

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30

**Chronic supplementation with a mitochondrial antioxidant (MitoQ)
improves vascular function in healthy late middle-aged and older adults**

Matthew J. Rossman¹, Jessica R. Santos-Parker¹, Chelsea A.C. Steward¹, Nina Z. Bispham¹,
Lauren M. Cuevas¹, Hannah L. Rosenberg¹, Kayla A. Woodward¹, Michel Chonchol², Rachel A.
Gioscia-Ryan¹, Michael P. Murphy³, and Douglas R. Seals^{1,2}

¹Department of Integrative Physiology, University of Colorado Boulder, Boulder, CO, USA

²Department of Medicine, University of Colorado Anschutz Medical Campus, Aurora, CO, USA

³MRC Mitochondrial Biology Unit, Cambridge, UK

Running Title: MitoQ treatment and vascular function with aging

Corresponding Author:

Matthew J. Rossman, Ph.D.
Department of Integrative Physiology
University of Colorado Boulder
354 UCB
Boulder, CO 80309, USA
Phone: 303-492-7702
E-mail: matthew.rossman@colorado.edu

31 **Abstract**

32 Excess reactive oxygen species production by mitochondria is a key mechanism of age-related
33 vascular dysfunction. Our laboratory has shown that supplementation with the mitochondrial-
34 targeted antioxidant MitoQ improves vascular endothelial function by reducing mitochondrial
35 reactive oxygen species and ameliorates arterial stiffening in old mice, but the effects in humans
36 are unknown. Here we sought to translate our preclinical findings to humans and determine the
37 safety and efficacy of MitoQ. Twenty healthy older adults (60-79 years) with impaired
38 endothelial function (brachial artery flow-mediated dilation <6%) underwent six weeks of oral
39 supplementation with MitoQ (20 mg/day) or placebo in a randomized, placebo-controlled,
40 double-blind, crossover design study. MitoQ was well tolerated and plasma MitoQ was higher
41 after the treatment vs. placebo period ($P<0.05$). Brachial artery flow-mediated dilation was 42%
42 higher after MitoQ vs. placebo ($P<0.05$); the improvement was associated with amelioration of
43 mitochondrial reactive oxygen species-related suppression of endothelial function (assessed as
44 the increase in flow-mediated dilation with acute, supra-therapeutic MitoQ [160 mg]
45 administration, $n=9$, $P<0.05$). Aortic stiffness (carotid-femoral pulse wave velocity) was lower
46 after MitoQ vs. placebo ($P<0.05$) in participants with elevated baseline levels (carotid-femoral
47 pulse wave velocity >7.60 m/s, $n=11$). Plasma oxidized low-density lipoprotein, a marker of
48 oxidative stress, also was lower after MitoQ vs. placebo ($P<0.05$). Participant characteristics,
49 endothelium-independent dilation (sublingual nitroglycerin) and circulating markers of
50 inflammation were not different (all $P>0.1$). These findings in humans extend earlier preclinical
51 observations and suggest that MitoQ and other therapeutic strategies targeting mitochondrial
52 reactive oxygen species may hold promise for treating age-related vascular dysfunction.

53

54 **Keywords:** aging, reactive oxygen species, endothelial function, arterial stiffness, mitochondria

55

56

57 **Introduction**

58 Cardiovascular diseases (CVD) remain the leading cause of morbidity and mortality in
59 developed societies.^{1,2} Advancing age is the primary risk factor for CVD, which is largely
60 mediated by adverse changes to arteries.^{1,3} Two features of vascular aging that are key
61 antecedents to CVD are the development of endothelial dysfunction, as assessed by reduced
62 endothelium-dependent dilation (EDD), and stiffening of the large elastic arteries (i.e., aortic
63 stiffening).^{3,4} Vascular dysfunction with age is a consequence of excessive superoxide-related
64 oxidative stress, much of which is of mitochondrial origin.⁵⁻⁷ Given the projected increase in
65 CVD prevalence in the coming decades, driven mainly by increases in the number of middle-
66 aged and older (MA/O) adults,^{8,9} identifying novel strategies that reduce excess mitochondrial
67 reactive oxygen species (mtROS) to improve vascular function and reduce CVD risk in this
68 population is a biomedical priority.

69 MitoQ is a mitochondria-targeted antioxidant consisting of the naturally occurring
70 antioxidant ubiquinol attached to a lipophilic cation; the lipophilicity and positive charge of this
71 compound enable it to cross cell membranes and accumulate on the matrix facing the surface of
72 the mitochondrial inner membrane where it is optimally positioned to reduce mtROS.^{10,11}
73 Preclinical findings from our laboratory show that 4 weeks of oral supplementation of MitoQ
74 (drinking water) completely restores EDD in old mice to levels observed in young mice.⁶ The
75 improvement in EDD in old mice was mediated by a suppression of mtROS, as indicated by an
76 increase in EDD with acute MitoQ administration in untreated, but not in MitoQ-treated,
77 animals.⁶ More recently, we reported that 4 weeks of MitoQ treatment also ameliorated age-
78 related increases in aortic stiffness in old mice, as indicated by a reduction in aortic pulse wave
79 velocity (PWV).¹² MitoQ supplementation did not impact endothelial function or aortic stiffness in
80 young mice.^{6,12}

81 MitoQ has been used safely in Phase II clinical trials for Parkinson's and liver disease.¹³
82 ¹⁴ Moreover, MitoQ is now available as a dietary supplement and recently was administered
83 chronically (3 weeks) to healthy young adults without adverse effects.¹⁵ However, presently the
84 efficacy of chronic MitoQ supplementation for improving vascular function in healthy MA/O
85 adults is unknown. Accordingly, we sought to translate our preclinical findings to humans by
86 conducting the first randomized, double-blind, placebo-controlled clinical trial with MitoQ in
87 healthy late MA/O humans (crossover design with 6-week intervention arms). EDD, as
88 measured by nitric oxide (NO)-dependent brachial artery flow-mediated dilation (BAFMD), was
89 our primary outcome. To gain mechanistic insight into the possible role of reduced tonic
90 suppression of EDD by mtROS in mediating improvements in endothelial function with MitoQ
91 supplementation, we used a novel functional bioassay in which the acute change in BAFMD in
92 response to a single supra-therapeutic dose of oral MitoQ was compared after both the active
93 treatment and placebo phases of the trial. Based on our recent preclinical findings in mice,¹² we
94 also took the opportunity to evaluate the impact of MitoQ on aortic stiffness, assessed
95 translationally using carotid-femoral PWV (CFPWV). Given that this trial was the first conducted
96 in healthy MA/O adults, safety, tolerability and plasma levels of MitoQ with treatment also were
97 evaluated. Lastly, we assessed changes in circulating markers of oxidative stress and
98 inflammation for additional mechanistic insight.

99

100 **Materials and Methods**

101 All procedures were reviewed and approved by the Institutional Review Board at the
102 University of Colorado Boulder. The nature, benefits, and risks of all study procedures were
103 explained to volunteers and their written informed consent was obtained before participation in
104 the study. This trial was registered on ClinicalTrials.gov (NCT02597023). All measurements
105 were performed at the University of Colorado Boulder Clinical Translational Research Center

106 (CTRC). The Food and Drug Administration categorized MitoQ as a dietary supplement for the
107 manner utilized in this study and, as such, deemed that an IND was not needed. The data
108 supporting the findings of this study are available from the corresponding author upon
109 reasonable request.

110 **Participants.** Late MA/O men and postmenopausal women aged 60 to 79 years from
111 Boulder County, Colorado and the surrounding areas were studied. All participants were non-
112 smokers and free of clinical diseases, including peripheral arterial disease (ankle-brachial index
113 >0.90) and overt CVD as determined by medical history, physical examination, blood
114 chemistries, and blood pressure and electrocardiogram at rest and during incremental treadmill
115 exercise. All participants demonstrated age-associated impairments in endothelial function at
116 screening, defined as BAFMD <6%. Potential participants were excluded if they had abnormal
117 blood chemistries, alcohol dependence, uncontrolled thyroid disease, severe obesity (body
118 mass index [BMI] >40 kg/m²), or were not weight stable (defined as >2.5 kg change in body
119 mass) for at least 3 months prior to enrolling in the study.

120 **Study design, randomization, and intervention.** The study design consisted of a 2 x
121 6-week randomized, double-blind, placebo-controlled, crossover clinical trial. To optimize the
122 potential for observing effects of treatment, participants consumed MitoQ at the upper limit of
123 the manufacturer recommended dose (20 mg, 1x/day; MitoQ Limited) or identical placebo
124 capsules for 6 weeks before crossing over to the other treatment arm in a randomly determined
125 order. Randomization was performed by a member of the study team not involved in the
126 assessment of outcomes, and a block randomization scheme stratified for age and sex was
127 used. MitoQ or placebo capsules were taken once each morning with breakfast. Every 2 weeks
128 during the active treatment and placebo phases, in-person check-in visits were performed to
129 exchange intervention capsules (a precise number of capsules were allocated until the
130 participant's next visit) and to assess participant adherence by survey and pill count. Tolerability
131 and side effects were also evaluated during these check-in visits; treatment-emergent adverse

132 events were documented throughout the study by the CTRC staff and reported to the CTRC
133 Safety Monitoring Committee and the Institutional Review Board.

134 **Measurements.** All measurements were performed following a 12-hour fast from food
135 (water allowed) and caffeine, a 24-hour abstention from alcohol, physical activity, prescription
136 medications and the study compound (MitoQ and placebo) and a 48-hour abstention from over
137 the counter medications and supplements.¹⁶ A single study investigator who was blinded to
138 treatment condition of the subject performed all vascular data acquisition and analysis.

139 **Participant characteristics and clinical blood assays.** BMI was determined by
140 anthropometry¹⁷ and arterial systolic and diastolic blood pressures were assessed in triplicate
141 over the brachial artery at rest with a semi-automated device (Dinamap XL, Johnson &
142 Johnson) at the end of each intervention phase and at check-in visits. Leisure time physical
143 activity was determined by the Modifiable Activity Questionnaire at the end of each intervention
144 phase.¹⁸

145 Blood samples were drawn from an intravenous catheter the cubital vein at screening
146 and after each treatment arm. The Colorado Clinical and Translational Sciences Institute CTRC
147 Core Laboratory and Boulder Community Hospital Clinical Laboratory performed the following
148 blood assays as previously described.¹⁹ Fasting serum lipids were determined with standard
149 assays. Fasting plasma glucose was measured by reflective spectrophotometry (Ortho Clinical
150 Diagnostics). Plasma oxidized low-density lipoprotein (LDL) and serum interleukin (IL)-6 were
151 assessed by ELISA (Mercodia). Serum high-sensitivity C-reactive protein was measured by
152 immunoturbidimetry (Beckman Coulter).

153 **Plasma MitoQ levels.** EDTA-treated plasma collected 24-26 hours after capsule
154 ingestion following each 6-week intervention period was obtained to determine the effects of
155 chronic MitoQ supplementation on circulating MitoQ levels in plasma. Plasma samples were
156 analyzed by reversed-phase liquid chromatography using gradient elution with acetonitrile,

157 water, and formic acid and the deuterated compound (d_{15} -MitoQ) was used as an internal
158 standard, as previously described.²⁰

159 **Vascular endothelial function.** EDD was determined by NO-dependent BAFMD (using
160 a five-minute forearm cuff occlusion) using high-resolution ultrasonography (Toshiba Xario XG),
161 as described previously.^{16, 21} BAFMD was measured at screening to establish baseline
162 endothelial dysfunction and after each 6-week supplementation period. BAFMD is reported as
163 percentage and absolute change from baseline diameter.¹⁶ BAFMD shear rate was calculated
164 as: $[8 \times \text{mean velocity (m/s)}] / \text{occlusion diameter (m)}$.¹⁶ Brachial artery dilation following 0.4 mg
165 sublingual nitroglycerin was measured after each 6-week supplementation period to control for
166 changes in smooth muscle sensitivity to NO. Dilation with nitroglycerin is reported as percent
167 change from baseline diameter. Brachial artery diameters and blood velocities were captured
168 and analyzed by Vascular Research Tools 5.10.9 (Medical Imaging Applications).

169 **MtROS-mediated suppression of vascular endothelial function.** After each 6-week
170 treatment arm, BAFMD was measured before and 1 hour after an acute 160 mg oral dose of
171 MitoQ, used to temporarily and reversibly reduce mtROS. The difference in FMD after vs. before
172 acute oral MitoQ was taken as a measure of tonic suppression of EDD by mtROS.

173 **Aortic stiffness.** Aortic stiffness was assessed by CFPWV, as previously described.²²
174 Briefly, CFPWV was determined by applanation tonometry with simultaneous electrocardiogram
175 gating of the R-wave to measure the time delay between the foot of the carotid and femoral
176 arterial pressure waves; PWV was calculated as the distance between arterial sites (m) divided
177 by the arterial pressure wave transit time (s) at each site, with automated software (Non-
178 Invasive Hemodynamics Workstation, Cardiovascular Engineering Inc.).

179 **Data analysis.** Statistical analyses were performed with G*Power 3.1 and GraphPad
180 Prism version 7. Sample size was estimated based on an effect size to detect significant group
181 differences in our primary outcome variable (FMD: 0.7) calculated from our laboratory's previous
182 intervention studies.²³⁻²⁶ Our sample size estimate indicated that n=18 participants would be

183 adequate to detect a difference with >80% power at the $\alpha=0.05$ level; to account for a potentially
184 lower effect size and subject attrition, we enrolled 24 participants in the study. The impact of
185 MitoQ on outcome variables was evaluated with paired t-tests. Given the crossover design, we
186 also tested for the presence of a carryover effect for each of the outcomes using a mixed model
187 ANOVA, with treatment order as a between group factor (no carryover effects were observed
188 between conditions). The acute effect of supra-therapeutic MitoQ administration on FMD
189 following chronic MitoQ and placebo supplementation was assessed with a 2-way repeated
190 measures ANOVA and Least Significant Differences *post-hoc* analysis if a significant interaction
191 was found. Data are expressed as mean \pm standard error (SEM). Statistical significance was set
192 *a priori* at $\alpha=0.05$.

193

194 **Results**

195 ***Participants.*** Fifty-five individuals were consented for the study. Twenty-six did not meet
196 inclusion criteria. Five individuals withdrew from the study prior to randomization due to the time
197 commitment (n=4) or study restrictions (n=1). Of the remaining 24 participants, 13 participants
198 were randomized to Group A, which received MitoQ first followed by placebo, and 11 subjects
199 were randomized to Group B, which received placebo first followed by MitoQ. Three participants
200 withdrew from Group A (procedure invasiveness, non-study related injury, and personal
201 emergency). One participant was excluded from Group B because of an abnormal ECG (atrial
202 fibrillation). The participant characteristics and clinical blood markers of the 20 participants who
203 completed the trial are shown in Table 1. Anthropometrics, brachial artery blood pressure,
204 fasting blood lipids, glucose and physical activity levels were unchanged with the intervention
205 (Table 1).

206 ***Safety and tolerability.*** Adherence to the intervention was excellent, with all
207 participants consuming greater than 90% of all MitoQ and placebo capsules administered. The

208 20 mg/day dose of MitoQ was well tolerated, and no treatment-related serious adverse events
209 occurred. A total of 7 treatment-emergent adverse events were reported by 7 of the 24
210 participants who were enrolled in the study. All self-reported adverse events were mild in
211 severity and included gastrointestinal discomfort (n=1) during the MitoQ condition and
212 gastrointestinal discomfort (n=2) and diarrhea (n=1) under the placebo condition; diarrhea (n=2)
213 and vomiting (n=1) were reported following acute administration of 160 mg of MitoQ. None of
214 the enrolled participants dropped out of the study because of side effects.

215 **Plasma MitoQ.** Plasma levels of MitoQ were higher after 6 weeks of MitoQ
216 supplementation vs. placebo (Figure 1). The observed levels of MitoQ in plasma are consistent
217 with the known pharmacokinetic profile of orally ingested MitoQ.^{10, 11} Moreover, the reported
218 plasma MitoQ levels likely represent the *lowest* values achieved during the MitoQ
219 supplementation phase because they were assessed 24-26 hours after the most recent dose
220 (~1 hour prior to the subsequent dose).

221 **Vascular endothelial function.** BAFMD was 42% higher following chronic MitoQ
222 supplementation vs. placebo (17/20 subjects had higher values after MitoQ vs. placebo, Figure
223 2). A post-hoc power analysis revealed an effect size of 0.66 and a statistical power of 80% for
224 the observed difference in BAFMD (primary outcome). Other brachial artery parameters were
225 not different between conditions (Supplementary Table 1). Smooth muscle sensitivity to NO
226 (endothelium-independent dilation), assessed by brachial artery dilation with sublingual
227 nitroglycerin, was not different between conditions (24±2% vs. 25±3%, for placebo and MitoQ,
228 respectively, P>0.1).

229 **MtROS-mediated suppression of vascular endothelial function.** Oral administration
230 of a single 160 mg dose of MitoQ increased BAFMD acutely (compared with the pre-
231 administration baseline level) after the placebo supplementation phase, but not after the MitoQ
232 supplementation phase (n=9; Figure 3). These results are consistent with the idea of reduced
233 mtROS-mediated suppression of EDD following 6 weeks of chronic MitoQ supplementation.

234 **Aortic stiffness.** There were no differences between conditions in CFPWV in the group
235 as a whole (placebo: 8.34 ± 0.46 m/s vs. MitoQ: 8.28 ± 0.41 m/s, $P > 0.1$). However, the overall
236 group consisted of a range of individual subject values in the untreated (placebo arm) condition,
237 and our previous preclinical study¹² found that MitoQ only reduced CFPWV in old mice (i.e., the
238 group with elevated baseline aortic PWV); there was no effect in young animals. Accordingly, to
239 determine if MitoQ treatment reduced CFPWV in subjects with elevated levels of CFPWV in the
240 untreated state, we separated our subjects into 2 subgroups based on the recent Framingham
241 Heart Study reported cutoff for “healthy vascular aging” of < 7.60 m/s, which is the mean +2
242 standard deviations value from a reference sample of individuals < 30 years old without CVD risk
243 factors.²⁷ The subgroup of our subjects with elevated aortic stiffness in the untreated state
244 ($n=11$) was slightly older (69 ± 1 vs. 65 ± 1 years, $P < 0.05$), and had higher systolic blood pressure
245 (118 ± 2 vs. 105 ± 2 mmHg, $P < 0.05$) and CFPWV (9.74 ± 0.44 vs. 6.52 ± 0.16 m/s, $P < 0.05$) values,
246 but the cohorts were otherwise not different. CFPWV was lower following MitoQ
247 supplementation in the cohort with CFPWV values > 7.60 m/s (9.25 ± 0.39 m/s; $P < 0.05$), but was
248 unaltered in the cohort with CFPWV values < 7.60 m/s (6.71 ± 0.33 m/s; $P > 0.1$) (Figure 4). MitoQ
249 did not differentially affect blood pressure in the 2 subgroups.

250 **Circulating markers of oxidative stress and inflammation.** Plasma concentrations of
251 oxidized LDL, a circulating marker of oxidative stress, were 13% lower following 6 weeks of
252 MitoQ supplementation (Figure 5). There were no differences in circulating markers of
253 inflammation, as assessed by C-reactive protein (placebo: 1.7 ± 0.4 mg/L vs. MitoQ: 1.5 ± 0.3
254 mg/L, $P > 0.1$) and IL-6 (placebo: 1.3 ± 0.1 mg/L vs. MitoQ: 1.4 ± 0.2 mg/L, $P > 0.1$).

255

256 **Discussion**

257 In the current study, we translated our recent preclinical findings to humans using a
258 rigorous randomized, placebo-controlled, double-blind crossover study design to evaluate the

259 effects of the mitochondrial-targeted antioxidant MitoQ on vascular function in healthy late MA/O
260 adults. We show that chronic administration of MitoQ increased plasma MitoQ levels and was
261 safe and well-tolerated over the treatment duration (6 weeks) studied here. Most importantly, we
262 found that supplementation with MitoQ improved our primary outcome, BAFMD (endothelial
263 function), in this group with normal (modest) age-related impairments in baseline FMD, and
264 present evidence that this improvement was mediated, at least in part, by a suppression of
265 mtROS. MitoQ treatment also reduced aortic stiffness in participants exhibiting age-related
266 aortic stiffening in the untreated state, and decreased plasma oxidized LDL, a marker of
267 oxidative stress, without altering circulating markers of inflammation or traditional CVD risk
268 factors. Collectively, these findings provide support for the concept that MitoQ, and perhaps
269 other strategies targeting mtROS, may be novel therapeutic options for improving vascular
270 function and reducing the risk of age-related CVD.

271 Preclinical findings from our laboratory show that oral MitoQ supplementation restores
272 age-related decreases in EDD in old mice.⁶ To our knowledge, no prior studies have evaluated
273 supplementation with MitoQ, or any other mitochondria-specific antioxidant, for improving
274 vascular endothelial function in humans. In the present investigation, we extend our previous
275 findings on aging in mice to healthy MA/O adults, and demonstrate for the first time that 6 weeks
276 of MitoQ supplementation improves BAFMD, a measure of NO-mediated EDD and independent
277 predictor of incident CVD risk.^{28, 29} The improvement in BAFMD with MitoQ occurred without a
278 change in endothelium-independent dilation, indicating unaltered vascular smooth muscle
279 sensitivity to NO and, therefore, that the increase in FMD with treatment reflects an
280 improvement in endothelium-specific NO-mediated dilation. BAFMD was improved by 42% with
281 MitoQ in the overall group, and increases were observed in both men and women. This
282 magnitude of improvement in BAFMD is within the range produced by healthy lifestyle
283 interventions known for exerting a strong physiological stimulus, such as caloric restriction-
284 based weight loss (~30% improvement)²³ and aerobic exercise (~50% improvement in men),³⁰

285 when applied for longer durations (up to 3 months) than the present 6-week treatment period.
286 Given that most MA/O adults do not meet current guidelines for healthy lifestyle behaviors,³¹⁻³³
287 MitoQ supplementation may represent an alternative or complementary pharmacological
288 strategy for enhancing vascular endothelial function in this group.

289 We found previously that acute *ex vivo* incubation with MitoQ increased EDD of isolated
290 arterial segments from old mice,⁶ and others have reported similar results in skeletal muscle
291 feed arteries biopsied from older adult humans,⁷ both of which indicate that age-related
292 impairments in EDD are mediated by excess mtROS. Furthermore, in our preclinical study,
293 chronic MitoQ supplementation ameliorated excess mtROS-mediated suppression of EDD in old
294 mice, as shown by no change in EDD with acute *ex vivo* incubation of isolated arterial segments
295 with MitoQ after 4 weeks of oral treatment with MitoQ.⁶ In the current study, we extend these
296 findings to an *in vivo* setting in humans, as we observed an improvement in EDD in response to
297 an acute supra-therapeutic dose of MitoQ following the placebo phase, which revealed the
298 presence of tonic inhibition of EDD (BAFMD) by mtROS. The inhibition of EDD by mtROS was
299 abolished following chronic MitoQ supplementation, as indicated by a lack of further increase in
300 EDD with acute supra-therapeutic administration of MitoQ. These observations in older humans
301 support our previous observations in old mice that MitoQ improves vascular endothelial function,
302 at least in part, by reducing mitochondria-derived oxidative stress.

303 Our preclinical findings show that MitoQ treatment reduced aortic stiffness, as assessed
304 by aortic PWV, in old mice.¹² MitoQ did not alter aortic stiffness in young mice, suggesting
305 favorable effects of MitoQ only in a setting of age-related aortic stiffening.¹² The current study
306 translates these findings to humans by showing that MitoQ lowers CFPWV in late MA/O adults
307 exhibiting age-related aortic stiffening based on recently published cutoffs for CFPWV from the
308 Framingham Heart Study.²⁷ This finding has potentially important clinical implications in that
309 CFPWV is the gold-standard *in vivo* measure of aortic stiffness and an independent predictor of
310 CVD risk and other common disorders of aging, including cognitive impairment, chronic kidney

311 disease and frailty.^{22, 34-37} MitoQ did not alter aortic stiffness in participants with CFPWV <7.60
312 m/s, consistent with our previous results in young mice.¹²

313 The mechanisms responsible for the de-stiffening effects of MitoQ in our subjects with
314 elevated CFPWV are not obvious. Endothelial function affects arterial stiffness by influencing
315 vascular smooth muscle tone^{22, 38} and average improvements in BAFMD tended to be greater
316 (51% vs. 36% increase) in the subgroup with elevated initial CFPWV levels, which may have
317 contributed to their reductions in CFPWV. Our recent findings in mice indicate that MitoQ
318 treatment improved aortic stiffness in old animals by influencing the elastin component of
319 intrinsic wall stiffness.¹² However, the 6-week supplementation period in the present study in
320 humans seems too brief to induce changes in structural proteins in the aortic wall and, instead,
321 suggests functional influences on smooth muscle tone, perhaps linked to reductions in oxidative
322 stress, as we have reported previously in postmenopausal estrogen-deficient women.³⁹
323 Importantly, blood pressure was unaltered in the subgroup demonstrating reductions in CFPWV
324 with MitoQ treatment, indicating that this was not a mechanism. Overall, although future studies
325 are needed to elucidate responsible mechanisms, our findings provide the first evidence for
326 chronic MitoQ supplementation lowering aortic stiffness in late MA/O adults with age-related
327 aortic stiffening. These data have important implications for reducing the risk of CVD and
328 numerous other age-related disorders linked with elevated aortic stiffness.

329 In our preclinical studies in mice, MitoQ supplementation normalized age-related
330 increases in oxidative stress without affecting pro-inflammatory cytokine levels in old animals.^{6,}
331 ¹² Consistent with this notion, in the present study MitoQ supplementation was associated with a
332 decrease in plasma oxidized LDL, a circulating marker of oxidative stress, as well as with
333 amelioration of mtROS-mediated suppression of EDD. MitoQ supplementation did not alter
334 circulating markers of inflammation in the current study; moreover, BMI, blood lipids, glucose
335 and blood pressure were unchanged with MitoQ. These data are in agreement with the lack of
336 change in these potential modulatory factors observed in our preclinical studies^{6, 12} and suggest

337 that improvements in vascular function observed with MitoQ were independent of overt changes
338 in traditional CVD risk factors and inflammation. Taken together, these observations support a
339 reduction in oxidative stress as an important mechanism underlying improvements in vascular
340 function with MitoQ.

341 A limitation of the current study is that we did not directly assess the effect of MitoQ on
342 ROS production by mitochondria. However, the ability of MitoQ to both chronically and acutely
343 reduce mtROS in the vasculature is supported by: 1) our preclinical study showing that chronic
344 MitoQ supplementation in old mice reduced aortic mitochondrial superoxide,⁶ assessed with a
345 mitochondria-specific spin probe and electron paramagnetic resonance (EPR) spectroscopy -
346 the gold-standard and most direct measure of ROS;⁴⁰ and 2) *ex vivo* experiments in biopsied
347 skeletal muscle feed arteries from humans demonstrating that 1 hour of MitoQ incubation
348 improved EDD, which was associated with reduced EPR spectroscopy-based measures of
349 mitochondrial superoxide levels.⁷ Regarding the latter assessments of mtROS in human tissue,
350 we could not perform these invasive analyses in the current clinical intervention study; however,
351 our innovative acute MitoQ administration paradigm is a direct translation of this experimental
352 model to humans *in vivo* and provides evidence for reduced mtROS as a likely mechanism of
353 action of chronic MitoQ supplementation.

354

355 **Perspectives**

356 Here, we demonstrate for the first time that supplementation with the mitochondria-
357 targeted antioxidant MitoQ is safe and well tolerated in late MA/O adults, improves vascular
358 endothelial function (likely by suppressing excess mtROS), reduces aortic stiffness in MA/O
359 adults with elevated initial levels, and decreases oxidized LDL, a circulating marker of oxidative
360 stress. Collectively, these findings establish the experimental basis for conducting a larger scale
361 clinical trial in older adults or clinical populations, particularly those associated with endothelial
362 dysfunction and/or elevated aortic stiffness. In the broadest terms, our results provide initial

363 support for the idea that MitoQ and, potentially other mitochondria-targeted antioxidants, may be
364 an effective treatment for improving vascular function and possibly decreasing the risk of CVD
365 and other clinical disorders of aging, including cognitive dysfunction and chronic kidney disease.
366

367 **Acknowledgements**

368 The authors thank the staff of the University of Colorado Boulder CTRC for their
369 technical assistance.
370

371 **Funding**

372 This work was supported by NIH awards AG049451, AG000279, AG053009, Colorado
373 CTSA UL1 TR001082 and an industry contract with MitoQ Limited. MPM is supported by UK
374 MRC MC_U105663142 and as a Wellcome Trust Investigator (110159/Z/15/Z).

375 Contents are the authors' sole responsibility and do not necessarily represent official
376 NIH views.
377

378 **Disclosures**

379 MPM is on the scientific advisory board of Antipodean Pharmaceuticals. All other
380 authors have no declarations of interest to disclose.
381

382 **Author Contributions**

383 Initial study design [MJR, JSP, RGR, MC, DRS], data acquisition/analysis [MJR, JSP,
384 CCS, LMC, HLR, NZB, KW, MPM], data interpretation [MJR, MPM, DRS], creating
385 tables/figures [MJR], writing initial draft [MJR], editing and approving final draft [MJR, JSP, CCS,
386 NZB, LMC, HLR, RGR, MC, MPM, DRS].

387 **References**

- 388 1. Benjamin EJ, Blaha MJ, Chiuve SE, et al. Heart disease and stroke statistics-2017
389 update: A report from the american heart association. *Circulation*. 2017;135:e146-e603
- 390 2. Weintraub WS, Daniels SR, Burke LE, et al. Value of primordial and primary prevention
391 for cardiovascular disease: A policy statement from the american heart association.
392 *Circulation*. 2011;124:967-990
- 393 3. Lakatta EG, Levy D. Arterial and cardiac aging: Major shareholders in cardiovascular
394 disease enterprises: Part i: Aging arteries: A "set up" for vascular disease. *Circulation*.
395 2003;107:139-146
- 396 4. Widlansky ME, Gokce N, Keaney JF, Jr., Vita JA. The clinical implications of endothelial
397 dysfunction. *J Am Coll Cardiol*. 2003;42:1149-1160
- 398 5. Seo AY, Joseph AM, Dutta D, Hwang JC, Aris JP, Leeuwenburgh C. New insights into
399 the role of mitochondria in aging: Mitochondrial dynamics and more. *J Cell Sci*.
400 2010;123:2533-2542
- 401 6. Gioscia-Ryan RA, LaRocca TJ, Sindler AL, Zigler MC, Murphy MP, Seals DR.
402 Mitochondria-targeted antioxidant (mitoq) ameliorates age-related arterial endothelial
403 dysfunction in mice. *J Physiol*. 2014;592:2549-2561
- 404 7. Park SY, Kwon OS, Andtbacka RHI, Hyngstrom JR, Reese V, Murphy MP, Richardson
405 RS. Age-related endothelial dysfunction in human skeletal muscle feed arteries: The role
406 of free radicals derived from mitochondria in the vasculature. *Acta Physiol (Oxf)*.
407 2018;222

- 408 8. Heidenreich PA, Trogdon JG, Khavjou OA, et al. Forecasting the future of cardiovascular
409 disease in the united states: A policy statement from the american heart association.
410 *Circulation*. 2011;123:933-944
- 411 9. Olshansky SJ, Goldman DP, Zheng Y, Rowe JW. Aging in america in the twenty-first
412 century: Demographic forecasts from the macarthur foundation research network on an
413 aging society. *Milbank Q*. 2009;87:842-862
- 414 10. Murphy MP, Smith RA. Targeting antioxidants to mitochondria by conjugation to
415 lipophilic cations. *Annu Rev Pharmacol Toxicol*. 2007;47:629-656
- 416 11. Smith RA, Murphy MP. Animal and human studies with the mitochondria-targeted
417 antioxidant mitoq. *Ann N Y Acad Sci*. 2010;1201:96-103
- 418 12. Gioscia-Ryan RA, Battson ML, Cuevas LM, Eng JS, Murphy MP, Seals DR.
419 Mitochondria-targeted antioxidant therapy with mitoq ameliorates aortic stiffening in old
420 mice. *J Appl Physiol (1985)*. 2017;jap 00670 02017
- 421 13. Gane EJ, Weilert F, Orr DW, Keogh GF, Gibson M, Lockhart MM, Frampton CM, Taylor
422 KM, Smith RA, Murphy MP. The mitochondria-targeted anti-oxidant mitoquinone
423 decreases liver damage in a phase ii study of hepatitis c patients. *Liver Int*.
424 2010;30:1019-1026
- 425 14. Snow BJ, Rolfe FL, Lockhart MM, Frampton CM, O'Sullivan JD, Fung V, Smith RA,
426 Murphy MP, Taylor KM, Protect Study G. A double-blind, placebo-controlled study to
427 assess the mitochondria-targeted antioxidant mitoq as a disease-modifying therapy in
428 parkinson's disease. *Mov Disord*. 2010;25:1670-1674

- 429 15. Shill DD, Southern WM, Willingham TB, Lansford KA, McCully KK, Jenkins NT.
430 Mitochondria-specific antioxidant supplementation does not influence endurance
431 exercise training-induced adaptations in circulating angiogenic cells, skeletal muscle
432 oxidative capacity or maximal oxygen uptake. *J Physiol.* 2016;594:7005-7014
- 433 16. Harris RA, Nishiyama SK, Wray DW, Richardson RS. Ultrasound assessment of flow-
434 mediated dilation. *Hypertension.* 2010;55:1075-1085
- 435 17. Lohman TG RAaMR. Anthropometric standardization reference manual. *Human*
436 *Kinetics.* 1988
- 437 18. Pereira MA, FitzGerald SJ, Gregg EW, Joswiak ML, Ryan WJ, Suminski RR, Utter AC,
438 Zmuda JM. A collection of physical activity questionnaires for health-related research.
439 *Med Sci Sport Exerc.* 1997;29:S1-205
- 440 19. DeVan AE, Johnson LC, Brooks FA, et al. Effects of sodium nitrite supplementation on
441 vascular function and related small metabolite signatures in middle-aged and older
442 adults. *J Appl Physiol (1985).* 2016;120:416-425
- 443 20. Li Y, Zhang H, Fawcett JP, Tucker IG. Quantitation and metabolism of mitoquinone, a
444 mitochondria-targeted antioxidant, in rat by liquid chromatography/tandem mass
445 spectrometry. *Rapid Commun Mass Spectrom.* 2007;21:1958-1964
- 446 21. Eskurza I, Monahan KD, Robinson JA, Seals DR. Effect of acute and chronic ascorbic
447 acid on flow-mediated dilatation with sedentary and physically active human ageing. *J*
448 *Physiol.* 2004;556:315-324

- 449 22. Townsend RR, Wilkinson IB, Schiffrin EL, et al. Recommendations for improving and
450 standardizing vascular research on arterial stiffness: A scientific statement from the
451 american heart association. *Hypertension*. 2015;66:698-722
- 452 23. Pierce GL, Beske SD, Lawson BR, Southall KL, Benay FJ, Donato AJ, Seals DR. Weight
453 loss alone improves conduit and resistance artery endothelial function in young and
454 older overweight/obese adults. *Hypertension*. 2008;52:72-79
- 455 24. Seals DR, Tanaka H, Clewenger CM, Monahan KD, Reiling MJ, Hiatt WR, Davy KP,
456 DeSouza CA. Blood pressure reductions with exercise and sodium restriction in
457 postmenopausal women with elevated systolic pressure: Role of arterial stiffness. *J Am
458 Coll Cardiol*. 2001;38:506-513
- 459 25. Jablonski KL, Racine ML, Geolfos CJ, Gates PE, Chonchol M, McQueen MB, Seals DR.
460 Dietary sodium restriction reverses vascular endothelial dysfunction in middle-aged/older
461 adults with moderately elevated systolic blood pressure. *J Am Coll Cardiol*. 2013;61:335-
462 343
- 463 26. Santos-Parker JR, Strahler TR, Bassett CJ, Bispham NZ, Chonchol MB, Seals DR.
464 Curcumin supplementation improves vascular endothelial function in healthy middle-
465 aged and older adults by increasing nitric oxide bioavailability and reducing oxidative
466 stress. *Aging (Albany NY)*. 2017;9:187-208
- 467 27. Niiranen TJ, Lyass A, Larson MG, Hamburg NM, Benjamin EJ, Mitchell GF, Vasan RS.
468 Prevalence, correlates, and prognosis of healthy vascular aging in a western community-
469 dwelling cohort: The framingham heart study. *Hypertension*. 2017;70:267-274

- 470 28. Yeboah J, Crouse JR, Hsu FC, Burke GL, Herrington DM. Brachial flow-mediated
471 dilation predicts incident cardiovascular events in older adults: The cardiovascular health
472 study. *Circulation*. 2007;115:2390-2397
- 473 29. Yeboah J, Folsom AR, Burke GL, Johnson C, Polak JF, Post W, Lima JA, Crouse JR,
474 Herrington DM. Predictive value of brachial flow-mediated dilation for incident
475 cardiovascular events in a population-based study: The multi-ethnic study of
476 atherosclerosis. *Circulation*. 2009;120:502-509
- 477 30. Pierce GL, Eskurza I, Walker AE, Fay TN, Seals DR. Sex-specific effects of habitual
478 aerobic exercise on brachial artery flow-mediated dilation in middle-aged and older
479 adults. *Clin Sci*. 2010;120:13-23
- 480 31. Douketis JD, Macie C, Thabane L, Williamson DF. Systematic review of long-term
481 weight loss studies in obese adults: Clinical significance and applicability to clinical
482 practice. *Int J Obes (Lond)*. 2005;29:1153-1167
- 483 32. Saida T, Juul Sorensen T, Langberg H. Long-term exercise adherence after public
484 health training in at-risk adults. *Ann Phys Rehabil Med*. 2017;60:237-243
- 485 33. Wing RR, Phelan S. Long-term weight loss maintenance. *Am J Clin Nutri*.
486 2005;82:222S-225S
- 487 34. Ben-Shlomo Y, Spears M, Boustred C, et al. Aortic pulse wave velocity improves
488 cardiovascular event prediction: An individual participant meta-analysis of prospective
489 observational data from 17,635 subjects. *J Am Coll Cardiol*. 2014:636-646

- 490 35. Mitchell GF, Hwang SJ, Vasani RS, Larson MG, Pencina MJ, Hamburg NM, Vita JA,
491 Levy D, Benjamin EJ. Arterial stiffness and cardiovascular events: The framingham heart
492 study. *Circulation*. 2010;121:505-511
- 493 36. Pase MP, Beiser A, Himali JJ, Tsao C, Satizabal CL, Vasani RS, Seshadri S, Mitchell
494 GF. Aortic stiffness and the risk of incident mild cognitive impairment and dementia.
495 *Stroke*. 2016;47:2256-2261
- 496 37. Abbatecola AM, Chiodini P, Gallo C, Lakatta E, Sutton-Tyrrell K, Tyllavsky FA,
497 Goodpaster B, de Rekeneire N, Schwartz AV, Paolisso G, Harris T, Health ABCs. Pulse
498 wave velocity is associated with muscle mass decline: Health abc study. *Age (Dordr)*.
499 2012;34:469-478
- 500 38. Wilkinson IB, MacCallum H, Cockcroft JR, Webb DJ. Inhibition of basal nitric oxide
501 synthesis increases aortic augmentation index and pulse wave velocity in vivo. *Br J Clin*
502 *Pharmacol*. 2002;53:189-192
- 503 39. Moreau KL, Gavin KM, Plum AE, Seals DR. Ascorbic acid selectively improves large
504 elastic artery compliance in postmenopausal women. *Hypertension*. 2005;45:1107-1112
- 505 40. Griendling KK, Touyz RM, Zweier JL, Dikalov S, Chilian W, Chen YR, Harrison DG,
506 Bhatnagar A, American Heart Association Council on Basic Cardiovascular S.
507 Measurement of reactive oxygen species, reactive nitrogen species, and redox-
508 dependent signaling in the cardiovascular system: A scientific statement from the
509 american heart association. *Circ Res*. 2016;119:e39-75
- 510
- 511
- 512

513 **Novelty and Significance**

514
515 **What Is New?**

- 516 • This is the first trial in humans to show that 6 weeks of daily oral supplementation with
517 the mitochondrial-targeted antioxidant MitoQ improves vascular endothelial function.
- 518 • We provide the first *in vivo* evidence in humans that MitoQ improves endothelial function
519 by suppressing mitochondrial-derived oxidative stress.
- 520 • We provide the initial evidence in humans that MitoQ supplementation reduces aortic
521 stiffness in adults with elevated baseline levels.

522
523 **What Is Relevant?**

- 524 • Older individuals have a greater risk for cardiovascular diseases largely because of
525 vascular dysfunction, including reduced endothelial function and aortic stiffening. Thus, it
526 is important to establish evidence-based therapeutic options to improve vascular
527 function in this group.
- 528 • This study demonstrates that mitochondrial-targeted antioxidant MitoQ improves
529 endothelial function and aortic stiffness in healthy late middle-aged and older adults with
530 impaired baseline vascular function, thus establishing initial evidence for efficacy.

531
532 **Summary**

- 533 • MitoQ supplementation improved vascular endothelial function in healthy late middle-
534 aged and older adults by reducing the tonic suppressive effects of excessive
535 mitochondrial-specific reactive oxygen species. MitoQ also reduced aortic stiffness in
536 individuals with age-related arterial stiffening.

537
538

539 **Figure Legends**

540

541 **Figure 1.** Plasma levels of MitoQ after 6 weeks of placebo or MitoQ supplementation,
542 assessed ~24 hours after the last dose. Values are presented as mean±SEM. *P<0.05 vs.
543 placebo.

544

545 **Figure 2.** Brachial artery flow-mediated dilation (BAFMD) expressed as percent (left) and
546 absolute (right) change after 6 weeks of placebo or MitoQ supplementation. Values are
547 presented as mean±SEM, with individual responses below. *P<0.05 vs. placebo.

548

549 **Figure 3.** Brachial artery flow-mediated dilation (BAFMD) before (-) and 1 hour after (+) acute
550 ingestion of 160 mg of MitoQ following 6 weeks of placebo or MitoQ supplementation assessed
551 in a subset of n=9 participants. Values are presented as mean±SEM. ‡P<0.05 vs. before
552 ingestion of 160 mg MitoQ; *P<0.05 vs. placebo.

553

554 **Figure 4.** Carotid-femoral pulse wave velocity (CFPWV) after 6 weeks of placebo or MitoQ
555 supplementation. Participants were separated into 2 subgroups, based on placebo (untreated)
556 CFPWV values above and below 7.60 m/s (demarcated by the dashed line). Values are
557 presented as mean±SEM and individual responses are depicted. *P<0.05 vs. placebo.

558

559 **Figure 5.** Plasma levels of oxidized low-density lipoprotein (LDL), a circulating marker of
560 oxidative stress, after 6 weeks of placebo or MitoQ supplementation. Values are presented as
561 mean±SEM. *P<0.05 vs. placebo.

562

563

564 **Table 1. Participant Characteristics**

<i>Characteristics</i>	Placebo	MitoQ
N, men/women	9/11	--
Age, years	68±1	--
Body mass index, kg/m ²	23±1	23±1
Systolic blood pressure, mmHg	113±3	114±4
Diastolic blood pressure, mmHg	67±1	67±1
Resting heart rate, beats/min	62±2	61±1
Physical activity caloric expenditure, kcal/wk	5201±570	5326±726
Total cholesterol, mmol/L	4.9±0.2	4.9±0.2
High-density lipoprotein cholesterol, mmol/L	1.3±0.1	1.3±0.1
Low-density lipoprotein cholesterol, mmol/L	3.1±0.2	3.0±0.2
Triglycerides, mmol/L	1.2±0.2	1.2±0.1
Glucose, mmol/L	4.7±0.1	4.6±0.1

565 *Data are mean±SEM*

566

567

568

569

570

571

572

573