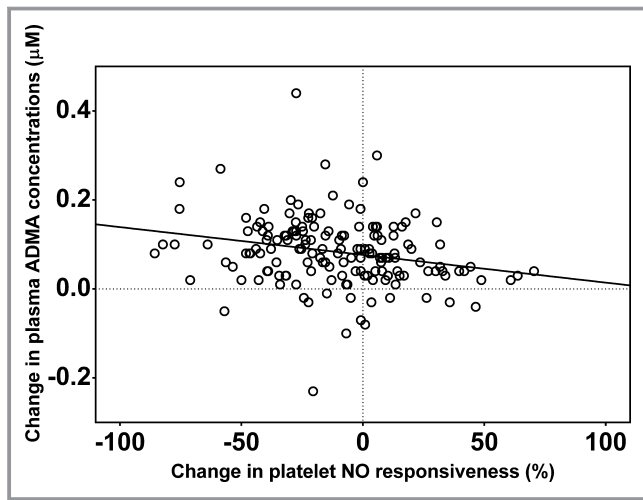
Figure 4. Correlation between changes in platelet NO responsiveness and plasma ADMA concentrations ($r=-0.2$; $P=0.013$). ADMA indicates asymmetric dimethylarginine; NO, nitric oxide.

Discussion

The main findings of the current study are:

1. Aging is associated with both increases in ADP-induced platelet aggregation and plasma ADMA concentrations, as well as with reductions in platelet NO responsiveness—all important risk factors for cardiovascular events (CVEs).
2. Female gender is associated with higher ADP-induced platelet aggregation, lower platelet NO responsiveness, greater arterial stiffness, and, critically, with more-pronounced fall in platelet NO responsiveness with time.
3. There is a significant relationship between deterioration in platelet NO responsiveness and increases in ADMA concentrations.
4. Use of ACEIs/ARBs is associated with better platelet NO responsiveness and lower arterial stiffness.

It is important to note that postulated mechanisms underlying normal aging include increased oxidative stress (OS),[25] which affects NO signaling.[7,9] However, previous studies have not focused on a central role for NO/soluble guanylate cyclase (sGC) signaling cascade in CV aging.

Our most central finding, that there is significant impairment of platelet NO responsiveness with aging, is consistent with effects of incremental OS. The phenomenon of NO resistance appears to reflect, from a biochemical point of view, the combined effect of scavenging of NO, primarily by superoxide anion, and oxidative dysfunction or inactivation of sGC.[7] Thioredoxin (TRX)-interacting protein (TXNIP), which functions as an inhibitor of TRX, a major antioxidant defense protein involved in the maintenance of cellular physiology and survival, may function within the CV system as a physiological antagonist to NO signaling[26] and thus play a direct role in the observed impairment of platelet NO responsiveness. Furthermore, we have recently demonstrated that platelet TXNIP content increases with age, and in this cohort, higher platelet TXNIP content predicted worse platelet NO responsiveness.[9] It is also possible that thrombospondin 1 (TSP-1),[27] which is released from platelet alpha granules, may contribute to platelet NO resistance observed in our study. Indeed, similarly to TXNIP, TSP-1 has recently been shown to increase with age.[28] It has also been shown to lead to NADPH oxidase activation[29] and subsequent reactive oxygen species generation as well as inhibition of sGC.[30] The current experiments did not permit delineation of the extent of NO scavenging versus sGC dysfunction. Interestingly, a number of agents, such as ACEI, perhexiline, and, possibly, statins, ameliorate platelet NO resistance.[7] The previously described effects of ACEIs include not only reversal of NO resistance,[31] but also reduction in arterial stiffness.[32] The design of the current study did not permit comparison of aging of NO responsiveness in blood vessels versus platelets. However, it has been established that NO resistance, whether at the vascular[33] or platelet[13] level, is an independent marker of risk of coronary events.

Furthermore, we identified 2 factors associated with accelerated decline of platelet NO responsiveness—female gender and lower vitamin D levels. Females are thought to be protected from coronary disease before menopause, while postmenopausally they rapidly catch up with their male counterparts and indeed generally have worse CV outcomes, compared to males.[34] The finding of more-significant impairment of platelet NO responsiveness could offer a partial explanation for this epidemiologically observed risk. Interestingly, we have also demonstrated that this process starts premenopausally, in the fifth decade of life,[15] and current results suggest that it then takes on an accelerated course. Indeed, as previously reported,[35] female gender was also a correlate of higher baseline ADP-induced platelet aggregation, lending further support toward an increased prothrombotic diathesis. The mechanism(s) underlying these phenomena are worthy of specific investigation.

Vitamin D deficiency has also been associated with a number of CVD states.[36] The mechanism for this association is not absolutely clear, but the observed association between low vitamin D levels and higher ADMA concentrations in this cohort[12] may provide a partial explanation.

While ADMA concentrations have previously been shown to increase with age in the healthy population,[37] longitudinal evaluation of ADMA concentrations in relatively unselected patient cohorts has not been undertaken. Interestingly, in a cross-sectional study aimed to derive reference ranges for plasma ADMA concentration in healthy individuals, Hov et al.

found that ADMA concentrations had a very modest correlation with age in women, but not in men.[38] While, in our study, female gender was a univariate predictor of higher ADMA, the gender per se had no effect on the age–ADMA relationship (Figure 5). There are 2 potential explanations for this discrepancy. First, the study by Hov et al. included healthy individuals, with no significant comorbidities and not on any medications other than hormonal supplements, whereas our cohort represented an unselected aging Western population, including subjects with diabetes, heart disease, and those taking multiple medications. This may have obscured the relatively small effect of female gender on the age–ADMA relationship noted by Hov et al.[38] Second, the study by Hov et al. had a substantial proportion of premenopausal women, and estrogen is known to reduce ADMA concentrations[39]; our study only included subjects above the age of 55, so the majority of women are postmenopausal and ADMA-lowering effects of estrogens would not be observed.

Importantly, higher ADMA concentrations are found in renal insufficiency[40] or in patients with coronary risk factors (CRFs).[41–43] Indeed, consistent with the previous literature, in our population, renal function was a determinant of increasing ADMA concentrations, as was the presence of diabetes, yet no other CRFs appear to predict increases in ADMA concentrations with age in our population cohort. The increase in ADMA concentrations was not primarily driven by deterioration in renal function because there was no reduction in renal function over the follow-up period. This suggests that other factors, namely, enzymatic generation of ADMA by protein arginine methyltransferase–1 and/or metabolic clearance of

ADMA by dimethylarginine dimethylaminohydrolase (DDAH)–1 and -2,[44] may have contributed to the observed changes.

In this regard, an intriguing finding of the current experiments was the inverse correlation between changes in platelet NO responsiveness and those in plasma ADMA concentrations. Two possible explanations are available for this correlation. First, DDAH is known to be susceptible to OS[45] and appears to be activated, in part, by NO.[46] Thus, it is possible that OS might similarly affect DDAH activity and platelet NO signaling, as well as an NO-based feedback mechanism at the level of DDAH. A second explanation would relate to the precipitation of NOS uncoupling by ADMA.[47] While NO responsiveness is superficially NOS independent, increased superoxide production within platelets as a result of NOS uncoupling would contribute to NO resistance. A schematic of potential interactions between ADMA and NO signaling is depicted in Figure 6.

The detection of platelet hyporesponsiveness to NO utilizing whole-blood aggregometry is an important aspect of study design: The observed abnormalities may reflect not only disordered platelet function per se, but also interactions with neutrophil-derived NAD(P)H oxidase[48] and/or "uncoupled" NOS[49]-derived superoxide radical release. We have utilized SNP as an NO donor in our study because it represents a
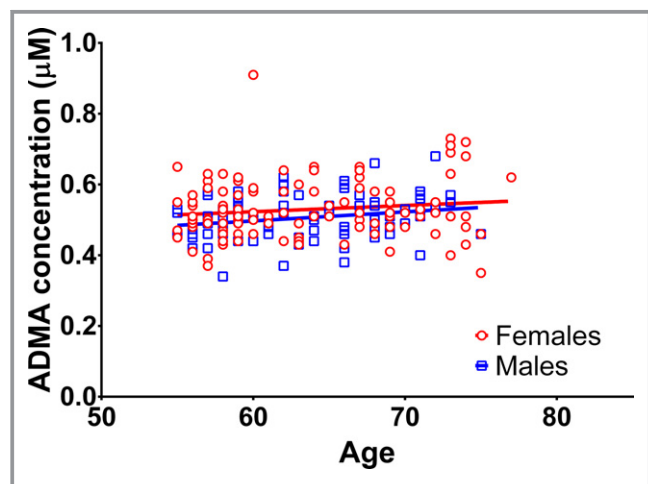
Figure 5. Relationship between baseline age and plasma ADMA concentrations stratified by gender. While females have higher ADMA concentrations (ANCOVA; F=4.8; P=0.03), there is now gender effect upon age–ADMA concentrations relationship (ANCOVA; F=0.2; P=0.8). ADMA indicates asymmetric dimethylarginine.
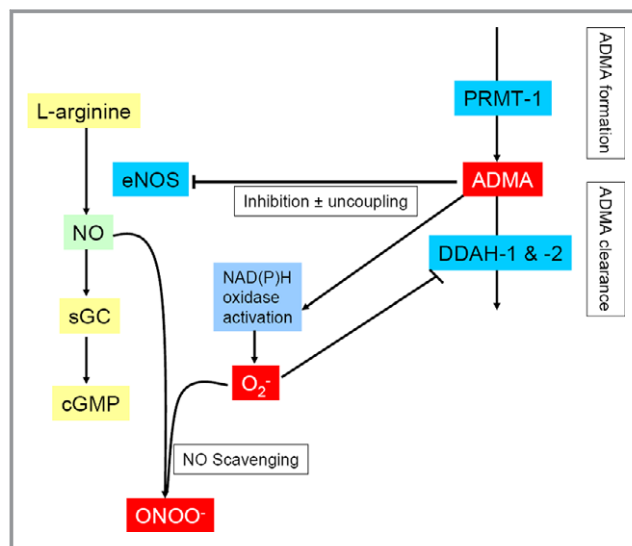
Figure 6. Interactions between integrity of NO signaling and increased ADMA clearance: potential pathways. ADMA can directly inhibit and uncouple eNOS resulting in less NO and more superoxide (O_2^-) generation. ADMA can activate NAD(P)H oxidase, resulting in increased superoxide production, which, in turn, scavenges available NO, with the end result being reduced NO availability and signaling. Increased superoxide can inactivate DDAH-1 and -2, which, in turn, reduces ADMA clearance, completing a vicious cycle of high ADMA begetting more ADMA. ADMA indicates asymmetric dimethylarginine; DDAH, dimethylarginine dimethylaminohydrolase; eNOS, endothelial nitric oxide synthase; NO, nitric oxide.

tolerance-independent donor, which has been used extensively in the past to characterize NO resistance at the platelet level[9,15,31,50,51] and which exerts largely sGC-dependent antiaggregatory effects.[7] Importantly, its use permits exclusion of both nitrosylation-mediated effects and activation of the redox-dependent site on guanosine 3',5'-monophosphate (cGMP)-dependent protein kinase as substantial modulators of observed changes in platelet responsiveness.[52] The drawbacks for the use of SNP are mainly its relative inconvenience as a result of its half-life in whole blood and the fact that it may not be a "pure NO" donor.

The current study has a number of potential limitations. The central issue is that the study was predominantly phenomenological, without detailed parallel mechanistic evaluation at the molecular level. In particular, the mechanism(s) underlying changes in ADMA kinetics, the potential effect of these changes on NOS function, and the integrity of sGC would be important areas for detailed evaluation. The potential gain from such an evaluation would be primarily to delineate the optimal approach for amelioration and/or circumvention of the aging-associated changes in NO signaling.

Second, the study was not designed to evaluate the effect of the observed changes in incidence of CVEs in this population. To achieve such an objective, a much larger population would have had to be followed up for a longer period of time. While there is abundant evidence that elevation of ADMA concentrations[24] and presence of NO resistance[13] are prognostic markers in various populations, it would be worthwhile to identify the extent of association in this "normal" cohort. An additional limitation of the study is that the data were only collected at baseline and end of the study. Thus, it is possible that the parameters measured, while worsening over time, have a much more waxing and waning course than one of continuous progressive decline.

Conclusions

Previous studies have reported a relationship between age and ADMA concentrations[37] as well as between age and platelet hyperaggregability.[53] Little is known about platelet NO responsiveness and age in unselected subjects, other than the phenomenon of age-related reduction in platelet cGMP.[54] These parameters have not been assessed simultaneously in the past nor have they been followed up in the same cohort. Thus, the novelty of the current study is that it longitudinally evaluated changes in platelet aggregability, NO responsiveness, arterial stiffness, and ADMA concentrations in the same cohort of aging, but otherwise unselected, subjects and correlated these with one another. The current findings demonstrate that (1) NO-related parameters of vascular and platelet function deteriorate with aging,

(2) there is a correlation between the degree of deterioration of platelet (NO responsiveness) and vascular (ADMA levels) NO-related function, and (3) female gender is a particular risk for worsening of platelet NO responsiveness over time. Our observations of aging of the NO system in platelets and the vasculature may have important parallels in other organs. Notably, the phenomenon of impairment of LV diastolic function becomes increasingly frequent with aging,[55] and there is increasing evidence that impaired NO signaling is important as a modulator of this form of heart failure.[10,56] Both this issue, and also that of impairment of the anti-inflammatory/antiapoptotic effects of NO[57] would represent technically more challenging areas of investigation, which are nevertheless increasingly relevant, given our current results. Additionally, correlation between rising ADMA concentrations and worsening platelet SNP responsiveness have never been reported on and may represent an interesting direction for future research to determine whether these are inter-related or parallel effects.

Disclosures

None.

References

1. Department of Economic and Social Affairs. Population Division. United Nations. *World Population Ageing: 1950–2050*. Department of Economic and Social Affairs. Population Division. United Nations; 2001.

2. Kovacic JC, Moreno P, Hachinski V, Nabel EG, Fuster V. Cellular senescence, vascular disease, and aging: Part 1 of a 2-part review. *Circulation*. 2011;123: 1650–1660.

3. Fleg JL, Gerstenblith G, Zonderman AB, Becker LC, Weisfeldt ML, Costa PT, Jr, Lakatta EG. Prevalence and prognostic significance of exercise-induced silent myocardial ischemia detected by thallium scintigraphy and electrocardiography in asymptomatic volunteers. *Circulation*. 1990;81:428–436.

4. Ngo DT, Sverdlov AL, Willoughby SR, Nightingale AK, Chirkov YY, McNeil JJ, Horowitz JD. Determinants of occurrence of aortic sclerosis in an aging population. *JACC Cardiovasc Imaging*. 2009;2:919–927.

5. Lakatta EG. Arterial and cardiac aging: major shareholders in cardiovascular disease enterprises: Part III: cellular and molecular clues to heart and arterial aging. *Circulation*. 2003;107:490–497.

6. Lakatta EG, Levy D. Arterial and cardiac aging: major shareholders in cardiovascular disease enterprises: Part II: the aging heart in health: links to heart disease. *Circulation*. 2003;107:346–354.

7. Chirkov YY, Horowitz JD. Impaired tissue responsiveness to organic nitrates and nitric oxide: a new therapeutic frontier? *Pharmacol Ther*. 2007;116:287–305.

8. Gao Y. The multiple actions of NO. *Pflugers Arch*. 2010;459:829–839.

9. Sverdlov AL, Chan WPA, Procter NEK, Chirkov YY, Ngo DTM, Horowitz JD. Reciprocal regulation of no signaling and txnip expression in humans: impact of aging and ramipril therapy. *Int J Cardiol*. 2013;168:4624–4630.

10. Sverdlov AL, Ngo DT, Nightingale AK, Rajendran S, Mishra K, Heresztyn T, Ritchie RH, Marwick TH, Frenneaux MP, Horowitz JD. The endogenous nos inhibitor asymmetric dimethylarginine (ADMA) predicts LV mass independent of afterload. *Nitric Oxide*. 2011;25:41–46.

11. Sibal L, Agarwal SC, Home PD, Boger RH. The role of asymmetric dimethylarginine (ADMA) in endothelial dysfunction and cardiovascular disease. *Curr Cardiol Rev*. 2010;6:82–90.

12. Ngo DT, Sverdlov AL, McNeil JJ, Horowitz JD. Does vitamin D modulate asymmetric dimethylarginine and c-reactive protein concentrations? *Am J Med*. 2010;123:335–341.

13. Willoughby SR, Stewart S, Holmes AS, Chirkov YY, Horowitz JD. Platelet nitric oxide responsiveness: a novel prognostic marker in acute coronary syndromes. *Arterioscler Thromb Vasc Biol*. 2005;25:2661–2666.

14. Marliss EB, Chevalier S, Gougeon R, Morais JA, Lamarche M, Adegoke OA, Wu G. Elevations of plasma methylarginines in obesity and ageing are related to insulin sensitivity and rates of protein turnover. *Diabetologia*. 2006;49:351–359.

15. Chan WP, Ngo DT, Sverdlov AL, Rajendran S, Stafford I, Heresztyn T, Chirkov YY, Horowitz JD. Premature aging of cardiovascular/platelet function in polycystic ovarian syndrome. *Am J Med*. 2013;126:e641–e647.

16. Grant JF, Chittleborough CR, Taylor AW, Dal GRANDE E, Wilson DH, Phillips PJ, Adams RJ, Cheek J, Price K, Gill T, Ruffin RE. The North West Adelaide Health Study: detailed methods and baseline segmentation of a cohort for selected chronic diseases. *Epidemiol Perspect Innov*. 2006;3:4

17. Chirkov YY, Holmes AS, Willoughby SR, Stewart S, Horowitz JD. Association of aortic stenosis with platelet hyperaggregability and impaired responsiveness to nitric oxide. *Am J Cardiol*. 2002;90:551–554.

18. Wilkinson IB, Cockcroft JR, Webb DJ. Pulse wave analysis and arterial stiffness. *J Cardiovasc Pharmacol*. 1998;32(suppl 3):S33–S37.

19. Wilkinson IB, MacCallum H, Flint L, Cockcroft JR, Newby DE, Webb DJ. The influence of heart rate on augmentation index and central arterial pressure in humans. *J Physiol*. 2000;525(Pt 1):263–270.

20. Weber T, Auer J, O'Rourke MF, Kvas E, Lassnig E, Berent R, Eber B. Arterial stiffness, wave reflections, and the risk of coronary artery disease. *Circulation*. 2004;109:184–189.

21. Heresztyn T, Worthley MI, Horowitz JD. Determination of L-arginine and NG, NG – and NG, NG′ -dimethyl-L-arginine in plasma by liquid chromatography as accq-fluor fluorescent derivatives. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2004;805:325–329.

22. Geiger J. Inhibitors of platelet signal transduction as anti-aggregatory drugs. *Expert Opin Investig Drugs*. 2001;10:865–890.

23. McEniery CM, Wallace S, Mackenzie IS, McDonnell B, Yasmin, Newby DE, Cockcroft JR, Wilkinson IB. Endothelial function is associated with pulse pressure, pulse wave velocity, and augmentation index in healthy humans. *Hypertension*. 2006;48:602–608

24. Horowitz JD, Heresztyn T. An overview of plasma concentrations of asymmetric dimethylarginine (ADMA) in health and disease and in clinical studies: methodological considerations. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2007;851:42–50.

25. Muller FL, Lustgarten MS, Jang Y, Richardson A, Van Remmen H. Trends in oxidative aging theories. *Free Radic Biol Med*. 2007;43:477–503.

26. Lee S, Kim SM, Lee RT. Thioredoxin and thioredoxin target proteins: from molecular mechanisms to functional significance. *Antioxid Redox Signal*. 2013;18:1165–1207.

27. Roberts DD, Miller TW, Rogers NM, Yao M, Isenberg JS. The matricellular protein thrombospondin-1 globally regulates cardiovascular function and responses to stress via CD47. *Matrix Biol*. 2012;31:162–169.

28. Rogers NM, Roberts DD, Isenberg JS. Age-associated induction of cell membrane CD47 limits basal and temperature-induced changes in cutaneous blood flow. *Ann Surg*. 2013;258:184–191.

29. Yao M, Rogers NM, Csanyi G, Rodriguez AI, Ross MA, St CroixC, Knupp H, Novelli EM, Thomson AW, Pagano PJ, Isenberg JS. Thrombospondin-1 activation of signal-regulatory protein-alpha stimulates reactive oxygen species production and promotes renal ischemia reperfusion injury. *J Am Soc Nephrol*. 2014;25:1171–1186.

30. Miller TW, Isenberg JS, Roberts DD. Thrombospondin-1 is an inhibitor of pharmacological activation of soluble guanylate cyclase. *Br J Pharmacol*. 2010;159:1542–1547.

31. Willoughby SR, Rajendran S, Chan WP, Procter N, Leslie S, Liberts EA, Heresztyn T, Chirkov YY, Horowitz JD. Ramipril sensitizes platelets to nitric oxide: implications for therapy in high-risk patients. *J Am Coll Cardiol*. 2012;60:887–894.

32. Ngo DT, Sverdlov AL, McNeil JJ, Horowitz JD. Correlates of arterial stiffness in an ageing population: role of asymmetric dimethylarginine. *Pharmacol Res*. 2009;60:503–507.

33. Schachinger V, Britten MB, Zeiher AM. Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease. *Circulation*. 2000;101:1899–1906.

34. Lee LV, Foody JM. Women and heart disease. *Cardiol Clin*. 2011;29:35–45.

35. Yee DL, Sun CW, Bergeron AL, Dong JF, Bray PF. Aggregometry detects platelet hyperreactivity in healthy individuals. *Blood*. 2005;106:2723–2729.

36. McGreevy C, Williams D. New insights about vitamin D and cardiovascular disease: a narrative review. *Ann Intern Med*. 2011;155:820–826.

37. Kielstein JT, Bode-Boger SM, Frolich JC, Ritz E, Haller H, Fliser D. Asymmetric dimethylarginine, blood pressure, and renal perfusion in elderly subjects. *Circulation*. 2003;107:1891–1895.

38. Hov GG, Sagen E, Bigonah A, Asberg A. Health-associated reference values for arginine, asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) measured with high-performance liquid chromatography. *Scand J Clin Lab Invest*. 2007;67:868–876.

39. Holden DP, Cartwright JE, Nussey SS, Whitley GS. Estrogen stimulates dimethylarginine dimethylaminohydrolase activity and the metabolism of asymmetric dimethylarginine. *Circulation*. 2003;108:1575–1580.

40. Vallance P, Leone A, Calver A, Collier J, Moncada S. Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. *Lancet*. 1992;339:572–575.

41. Abbasi F, Asagmi T, Cooke JP, Lamendola C, McLaughlin T, Reaven GM, Stuehlinger M, Tsao PS. Plasma concentrations of asymmetric dimethylarginine are increased in patients with type 2 diabetes mellitus. *Am J Cardiol*. 2001;88:1201–1203.

42. Boger RH, Bode-Boger SM, Szuba A, Tsao PS, Chan JR, Tangphao O, Blaschke TF, Cooke JP. Asymmetric dimethylarginine (ADMA): a novel risk factor for endothelial dysfunction: Its role in hypercholesterolemia. *Circulation*. 1998;98:1842–1847.

43. Achan V, Broadhead M, Malaki M, Whitley G, Leiper J, MacAllister R, Vallance P. Asymmetric dimethylarginine causes hypertension and cardiac dysfunction in humans and is actively metabolized by dimethylarginine dimethylaminohydrolase. *Arterioscler Thromb Vasc Biol*. 2003;23:1455–1459.

44. Blackwell S. The biochemistry, measurement and current clinical significance of asymmetric dimethylarginine. *Ann Clin Biochem*. 2010;47:17–28.

45. Tain YL, Baylis C. Determination of dimethylarginine dimethylaminohydrolase activity in the kidney. *Kidney Int*. 2007;72:886–889.

46. Sakurada M, Shichiri M, Imamura M, Azuma H, Hirata Y. Nitric oxide upregulates dimethylarginine dimethylaminohydrolase-2 via cyclic GMP induction in endothelial cells. *Hypertension*. 2008;52:903–909.

47. Antoniades C, Shirodaria C, Leeson P, Antonopoulos A, Warrick N, Van-Assche T, Cunnington C, Tousoulis D, Pillai R, Ratnatunga C, Stefanadis C, Channon KM. Association of plasma asymmetrical dimethylarginine (ADMA) with elevated vascular superoxide production and endothelial nitric oxide synthase uncoupling: implications for endothelial function in human atherosclerosis. *Eur Heart J*. 2009;30:1142–1150.

48. Griendling KK, Sorescu D, Ushio-Fukai M. NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ Res*. 2000;86:494–501.

49. Satoh M, Fujimoto S, Haruna Y, Arakawa S, Horike H, Komai N, Sasaki T, Tsujioka K, Makino H, Kashihara N. NAD(P)H oxidase and uncoupled nitric oxide synthase are major sources of glomerular superoxide in rats with experimental diabetic nephropathy. *Am J Physiol Renal Physiol*. 2005;288: F1144–F1152.

50. Monteiro PF, Morganti RP, Delbin MA, Calixto MC, Lopes-Pires ME, Marcondes S, Zanesco A, Antunes E. Platelet hyperaggregability in high-fat fed rats: a role for intraplatelet reactive-oxygen species production. *Cardiovasc Diabetol*. 2012;11:5.

51. Sverdlov AL, Ngo DT, Chan WP, Chirkov YY, Gersh BJ, McNeil JJ, Horowitz JD. Determinants of aortic sclerosis progression: implications regarding impairment of nitric oxide signalling and potential therapeutics. *Eur Heart J*. 2012;33:2419–2425.

52. Burgoyne JR, Prysyazhna O, Rudyk O, Eaton P. cGMP-dependent activation of protein kinase g precludes disulfide activation: Implications for blood pressure control. *Hypertension*. 2012;60:1301–1308.

53. O'Donnell CJ, Larson MG, Feng D, Sutherland PA, Lindpaintner K, Myers RH, D'Agostino RA, Levy D, Tofler GH; Framingham Heart S. Genetic and

environmental contributions to platelet aggregation: the Framingham heart study. *Circulation*. 2001;103:3051–3056.

54. Origlia C, Pescarmona G, Capizzi A, Cogotti S, Gambino R, Cassader M, Benso A, Granata R, Martina V. Platelet cGMP inversely correlates with age in healthy subjects. *J Endocrinol Invest*. 2004;27:RC1–RC4

55. Kane GC, Karon BL, Mahoney DW, Redfield MM, Roger VL, Burnett JC, Jr, Jacobsen SJ, Rodeheffer RJ. Progression of left ventricular diastolic dysfunction and risk of heart failure. *JAMA*. 2011;306:856–863.

56. Silberman GA, Fan TH, Liu H, Jiao Z, Xiao HD, Lovelock JD, Boulden BM, Widder J, Fredd S, Bernstein KE, Wolska BM, Dikalov S, Harrison DG, Dudley SC, Jr. Uncoupled cardiac nitric oxide synthase mediates diastolic dysfunction. *Circulation*. 2010;121:519–528.

57. Ritchie RH, Irvine JC, Rosenkranz AC, Patel R, Wendt IR, Horowitz JD, Kemp-Harper BK. Exploiting cGMP-based therapies for the prevention of left ventricular hypertrophy: NO* and beyond. *Pharmacol Ther*. 2009;124:279–300.