blood advances

American Society of Hematology
2021 L Street NW, Suite 900,
Washington, DC 20036
Phone: 202-776-0544 | Fax 202-776-0545
bloodadvances@hematology.org

Isoquercetin for thromboinflammation in Sickle Cell Disease: a randomized double-blind placebo-controlled trial

Tracking no: ADV-2023-011542R1

Maria Lizarralde-Iragorri (NIH, United States) Bindu Parachalil Gopalan (NHLBI/NIH, United States) Brenda Merriweather (Boston Medical Center, United States) Jennifer Brooks (NHLBI/NIH, United States) Mai Hill (NHLBI/NIH, United States) Dianna Lovins (NHLBI/NIH, United States) Ruth Pierre-Charles (NHLBI/NIH, United States) Ann Cullinane (DLM/NIH, United States) Alina Dulau-Florea (NIH Clinical Center, United States) Duck-Yeon Lee (NHLBI/NIH, United States) Rafael Villasmil (NEI/NIH, United States) Neal Jeffries (National Heart, Lung, and Blood Institute, United States) Arun Shet (NHLBI/NIH, United States)

Abstract:
Data from a small trial in cancer patients suggest that isoquercetin treatment lowered thrombosis biomarkers and prevented clinical thrombosis but no studies of isoquercetin have been conducted to target thromboinflammation in adults with sickle cell disease (SCD). We conducted a randomized, double-blind, placebo-controlled trial in adults with steady state SCD (HbSS or HbSß0thal or HbSß+thal or HbSC). The primary outcome was the change in plasma soluble P-selectin (sP-selectin) post-treatment compared to baseline, analyzed in the intention-to-treat population. Between November 2019 and July 2022, 46 patients (age 40 {plus minus} 11 years, 56% female, 75% under hydroxyurea treatment) were randomized to receive isoquercetin (n=23) or placebo (n=23). Isoquercetin was well tolerated and all the adverse events (AEs=21) or serious AEs (14) recorded were not attributable to the study drug. The mean post-treatment change for sP-selectin showed no significant difference between the treatment groups (IQ=0.10 {plus minus} 6.53 vs. placebo=0.74 {plus minus} 4.54; p=0.64). In isoquercetin treated patients, whole blood coagulation (p=0.03) and collagen-induced platelet aggregation (p=0.03) were significantly reduced from the baseline. Inducible mononuclear cell tissue factor gene expression and plasma PDI reductase activity were also significantly inhibited (p= 0.003 and 0.02 respectively). Short term fixed dose isoquercetin in patients with SCD was safe with no off-target bleeding and was associated with changes from the baseline in the appropriate direction for several biomarkers of thromboinflammation. The trial is registered with clinicaltrials.gov (NCT04514510).

Conflict of interest: No COI declared

COI notes:

Preprint server: No;

Author contributions and disclosures: M.A.L.I. and B.P.G. managed conduct of samples and performance of laboratory assays and contributed to data analysis and drafting of the manuscript; A.S.S. developed the protocol and, with the help of study statistician N.J. analyzed results, generated a clinical study report, and prepared the manuscript; A.S.S. was the principal investigator and provided medical oversight of the trial; A.DF., A.C., R.V., D-Y. L. conducted laboratory assays and contributed to data analysis; B.M., J.B., M.H., D.L., and R.P-C. participated in protocol review, patient recruitment, patient management, and collection of clinical outcomes; and all authors reviewed the manuscript with opportunity to provide input.

Non-author contributions and disclosures: No;

Agreement to Share Publication-Related Data and Data Sharing Statement: Individual participant data will not be shared. Original experimental and clinical trial data can be obtained by contacting the corresponding author.

Clinical trial registration information (if any): NCT04514510
https://classic.clinicaltrials.gov/ct2/show/NCT04514510

Isoquercetin for thromboinflammation in Sickle Cell Disease: a randomized double-blind placebo-controlled trial

*Maria A. Lizarralde-Iragorri[1], *Bindu Parachalil Gopalan[1], Brenda Merriweather[2], Jennifer Brooks[3], Mai Hill[3], Dianna Lovins[3], Ruth Pierre-Charles[3], Ann Cullinane[4], Alina Dulau-Florea[4], Duck-Yeon Lee[5], Rafael Villasmil[6], Neal Jeffries[7], Arun S. Shet[1]

[1]Sickle Thrombosis and Vascular Biology Lab, Sickle Cell Branch, National Heart Lung and Blood Institute, National Institutes of Health, Bethesda, MD, USA

[2]Sickle Cell Branch, National Heart Lung and Blood Institute, National Institutes of Health, Bethesda, MD, USA

[3]Office of the Clinical Director, National Heart Lung and Blood Institute, National Institutes of Health, Bethesda, MD, USA

[4] Department of Laboratory Medicine, Clinical Center, National Institutes of Health, Bethesda, MD, USA

[5]Biochemistry Core Facility, National Heart Lung and Blood Institute, National Institutes of Health, Bethesda, MD, USA

[6] Flow Cytometry Core Facility, National Eye Institute, National Institutes of Health, Bethesda, MD, USA

[7]Office of Biostatistics Research, National Heart Lung and Blood Institute, National Institutes of Health, Bethesda, MD, USA.

* M.A.L.I. and B.P.G. contributed equally to this study.

Abstract: 228 words

23 Main text: 4003 words

24 Figures: 5

25 Tables: 3

26 Number of references: 39

27

28 **Address for Correspondence:**

29 Arun S. Shet

30 Sickle Cell Branch, National Heart, Lung, and Blood Institute

31 10 Center Drive, Building 10, Room 6S241 MSC 1589

32 Bethesda, MD 20892-1589

33 Phone: 301-435-2345

34 Email: arun.shet@nih.gov

35

36 Individual participant data will not be shared. Original experimental and clinical trial data can be

37 obtained by contacting the corresponding author.

38

39 **Key points**

40 - Short-term fixed-dose isoquercetin did not lower plasma sP-selectin in adults with

41 steady state SCD.

42 - Isoquercetin treatment attenuated blood coagulation, platelet aggregation and

43 inducible tissue factor gene expression in adults with SCD.

44

ABSTRACT

Data from a small trial in cancer patients suggest that isoquercetin treatment lowered thrombosis biomarkers and prevented clinical thrombosis but no studies of isoquercetin have been conducted to target thromboinflammation in adults with sickle cell disease (SCD). We conducted a randomized, double-blind, placebo-controlled trial in adults with steady state SCD (HbSS or HbSß0thal or HbSß$^+$thal or HbSC). The primary outcome was the change in plasma soluble P-selectin (sP-selectin) post-treatment compared to baseline, analyzed in the intention-to-treat population. Between November 2019 and July 2022, 46 patients (age 40 ± 11 years, 56% female, 75% under hydroxyurea treatment) were randomized to receive isoquercetin (n=23) or placebo (n=23). Isoquercetin was well tolerated and all the adverse events (AEs=21) or serious AEs (14) recorded were not attributable to the study drug. The mean post-treatment change for sP-selectin showed no significant difference between the treatment groups (IQ=0.10 ± 6.53 vs. placebo=0.74 ± 4.54; p=0.64). In isoquercetin treated patients, whole blood coagulation (p=0.03) and collagen-induced platelet aggregation (p=0.03) were significantly reduced from the baseline. Inducible mononuclear cell tissue factor gene expression and plasma PDI reductase activity were also significantly inhibited (p= 0.003 and 0.02 respectively). Short term fixed dose isoquercetin in patients with SCD was safe with no off-target bleeding and was associated with changes from the baseline in the appropriate direction for several biomarkers of thromboinflammation. The trial is registered with clinicaltrials.gov (NCT04514510).

67

68

69

70 **INTRODUCTION**

71 Sickle cell disease (SCD) is an inherited hemoglobin (Hb) disorder wherein a single-nucleotide
72 change in the β-globin gene leads to production of variant hemoglobin HbS ($\beta^{Glu6Val}$), that under
73 hypoxic conditions polymerizes causing "sickling" of red blood cells (RBCs).[1,2] Disease
74 manifestations characteristically include episodic disease flares termed acute vaso-occlusive
75 crises (VOCs), that accumulate over time and lead to vascular complications, end-organ damage
76 and reduce adult life expectancy. Although the backbone of treatment for SCD is hydroxyurea,
77 three new drugs (crizanlizumab, L-glutamine, and Voxelotor) were recently approved by the US
78 Food and Drug Administration. Yet, most of these disease-modifying therapies fail to
79 substantially reduce VOC frequency and are not widely available to the global sickle cell
80 community, leaving patients with this disease vulnerable to devastating complications.

81

82 HbS polymerization is seen as the primary driver of SCD pathophysiology, but a cascade of
83 interrelated events including hemolysis; activation and adhesion of neutrophils, monocytes,
84 platelets, and endothelial cells; sterile inflammation; and abnormal coagulation are increasingly
85 recognized as important contributors to clinical disease.[3] An inherent hypercoagulable state
86 heightens the risk for arterial and venous thrombosis in sickle cell patients, both outcomes that
87 are associated with higher mortality.[4-6] Unfortunately, even hydroxyurea treated patients
88 experience recurrent venous thromboembolism (VTE) necessitating lifelong anticoagulation,

89 which increases the risk of life threatening bleeding.[7] Thus, treatments targeting
90 thromboinflammatory pathophysiology in SCD with agents that lack bleeding side effects and
91 are widely implementable are a priority to investigate.
92
93 Accumulated evidence points to tissue factor (TF)-initiated thromboinflammation in both
94 patients,[8,9] and animal models of SCD, that favors systemic thrombin generation,[10] stasis,[11] and
95 end-organ damage.[12] Patients with SCD display "blood-borne" TF on the surface of
96 monocytes,[13] and vascular endothelial cells,[8] and on microvesicles derived from these cells,[14]
97 which increases further during VOC. TF-initiated coagulation is also regulated post
98 translationally by a vascular thiol-isomerase, protein disulfide isomerase (PDI).[15] In animal
99 models, release of endothelial and platelets derived PDI into the vasculature following vessel
100 injury as occurs during thromboinflammation facilitates TF-initiated thrombosis.[16] Our *in vitro*
101 studies demonstrate robust inhibition of monocyte and endothelial cell-surface TF expression
102 and cell-surface PDI reductase activity by a flavonoid, quercetin. Since isoquercetin the oral
103 bioavailable glucoside form of quercetin improved thrombosis biomarkers in cancer patients
104 without inducing bleeding[17] we tested its safety and efficacy to modulate
105 thromboinflammatory pathophysiology in SCD.
106
107 **METHODS**
108
109 ***Study design***
110 This was an investigator-initiated single-center, randomized, double-blind, placebo-controlled
111 phase II study conducted between 19th November 2019 and 7th July 2022. The trial consisted of

112 a 4-week screening phase and a 4-week (28 – 35 days) blinded treatment phase, followed by a
113 4-week follow up phase for assessing safety and adverse events. The study protocol was
114 approved by the NIH institutional review board and an FDA investigational new drug
115 (IND#150896) application and was registered with clinicaltrials.gov (NCT04514510).
116
117 ***Study participants***
118 Participants were ≥ 18 years of age with SCD defined by hemoglobin electrophoresis (HbSS or
119 HbSß^{0}thal or HbSß$^+$thal or HbSC) who were in their steady state defined as having no significant
120 complications (VOC or acute condition requiring hospitalization) or a blood transfusion
121 occurring within 2 months of the baseline visit. Patients receiving hydroxyurea were required to
122 be on a stable dose for ≥ 12 weeks prior to the baseline visit. Participants with a history of a
123 recent VOC or blood transfusion (< 2 months), VTE event (<3 month) or actively receiving
124 crizanlizumab therapy were excluded.
125
126 ***Randomization and blinding***
127 Participants meeting eligibility criteria were randomly assigned in a 1:1 ratio to receive 28-35
128 doses of either 1000 mg isoquercetin or identically matching placebo. The randomization
129 allocation was prepared by a statistician who was not part of the study team and shared
130 directly with the NIH pharmacy team who assumed responsibility for dispensing study drug per
131 allocation assignment. Participants and the study team remained blinded throughout study
132 conduct and during analysis of results. Unblinding of treatment allocations occurred after the
133 data was curated by the study team.

134

135 ***Intervention***

136 Isoquercetin dose and exposure duration were determined based on prior clinical experience
137 from a phase 2 clinical trial of isoquercetin in cancer-induced hypercoagulability[17]. The duration
138 of study drug exposure was reduced to 4 weeks to minimize confounding by the occurrence of
139 frequent VOC. Participants in the intervention group took oral isoquercetin (Quercis Pharma
140 AG, Zug, Switzerland) 1000mg administered orally once daily for at least 4 weeks ranging from
141 28 to a maximum of 35 days. Isoquercetin was supplied as capsules of 250 mg active dosage
142 strength isoquercetin blended with 62 mg of Vitamin C/Ascorbic acid and 5 mg of Vitamin
143 B3/Nicotinic acid. Participants in the control group took identically formulated placebo
144 containing 62 mg of Vitamin C/Ascorbic acid and 5 mg of Vitamin B3/Nicotinic acid.

145

146 ***Sample collection***

147 Blood samples for the primary and secondary endpoints were obtained at baseline and post-
148 treatment. Platelet free plasma (PFP) was prepared from citrated anticoagulated blood by
149 double centrifugation at 2,500 x *g* for 15 min within 30 min of sample collection and stored at –
150 80° C until batch analysis. Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA
151 anticoagulated blood by gradient centrifugation (Histopaque-1077, Sigma-Aldrich) and stored at
152 –80° C until analysis.

153

154 ***Primary outcome***

155 The primary endpoint of the trial was the change in plasma soluble P-selectin (sP-selectin) after
156 4 weeks of treatment from baseline in the isoquercetin group compared with placebo assessed
157 by ELISA (Human P-Selectin/CD62P Quantikine ELISA Kit, R&D Systems, #SPSE00). To adhere to
158 the intention to treat principle, for every patient that was randomized, we attempted to obtain
159 28-35 day endpoint measurements despite occurrence of expected and/or unexpected events
160 due to the underlying disease.

161

162 ***Secondary outcomes***

163 ***Safety***

164 Safety assessments performed during screening (visit #1), baseline (visit #2), post treatment
165 (visit #3) and at the end of study (visit #4) included SCD focused medical history, concomitant
166 medication use, side effects, physical examination, vital signs, adverse events (AEs) and serious
167 AEs (SAEs) and clinical laboratory tests (comprehensive metabolic panel, complete and
168 differential blood count, and urinalysis). The medical records of SAEs were obtained and
169 reviewed to determine study relatedness. The principal investigator and study team reviewed
170 the safety parameters and determined whether the intervention required modification as
171 defined in the study protocol.

172

173 ***Biomarkers of thromboinflammatory pathophysiology***

174 **Whole blood coagulability**: Thromboelastography (TEG) was performed in citrate
175 anticoagulated whole blood within 30 min of phlebotomy using the TEG 5000® Analyzer
176 (Haemonetics UK Ltd., Coventry, UK). The parameters of interest included reaction time (R), clot

177 kinetics (K), rate of clot formation (α-angle), maximal amplitude (MA) and coagulation index
178 (CI). CI > +3.0 indicates hypercoagulability and CI < -3.0 indicates hypocoagulability.[18]

179 **Platelet aggregation**: Platelet aggregometry was performed using whole blood optical
180 lumiaggregometry (Model 700 Whole Blood/Optical Lumi-aggregometer, CHRONO-LOG).
181 Maximal platelet aggregation using electrical impedance in whole blood samples was
182 determined following stimulation by various platelet agonists under conditions of continuous
183 stirring (1200 rpm).[19]

184 **Plasma TF$^+$ microvesicles:** Tissue factor positive microvesicles (TF+ MVs) in PFP were detected
185 and enumerated by flow cytometry using a high resolution flow cytometer (CytoFLEX, Beckman
186 Coulter Inc. CA, USA) equipped with a 405 nm laser (violet) using published methods with minor
187 modifications.[14] MVs isolated from plasma were visualized by scanning electron microscopy to
188 confirm their vesicular structure.

189 **Microvesicle-associated TF procoagulant activity [PCA]:** MVs isolated from PFP (20,000x*g* for
190 60 minutes) were utilized to determine MV associated TF PCA using a more sensitive
191 fluorogenic substrate (Pefafluor Fxa) as described previously.[20]

192 **Thrombin and fibrin generation:** D-dimer levels were measured using a latex-
193 immunoturbidimetric assay (STA® - Liatest® D-Di/Diagnostica Stago). Thrombin anti-thrombin
194 complexes [TAT], were measured by ELISA following manufacturer's instructions (Human
195 Thrombin-Antithrombin complex ELISA kit, abcam, #ab108907).

196 **Plasma PDI antigen and activity:** PDI antigen was measured in PFP by ELISA (Human P4HB Pair
197 Set, SinoBiological, #SEK10827). PDI reductase activity was measured in PFP using the Di-Eosin
198 GSSG assay as described previously (supplemental data).[21,22]

199 **Mononuclear cell TF-mRNA expression**: Lipopolysaccharide (LPS) induced TF gene expression
200 was assessed using stored PBMCs obtained at baseline and post-treatment only in participants
201 from the isoquercetin group (n=22). Briefly, PBMCs were suspended in RPMI with 10% FBS at a
202 concentration of 3×10^6 cells/ml and stimulated with 100 ng LPS (Escherichia Coli O26:B6,
203 Invitrogen) for 3 hours. Subsequently, the total cell derived RNA was extracted using Trizol
204 (Invitrogen, Life Technologies, # 15596-026) and subject to qRT–PCR (iTaq™ Universal SYBR®
205 Green One-Step Kit, Biorad, Hercules, California, USA, #1725150) according to the kit
206 instructions. The TF gene mRNA level was normalized to GAPDH (primers sequences available in
207 supplemental Table 2) and TF gene expression was compared to unstimulated mononuclear cell
208 TF gene expression and presented as the fold change using the $2^{-\Delta\Delta CT}$ method.[23]

209 **Adherence and plasma quercetin measurement:** Subject adherence was enhanced using an
210 electronic pill dispenser and objectively determined by pill counts performed by the research
211 team**.** Using random non-fasting blood samples obtained post treatment, plasma quercetin
212 levels were determined by liquid chromatography–tandem mass spectrometry as described
213 previously (LC-MS/MS).[24]

214

215 ***Statistical Analysis***

216 We hypothesized a 25% reduction in sP-selectin (i.e., a 7.25 ng/ml decline from an average
217 value of 29 ng/ml) as the treatment effect for isoquercetin to achieve a clinically meaningful
218 reduction in thrombosis risk using basal plasma sP-selectin levels in banked samples from
219 steady state SCD patients (n=29) recruited under NIH protocol (17-H-0056). Under these
220 conditions, a total of 40 participants (20 per group) were required to obtain the power of 90%

221 using an analysis of covariance (ANCOVA) model. This number was increased to 46 to account
222 for potential diluting effects of possible treatment non-compliance and/or study dropouts and
223 to provide adequate power to test our hypothesis in the subgroup of per-protocol patients,
224 who avoided acute crises that could have distorted their plasma sP-selectin and other
225 measurements. The statistical analysis was performed on an intention to treat principle. The
226 primary endpoint was the change in plasma sP-selectin when comparing the baseline to the
227 post-treatment level after 28 days among subjects in the isoquercetin group versus the
228 placebo. We used an ANCOVA model with follow-up sP-selectin measurements as the
229 dependent variable with baseline measurements and treatment assignment as the covariates.
230 To address missing data from the participant/s lost to follow-up, a multiple imputation
231 procedure was developed without knowledge of the treatment assignment and performed for
232 the primary endpoint. A per-protocol analysis was also conducted for the primary endpoint
233 after the exclusion of participant/s failing to remain in steady state during the intervention
234 period (experienced VOC, infection, and/or received red blood cell transfusion) or not receiving
235 the intervention. Significance was evaluated using a two-sided test with an alpha level of 0.05.
236 Differences in post-treatment measures of secondary endpoints were assessed either with
237 analysis of covariance (ANCOVA) and for non-Gaussian distributed data, either with the
238 Wilcoxon rank-sum test or the Spearman test. *In vitro* study endpoint differences were
239 analyzed using ANOVA, t-test and paired t-tests.
240
241 **RESULTS**

242 Out of 168 eligible individuals with SCD approached, 52 did not meet the eligibility criteria and
243 70 were not enrolled for other reasons (Supplemental Figure 1). This resulted in 46 participants
244 randomly allocated to receive either 1000 mg of isoquercetin (n=23) or placebo (n=23) daily for
245 a minimum of 28 days to a maximum of 35 days (Supplemental Figure 1). Attrition due to
246 screen failure in the isoquercetin group (n=1) and loss to follow-up in the placebo group (n=1)
247 resulted in 22 participants per study group providing post-treatment measurements. At
248 baseline, clinical and laboratory parameters and thrombosis biomarkers were relatively well
249 balanced between the study groups (Table 1). The mean age of the study participants was 40 ±
250 11 years, and 56% were female. Most participants had HbSS genotype and received disease
251 modifying therapy with hydroxyurea (75%; average dose = 18 ± 8 mg/kg). A subgroup of
252 patients reported prior history of thrombosis (venous thrombosis n=13; arterial thrombosis
253 n=6) and while on study received treatment with either systemic anticoagulants (n=4) or aspirin
254 (n=6). During the intervention period, 22% of the participants experienced acute VOC (5 in each
255 study group) that occasionally required blood transfusions (n=2).
256
257 Steady state SCD patients in both groups had comparable sP-selectin levels (ng/ml, IQ: 30 ± 9.9
258 vs. placebo: 32 ± 8.3 ; *p*=0.56) that were significantly elevated when compared with ethnic
259 matched healthy controls (Supplemental Figure 2). After 28 - 35 days of treatment, plasma sP-
260 selectin levels remained elevated (IQ: 30 ± 10.3 ng/ml vs. placebo: 33 ± 9.2; *p*=0.42), and the
261 primary analysis revealed no differences in mean change post-treatment from baseline (mean
262 change from baseline ± SD: IQ: 0.10 ± 6.53 ng/ml vs. placebo: 0.74 ± 4.54; *p*=0.64) (Figure 1).
263 Per protocol analysis conducted after excluding patients that experienced VOC (n=10; 5 in each

264 study group) or failed to receive the intervention (n=2) also did not reveal differences (*p*=0.61).
265 Although the sample size was small, a sensitivity analysis conducted for the presence of a
266 treatment interaction with hydroxyurea or prior history of VTE revealed no evidence of
267 interactions (*p*=0.48 and 0.90 respectively).
268
269 A total of 21 AEs were reported in 15 patients, 10 in 8 patients treated with isoquercetin, and
270 11 in 7 patients that received placebo (Table 2). The majority of AEs in the IQ group were
271 moderate, except one that was severe. Two severe grade AEs were reported in the placebo
272 group. None of the AEs in either the IQ or the placebo group were attributable to study drug.
273 Fourteen SAEs were reported in 10 patients, including 8 in 6 patients from the IQ group, and 6
274 in 4 patients in the placebo group. All SAEs excepting one were due to VOC and were
275 attributable to the underlying SCD and not to study drug exposure. One incident of retinal
276 detachment occurring in the IQ group was deemed secondary to high myopia. Comparison of
277 the baseline and post-treatment clinical and laboratory parameters (Table 3) did not reveal any
278 organ toxicity. Importantly, there were no off-target bleeding side effects detected. Overall,
279 short term fixed dose isoquercetin was safe and well tolerated.
280
281 Consistent with prior reports,[18] TEG determined whole blood coagulation in subjects from both
282 study groups was significantly elevated compared to ethnic matched healthy controls (CI, IQ:
283 3.0 ± 1.5 and placebo: 3.3 ± 1.5 vs. healthy ethnic matched controls: 2.5 ± 0.8; *p*= 0.02). After
284 isoquercetin treatment, almost all TEG parameters demonstrated a significant change in the
285 appropriate direction from the baseline compared to placebo (mean change in CI from baseline

286 ± SD: IQ: -0.29 ± 1.30, placebo: 0.43 ± 1.35; p=0.03) (Figure 2 and Table 3). Isoquercetin
287 treatment also significantly reduced platelet aggregation responses following activation with
288 low dose collagen (mean change from baseline in impedance ± SD: IQ: -3.71 ± 7.30 ohms,
289 placebo: -0.71 ± 8.39; p=0.03) although platelet aggregation induced by more potent platelet
290 agonists was unaffected (Figure 3 and Table 3). The effects of isoquercetin treatment on whole
291 blood coagulation and collagen induced platelet aggregation persisted after exclusion of
292 participants receiving either anticoagulants (TEG, IQ=2; placebo=2) or aspirin (platelet
293 aggregation, IQ=3; placebo=3). Plasma D-dimer levels reflective of the sickle hypercoagulable
294 state were elevated at baseline above the normal range (Table 1) but isoquercetin treatment
295 did not affect either d-dimers (mean change from baseline ± SD: IQ: 0.15 ± 0.92 mcg/L; placebo:
296 -0.10 ± 2.1; p=0.81) or TAT complexes (mean change from baseline ± SD: IQ: 0.09 ± 1.6 ng/ml;
297 placebo: -0.05 ± 1.3. p=0.91) (Table 3).

298

299 Because agonist-induced TF expression in patient-derived monocytes and cultured endothelial
300 cells (Supplemental Figure 3) was significantly inhibited by quercetin treatment *in vitro*, we
301 expected a reduction in the number of plasma TF^+ MVs in the isoquercetin treated subjects.
302 Surprisingly, TF^+ MVs were reduced in both groups, but the placebo rather than the
303 isoquercetin treated group demonstrated a significantly greater reduction in TF^+ MVs (mean
304 change from baseline ± SD: IQ: -1.2 ± 5.9 x10^3/mL; placebo: -1.7 ± 4.0 x10 ^{3}/mL; p=0.02) (Figure
305 4A and Table 3). However, in line with our hypothesis, TF^+ MVs isolated from the plasma of
306 isoquercetin treated patients accelerated coagulation *in vitro* less rapidly compared with
307 placebo, although this difference was not significant (mean change from baseline ± SD: IQ: -427

308 ± 1868 fMoles; placebo: -43 ± 1404; *p*=0.51) (Figure 4B and Table 3). To address these
309 discrepant findings, we evaluated inducible monocyte TF mRNA expression in paired samples of
310 PBMCs obtained from the isoquercetin treated group at baseline (BL) and post-isoquercetin
311 treatment (PT). Reassuringly, isoquercetin treatment significantly attenuated TF mRNA
312 expression *ex vivo* in LPS stimulated PBMCs (TF mRNA fold change mean ± SD, BL: 4.67 ± 6.55
313 vs. PT: 1.91 ± 3.03; *p*=0.007) (Figure 4C).
314
315 In SCD patients, both plasma PDI antigen (ng/ml, SS: 3.0 ± 4.5 vs. control (AA): 1.2 ± 2.2;
316 *p*<0.0001) and PDI reductase activity (SS: 19.4 ± 6.7 pMol/min/µl vs. AA: 14.9 ± 1.1; *p*=0.04)
317 were significantly elevated compared to ethnic matched controls (Supplemental Figure 4).
318 Although there was no discernable effect on plasma PDI antigen (Figure 5A), isoquercetin
319 treatment was associated with a significantly lowered plasma PDI reductase activity compared
320 to placebo (mean change from baseline ± SD: IQ: -3.1 ± 10.9 pMol/min/µl; placebo: 1.9 ± 9.3;
321 *p*=0.02) (Figure 5B).
322
323 Study drug adherence was above average and subjects in both study groups exhibited similar
324 levels of adherence (IQ = 96% vs placebo = 97%; *p*=0.24). Consistent with these adherence data,
325 post treatment steady state plasma quercetin measurements were significantly higher in the
326 isoquercetin treated group compared to placebo (mean ± SD: IQ: 253 ± 330 ng/ml vs. placebo:
327 15 ± 17; p <0.0001) (Supplemental Figure 5).
328
329 **DISCUSSION**

330 In this phase 2 randomized double-blind placebo-controlled trial conducted in patients with
331 steady state SCD, we show that short term fixed dosage isoquercetin is safe, well tolerated and
332 attenuates several biomarkers reflective of sickle thromboinflammatory pathology. Specifically,
333 this is the first report to demonstrate that isoquercetin treatment in patients with SCD: (1) does
334 not substantially reduce basally elevated plasma sP-selectin, (2) reduces whole blood
335 coagulation and platelet aggregation in response to submaximal stimulation with collagen, (3)
336 inhibits plasma PDI reductase activity, and (4) reduces LPS-induced TF mRNA expression in
337 peripheral blood mononuclear cells.
338
339 Our safety results are in line with other phase 1 clinical trials that report no drug-linked severe
340 adverse effects using isoquercetin at the daily dosage of 1 g/d validating its safety in SCD
341 patients.[17,25] Moreover, the lack of increased frequency of VOC in the treatment group suggests
342 that high dose isoquercetin is both safe and tolerable in this patient population. Firmly
343 establishing the safety of high dose flavonoids in patients with SCD can now pave the way for
344 escalating dose trials to test whether isoquercetin can definitively attenuate
345 thromboinflammatory pathophysiology in SCD given that a prior study has tested up to 5 g/d. [26]
346
347 Unlike the study in active cancer patients,[17] short term fixed dosage isoquercetin treatment in
348 steady state patients with SCD neither met its primary endpoint of reducing basal plasma sP-
349 selectin levels nor impacted plasma D-dimers. Several reasons may explain these findings, in
350 addition to differences in the disease population and duration of isoquercetin exposure (28 vs
351 52 days). First, the anticipated effect size of treatment on sP-selectin may have been

352 overestimated, so the small sample size did not provide adequate statistical power to detect a
353 smaller, more modest change from the baseline in sP-selectin. Second, the study cohort had
354 noticeably lower sP-selectin levels compared to previously reported values,[27] reflecting
355 inherent biological differences in the patient population studied. Similarly, D-dimers were lower
356 in this steady state cohort suggesting that optimally dosed hydroxyurea treatment possibly
357 attenuated sickle hypercoagulability, hindering the detection of hypothesized differences
358 between the treatment groups.
359
360 The sickle hypercoagulable state is accompanied by increased incident and recurrent venous
361 thrombosis requiring treatment with anticoagulation.[28] However, systemic anticoagulant use is
362 associated with a 21% increased incidence of clinically relevant major bleeding in patients with
363 SCD.[7] By lacking off-target bleeding side effects, isoquercetin offers some safety advantages for
364 managing hypercoagulability in SCD patients. Importantly, most TEG parameters previously
365 shown to reflect the sickle hypercoagulable state[18] were significantly impacted by isoquercetin
366 treatment, although it should be recognized that TEG has not been shown to predict
367 thrombotic risk. Similarly, isoquercetin treatment significantly reduced platelet aggregation
368 responses to stimulation with low dose collagen, consistent with its known antiplatelet effect.[18]
369 Since SCD patients in their steady state exhibit higher basal platelet activation when compared
370 with ethnic matched healthy controls[29], the significance of these findings could be probed for
371 clinical relevance in future studies of isoquercetin treatment. Taken together, our data provide
372 valuable safety and efficacy signals that this relatively inexpensive medication hold for
373 managing the thromboinflammatory complications of SCD in conjunction with standard care.

374

375 Heme-induced monocyte TF expression *in vitro*[30] mediated possibly via damage-associated
376 molecular pattern responses, links hemolysis-induced monocyte TF expression *in vivo* during
377 VOC.[14,31] Sickle cell patients also display monocyte activation[32] and tissue factor expression,[8,13]
378 that increase further during VOC.[31] Quercetin robustly inhibited heme and LPS induced TF
379 expression in monocytes from sickle cell patients *in vitro* (Appendix Figure 2A) yet short term
380 fixed dose isoquercetin treatment did not significantly impact blood borne TF antigen assessed
381 by the number of circulating plasma TF^+ MV, possibly reflecting the inherent variability
382 observed with this biomarker.[33] In contrast, TF procoagulant activity a more reliable indicator of
383 TF's functional ability to initiate coagulation *in vivo* showed a non-significant reduction in the
384 treatment group compared to placebo. Furthermore, inducible TF gene expression in peripheral
385 blood monocytes, an important source of in the blood of patients with SCD was significantly
386 inhibited by short term fixed dose isoquercetin treatment. Taken together, these data suggest
387 that higher doses of isoquercetin treatment for a more prolonged duration (>28 days) could
388 achieve clinically relevant suppression of blood borne TF induced by heme and mitigate sickle
389 thromboinflammatory pathophysiology.

390

391 We report plasma PDI levels with detectable plasma PDI reductase activity in sickle cell patients
392 that were significantly higher than ethnic matched healthy controls (Supplemental Figure 4).
393 Plasma PDI is possibly elevated in SCD because of intravascular secretion of intracellular PDI
394 from activated platelets[34] and endothelial cells.[35] Alternately, endothelial cell/platelet injury or
395 RBC hemolysis during VOC could lead to intracellular PDI leaking into the plasma. While the

396 exact explanation for the source of elevated PDI in sickle plasma is presently unclear, it is of
397 considerable interest given the role that cell surface PDI reductase plays in sickle erythrocyte
398 dehydration,[36] platelet activation, and neutrophil recruitment in animal models of SCD.[37] Akin
399 to its effect in patients with cancer, isoquercetin decreased plasma PDI reductase activity in
400 patients with SCD, albeit more modestly[17] due to methodological differences between the two
401 studies. By targeting PDI reductase activity, which allosterically activates TF, isoquercetin
402 treatment can impact TF-initiated coagulation in SCD at two different levels: i) at the
403 translational level as discussed above, by inhibiting inducible TF expression on the surface of
404 monocytes, and ii) at the posttranslational level, by inhibiting PDI reductase activity.[38]
405
406 With respect to drug dosing, the quercetin concentrations observed in this study were
407 comparable but noticeably lower than post-supplementation plasma quercetin concentrations
408 observed in healthy participants (253 ± 330 vs. 427.1 ± 89.2 ng/ml) consuming similar quantities
409 of isoquercetin for 28 days.[39] This was probably due to a higher clearance rate in SCD patients
410 because of glomerular hyperfiltration but could also reflect lower dietary flavonoid intake.
411
412 This study has several strengths. First, it utilized a randomized, double-blind, placebo-controlled
413 design to reduce potential biases. Second, it included a representative population of adults with
414 steady-state SCD with hallmarks of severe disease (VOC frequency 3/year and a history or prior
415 arterial and/or venous thrombosis) receiving optimally dosed hydroxyurea treatment. Third, it
416 assessed a relatively inexpensive oral treatment that broadly targeted thromboinflammatory
417 pathophysiology and assessed a range of thromboinflammation biomarkers relevant to sickle

418 cell pathobiology. However, the limitations are worth considering. Using fixed instead of
419 escalating dose isoquercetin possibly hampered detection of the hypothesized differences in
420 plasma sP-selectin. The limitations notwithstanding, this study provides an important signal of
421 safety and efficacy upon which future clinical trials can be designed.
422
423 In conclusion, these data confirm the utility of a relatively inexpensive and safe oral flavonoid
424 with demonstrable efficacy in reducing several thromboinflammatory biomarkers in SCD. Taken
425 together, these findings suggest that trials of higher dose isoquercetin for more extended
426 treatment durations are required in patients with SCD.
427

ACKNOWLEDGMENTS

This study was sponsored by the intramural research program of the National Heart Lung and Blood Institute (ZIA#HL006256 & HL006241), and the sponsor had no role in the overall conduct of the study as well as the collection, analyses, and interpretation of the data. The content of this publication does not necessarily reflect the views or polices of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the US government. The authors thank all the study participants and their families.

AUTHORSHIP CONTRIBUTIONS

M.A.L.I. and B.P.G. managed conduct of samples and performance of laboratory assays and contributed to data analysis and drafting of the manuscript; A.S.S. developed the protocol and, with the help of study statistician N.J. analyzed results, generated a clinical study report, and prepared the manuscript; A.S.S. was the principal investigator and provided medical oversight of the trial; A.DF., A.C., R.V., D-Y. L. conducted laboratory assays and contributed to data analysis; B.M., J.B., M.H., D.L., and R.P-C. participated in protocol review, patient recruitment, patient management, and collection of clinical outcomes; and all authors reviewed the manuscript with opportunity to provide input.

DISCLOSURES

The authors have no conflicts of interest to disclose.

452 **REFERENCES**

453 1. Pauling L, Itano HA, et al. Sickle cell anemia a molecular disease. *Science*.
454 1949;110(2865):543-548.
455 2. Ingram VM. Gene mutations in human haemoglobin: the chemical difference between
456 normal and sickle cell haemoglobin. *Nature*. 1957;180(4581):326-328.
457 3. Conran N, De Paula EV. Thromboinflammatory mechanisms in sickle cell disease -
458 challenging the hemostatic balance. *Haematologica*. 2020.
459 4. Ohene-Frempong K, Weiner SJ, Sleeper LA, et al. Cerebrovascular accidents in sickle cell
460 disease: rates and risk factors. *Blood*. 1998;91(1):288-294.
461 5. Naik RP, Streiff MB, Haywood C, Jr., Segal JB, Lanzkron S. Venous thromboembolism
462 incidence in the Cooperative Study of Sickle Cell Disease. *J Thromb Haemost*. 2014;12(12):2010-
463 2016.
464 6. Brunson A, Lei A, Rosenberg AS, White RH, Keegan T, Wun T. Increased incidence of VTE
465 in sickle cell disease patients: risk factors, recurrence and impact on mortality. *Br J Haematol*.
466 2017;178(2):319-326.
467 7. Hariharan N, Brunson A, Mahajan A, Keegan THM, Wun T. Bleeding in patients with
468 sickle cell disease: a population-based study. *Blood Adv*. 2020;4(5):793-802.
469 8. Solovey A, Gui L, Key NS, Hebbel RP. Tissue factor expression by endothelial cells in
470 sickle cell anemia. *J Clin Invest*. 1998;101(9):1899-1904.
471 9. Key NS, Slungaard A, Dandelet L, et al. Whole blood tissue factor procoagulant activity is
472 elevated in patients with sickle cell disease. *Blood*. 1998;91(11):4216-4223.
473 10. Chantrathammachart P, Mackman N, Sparkenbaugh E, et al. Tissue factor promotes
474 activation of coagulation and inflammation in a mouse model of sickle cell disease. *Blood*.
475 2012;120(3):636-646.
476 11. Sparkenbaugh EM, Chen C, Brzoska T, et al. Thrombin-mediated activation of PAR-1
477 contributes to microvascular stasis in mouse models of sickle cell disease. *Blood*. 2020.
478 12. Arumugam PI, Mullins ES, Shanmukhappa SK, et al. Genetic diminution of circulating
479 prothrombin ameliorates multiorgan pathologies in sickle cell disease mice. *Blood*.
480 2015;126(15):1844-1855.
481 13. Setty BN, Key NS, Rao AK, et al. Tissue factor-positive monocytes in children with sickle
482 cell disease: correlation with biomarkers of haemolysis. *Br J Haematol*. 2012;157(3):370-380.
483 14. Shet AS, Aras O, Gupta K, et al. Sickle blood contains tissue factor-positive microparticles
484 derived from endothelial cells and monocytes. *Blood*. 2003;102(7):2678-2683.
485 15. Langer F, Ruf W. Synergies of phosphatidylserine and protein disulfide isomerase in
486 tissue factor activation. *Thromb Haemost*. 2014;111(4):590-597.
487 16. Reinhardt C, von Bruhl ML, Manukyan D, et al. Protein disulfide isomerase acts as an
488 injury response signal that enhances fibrin generation via tissue factor activation. *J Clin Invest*.
489 2008;118(3):1110-1122.
490 17. Zwicker JI, Schlechter BL, Stopa JD, et al. Targeting protein disulfide isomerase with the
491 flavonoid isoquercetin to improve hypercoagulability in advanced cancer. *JCI Insight*. 2019;4(4).
492 18. Wijnberge M, Parmar K, Kesse-Adu R, Howard J, Cohen AT, Hunt BJ. The utility of
493 thromboelastography and thrombin generation in assessing the prothrombotic state of adults
494 with sickle cell disease. *Thromb Res*. 2017;158:113-120.

495 19. Zeck J, Schallheim J, Lew SQ, DePalma L. Whole blood platelet aggregation and release
496 reaction testing in uremic patients. *Biomed Res Int*. 2013;2013:486290.
497 20. Vallier L, Bouriche T, Bonifay A, et al. Increasing the sensitivity of the human
498 microvesicle tissue factor activity assay. *Thromb Res*. 2019;182:64-74.
499 21. Raturi A, Mutus B. Characterization of redox state and reductase activity of protein
500 disulfide isomerase under different redox environments using a sensitive fluorescent assay.
501 *Free Radic Biol Med*. 2007;43(1):62-70.
502 22. Bekendam RaF, R. Functional Disulphide Bonds: Humana Press; 2019.
503 23. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method.
504 *Nat Protoc*. 2008;3(6):1101-1108.
505 24. Wang L, Morris ME. Liquid chromatography-tandem mass spectroscopy assay for
506 quercetin and conjugated quercetin metabolites in human plasma and urine. *J Chromatogr B*
507 *Analyt Technol Biomed Life Sci*. 2005;821(2):194-201.
508 25. Buonerba C, De Placido P, Bruzzese D, et al. Isoquercetin as an Adjunct Therapy in
509 Patients With Kidney Cancer Receiving First-Line Sunitinib (QUASAR): Results of a Phase I Trial.
510 *Front Pharmacol*. 2018;9:189.
511 26. Lu NT, Crespi CM, Liu NM, et al. A Phase I Dose Escalation Study Demonstrates
512 Quercetin Safety and Explores Potential for Bioflavonoid Antivirals in Patients with Chronic
513 Hepatitis C. *Phytother Res*. 2016;30(1):160-168.
514 27. Kato GJ, Martyr S, Blackwelder WC, et al. Levels of soluble endothelium-derived
515 adhesion molecules in patients with sickle cell disease are associated with pulmonary
516 hypertension, organ dysfunction, and mortality. *Br J Haematol*. 2005;130(6):943-953.
517 28. Shet AS, Wun T. How I diagnose and treat venous thromboembolism in sickle cell
518 disease. *Blood*. 2018;132(17):1761-1769.
519 29. Tomer A, Harker LA, Kasey S, Eckman JR. Thrombogenesis in sickle cell disease. *J Lab Clin*
520 *Med*. 2001;137(6):398-407.
521 30. Belcher JD, Chen C, Nguyen J, et al. Heme triggers TLR4 signaling leading to endothelial
522 cell activation and vaso-occlusion in murine sickle cell disease. *Blood*. 2014;123(3):377-390.
523 31. Guarda CC, Silveira-Mattos PSM, Yahouedehou S, et al. Hydroxyurea alters circulating
524 monocyte subsets and dampens its inflammatory potential in sickle cell anemia patients. *Sci*
525 *Rep*. 2019;9(1):14829.
526 32. Belcher JD, Marker PH, Weber JP, Hebbel RP, Vercellotti GM. Activated monocytes in
527 sickle cell disease: potential role in the activation of vascular endothelium and vaso-occlusion.
528 *Blood*. 2000;96(7):2451-2459.
529 33. Hebbel RP, Key NS. Microparticles in sickle cell anaemia: promise and pitfalls. *Br J*
530 *Haematol*. 2016;174(1):16-29.
531 34. Sharda A, Kim SH, Jasuja R, et al. Defective PDI release from platelets and endothelial
532 cells impairs thrombus formation in Hermansky-Pudlak syndrome. *Blood*. 2015;125(10):1633-
533 1642.
534 35. Jasuja R, Furie B, Furie BC. Endothelium-derived but not platelet-derived protein
535 disulfide isomerase is required for thrombus formation in vivo. *Blood*. 2010;116(22):4665-4674.
536 36. Prado GN, Romero JR, Rivera A. Endothelin-1 receptor antagonists regulate cell surface-
537 associated protein disulfide isomerase in sickle cell disease. *FASEB J*. 2013;27(11):4619-4629.

538 37. Li J, Kim K, Jeong SY, et al. Platelet Protein Disulfide Isomerase Promotes Glycoprotein
539 Ibalpha-Mediated Platelet-Neutrophil Interactions Under Thromboinflammatory Conditions.
540 *Circulation*. 2019;139(10):1300-1319.
541 38. Chen F, Zhao Z, Zhou J, Lu Y, Essex DW, Wu Y. Protein disulfide isomerase enhances
542 tissue factor-dependent thrombin generation. *Biochem Biophys Res Commun*. 2018;501(1):172-
543 177.
544 39. Conquer JA, Maiani G, Azzini E, Raguzzini A, Holub BJ. Supplementation with quercetin
545 markedly increases plasma quercetin concentration without effect on selected risk factors for
546 heart disease in healthy subjects. *J Nutr*. 1998;128(3):593-597.
547

1 **Isoquercetin for thromboinflammation in Sickle Cell Disease: a randomized double-blind**

2 **placebo-controlled trial**

3

4 *Maria A. Lizarralde-Iragorri[1], *Bindu Parachalil Gopalan[1], Brenda Merriweather[2], Jennifer

5 Brooks[3], Mai Hill[3], Dianna Lovins[3], Ruth Pierre-Charles[3], Ann Cullinane[4], Alina Dulau-Florea[4],

6 Duck-Yeon Lee[5], Rafael Villasmil[6], Neal Jeffries[7], Arun S. Shet[1]

7 [1]Sickle Thrombosis and Vascular Biology Lab, Sickle Cell Branch, National Heart Lung and Blood

8 Institute, National Institutes of Health, Bethesda, MD, USA

9 [2]Sickle Cell Branch, National Heart Lung and Blood Institute, National Institutes of Health,

10 Bethesda, MD, USA

11 [3]Office of the Clinical Director, National Heart Lung and Blood Institute, National Institutes of

12 Health, Bethesda, MD, USA

13 [4] Department of Laboratory Medicine, Clinical Center, National Institutes of Health, Bethesda,

14 MD, USA

15 [5]Biochemistry Core Facility, National Heart Lung and Blood Institute, National Institutes of

16 Health, Bethesda, MD, USA

17 [6] Flow Cytometry Core Facility, National Eye Institute, National Institutes of Health, Bethesda,

18 MD, USA

19 [7]Office of Biostatistics Research, National Heart Lung and Blood Institute, National Institutes of

20 Health, Bethesda, MD, USA.

21 * M.A.L.I. and B.P.G. contributed equally to this study.

22 Abstract: 228 words

23 Main text: 4003 words

24 Figures: 5

25 Tables: 3

26 Number of references: 39

27

28 **Address for Correspondence:**

29 Arun S. Shet

30 Sickle Cell Branch, National Heart, Lung, and Blood Institute

31 10 Center Drive, Building 10, Room 6S241 MSC 1589

32 Bethesda, MD 20892-1589

33 Phone: 301-435-2345

34 Email: arun.shet@nih.gov

35

36 **Key points**

37 • Short-term fixed-dose isoquercetin did not lower plasma sP-selectin in adults with
38 steady state SCD.

39 • Isoquercetin treatment attenuated blood coagulation, platelet aggregation and
40 inducible tissue factor gene expression in adults with SCD.

41

42 **ABSTRACT**

43 Data from a small trial in cancer patients suggest that isoquercetin treatment lowered

44 thrombosis biomarkers and prevented clinical thrombosis but no studies of isoquercetin have

been conducted to target thromboinflammation in adults with sickle cell disease (SCD). We conducted a randomized, double-blind, placebo-controlled trial in adults with steady state SCD (HbSS or HbSß0thal or HbSß$^{+}$thal or HbSC). The primary outcome was the change in plasma soluble P-selectin (sP-selectin) post-treatment compared to baseline, analyzed in the intention-to-treat population. Between November 2019 and July 2022, 46 patients (age 40 ± 11 years, 56% female, 75% under hydroxyurea treatment) were randomized to receive isoquercetin (n=23) or placebo (n=23). Isoquercetin was well tolerated and all the adverse events (AEs=21) or serious AEs (14) recorded were not attributable to the study drug. The mean post-treatment change for sP-selectin showed no significant difference between the treatment groups (IQ=0.10 ± 6.53 vs. placebo=0.74 ± 4.54; p=0.64). In isoquercetin treated patients, whole blood coagulation (p=0.03) and collagen-induced platelet aggregation (p=0.03) were significantly reduced from the baseline. Inducible mononuclear cell tissue factor gene expression and plasma PDI reductase activity were also significantly inhibited (p= 0.003 and 0.02 respectively). Short term fixed dose isoquercetin in patients with SCD was safe with no off-target bleeding and was associated with changes from the baseline in the appropriate direction for several biomarkers of thromboinflammation. The trial is registered with clinicaltrials.gov (NCT04514510).

INTRODUCTION

Sickle cell disease (SCD) is an inherited hemoglobin (Hb) disorder wherein a single-nucleotide change in the β-globin gene leads to production of variant hemoglobin HbS ($\beta^{Glu6Val}$), that under hypoxic conditions polymerizes causing "sickling" of red blood cells (RBCs).[1,2] Disease manifestations characteristically include episodic disease flares termed acute vaso-occlusive crises (VOCs), that accumulate over time and lead to vascular complications, end-organ damage and reduce adult life expectancy. Although the backbone of treatment for SCD is hydroxyurea, three new drugs (crizanlizumab, L-glutamine, and Voxelotor) were recently approved by the US Food and Drug Administration. Yet, most of these disease-modifying therapies fail to substantially reduce VOC frequency and are not widely available to the global sickle cell community, leaving patients with this disease vulnerable to devastating complications.

HbS polymerization is seen as the primary driver of SCD pathophysiology, but a cascade of interrelated events including hemolysis; activation and adhesion of neutrophils, monocytes, platelets, and endothelial cells; sterile inflammation; and abnormal coagulation are increasingly recognized as important contributors to clinical disease.[3] An inherent hypercoagulable state heightens the risk for arterial and venous thrombosis in sickle cell patients, both outcomes that are associated with higher mortality.[4-6] Unfortunately, even hydroxyurea treated patients experience recurrent venous thromboembolism (VTE) necessitating lifelong anticoagulation, which increases the risk of life threatening bleeding.[7] Thus, treatments targeting thromboinflammatory pathophysiology in SCD with agents that lack bleeding side effects and are widely implementable are a priority to investigate.

89

90 Accumulated evidence points to tissue factor (TF)-initiated thromboinflammation in both
91 patients,[8,9] and animal models of SCD, that favors systemic thrombin generation,[10] stasis,[11] and
92 end-organ damage.[12] Patients with SCD display “blood-borne” TF on the surface of
93 monocytes,[13] and vascular endothelial cells,[8] and on microvesicles derived from these cells,[14]
94 which increases further during VOC. TF-initiated coagulation is also regulated post
95 translationally by a vascular thiol-isomerase, protein disulfide isomerase (PDI).[15] In animal
96 models, release of endothelial and platelets derived PDI into the vasculature following vessel
97 injury as occurs during thromboinflammation facilitates TF-initiated thrombosis.[16] Our *in vitro*
98 studies demonstrate robust inhibition of monocyte and endothelial cell-surface TF expression
99 and cell-surface PDI reductase activity by a flavonoid, quercetin. Since isoquercetin the oral
100 bioavailable glucoside form of quercetin improved thrombosis biomarkers in cancer patients
101 without inducing bleeding[17] we tested its safety and efficacy to modulate
102 thromboinflammatory pathophysiology in SCD.

103

104 **METHODS**

105

106 ***Study design***

107 This was an investigator-initiated single-center, randomized, double-blind, placebo-controlled
108 phase II study conducted between 19th November 2019 and 7th July 2022. The trial consisted of
109 a 4-week screening phase and a 4-week (28 – 35 days) blinded treatment phase, followed by a
110 4-week follow up phase for assessing safety and adverse events. The study protocol was

111 approved by the NIH institutional review board and an FDA investigational new drug

112 (IND#150896) application and was registered with clinicaltrials.gov (NCT04514510).

113

114 ***Study participants***

115 Participants were ≥ 18 years of age with SCD defined by hemoglobin electrophoresis (HbSS or

116 $HbSß^0thal$ or $HbSß^+thal$ or HbSC) who were in their steady state defined as having no significant

117 complications (VOC or acute condition requiring hospitalization) or a blood transfusion

118 occurring within 2 months of the baseline visit. Patients receiving hydroxyurea were required to

119 be on a stable dose for ≥ 12 weeks prior to the baseline visit. Participants with a history of a

120 recent VOC or blood transfusion (< 2 months), VTE event (<3 month) or actively receiving

121 crizanlizumab therapy were excluded.

122

123 ***Randomization and blinding***

124 Participants meeting eligibility criteria were randomly assigned in a 1:1 ratio to receive 28-35

125 doses of either 1000 mg isoquercetin or identically matching placebo. The randomization

126 allocation was prepared by a statistician who was not part of the study team and shared

127 directly with the NIH pharmacy team who assumed responsibility for dispensing study drug per

128 allocation assignment. Participants and the study team remained blinded throughout study

129 conduct and during analysis of results. Unblinding of treatment allocations occurred after the

130 data was curated by the study team.

131

132 ***Intervention***

133 Isoquercetin dose and exposure duration were determined based on prior clinical experience
134 from a phase 2 clinical trial of isoquercetin in cancer-induced hypercoagulability[17]. The duration
135 of study drug exposure was reduced to 4 weeks to minimize confounding by the occurrence of
136 frequent VOC. Participants in the intervention group took oral isoquercetin (Quercis Pharma
137 AG, Zug, Switzerland) 1000mg administered orally once daily for at least 4 weeks ranging from
138 28 to a maximum of 35 days. Isoquercetin was supplied as capsules of 250 mg active dosage
139 strength isoquercetin blended with 62 mg of Vitamin C/Ascorbic acid and 5 mg of Vitamin
140 B3/Nicotinic acid. Participants in the control group took identically formulated placebo
141 containing 62 mg of Vitamin C/Ascorbic acid and 5 mg of Vitamin B3/Nicotinic acid.

142

143 ***Sample collection***

144 Blood samples for the primary and secondary endpoints were obtained at baseline and post-
145 treatment. Platelet free plasma (PFP) was prepared from citrated anticoagulated blood by
146 double centrifugation at 2,500 x *g* for 15 min within 30 min of sample collection and stored at –
147 80° C until batch analysis. Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA
148 anticoagulated blood by gradient centrifugation (Histopaque-1077, Sigma-Aldrich) and stored at
149 –80° C until analysis.

150

151 ***Primary outcome***

152 The primary endpoint of the trial was the change in plasma soluble P-selectin (sP-selectin) after
153 4 weeks of treatment from baseline in the isoquercetin group compared with placebo assessed
154 by ELISA (Human P-Selectin/CD62P Quantikine ELISA Kit, R&D Systems, #SPSE00). To adhere to

the intention to treat principle, for every patient that was randomized, we attempted to obtain 28-35 day endpoint measurements despite occurrence of expected and/or unexpected events due to the underlying disease.

Secondary outcomes

Safety

Safety assessments performed during screening (visit #1), baseline (visit #2), post treatment (visit #3) and at the end of study (visit #4) included SCD focused medical history, concomitant medication use, side effects, physical examination, vital signs, adverse events (AEs) and serious AEs (SAEs) and clinical laboratory tests (comprehensive metabolic panel, complete and differential blood count, and urinalysis). The medical records of SAEs were obtained and reviewed to determine study relatedness. The principal investigator and study team reviewed the safety parameters and determined whether the intervention required modification as defined in the study protocol.

Biomarkers of thromboinflammatory pathophysiology

Whole blood coagulability: Thromboelastography (TEG) was performed in citrate anticoagulated whole blood within 30 min of phlebotomy using the TEG 5000® Analyzer (Haemonetics UK Ltd., Coventry, UK). The parameters of interest included reaction time (R), clot kinetics (K), rate of clot formation (α-angle), maximal amplitude (MA) and coagulation index (CI). CI > +3.0 indicates hypercoagulability and CI < -3.0 indicates hypocoagulability.[18]

176 **Platelet aggregation**: Platelet aggregometry was performed using whole blood optical
177 lumiaggregometry (Model 700 Whole Blood/Optical Lumi-aggregometer, CHRONO-LOG).
178 Maximal platelet aggregation using electrical impedance in whole blood samples was
179 determined following stimulation by various platelet agonists under conditions of continuous
180 stirring (1200 rpm).[19]

181 **Plasma TF^{+} microvesicles:** Tissue factor positive microvesicles (TF+ MVs) in PFP were detected
182 and enumerated by flow cytometry using a high resolution flow cytometer (CytoFLEX, Beckman
183 Coulter Inc. CA, USA) equipped with a 405 nm laser (violet) using published methods with minor
184 modifications.[14] MVs isolated from plasma were visualized by scanning electron microscopy to
185 confirm their vesicular structure.

186 **Microvesicle-associated TF procoagulant activity [PCA]:** MVs isolated from PFP (20,000x*g* for
187 60 minutes) were utilized to determine MV associated TF PCA using a more sensitive
188 fluorogenic substrate (Pefafluor Fxa) as described previously.[20]

189 **Thrombin and fibrin generation:** D-dimer levels were measured using a latex-
190 immunoturbidimetric assay (STA® - Liatest® D-Di/Diagnostica Stago). Thrombin anti-thrombin
191 complexes [TAT], were measured by ELISA following manufacturer's instructions (Human
192 Thrombin-Antithrombin complex ELISA kit, abcam, #ab108907).

193 **Plasma PDI antigen and activity:** PDI antigen was measured in PFP by ELISA (Human P4HB Pair
194 Set, SinoBiological, #SEK10827). PDI reductase activity was measured in PFP using the Di-Eosin
195 GSSG assay as described previously (supplemental data).[21,22]

196 **Mononuclear cell TF-mRNA expression**: Lipopolysaccharide (LPS) induced TF gene expression
197 was assessed using stored PBMCs obtained at baseline and post-treatment only in participants
198 from the isoquercetin group (n=22). Briefly, PBMCs were suspended in RPMI with 10% FBS at a
199 concentration of 3×10^6 cells/ml and stimulated with 100 ng LPS (Escherichia Coli O26:B6,
200 Invitrogen) for 3 hours. Subsequently, the total cell derived RNA was extracted using Trizol
201 (Invitrogen, Life Technologies, # 15596-026) and subject to qRT–PCR (iTaq™ Universal SYBR®
202 Green One-Step Kit, Biorad, Hercules, California, USA, #1725150) according to the kit
203 instructions. The TF gene mRNA level was normalized to GAPDH (primers sequences available in
204 supplemental Table 2) and TF gene expression was compared to unstimulated mononuclear cell
205 TF gene expression and presented as the fold change using the $2^{-\Delta\Delta CT}$ method.[23]

206 **Adherence and plasma quercetin measurement:** Subject adherence was enhanced using an
207 electronic pill dispenser and objectively determined by pill counts performed by the research
208 team**.** Using random non-fasting blood samples obtained post treatment, plasma quercetin
209 levels were determined by liquid chromatography–tandem mass spectrometry as described
210 previously (LC-MS/MS).[24]

211

212 ***Statistical Analysis***

213 We hypothesized a 25% reduction in sP-selectin (i.e., a 7.25 ng/ml decline from an average
214 value of 29 ng/ml) as the treatment effect for isoquercetin to achieve a clinically meaningful
215 reduction in thrombosis risk using basal plasma sP-selectin levels in banked samples from
216 steady state SCD patients (n=29) recruited under NIH protocol (17-H-0056). Under these
217 conditions, a total of 40 participants (20 per group) were required to obtain the power of 90%

218 using an analysis of covariance (ANCOVA) model. This number was increased to 46 to account
219 for potential diluting effects of possible treatment non-compliance and/or study dropouts and
220 to provide adequate power to test our hypothesis in the subgroup of per-protocol patients,
221 who avoided acute crises that could have distorted their plasma sP-selectin and other
222 measurements. The statistical analysis was performed on an intention to treat principle. The
223 primary endpoint was the change in plasma sP-selectin when comparing the baseline to the
224 post-treatment level after 28 days among subjects in the isoquercetin group versus the
225 placebo. We used an ANCOVA model with follow-up sP-selectin measurements as the
226 dependent variable with baseline measurements and treatment assignment as the covariates.
227 To address missing data from the participant/s lost to follow-up, a multiple imputation
228 procedure was developed without knowledge of the treatment assignment and performed for
229 the primary endpoint. A per-protocol analysis was also conducted for the primary endpoint
230 after the exclusion of participant/s failing to remain in steady state during the intervention
231 period (experienced VOC, infection, and/or received red blood cell transfusion) or not receiving
232 the intervention. Significance was evaluated using a two-sided test with an alpha level of 0.05.
233 Differences in post-treatment measures of secondary endpoints were assessed either with
234 analysis of covariance (ANCOVA) and for non-Gaussian distributed data, either with the
235 Wilcoxon rank-sum test or the Spearman test. *In vitro* study endpoint differences were
236 analyzed using ANOVA, t-test and paired t-tests.
237

238 **RESULTS**

239 Out of 168 eligible individuals with SCD approached, 52 did not meet the eligibility criteria and
240 70 were not enrolled for other reasons (Supplemental Figure 1). This resulted in 46 participants
241 randomly allocated to receive either 1000 mg of isoquercetin (n=23) or placebo (n=23) daily for
242 a minimum of 28 days to a maximum of 35 days (Supplemental Figure 1). Attrition due to
243 screen failure in the isoquercetin group (n=1) and loss to follow-up in the placebo group (n=1)
244 resulted in 22 participants per study group providing post-treatment measurements. At
245 baseline, clinical and laboratory parameters and thrombosis biomarkers were relatively well
246 balanced between the study groups (Table 1). The mean age of the study participants was 40 ±
247 11 years, and 56% were female. Most participants had HbSS genotype and received disease
248 modifying therapy with hydroxyurea (75%; average dose = 18 ± 8 mg/kg). A subgroup of
249 patients reported prior history of thrombosis (venous thrombosis n=13; arterial thrombosis
250 n=6) and while on study received treatment with either systemic anticoagulants (n=4) or aspirin
251 (n=6). During the intervention period, 22% of the participants experienced acute VOC (5 in each
252 study group) that occasionally required blood transfusions (n=2).

253

254 Steady state SCD patients in both groups had comparable sP-selectin levels (ng/ml, IQ: 30 ± 9.9
255 vs. placebo: 32 ± 8.3 ; *p*=0.56) that were significantly elevated when compared with ethnic
256 matched healthy controls (Supplemental Figure 2). After 28 - 35 days of treatment, plasma sP-
257 selectin levels remained elevated (IQ: 30 ± 10.3 ng/ml vs. placebo: 33 ± 9.2; *p*=0.42), and the
258 primary analysis revealed no differences in mean change post-treatment from baseline (mean
259 change from baseline ± SD: IQ: 0.10 ± 6.53 ng/ml vs. placebo: 0.74 ± 4.54; *p*=0.64) (Figure 1).
260 Per protocol analysis conducted after excluding patients that experienced VOC (n=10; 5 in each

261 study group) or failed to receive the intervention (n=2) also did not reveal differences (*p*=0.61).
262 Although the sample size was small, a sensitivity analysis conducted for the presence of a
263 treatment interaction with hydroxyurea or prior history of VTE revealed no evidence of
264 interactions (*p*=0.48 and 0.90 respectively).
265
266 A total of 21 AEs were reported in 15 patients, 10 in 8 patients treated with isoquercetin, and
267 11 in 7 patients that received placebo (Table 2). The majority of AEs in the IQ group were
268 moderate, except one that was severe. Two severe grade AEs were reported in the placebo
269 group. None of the AEs in either the IQ or the placebo group were attributable to study drug.
270 Fourteen SAEs were reported in 10 patients, including 8 in 6 patients from the IQ group, and 6
271 in 4 patients in the placebo group. All SAEs excepting one were due to VOC and were
272 attributable to the underlying SCD and not to study drug exposure. One incident of retinal
273 detachment occurring in the IQ group was deemed secondary to high myopia. Comparison of
274 the baseline and post-treatment clinical and laboratory parameters (Table 3) did not reveal any
275 organ toxicity. Importantly, there were no off-target bleeding side effects detected. Overall,
276 short term fixed dose isoquercetin was safe and well tolerated.
277
278 Consistent with prior reports,[18] TEG determined whole blood coagulation in subjects from both
279 study groups was significantly elevated compared to ethnic matched healthy controls (CI, IQ:
280 3.0 ± 1.5 and placebo: 3.3 ± 1.5 vs. healthy ethnic matched controls: 2.5 ± 0.8; *p*= 0.02). After
281 isoquercetin treatment, almost all TEG parameters demonstrated a significant change in the
282 appropriate direction from the baseline compared to placebo (mean change in CI from baseline

283 ± SD: IQ: -0.29 ± 1.30, placebo: 0.43 ± 1.35; *p*=0.03) (Figure 2 and Table 3). Isoquercetin
284 treatment also significantly reduced platelet aggregation responses following activation with
285 low dose collagen (mean change from baseline in impedance ± SD: IQ: -3.71 ± 7.30 ohms,
286 placebo: -0.71 ± 8.39; *p*=0.03) although platelet aggregation induced by more potent platelet
287 agonists was unaffected (Figure 3 and Table 3). The effects of isoquercetin treatment on whole
288 blood coagulation and collagen induced platelet aggregation persisted after exclusion of
289 participants receiving either anticoagulants (TEG, IQ=2; placebo=2) or aspirin (platelet
290 aggregation, IQ=3; placebo=3). Plasma D-dimer levels reflective of the sickle hypercoagulable
291 state were elevated at baseline above the normal range (Table 1) but isoquercetin treatment
292 did not affect either d-dimers (mean change from baseline ± SD: IQ: 0.15 ± 0.92 mcg/L; placebo:
293 -0.10 ± 2.1; *p*=0.81) or TAT complexes (mean change from baseline ± SD: IQ: 0.09 ± 1.6 ng/ml;
294 placebo: -0.05 ± 1.3. *p*=0.91) (Table 3).
295
296 Because agonist-induced TF expression in patient-derived monocytes and cultured endothelial
297 cells (Supplemental Figure 3) was significantly inhibited by quercetin treatment *in vitro*, we
298 expected a reduction in the number of plasma TF^+ MVs in the isoquercetin treated subjects.
299 Surprisingly, TF^+ MVs were reduced in both groups, but the placebo rather than the
300 isoquercetin treated group demonstrated a significantly greater reduction in TF^+ MVs (mean
301 change from baseline ± SD: IQ: -1.2 ± 5.9 $x10^3$/mL; placebo: -1.7 ± 4.0 $x10^3$/mL; *p*=0.02) (Figure
302 4A and Table 3). However, in line with our hypothesis, TF^+ MVs isolated from the plasma of
303 isoquercetin treated patients accelerated coagulation *in vitro* less rapidly compared with
304 placebo, although this difference was not significant (mean change from baseline ± SD: IQ: -427

305 ± 1868 fMoles; placebo: -43 ± 1404; *p*=0.51) (Figure 4B and Table 3). To address these
306 discrepant findings, we evaluated inducible monocyte TF mRNA expression in paired samples of
307 PBMCs obtained from the isoquercetin treated group at baseline (BL) and post-isoquercetin
308 treatment (PT). Reassuringly, isoquercetin treatment significantly attenuated TF mRNA
309 expression *ex vivo* in LPS stimulated PBMCs (TF mRNA fold change mean ± SD, BL: 4.67 ± 6.55
310 vs. PT: 1.91 ± 3.03; *p*=0.007) (Figure 4C).
311
312 In SCD patients, both plasma PDI antigen (ng/ml, SS: 3.0 ± 4.5 vs. control (AA): 1.2 ± 2.2;
313 *p*<0.0001) and PDI reductase activity (SS: 19.4 ± 6.7 pMol/min/µl vs. AA: 14.9 ± 1.1; *p*=0.04)
314 were significantly elevated compared to ethnic matched controls (Supplemental Figure 4).
315 Although there was no discernable effect on plasma PDI antigen (Figure 5A), isoquercetin
316 treatment was associated with a significantly lowered plasma PDI reductase activity compared
317 to placebo (mean change from baseline ± SD: IQ: -3.1 ± 10.9 pMol/min/µl; placebo: 1.9 ± 9.3;
318 *p*=0.02) (Figure 5B).
319
320 Study drug adherence was above average and subjects in both study groups exhibited similar
321 levels of adherence (IQ = 96% vs placebo = 97%; *p*=0.24). Consistent with these adherence data,
322 post treatment steady state plasma quercetin measurements were significantly higher in the
323 isoquercetin treated group compared to placebo (mean ± SD: IQ: 253 ± 330 ng/ml vs. placebo:
324 15 ± 17; p <0.0001) (Supplemental Figure 5).
325
326 **DISCUSSION**

327 In this phase 2 randomized double-blind placebo-controlled trial conducted in patients with
328 steady state SCD, we show that short term fixed dosage isoquercetin is safe, well tolerated and
329 attenuates several biomarkers reflective of sickle thromboinflammatory pathology. Specifically,
330 this is the first report to demonstrate that isoquercetin treatment in patients with SCD: (1) does
331 not substantially reduce basally elevated plasma sP-selectin, (2) reduces whole blood
332 coagulation and platelet aggregation in response to submaximal stimulation with collagen, (3)
333 inhibits plasma PDI reductase activity, and (4) reduces LPS-induced TF mRNA expression in
334 peripheral blood mononuclear cells.
335
336 Our safety results are in line with other phase 1 clinical trials that report no drug-linked severe
337 adverse effects using isoquercetin at the daily dosage of 1 g/d validating its safety in SCD
338 patients.[17,25] Moreover, the lack of increased frequency of VOC in the treatment group suggests
339 that high dose isoquercetin is both safe and tolerable in this patient population. Firmly
340 establishing the safety of high dose flavonoids in patients with SCD can now pave the way for
341 escalating dose trials to test whether isoquercetin can definitively attenuate
342 thromboinflammatory pathophysiology in SCD given that a prior study has tested up to 5 g/d. [26]
343
344 Unlike the study in active cancer patients,[17] short term fixed dosage isoquercetin treatment in
345 steady state patients with SCD neither met its primary endpoint of reducing basal plasma sP-
346 selectin levels nor impacted plasma D-dimers. Several reasons may explain these findings, in
347 addition to differences in the disease population and duration of isoquercetin exposure (28 vs
348 52 days). First, the anticipated effect size of treatment on sP-selectin may have been

349 overestimated, so the small sample size did not provide adequate statistical power to detect a
350 smaller, more modest change from the baseline in sP-selectin. Second, the study cohort had
351 noticeably lower sP-selectin levels compared to previously reported values,[27] reflecting
352 inherent biological differences in the patient population studied. Similarly, D-dimers were lower
353 in this steady state cohort suggesting that optimally dosed hydroxyurea treatment possibly
354 attenuated sickle hypercoagulability, hindering the detection of hypothesized differences
355 between the treatment groups.

356

357 The sickle hypercoagulable state is accompanied by increased incident and recurrent venous
358 thrombosis requiring treatment with anticoagulation.[28] However, systemic anticoagulant use is
359 associated with a 21% increased incidence of clinically relevant major bleeding in patients with
360 SCD.[7] By lacking off-target bleeding side effects, isoquercetin offers some safety advantages for
361 managing hypercoagulability in SCD patients. Importantly, most TEG parameters previously
362 shown to reflect the sickle hypercoagulable state[18] were significantly impacted by isoquercetin
363 treatment, although it should be recognized that TEG has not been shown to predict
364 thrombotic risk. Similarly, isoquercetin treatment significantly reduced platelet aggregation
365 responses to stimulation with low dose collagen, consistent with its known antiplatelet effect.[18]
366 Since SCD patients in their steady state exhibit higher basal platelet activation when compared
367 with ethnic matched healthy controls[29], the significance of these findings could be probed for
368 clinical relevance in future studies of isoquercetin treatment. Taken together, our data provide
369 valuable safety and efficacy signals that this relatively inexpensive medication hold for
370 managing the thromboinflammatory complications of SCD in conjunction with standard care.

371

372 Heme-induced monocyte TF expression *in vitro*[30] mediated possibly via damage-associated
373 molecular pattern responses, links hemolysis-induced monocyte TF expression *in vivo* during
374 VOC.[14,31] Sickle cell patients also display monocyte activation[32] and tissue factor expression,[8,13]
375 that increase further during VOC.[31] Quercetin robustly inhibited heme and LPS induced TF
376 expression in monocytes from sickle cell patients *in vitro* (Appendix Figure 2A) yet short term
377 fixed dose isoquercetin treatment did not significantly impact blood borne TF antigen assessed
378 by the number of circulating plasma TF^+ MV, possibly reflecting the inherent variability
379 observed with this biomarker.[33] In contrast, TF procoagulant activity a more reliable indicator of
380 TF's functional ability to initiate coagulation *in vivo* showed a non-significant reduction in the
381 treatment group compared to placebo. Furthermore, inducible TF gene expression in peripheral
382 blood monocytes, an important source of in the blood of patients with SCD was significantly
383 inhibited by short term fixed dose isoquercetin treatment. Taken together, these data suggest
384 that higher doses of isoquercetin treatment for a more prolonged duration (>28 days) could
385 achieve clinically relevant suppression of blood borne TF induced by heme and mitigate sickle
386 thromboinflammatory pathophysiology.

387

388 We report plasma PDI levels with detectable plasma PDI reductase activity in sickle cell patients
389 that were significantly higher than ethnic matched healthy controls (Supplemental Figure 4).
390 Plasma PDI is possibly elevated in SCD because of intravascular secretion of intracellular PDI
391 from activated platelets[34] and endothelial cells.[35] Alternately, endothelial cell/platelet injury or
392 RBC hemolysis during VOC could lead to intracellular PDI leaking into the plasma. While the

393 exact explanation for the source of elevated PDI in sickle plasma is presently unclear, it is of
394 considerable interest given the role that cell surface PDI reductase plays in sickle erythrocyte
395 dehydration,[36] platelet activation, and neutrophil recruitment in animal models of SCD.[37] Akin
396 to its effect in patients with cancer, isoquercetin decreased plasma PDI reductase activity in
397 patients with SCD, albeit more modestly[17] due to methodological differences between the two
398 studies. By targeting PDI reductase activity, which allosterically activates TF, isoquercetin
399 treatment can impact TF-initiated coagulation in SCD at two different levels: i) at the
400 translational level as discussed above, by inhibiting inducible TF expression on the surface of
401 monocytes, and ii) at the posttranslational level, by inhibiting PDI reductase activity.[38]

402

403 With respect to drug dosing, the quercetin concentrations observed in this study were
404 comparable but noticeably lower than post-supplementation plasma quercetin concentrations
405 observed in healthy participants (253 ± 330 vs. 427.1 ± 89.2 ng/ml) consuming similar quantities
406 of isoquercetin for 28 days.[39] This was probably due to a higher clearance rate in SCD patients
407 because of glomerular hyperfiltration but could also reflect lower dietary flavonoid intake.

408

409 This study has several strengths. First, it utilized a randomized, double-blind, placebo-controlled
410 design to reduce potential biases. Second, it included a representative population of adults with
411 steady-state SCD with hallmarks of severe disease (VOC frequency 3/year and a history or prior
412 arterial and/or venous thrombosis) receiving optimally dosed hydroxyurea treatment. Third, it
413 assessed a relatively inexpensive oral treatment that broadly targeted thromboinflammatory
414 pathophysiology and assessed a range of thromboinflammation biomarkers relevant to sickle

415 cell pathobiology. However, the limitations are worth considering. Using fixed instead of
416 escalating dose isoquercetin possibly hampered detection of the hypothesized differences in
417 plasma sP-selectin. The limitations notwithstanding, this study provides an important signal of
418 safety and efficacy upon which future clinical trials can be designed.
419
420 In conclusion, these data confirm the utility of a relatively inexpensive and safe oral flavonoid
421 with demonstrable efficacy in reducing several thromboinflammatory biomarkers in SCD. Taken
422 together, these findings suggest that trials of higher dose isoquercetin for more extended
423 treatment durations are required in patients with SCD.
424

ACKNOWLEDGMENTS

This study was sponsored by the intramural research program of the National Heart Lung and Blood Institute (ZIA#HL006256 & HL006241), and the sponsor had no role in the overall conduct of the study as well as the collection, analyses, and interpretation of the data. The content of this publication does not necessarily reflect the views or polices of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the US government. The authors thank all the study participants and their families.

AUTHORSHIP CONTRIBUTIONS

M.A.L.I. and B.P.G. managed conduct of samples and performance of laboratory assays and contributed to data analysis and drafting of the manuscript; A.S.S. developed the protocol and, with the help of study statistician N.J. analyzed results, generated a clinical study report, and prepared the manuscript; A.S.S. was the principal investigator and provided medical oversight of the trial; A.DF., A.C., R.V., D-Y. L. conducted laboratory assays and contributed to data analysis; B.M., J.B., M.H., D.L., and R.P-C. participated in protocol review, patient recruitment, patient management, and collection of clinical outcomes; and all authors reviewed the manuscript with opportunity to provide input.

DISCLOSURES

The authors have no conflicts of interest to disclose.

449 **REFERENCES**

450 1. Pauling L, Itano HA, et al. Sickle cell anemia a molecular disease. *Science*.
451 1949;110(2865):543-548.
452 2. Ingram VM. Gene mutations in human haemoglobin: the chemical difference between
453 normal and sickle cell haemoglobin. *Nature*. 1957;180(4581):326-328.
454 3. Conran N, De Paula EV. Thromboinflammatory mechanisms in sickle cell disease -
455 challenging the hemostatic balance. *Haematologica*. 2020.
456 4. Ohene-Frempong K, Weiner SJ, Sleeper LA, et al. Cerebrovascular accidents in sickle cell
457 disease: rates and risk factors. *Blood*. 1998;91(1):288-294.
458 5. Naik RP, Streiff MB, Haywood C, Jr., Segal JB, Lanzkron S. Venous thromboembolism
459 incidence in the Cooperative Study of Sickle Cell Disease. *J Thromb Haemost*. 2014;12(12):2010-
460 2016.
461 6. Brunson A, Lei A, Rosenberg AS, White RH, Keegan T, Wun T. Increased incidence of VTE
462 in sickle cell disease patients: risk factors, recurrence and impact on mortality. *Br J Haematol*.
463 2017;178(2):319-326.
464 7. Hariharan N, Brunson A, Mahajan A, Keegan THM, Wun T. Bleeding in patients with
465 sickle cell disease: a population-based study. *Blood Adv*. 2020;4(5):793-802.
466 8. Solovey A, Gui L, Key NS, Hebbel RP. Tissue factor expression by endothelial cells in
467 sickle cell anemia. *J Clin Invest*. 1998;101(9):1899-1904.
468 9. Key NS, Slungaard A, Dandelet L, et al. Whole blood tissue factor procoagulant activity is
469 elevated in patients with sickle cell disease. *Blood*. 1998;91(11):4216-4223.
470 10. Chantrathammachart P, Mackman N, Sparkenbaugh E, et al. Tissue factor promotes
471 activation of coagulation and inflammation in a mouse model of sickle cell disease. *Blood*.
472 2012;120(3):636-646.
473 11. Sparkenbaugh EM, Chen C, Brzoska T, et al. Thrombin-mediated activation of PAR-1
474 contributes to microvascular stasis in mouse models of sickle cell disease. *Blood*. 2020.
475 12. Arumugam PI, Mullins ES, Shanmukhappa SK, et al. Genetic diminution of circulating
476 prothrombin ameliorates multiorgan pathologies in sickle cell disease mice. *Blood*.
477 2015;126(15):1844-1855.
478 13. Setty BN, Key NS, Rao AK, et al. Tissue factor-positive monocytes in children with sickle
479 cell disease: correlation with biomarkers of haemolysis. *Br J Haematol*. 2012;157(3):370-380.
480 14. Shet AS, Aras O, Gupta K, et al. Sickle blood contains tissue factor-positive microparticles
481 derived from endothelial cells and monocytes. *Blood*. 2003;102(7):2678-2683.
482 15. Langer F, Ruf W. Synergies of phosphatidylserine and protein disulfide isomerase in
483 tissue factor activation. *Thromb Haemost*. 2014;111(4):590-597.
484 16. Reinhardt C, von Bruhl ML, Manukyan D, et al. Protein disulfide isomerase acts as an
485 injury response signal that enhances fibrin generation via tissue factor activation. *J Clin Invest*.
486 2008;118(3):1110-1122.
487 17. Zwicker JI, Schlechter BL, Stopa JD, et al. Targeting protein disulfide isomerase with the
488 flavonoid isoquercetin to improve hypercoagulability in advanced cancer. *JCI Insight*. 2019;4(4).
489 18. Wijnberge M, Parmar K, Kesse-Adu R, Howard J, Cohen AT, Hunt BJ. The utility of
490 thromboelastography and thrombin generation in assessing the prothrombotic state of adults
491 with sickle cell disease. *Thromb Res*. 2017;158:113-120.

492 19. Zeck J, Schallheim J, Lew SQ, DePalma L. Whole blood platelet aggregation and release
493 reaction testing in uremic patients. *Biomed Res Int*. 2013;2013:486290.
494 20. Vallier L, Bouriche T, Bonifay A, et al. Increasing the sensitivity of the human
495 microvesicle tissue factor activity assay. *Thromb Res*. 2019;182:64-74.
496 21. Raturi A, Mutus B. Characterization of redox state and reductase activity of protein
497 disulfide isomerase under different redox environments using a sensitive fluorescent assay.
498 *Free Radic Biol Med*. 2007;43(1):62-70.
499 22. Bekendam RaF, R. Functional Disulphide Bonds: Humana Press; 2019.
500 23. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method.
501 *Nat Protoc*. 2008;3(6):1101-1108.
502 24. Wang L, Morris ME. Liquid chromatography-tandem mass spectroscopy assay for
503 quercetin and conjugated quercetin metabolites in human plasma and urine. *J Chromatogr B*
504 *Analyt Technol Biomed Life Sci*. 2005;821(2):194-201.
505 25. Buonerba C, De Placido P, Bruzzese D, et al. Isoquercetin as an Adjunct Therapy in
506 Patients With Kidney Cancer Receiving First-Line Sunitinib (QUASAR): Results of a Phase I Trial.
507 *Front Pharmacol*. 2018;9:189.
508 26. Lu NT, Crespi CM, Liu NM, et al. A Phase I Dose Escalation Study Demonstrates
509 Quercetin Safety and Explores Potential for Bioflavonoid Antivirals in Patients with Chronic
510 Hepatitis C. *Phytother Res*. 2016;30(1):160-168.
511 27. Kato GJ, Martyr S, Blackwelder WC, et al. Levels of soluble endothelium-derived
512 adhesion molecules in patients with sickle cell disease are associated with pulmonary
513 hypertension, organ dysfunction, and mortality. *Br J Haematol*. 2005;130(6):943-953.
514 28. Shet AS, Wun T. How I diagnose and treat venous thromboembolism in sickle cell
515 disease. *Blood*. 2018;132(17):1761-1769.
516 29. Tomer A, Harker LA, Kasey S, Eckman JR. Thrombogenesis in sickle cell disease. *J Lab Clin*
517 *Med*. 2001;137(6):398-407.
518 30. Belcher JD, Chen C, Nguyen J, et al. Heme triggers TLR4 signaling leading to endothelial
519 cell activation and vaso-occlusion in murine sickle cell disease. *Blood*. 2014;123(3):377-390.
520 31. Guarda CC, Silveira-Mattos PSM, Yahouedehou S, et al. Hydroxyurea alters circulating
521 monocyte subsets and dampens its inflammatory potential in sickle cell anemia patients. *Sci*
522 *Rep*. 2019;9(1):14829.
523 32. Belcher JD, Marker PH, Weber JP, Hebbel RP, Vercellotti GM. Activated monocytes in
524 sickle cell disease: potential role in the activation of vascular endothelium and vaso-occlusion.
525 *Blood*. 2000;96(7):2451-2459.
526 33. Hebbel RP, Key NS. Microparticles in sickle cell anaemia: promise and pitfalls. *Br J*
527 *Haematol*. 2016;174(1):16-29.
528 34. Sharda A, Kim SH, Jasuja R, et al. Defective PDI release from platelets and endothelial
529 cells impairs thrombus formation in Hermansky-Pudlak syndrome. *Blood*. 2015;125(10):1633-
530 1642.
531 35. Jasuja R, Furie B, Furie BC. Endothelium-derived but not platelet-derived protein
532 disulfide isomerase is required for thrombus formation in vivo. *Blood*. 2010;116(22):4665-4674.
533 36. Prado GN, Romero JR, Rivera A. Endothelin-1 receptor antagonists regulate cell surface-
534 associated protein disulfide isomerase in sickle cell disease. *FASEB J*. 2013;27(11):4619-4629.

535 37. Li J, Kim K, Jeong SY, et al. Platelet Protein Disulfide Isomerase Promotes Glycoprotein
536 Ibalpha-Mediated Platelet-Neutrophil Interactions Under Thromboinflammatory Conditions.
537 *Circulation*. 2019;139(10):1300-1319.
538 38. Chen F, Zhao Z, Zhou J, Lu Y, Essex DW, Wu Y. Protein disulfide isomerase enhances
539 tissue factor-dependent thrombin generation. *Biochem Biophys Res Commun*. 2018;501(1):172-
540 177.
541 39. Conquer JA, Maiani G, Azzini E, Raguzzini A, Holub BJ. Supplementation with quercetin
542 markedly increases plasma quercetin concentration without effect on selected risk factors for
543 heart disease in healthy subjects. *J Nutr*. 1998;128(3):593-597.
544

545 **Table 1. Demographics and clinical characteristics at baseline of enrolled patients**
546

	Isoquercetin (n = 23)	**Placebo (n = 23)**
Parameters	n (%)	n (%)
Age (years)	43 ± 12	38 ± 12
Body Mass Index (BMI) (Kg/m^2) ǂ	26·1 ± 4·9	24·4 ± 3·2
Sex §		
Female	12 (52)	14 (61)
Male	11 (48)	9 (39)
Genotype §		
SS	18 (78)	22 (96)
Others	5 (23)	1 (4)
Race §		
Black or African American	21 (91)	21 (95·5)
Multiple races	2 (9)	1 (4·5)
Ethnicity §		
Not-Latino or Hispanic	22 (96)	19 (86·5)
Latino or Hispanic	1 (4)	2 (9)
Unknown	0	1 (4.5)
History of VTE and VOC §		
VOCs (per year for the past 3 years)	3 (14)	3 (14)
VTE	5 (26)	8 (36)
Therapy Rx §		
Hydroxyurea	18 (78)	18 (82)
DOACs	2 (9)	2 (9)
Aspirin	3 (13)	3 (13.6)
Hematological Parameters ǂ		

Hemoglobin (g/dL)	8·9 ± 1·4	8·8 ± 1·6
Hemoglobin S (%)	72·6 ± 13·8	77·9 ± 9·0
Hemoglobin F (%)	11·1 ± 11·3	15·3 ± 9·9
Reticulocytes (10^3/µl)	217·8 ± 108·8	174 ± 124
Neutrophils (10^3/µl)	4·2 ± 2·0	4·0 ± 2·0
Monocytes (10^3/µl)	0·6 ± 0·7	0·6 ± 0·3
Platelets (10^3/µl)	217·8 ± 111·2	354·1 ± 120
Biochemical parameters [ǂ]		
hsCRP (mg/L)	7·5 ± 5·8	6·0 ± 6·0
DH (U/L)	392 ± 172	404 ± 135
Bilirubin total (mg/dL)	2·6 ± 2·0	2·5 ± 1·6
Biomarkers of Thrombotic risk [ǂ]		
Soluble P-selectin (ng/ml)	30 ± 9·9	32 ± 8·3
D-dimer (mcg/ml) [#]	1·5 ± 0·8	1·9 ± 2·3
TAT (ng/ml) [#]	2·7 ± 1·4	2·5 ± 0·9

[#] TAT and D-dimer values were obtained in n=22 in each group. [§]Number (%), [ǂ]Mean ± SD

550

Table 3. Summary of results obtained in the phase II isoquercetin Clinical Trial

	Isoquercetin (n = 22)	Placebo (n = 22)

	Serious Adverse Events (Total 14 SAEs in 10 patients)	
	Isoquercetin (n=23)	**Placebo (n=23)**
	(8 SAEs in 6 patients)	**(6 SAEs in 4 patients)**
Sickle cell anemia with Vaso-Occlusive Crisis (SCA with VOC)	5	4* (1 had 2 VOCs)
COVID-19	1* (also had SCA with VOC)	0
Lung infection	0	1* (also had SCA with VOC)
Priapism	1* (also had SCA with VOC)	0
Retinal detachment	1	0
	Adverse events (Total 21 AEs in 15 patients)	
	10 AEs in 8 patients	**11 AEs in 7 patients**
Sickle cell anemia with Vaso-Occlusive Crisis (SCA with VOC)	2	2
Chronic sickle cell pain	1	0
General disorder - Pain (not otherwise specified)	0	1
Infections and infestations - Other specify: COVID-19	1	1
CPK increased	1	1* (also had COVID-19)
Gallbladder pain	1	0
Rash acneiform	1	0
Reproductive system and breast disorders: Penile pain	1	0
Abdominal pain	1* (also had SCA with VOC)	0
Colitis	1* (also had Gall bladder pain)	0
Bloating	0	1
Headache	0	1* (also had bloating)
Blurred vision	0	1
Retinopathy	0	1* (also had blurred vision and dysmenorrhea)
Dysmenorrhea	0	1* (also had retinopathy and blurred vision))
Diarrhea	0	1
*multiple events in a single patient		

551
552

Laboratory Biomarkers	Baseline	Post-treatment	Baseline	Post-treatment
Plasma soluble P-selectin levels (ng/mL) ǂ	30.3 ± 9·9	30.4 ± 10·6	32.1 ± 8·4	32.8 ± 9·2
Thromboelastography ǂ				
Clot formation K-time (min)	4·6 ± 1·4	4·8 ± 1·3	4·3 ± 1·3	4·0 ± 1·0
Reaction time R (min)	1·2 ± 0·3	1·2 ± 0·3	1·3 ± 0·7	1·0 ± 0·2
α-angle (degree)	73·4 ± 3·4	72·5 ± 4·0	73·1 ± 5·5	75·2 ± 3·3
Maximal amplitude MA (mm)	71·3 ± 5·1	70·4 ± 6·7	71·1 ± 5·8	72·1 ± 5·6
Coagulation index (CI) (AU) *	3·0 ± 1·5	2·7 ± 1·7	3·3 ± 1·5	3·7 ± 1·3
Whole blood platelet aggregation (Impedance, ohms) ǂ				
Thrombin (0.1U)	31 ± 14·5	31·7 ± 13·3	35·8 ± 10·3	33·6 ± 12·9
ADP (10 μM)	13·1 ± 6·4	12·9 ± 6·2	17·7 ± 7·4	14·1 ± 7·2
Arachidonic acid (5mM)	14·1 ± 7·7	12·7 ± 8·1	12·2 ± 6·9	12·9 ± 7·1
Collagen (1μg/mL) *	12·4 ± 6·9	8·5 ± 5·6	13·6 ± 5·2	12·6 ± 6·2
Collagen (5 μg/mL)	14·2 ± 6.1	14·8 ± 4·9	18 ± 6·2	17·2 ± 5·9
Tissue Factor (TF) ǂǂ				
TF^+ microvesicles (number/mL)	1551 (404, 6379)	1723 (516, 7181)	2468 (603, 6827)	1515 (440, 3830)
TF^+ microvesicles PCA (fMol)	257 (148, 927)	362 (166, 834)	414 (122, 859)	498 (307, 923)
PBMC TF mRNA expression (fold change) ¶ *	1·41 (0·46, 6·11)	0·43 (0·29, 2·19)	NA	NA
Protein disulfide isomerase (PDI) ǂǂ				
Plasma PDI reductase activity (pMoles/min/μL) *	16 (14, 27)	16 (13, 21)	19 (16, 23)	20 (17, 25)
Plasma PDI antigen (ng/ml)	2·14 (1·61, 2·96)	2·18 (1·77, 3·31)	2·08 (1·88, 2·31)	2·23 (1·75, 2·72)
Other markers of coagulation ǂ				
D-dimer (mcg/mL)	1·5 ± 0·8	1·7 ± 1·1	1·6 ± 1·1	1·9 ± 1·8
Thrombin anti-thrombin complexes (TAT, ng/ml)	2·7 ± 1·4	2·7 ± 1·7	2·5 ± 0·9	2·4 ± 1·1
Plasma quercetin levels (ng/ml) ǂ	NA	253 ± 330	NA	15 ± 17

*ɣSAE not related with isoquercetin treatment; ǂMean ± SD; ǂǂMedian (IQR); ¶n=20; *statistically significant differences observed among the IQ and Placebo group. NA, Not Applicable. CI, Coagulation Index, AU, arbitrary units.* . Differences in post-treatment measures were assessed either with analysis of covariance (ANCOVA) and for non-Gaussian distributed data, either with the Wilcoxon rank-sum test or the Spearman test. Post-treatment value comparisons were intentionally not performed based on our a priori statistical analytical plan.

553

554

555 **FIGURE LEGENDS**
556
557
558
559
560 **Figure 1. Effect of isoquercetin on plasma soluble P-selectin.**
561 Mean change from baseline in plasma sP-selectin level in participants from the isoquercetin (IQ) group and placebo
562 group reveals no significant differences (IQ n=23/Placebo n=23; ANCOVA *p*=0.64).
563
564 **Figure 2. Effects of isoquercetin on whole blood coagulation.**
565 Mean change from baseline in A) Clot initiation time (reaction-time or R-time) and B) clot formation time (K- time)
566 showing significant prolongation from the baseline value following isoquercetin treatment (ANCOVA*p=0.04 and
567 *p=0.02 respectively). C) Fibrin crosslinking determined by the alpha-angle acuteness signified by the maximal
568 amplitude showing significant reduction from the baseline value following isoquercetin treatment (ANCOVA****
569 p=0.0001). However, D) clot strength (MA) was not affected by the isoquercetin treatment (p=0.09). E) Mean
570 change from the baseline in whole blood coagulation index (CI) assessed by thromboelastography showed a
571 significant reduction in the IQ group compared with placebo (ANCOVA**p*= 0.03, IQ n=22/Placebo n=22). AU,
572 Arbitrary Units
573
574 **Figure 3. Effects of isoquercetin on platelet aggregation.**
575 Mean agonist induced platelet aggregation shown as change from the baseline in impedance whole blood
576 aggregometry from patients from the IQ group (n=22) and the placebo group (n=22). Isoquercetin treatment only
577 show a significant effect on platelet aggregation following exposure to A) low doses of collagen (ANCOVA**p*=
578 0.02, IQ n=21/Placebo n=21), however, following standard platelet aggregation agonists such as B) Thrombin, C)
579 ADP, D) Arachidonic acid, and E) high dose of collagen (ANCOVA p=0.93; 0.72; 0.63; and 0.66; respectively) did
580 not show any effect.
581
582 **Figure 4. Effects of isoquercetin on tissue factor antigen, activity and gene expression.**
583 A) Mean change from the baseline in the number of TF^{+} microvesicles (MV) showing reduction in both isoquercetin
584 (IQ) and placebo groups but a significantly greater reduction in the placebo group (**p*=0.02). B) Mean change from
585 the baseline in TF^{+} microvesicle procoagulant activity (PCA) which is decreased in both groups but shows no
586 significant difference (*p*=0.51). C) LPS induced TF mRNA expression in PBMCs isolated from sickle cell patients
587 in the isoquercetin group at baseline (BL) and post-treatment (PT) showed a significant reduction in inducible TF
588 mRNA after isoquercetin treatment (***p*=0.003, n=20).
589
590 **Figure 5. Effect of isoquercetin on plasma PDI antigen and plasma PDI activity.**
591 A) Mean change from baseline in plasma PDI antigen was not different between the patients treated with IQ with the
592 ones treated with placebo (ANCOVA *p* = 0.52, IQ n=22/Placebo n=22). B) PDI reductase activity (mean change
593 from the baseline value) is significantly decreased in the patients treated with IQ compared with placebo (ANCOVA
594 **p* = 0.02, IQ n=22/Placebo n=22).
595

Figure 1

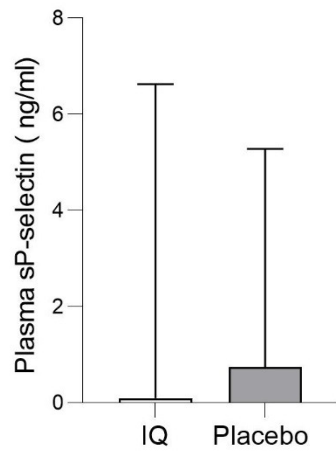

Figure 1. Effect of isoquercetin on plasma soluble P-selectin.

Figure 2

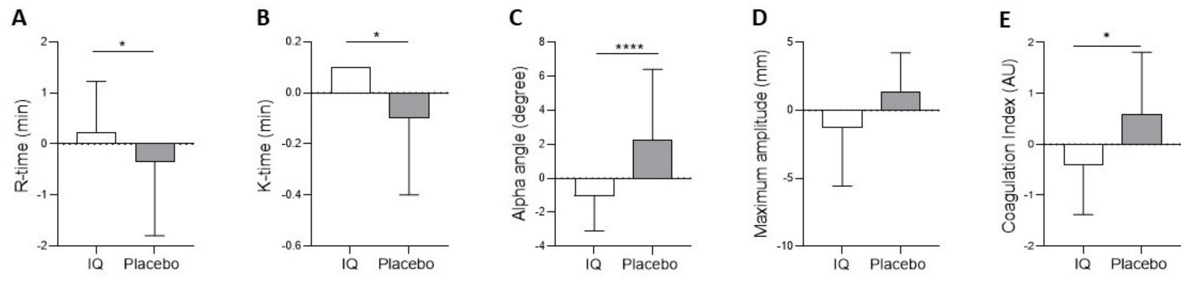

Figure 2. Effects of isoquercetin on whole blood coagulation.

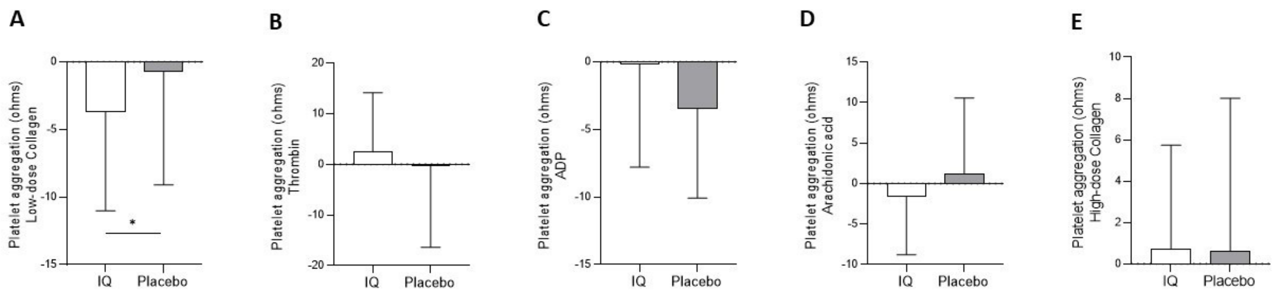

Figure 3. Effects of isoquercetin on platelet aggregation.

Figure 4

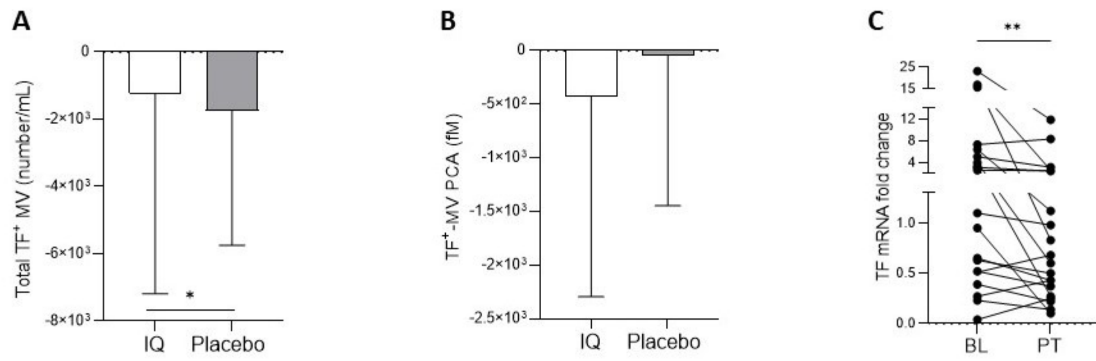

Figure 4. Effects of isoquercetin on tissue factor antigen, activity and gene expression.

Figure 5

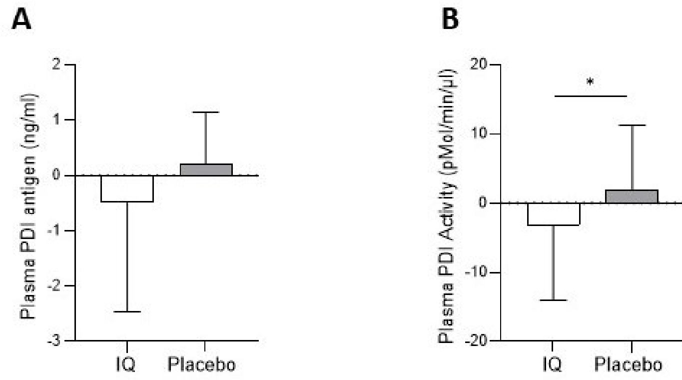

Figure 5. Effect of isoquercetin on plasma PDI antigen and plasma PDI activity.