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ORIGINAL ARTICLE

NOS3 (eNOS) mRNA in Whole Blood of Untreated Grade 1 Hypertension: A Case–Control Study with Reference-Gene Sensitivity Analysis

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ABSTRACT

Background: Reduced endothelial nitric oxide synthase (eNOS; gene NOS3) activity contributes to hypertension, but whether NOS3 transcripts in blood differ in early, untreated hypertension is unknown.

Objective: To compare whole-blood NOS3 mRNA between adults with untreated grade 1 hypertension and normotensive adults and to examine its dependence on the reference gene.

Methods: In this case–control study, 30 untreated adults with grade 1 hypertension and 30 normotensive adults aged 40–55 years were recruited in West Sumatra, Indonesia. NOS3 and GAPDH were quantified by SYBR Green qRT-PCR; ΔCq was compared by Mann–Whitney U test. Reference-gene dependence was assessed by covariance adjustment, slope and efficiency analyses and equivalence testing; age and sex adjustment was bounded over datasets consistent with group-level data.

Results: ΔCq was higher in hypertension (median 4.05 vs 2.18; p = 0.046; Hodges–Lehmann fold change 0.47, 95% CI 0.18–0.99; Livak 0.46, 0.19–1.10; Welch p = 0.081; post hoc power 41%). NOS3 Cq did not differ (p = 0.383), whereas GAPDH Cq was 1.30 cycles lower in hypertension. The NOS3–GAPDH slope was 0.19 (errors-in-variables upper bound 0.58; significance required ≥0.81). The GAPDH-adjusted fold change was 0.95 (0.71–1.27), equivalent within two-fold (p < 0.001). Adjusted age and sex effects were not identifiable.

Conclusion: Lower whole-blood NOS3 relative to GAPDH in untreated grade 1 hypertension tracked GAPDH Cq and was absent after GAPDH adjustment, so a change in NOS3 transcript abundance was not demonstrated. With a single reference gene, such measurements cannot be interpreted without multi-reference normalization.

1. Introduction

Hypertension is the most common modifiable cardiovascular risk factor worldwide. The number of adults aged 30–79 years with hypertension doubled between 1990 and 2019 to 1.28 billion. Treatment coverage in Indonesia was among the lowest of the 200 countries analyzed.[1] High systolic blood pressure ranked second among global risk factors for disability-adjusted life-years in 2021, accounting for 7.8% of the total.[2] Deaths attributable to it rose by 65.3% between 1990 and 2021, to 11 million, with the steepest burden in lower-middle-income countries.[3] In Indonesia, crude adult prevalence rose from 27.9% to 31.6% across the three national surveys from 2013 to 2023, while blood-pressure control improved only from 2.3% to 4.2%.[4] In the 2023 Indonesian Health Survey, 78.87% of 53,049 adults with hypertension were uncontrolled.[5] Three-quarters of cases in the 2018 national survey were undiagnosed.[6] Older age and female sex were strongly associated with hypertension in the 2023 survey,[7] central obesity raised its prevalence among Indonesian women by half,[8] and in a demographic surveillance site in Yogyakarta 40% of adults were hypertensive.[9] Early, untreated hypertension in middle-aged Indonesian women is therefore a large population in which hypertension is often undetected.

Endothelial nitric oxide synthase (eNOS), the product of the NOS3 gene, generates the nitric oxide (NO) that maintains vasodilator tone, limits platelet adhesion and restrains vascular inflammation. Its output is regulated at several levels. Laminar shear stress and Krüppel-like factor 2 induce NOS3 expression,[10] and shear stress also stabilizes NOS3 mRNA,[11] whereas inflammatory signaling destabilizes it: loss of endothelial OASL1 increased Erk1/2–NF-κB activity and lowered NOS3 mRNA;[12] in mice, endothelial lipid droplets destabilized NOS3 mRNA and raised blood pressure;[13] and in obesity-associated hypertension, NF-κB-induced miR-155-5p and miR-214-3p suppressed eNOS,[14] as did miR-155-5p in a rat model of renovascular hypertension.[15] At the protein level, tetrahydrobiopterin deficiency and RNA-methylation-dependent programming uncouple eNOS, which then produces superoxide instead of NO.[16,17] Clinically, flow-mediated dilation is already reduced in untreated and newly diagnosed hypertension,[18–20] and lower plasma nitrite and nitrate predict left-ventricular strain impairment and albuminuria.[21]

Most human evidence linking NOS3 to hypertension is genetic. A meta-analysis of 60 case–control studies estimated an odds ratio of 1.44 (95% CI 1.26–1.63) for the rs1799983 T allele,[22] and the intron-4 variable-number tandem repeat was also associated with hypertension.[23] Results in Asian populations are heterogeneous. In rural Indonesia the rs1799983 T allele was rare and linked to hypertension only after adjustment,[24] while studies from Thailand, China, India and Uzbekistan implicated different NOS3 variants.[25–28] Rare damaging NOS3 variants raise systolic pressure by about 3 mmHg.[29] A genotype, however, cannot show how much NOS3

a given person transcribes at a given time. Direct measurement of NOS3 mRNA in humans has been reported mainly in pre-eclampsia, where NOS3 was down-regulated 8.49-fold,[30] in circulating progenitor cells[31,32] and in peripheral leukocytes in fatty liver disease.[33] In peripheral blood leukocytes of 15 untreated patients with stage I–II hypertension, NOS3 mRNA did not differ from that of 25 healthy individuals.[34] Erythrocytes and platelets also contain functional eNOS, whose activity responds to shear, hypoxia and inflammation.[35–38] A blood-based NOS3 measurement is therefore feasible, but it reflects circulating cells and not the vessel wall.

Quantifying a low-abundance transcript in whole blood with the comparative quantification-cycle ($2^{-\Delta\Delta Cq}$) method rests on two assumptions: the reference gene must be stably expressed across the groups compared, and target and reference must also behave proportionally across the range of template input. The revised Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE 2.0) requires that both be demonstrated.[39] Single housekeeping genes can give opposite conclusions for the same target under hypoxic or disease conditions,[40] reference-gene rankings in blood and mononuclear cells depend on context,[41–44] and, when these assumptions fail, covariance-based normalization (analysis of covariance, ANCOVA) is more reliable than $2^{-\Delta\Delta Cq}$.[45] The leukocyte differential dominates whole-blood transcript levels.[46,47] Quantification at the edge of an assay's dynamic range is the least reliable.[48]

To our knowledge, no study published since 2021 has measured NOS3 mRNA in whole blood of untreated adults with grade 1 hypertension, and none has come from Indonesia. Blood transcriptome and epigenome-wide studies of blood pressure have not prioritized NOS3.[49–51] Indonesian research on the NO pathway has so far measured serum NO in 120 hypertensive patients in Aceh and in 200 smokers and non-smokers in Malang.[52,53] This study compared whole-blood NOS3 mRNA between adults aged 40–55 years with untreated grade 1 hypertension and normotensive adults recruited at RSUD Prof. Dr. M. Ali Hanafiah Batusangkar, West Sumatra. The aims were (1) to estimate the difference in GAPDH-normalized NOS3 expression with its uncertainty; (2) to determine whether the difference arose from the target or from the reference gene; (3) to establish what can be concluded about age- and sex-adjusted associations when these covariates are available only as group summaries; and (4) to specify the sample size and design required for a confirmatory study. The study therefore has a biological aim: to test the hypothesis that NOS3 expression is lower in grade 1 hypertension. It also has a methodological aim, which is to show how the conclusion of a single-reference qRT-PCR comparison in whole blood can be tested against the behavior of its reference gene.

2. Methods

2.1. Study design and setting

This was an analytical observational case–control study. It compared whole-blood NOS3 mRNA between adults with untreated grade 1 hypertension (cases) and normotensive adults (controls). Reporting followed the STROBE statement for case–control studies, and the molecular methods were reported against the MIQE 2.0 checklist as far as the data allowed (see Limitations).[39] No intervention was given. Participants were not followed up.

Recruitment took place from April to June 2026 at RSUD Prof. Dr. M. Ali Hanafiah Batusangkar, the regional general hospital of Tanah Datar Regency, West Sumatra, Indonesia. The Biomedical Laboratory, Faculty of Medicine, Universitas Andalas, Padang, carried out the molecular analyses from April to September 2026.

2.2. Participants and eligibility

Men and women aged 40–55 years were eligible. At the eligibility assessment, cases met the 2023 European Society of Hypertension (ESH) definition of grade 1 hypertension (systolic blood pressure 140–159 mmHg and/or diastolic blood pressure 90–99 mmHg, based on the mean of three seated readings) and were not taking antihypertensive medication.[54] Controls had optimal or normal blood pressure by the same guidelines (systolic <130 mmHg and diastolic <85 mmHg). They also had no history of hypertension and had never used antihypertensive drugs. Both groups excluded people with coronary heart disease, heart failure, stroke, kidney disease or diabetes mellitus. Any other condition or medication that the investigators judged likely to affect NOS3 expression was also an exclusion criterion. A participant was withdrawn if the blood sample was insufficient, if RNA failed the quality criteria, if cDNA synthesis or amplification failed after repetition, or if consent was withdrawn. Attendees who met the criteria were selected by purposive sampling.

The protocol set a target of 30 participants per group. To allow for 10% attrition, 34 per group were recruited. With 30 per group, a two-sided test at $\alpha = 0.05$ had 80% power to detect a standardized difference of 0.74. Using the pooled standard deviation observed in this study (2.46 cycles), this corresponds to about 1.8 cycles of ΔCq, or a 3.5-fold expression ratio. The power for the observed effect, and the sample sizes that a confirmatory study would need, are reported as results.

2.3. Variables and data collection

A sphygmomanometer was used to measure blood pressure in the seated position after rest. Three readings were taken, and their mean was recorded. Blood pressure at the sampling visit is reported in the Results. Age and sex were recorded for every participant. These three variables were analyzed as group summaries (medians with first and third quartiles, and counts). They were not linked to the individual qRT-PCR results, which were analyzed for every participant.

Peripheral venous blood (3–5 mL) was drawn into EDTA tubes, coded, and processed according to the laboratory's procedures, as detailed in Table 1. Total RNA was extracted with GENEzol™ reagent (Geneaid). Phase separation used 200 µL of chloroform per 1 mL of reagent, followed by centrifugation at 12,000–16,000 × g for 15 min at 4 °C. RNA was then precipitated with one volume of isopropanol and washed with 70% ethanol, and the pellet was dissolved in 20–50 µL of RNase-free water at 55–60 °C. Spectrophotometry (NanoDrop) gave RNA concentration and purity (A260/A280). All 60 samples met the laboratory's acceptance criteria. Complementary DNA was then synthesized from 500 ng of total RNA in 10 µL with the SensiFAST cDNA Synthesis Kit (Bioline), at 25 °C for 10 min, 42 °C for 15 min and 85 °C for 5 min.

Table 1. Specimen handling, assay design and reaction conditions (MIQE 2.0 items).

Step	Specification
Specimen	Peripheral venous whole blood, 3–5 mL in EDTA tubes; samples coded at collection and processed according to the laboratory's standard procedures
RNA extraction	GENEzol™ reagent (Geneaid); 200 µL chloroform per 1 mL reagent; centrifugation 12,000–16,000 × g, 15 min, 4 °C; isopropanol precipitation; 70% ethanol wash; pellet dissolved in 20–50 µL RNase-free water (55–60 °C, 10–15 min)
RNA quality	Concentration and A260/A280 purity (NanoDrop); all 60 samples met the laboratory acceptance criteria
Reverse transcription	SensiFAST cDNA Synthesis Kit (Bioline); 500 ng total RNA in 10 µL (2 µL 5× TransAmp buffer, 0.5 µL reverse transcriptase); 25 °C 10 min, 42 °C 15 min, 85 °C 5 min, hold 4 °C
qPCR chemistry	SensiFAST SYBR No-ROX 2× mix (Bioline); 10 µL reactions: 5 µL mix, 0.5 µL forward primer, 0.5 µL reverse primer, 1 µL cDNA (100 ng), 3 µL nuclease-free water
Cycling	95 °C 2 min; cycles of 95 °C 5 s and annealing 30 s at 53.2 °C (NOS3) or 60.1 °C (GAPDH), annealing temperatures set by gradient PCR; melt analysis 65–95 °C
NOS3 primers	F 5′-GGAAGCTGCAGGTGTTCG-3′; R 5′-ACTGTGATGGCCGAGCGAAG-3′; NM_000603.5 positions 907–1024; amplicon 118 bp (GC 56.8%); forward primer spans the exon 5–6 junction and reverse primer the exon 6–7 junction
GAPDH primers	F 5′-ATGGGTGTGAACCATGAGAAGTA-3′; R 5′-GGCAGTGATGGCATGGAC-3′; NM_002046.7 positions 473–625; amplicon 153 bp (GC 51.6%); exons 6–8 (two intervening introns)
Melt curves	NOS3: main peak 82–84 °C with a minor shoulder at 72–75 °C; GAPDH: single sharp peak 84–85 °C, uniform across samples
Quantification	Mean Cq of replicate reactions per sample; ΔCq = Cq(NOS3) − Cq(GAPDH); ΔΔCq relative to the mean normotensive ΔCq (2.80); relative expression 2−ΔΔCq (Equations 1–3)

Abbreviations: Cq, quantification cycle; F, forward; GC, guanine–cytosine content; MIQE, Minimum Information for Publication of Quantitative Real-Time PCR Experiments; R, reverse. Notes: Primer positions, amplicon length, GC content and exon structure were obtained by mapping the primer sequences to the NCBI RefSeq transcripts.

NOS3 and the reference gene GAPDH were amplified in 10-µL reactions. Each contained 5 µL of SensiFAST SYBR No-ROX 2× mix (Bioline), 0.5 µL of each primer, 1 µL of cDNA (100 ng) and 3 µL of nuclease-free water. Cycling began at 95 °C for 2 min. It continued with cycles of 95 °C for 5 s and annealing for 30 s at 53.2 °C (NOS3) or 60.1 °C (GAPDH), temperatures established by gradient PCR, and ended with melt-curve analysis from 65 to 95 °C. The NOS3 primers were designed with NCBI Primer-BLAST on RefSeq NM_000603.5. When mapped to the RefSeq transcripts, they occupied positions 907–1024 of NM_000603.5. The forward primer spanned the exon 5–6 junction and the reverse primer the exon 6–7 junction (118-bp amplicon, GC 56.8%). The GAPDH primers occupied positions 473–625 of NM_002046.7 across exons 6–8 (153 bp, GC 51.6%). Each primer matched its transcript once. For each sample, the mean quantification cycle (Cq) of replicate reactions was used, expressed to two decimal places. Table 1 summarizes the assay specifications.

ΔCq was calculated for each participant as the difference between the NOS3 and GAPDH quantification cycles (Equation 1). A higher ΔCq therefore indicates less NOS3 relative to GAPDH. ΔΔCq was calculated against the mean ΔCq of the normotensive group, which served as calibrator (Equation 2). The group fold change came from the difference in mean ΔCq (Equation 3).

$$(1)\quad \Delta Cq_i = Cq_{NOS3,i} - Cq_{GAPDH,i}$$

$$(2)\quad \Delta\Delta Cq_i = \Delta Cq_i - \overline{\Delta Cq}_{NT}$$

$$(3)\quad FC = 2^{-(\overline{\Delta Cq}_{HT} - \overline{\Delta Cq}_{NT})}$$

ΔCq is a $\log_2$ quantity. Statistical tests were therefore performed on ΔCq, and fold changes were obtained by back-transformation. The normotensive fold change of 1.00 is the calibrator by construction.

2.4. Statistical analysis

Continuous variables were described as mean ± standard deviation and as median (first to third quartile) with distribution-free 95% confidence intervals (CIs) of the median. Normality was assessed with the Shapiro–Wilk test. The protocol specified the Mann–Whitney U test as the primary test; ΔCq was not normally distributed in the normotensive group (Shapiro–Wilk p = 0.017), which supports the choice. Tied values received mid-ranks. The p value was obtained from the permutation distribution of U (400,000 relabelings) and from the tie-corrected normal approximation without continuity correction; the latter was used for all other Mann–Whitney tests. This was the single protocol-specified test, and all other estimates are supportive and were not adjusted for multiplicity. Its effect size was summarized by the rank-biserial correlation and by the Hodges–Lehmann shift with its Moses 95% CI, back-transformed to a fold change (Equation 4). To allow comparison with the $2^{-\Delta\Delta Cq}$ convention, the mean ΔΔCq was also estimated with the Welch t-test and back-transformed to a Livak fold change with its 95% CI. Hedges' g was calculated with the small-sample correction (Equation 5). Distribution-free confirmation came from a two-sided permutation test (200,000 random relabelings) of the differences in means and medians, and from a percentile bootstrap (20,000 resamples). The Levene test assessed equality of variances. Fisher's exact test was used to compare sex, and the Mann–Whitney U test to compare age and blood pressure.

$$(4)\quad \hat{\Delta} = \text{median}\{\Delta Cq_{HT,i} - \Delta Cq_{NT,j}\},\quad FC_{HL} = 2^{-\hat{\Delta}}$$

$$(5)\quad g = \left(1 - \frac{3}{4(n_1+n_2)-9}\right)\frac{\bar{x}_{HT} - \bar{x}_{NT}}{s_p}$$

We examined the dependence of the result on the reference gene in four ways. First, NOS3 Cq and GAPDH Cq were compared separately between groups. Second, NOS3 Cq was regressed on GAPDH Cq, because ΔCq normalization is valid only if the two change one-for-one (slope = 1). Third, the group difference in NOS3 was estimated by ANCOVA with GAPDH Cq as covariate (Equation 6). This model used heteroscedasticity-consistent (HC3) standard errors and a group × GAPDH interaction term, and Quade's rank ANCOVA served as a non-parametric check.[45] Fourth, the ΔCq comparison was repeated after four quality-based exclusions: negative ΔCq, GAPDH Cq ≥35, GAPDH Cq >33 and NOS3 Cq ≥35. Their thresholds were defined after data collection and are therefore post hoc. The comparison was also repeated after removing each sample in turn (leave-one-out). Fisher's exact test compared the frequency of late GAPDH amplification (Cq >33) and of negative ΔCq values between groups.

$$(6)\quad Cq_{NOS3,i} = \beta_0 + \beta_1 HT_i + \beta_2 Cq_{GAPDH,i} + \varepsilon_i,\quad FC_{ANCOVA} = 2^{-\beta_1}$$

Patient-level age and sex could not be linked to the qRT-PCR results. An age- and sex-adjusted model therefore could not be fitted to observed data. A sensitivity analysis was performed instead, over the space of datasets that agree with the observed marginals. An admissible dataset kept every observed Cq value. It assigned integer ages (40–55 years) and sex to the participants so that four quantities were reproduced exactly: the group quartiles of age (41.75, 48 and 51.25 years in cases; 42, 44 and 48.25 years in controls, weighted-average definition), the Mann–Whitney *p* value for age (0.060), and the numbers of men (four and two). Admissible age configurations were identified by random search of three million candidates. From these, 200,000 admissible datasets were sampled by random assignment of ages and sex to participants. Hill-climbing then searched for the most extreme adjusted effects (14 restarts per extreme, six from constructed concordant or discordant configurations and eight from random ones). These extremes are lower bounds on the attainable range. Two models were fitted to each dataset: ΔCq on group, age and sex, and NOS3 Cq on group, GAPDH Cq, age and sex. For each model we report the median, the 2.5th and 97.5th percentiles and the most extreme values of the group coefficient, together with the percentage of datasets in which it reached $p < 0.05$. An adjusted association was regarded as identified only if its whole attainable range lay on one side of zero.

We calculated post hoc power for the observed effect, and the sample sizes needed for 80% and 90% power across a range of standardized differences, for a two-sided independent-samples t-test ($\alpha = 0.05$). Because post hoc power follows from the observed *p* value, it is reported only to size a confirmatory study. All analyses were run in Python 3.14 with SciPy 1.18.1 and statsmodels 0.15.0. Statistical significance was set at $\alpha = 0.05$ (two-tailed). The *p* values are reported to three decimal places.

2.5. Ethical approval

The CMHC Research Center Ethics Committee, Indonesia, approved the protocol (approval No. 2026/0398), before recruitment began. All participants gave written informed consent after the study had been explained to them. The study followed the Declaration of Helsinki.

3. Results

3.1. Participants

Sixty-eight adults were screened, 34 in each blood-pressure stratum. In the hypertension stratum, two did not meet the inclusion criteria and two met an exclusion criterion. In the normotensive stratum, four declined or withdrew. Thirty participants with grade 1 hypertension and 30 normotensive participants were analyzed (Figure 1). No sample was lost at any laboratory step.

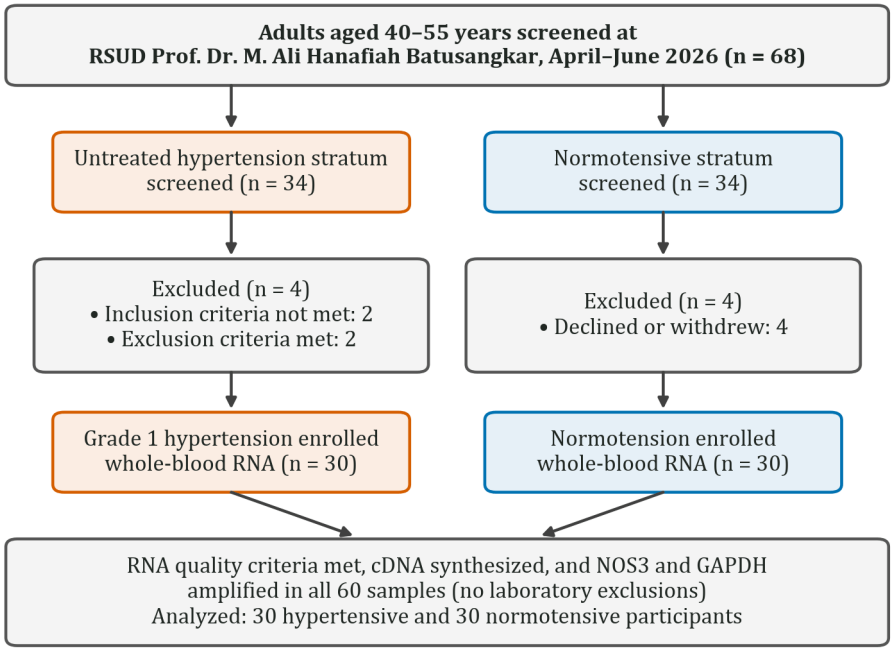


Figure 1. Participant flow (STROBE). Adults aged 40–55 years were screened in two blood-pressure strata; after exclusions, 30 participants with untreated grade 1 hypertension and 30 normotensive participants provided whole-blood RNA, and all 60 samples were amplified and analyzed.

Table 2 presents the characteristics of the participants. The median age was 48 years (41.75–51.25) in the hypertension group and 44 years (42.00–48.25) in the normotensive group ($p = 0.060$). Women made up 86.7% and 93.3% of the two groups ($p = 0.671$) and 90.0% overall (95% CI 79.9–95.3). Median systolic blood pressure was 150 mmHg (145–155) in the hypertension group and 110 mmHg (110–120) in the normotensive group. Median diastolic pressure was 90 mmHg (83.75–100) and 70 mmHg (70–80), respectively (both $p < 0.001$). The upper quartile of 100 mmHg indicates that about a quarter of the cases had diastolic readings in the grade 2 range at the sampling visit, although all had met the grade 1 criteria at eligibility. Four men with hypertension and two normotensive men were too few for any sex-specific analysis.

Table 2. Characteristics of the participants by blood-pressure group.

Characteristic	Grade 1 hypertension (*n* = 30)	Normotension (*n* = 30)	p
Age, years, median (Q1–Q3)	48 (41.75–51.25)	44 (42.00–48.25)	0.060[a]
Women, n (%) [Wilson 95% CI]	26 (86.7) [70.3–94.7]	28 (93.3) [78.7–98.2]	0.671[b]

Characteristic	Grade 1 hypertension (*n* = 30)	Normotension (*n* = 30)	p
Men, n (%)	4 (13.3)	2 (6.7)	
Systolic BP, mmHg, median (Q1–Q3)	150 (145–155)	110 (110–120)	<0.001[a]
Diastolic BP, mmHg, median (Q1–Q3)	90 (83.75–100)	70 (70–80)	<0.001[a]
Antihypertensive treatment	None (untreated)	None; no history of hypertension	—
Screened / excluded / analyzed, n	34 / 4 / 30	34 / 4 / 30	—

Abbreviations: BP, blood pressure; CI, confidence interval; Q1–Q3, first to third quartile. Notes: BP is the mean of three seated sphygmomanometer readings at the study visit. Overall, 54 of 60 participants were women (90.0%; Wilson 95% CI 79.9–95.3). [a] Mann–Whitney U test. [b] Fisher's exact test (odds ratio for male sex 2.15). Quartiles follow the weighted-average definition.

3.2. Quantification cycles

Table 3 summarizes the distributions of the quantification cycles. NOS3 amplified late in every sample. Cq values lay between 30.55 and 35.30 (medians 33.40 and 33.49 in the hypertension and normotensive groups), and two hypertensive samples had NOS3 Cq of at least 35. GAPDH Cq ranged widely, from 23.89 to 35.71, with medians of 29.77 and 31.26. ΔCq ranged from −1.50 to 7.23. Four samples (one hypertensive and three normotensive) had negative ΔCq, meaning that NOS3 crossed the threshold before GAPDH. The NOS3 melt curve showed a main peak at 82–84 °C with a minor shoulder at 72–75 °C. The GAPDH melt curve showed a single sharp peak at 84–85 °C.

Table 3. Quantification cycles of NOS3 and GAPDH and their difference (ΔCq) in whole blood.

Measure	HT mean ± SD	HT median (Q1–Q3)	HT 95% CI of median	HT range	NT mean ± SD	NT median (Q1–Q3)	NT 95% CI of median	NT range
Cq NOS3	33.15 ± 1.06	33.40 (32.26–33.66)	32.80–33.55	31.02 to 35.30	33.32 ± 0.81	33.49 (32.80–33.86)	33.06–33.75	30.55 to 34.40
Cq GAPDH	29.22 ± 2.52	29.77 (27.06–31.02)	27.85–30.79	23.89 to 33.25	30.52 ± 3.19	31.26 (27.37–33.27)	28.04–32.92	24.84 to 35.71
ΔCq (NOS3 − GAPDH)	3.93 ± 2.33	4.05 (2.12–6.13)	2.50–5.84	−0.84 to 7.23	2.80 ± 2.59	2.18 (0.55–5.60)	0.79–4.86	−1.50 to 6.82
Shapiro–Wilk p, ΔCq	0.052				0.017			
Samples with NOS3 Cq ≥ 35, n	2				0			
Samples with GAPDH Cq > 33, n	2				9			
Samples with ΔCq < 0, n	1				3			

Abbreviations: CI, confidence interval; Cq, quantification cycle; HT, grade 1 hypertension; NT, normotension; Q1–Q3, first to third quartile; SD, standard deviation. Notes: HT, n = 30; NT, n = 30. Cq values are means of replicates. Higher ΔCq indicates less NOS3 relative to GAPDH. Distribution-free 95% CIs of the median are order-statistic intervals (exact coverage 95.7%).

3.3. Comparison of ΔCq between groups

ΔCq was higher in the hypertension group, with a median of 4.05 (2.12–6.13) compared with 2.18 (0.55–5.60) in the normotensive group (Figure 2). The Mann–Whitney U test gave U = 315.0 and Z = −2.00. The p value was 0.046 by permutation and 0.046 by the tie-corrected normal approximation; Table 4 gives the details. The Hodges–Lehmann shift was 1.08 cycles (95% CI 0.01 to 2.44), corresponding to a fold change of 0.47 (95% CI 0.18–0.99). The rank-biserial correlation was 0.30. The mean ΔCq was 3.93 ± 2.33 and 2.80 ± 2.59, giving ΔΔCq = 1.13 cycles (95% CI −0.14 to 2.40; Welch t = 1.78, p = 0.081) and a Livak fold change of 0.46 (95% CI 0.19–1.10), that is, a point estimate 54% lower. Hedges' g was 0.45 (95% CI −0.06 to 0.97). Permutation of group labels gave p = 0.080 for the difference in means and p = 0.225 for the difference in medians (1.88 cycles). The bootstrap 95% CI of the fold change was 0.20–1.07.

Table 4. Comparison of whole-blood ΔCq between grade 1 hypertension and normotension.

Analysis	Estimate (95% CI)	Statistic	p
Mann–Whitney U test (primary)	Mean ranks 35.00 vs 26.00	U = 315.0; Z = −2.00	0.046[a]
Hodges–Lehmann shift in ΔCq, cycles	1.08 (0.01 to 2.44)	—	—
Fold change, 2−shift	0.47 (0.18–0.99)	—	—
Rank-biserial correlation	0.30	P(HT > NT) = 0.65	—
Welch t-test, mean ΔΔCq, cycles	1.13 (−0.14 to 2.40)	t = 1.78; df = 57.4	0.081
Fold change, 2−ΔΔCq (Livak)	0.46 (0.19–1.10); point estimate 54% lower	—	—
Hedges' *g*	0.45 (−0.06 to 0.97)	—	—
Permutation test, difference in means	1.13	200,000 permutations	0.080
Permutation test, difference in medians	1.88	200,000 permutations	0.225
Bootstrap, mean ΔΔCq, cycles	1.13 (−0.10 to 2.35)	20,000 resamples	—
Fold change, bootstrap	0.46 (0.20–1.07)	—	—
Equality of variances (Levene)	—	mean-centered / median-centered	0.277 / 0.365

Abbreviations: CI, confidence interval; Cq, quantification cycle; HT, grade 1 hypertension; NT, normotension; ΔΔCq, ΔCq of a participant minus the mean ΔCq of the normotensive group. Notes: Positive shifts and ΔΔCq indicate higher ΔCq (less NOS3 relative to GAPDH) in hypertension; fold change < 1 indicates lower relative NOS3. [a] Permutation p with mid-ranks for tied values (400,000 relabelings); Cq values to two decimal places; normal approximation with tie correction = 0.046. Hodges–Lehmann CI by the Moses method. The geometric mean of per-sample $2^{-\Delta\Delta Cq}$ was 0.46 in hypertension and 1.00 in normotension (calibrator).

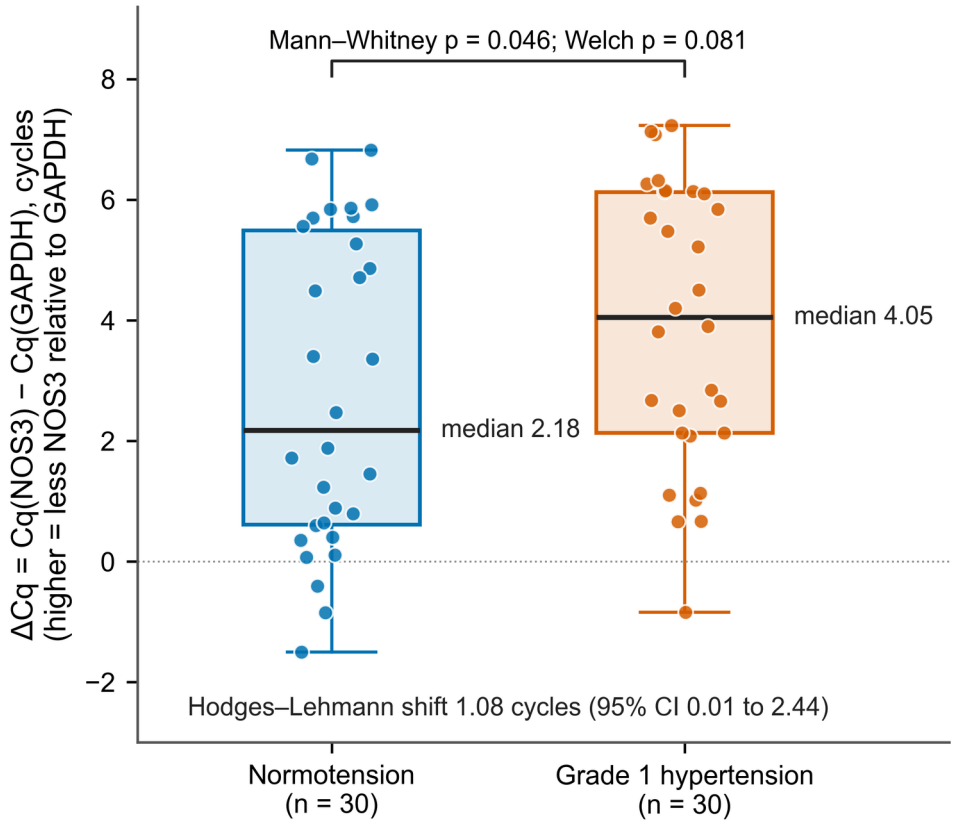


Figure 2. Whole-blood ΔCq of NOS3 relative to GAPDH by group. Boxes show the median and interquartile range, whiskers the range and points individual participants; higher ΔCq indicates less NOS3 relative to GAPDH. The Mann–Whitney (permutation, mid-ranks) and Welch p values and the Hodges–Lehmann shift with its 95% CI are shown.

3.4. Contribution of the target and reference genes

Figure 3 shows that the two components of ΔCq behaved differently. NOS3 Cq did not differ between the groups. The mean difference was −0.17 cycles (95% CI −0.66 to 0.32; p = 0.485), which corresponds to a relative NOS3 template of 1.13 (95% CI 0.80–1.58) in hypertension. GAPDH Cq was 1.30 cycles lower in the hypertension group (difference −1.30, 95% CI −2.79 to 0.18; Welch p = 0.085, Mann–Whitney p = 0.067). Its dispersion was larger in the normotensive group (standard deviation 3.19 vs 2.52 cycles; coefficient of variation 10.4% vs 8.6%; Levene p = 0.039). Late GAPDH amplification (Cq >33) occurred in 9 of 30 normotensive samples and in 2 of 30 hypertensive samples (p = 0.042); see Table 5.

Table 5. Reference-gene analyses: target and reference quantification cycles, normalization slope and covariance-adjusted comparison.

Analysis	Estimate (95% CI)	p
NOS3 Cq, hypertension − normotension, cycles	−0.17 (−0.66 to 0.32)	0.485[a]; 0.383[b]
Relative NOS3 template, HT/NT	1.13 (0.80–1.58)	—
GAPDH Cq, hypertension − normotension, cycles	−1.30 (−2.79 to 0.18)	0.085[a]; 0.067[b]
Relative GAPDH template, HT/NT	2.47 (0.88–6.91)	—
GAPDH Cq variance, HT vs NT	6.33 vs 10.17	0.039[c]
Correlation of ΔCq with GAPDH Cq (all samples)	r = −0.951; R^2 = 0.905	<0.001
Correlation of NOS3 Cq with GAPDH Cq	r = 0.571 (HT 0.378; NT 0.800)	<0.001
Common slope of NOS3 Cq on GAPDH Cq	0.19 (0.11–0.26)	<0.001[d]
Group-specific slopes	HT 0.16; NT 0.20	0.556[e]
Errors-in-variables bounds on the slope	0.19 (within-group OLS) to 0.58 (reverse regression); Deming 0.20	—
Group difference at slope 0.58	0.58 cycles	0.105[a]; 0.084[b]
Assumed slopes giving Mann–Whitney p < 0.05	0.81–1.14 (none for Welch over 0–1.20)	—
ANCOVA group effect on NOS3 Cq, cycles	0.07 (−0.35 to 0.49)	0.732; HC3 0.746
Fold change, ANCOVA-normalized	0.95 (0.71–1.27)	—
Equivalence (TOST), margin ±1 cycle (2-fold)	90% CI −0.28 to 0.42; fold change 0.75–1.21	<0.001
Quade rank ANCOVA	F = 0.079	0.780
GAPDH Cq > 33, HT vs NT, n/N	2/30 vs 9/30	0.042[f]
ΔCq < 0, HT vs NT, n/N	1/30 vs 3/30	0.612[f]

Analysis	Estimate (95% CI)	p
GAPDH Cq SD (CV), HT vs NT	2.52 (8.6%) vs 3.19 (10.4%)	—
Variance ratio, GAPDH Cq / NOS3 Cq (all samples)	9.6	—
ANCOVA residuals, Shapiro–Wilk	—	0.005

Abbreviations: ANCOVA, analysis of covariance; CI, confidence interval; CV, coefficient of variation; Cq, quantification cycle; HC3, heteroscedasticity-consistent standard error; HT, grade 1 hypertension; NT, normotension; SD, standard deviation; TOST, two one-sided tests. Notes: ΔCq normalization assumes that NOS3 Cq changes one-for-one with GAPDH Cq (slope = 1). ANCOVA: NOS3 Cq regressed on group and GAPDH Cq (R^2 = 0.328). TOST: equivalence of the ANCOVA group effect within ±1 cycle. [a] Welch t-test. [b] Mann–Whitney U test. [c] Levene test. [d] Test of slope = 1. [e] Group × GAPDH interaction. [f] Fisher's exact test.

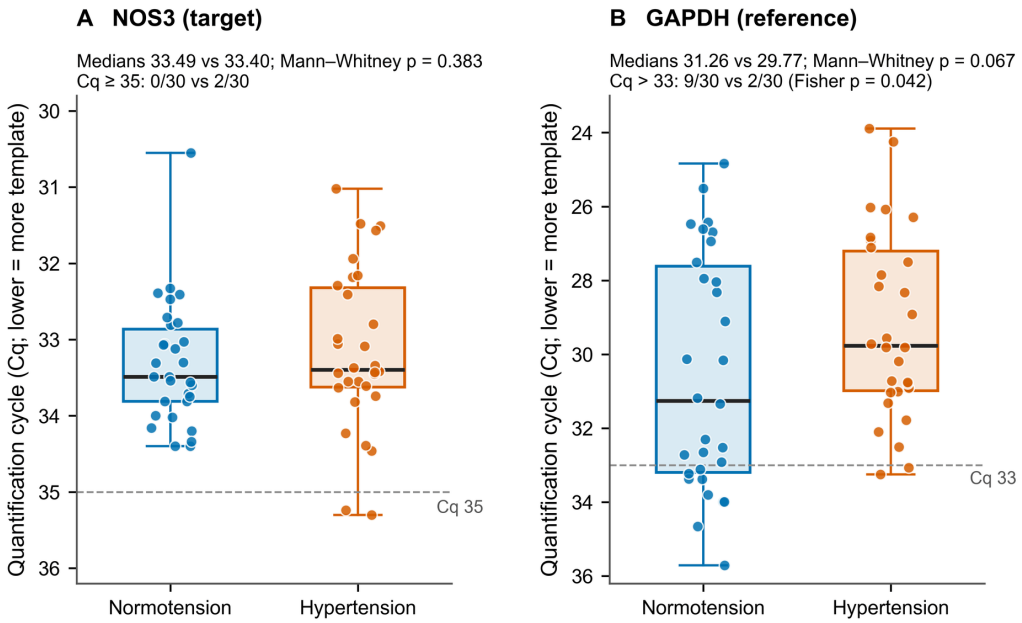


Figure 3. Quantification cycles of the target and reference genes. Panel A shows NOS3 Cq and panel B GAPDH Cq in each group (axes inverted so that higher positions indicate more template). NOS3 Cq did not differ between groups, whereas GAPDH Cq was lower in hypertension and late GAPDH amplification (Cq > 33) occurred mainly in normotensive samples.

NOS3 Cq increased with GAPDH Cq (r = 0.571). The common within-group slope was only 0.19 (95% CI 0.11–0.26), far from the slope of 1 assumed by ΔCq normalization (p < 0.001), and the slope did not differ between groups (interaction p = 0.556) (Figure 4). GAPDH Cq varied about 9.6 times as much as NOS3 Cq, so ΔCq tracked GAPDH Cq closely (r = −0.951; R^2 = 0.905) (Figure 5). This correlation is in part a mathematical consequence of computing ΔCq as a difference and is not by itself evidence about the group contrast. In the ANCOVA with GAPDH Cq as covariate, the group effect on NOS3 Cq was 0.07 cycles (95% CI −0.35 to 0.49; p = 0.732; HC3 p = 0.746), a fold change of 0.95 (95% CI 0.71–1.27). Quade's rank ANCOVA gave F = 0.079 (p = 0.780). The ANCOVA residuals were not normally distributed (Shapiro–Wilk p = 0.005), which is why heteroscedasticity-consistent and rank-based versions are reported.

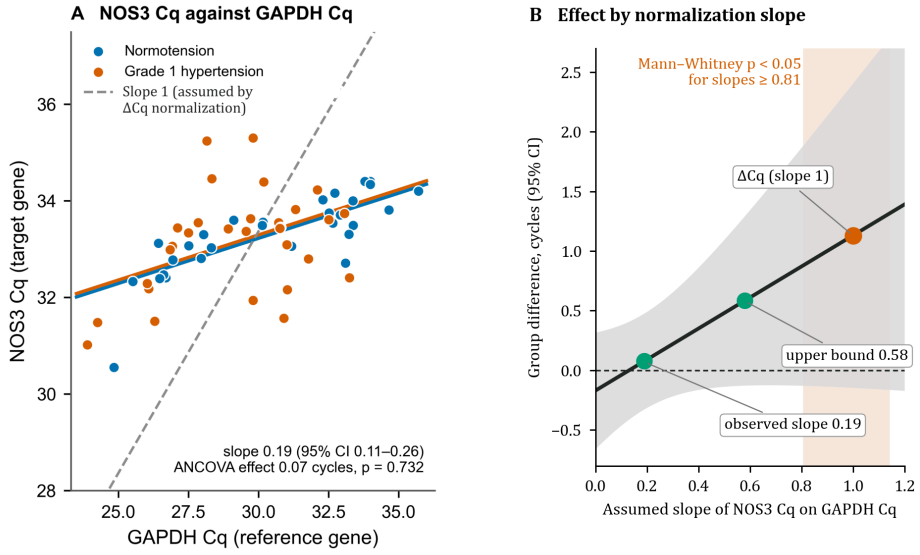


Figure 4. Dependence of the group comparison on the normalization slope. Panel A: NOS3 Cq against GAPDH Cq with the parallel group lines of the covariance-adjusted (ANCOVA) model and a dashed line of slope 1, the relation assumed by ΔCq normalization; the observed common slope is 0.19. Panel B: group difference in NOS3 Cq − slope × GAPDH Cq (hypertension minus normotension) with its Welch 95% CI across assumed slopes; markers show the observed slope, the errors-in-variables upper bound (0.58) and ΔCq (slope 1), and shading the slopes for which the Mann–Whitney test gives p < 0.05.

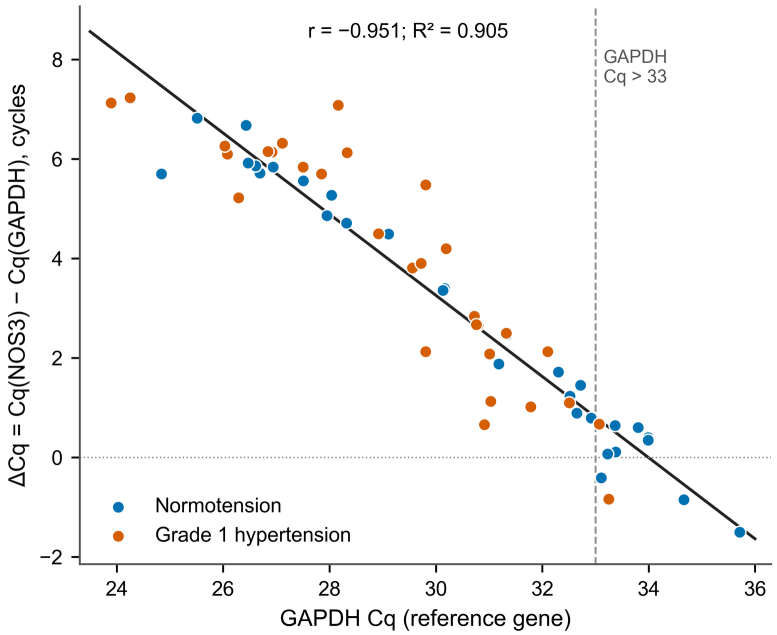


Figure 5. Dependence of ΔCq on the reference gene. ΔCq is plotted against GAPDH Cq for every participant with the least-squares line (R^2 = 0.905). Because ΔCq is computed as NOS3 Cq minus GAPDH Cq and GAPDH Cq varies about 9.6 times as much as NOS3 Cq, this correlation partly reflects mathematical coupling; the group contrast is evaluated in Figures 3 and 4. The dashed vertical line marks GAPDH Cq 33.

Random error in GAPDH Cq attenuates a regression slope, so the slope was also bounded under errors in variables. The within-group regression of NOS3 on GAPDH gave 0.19, Deming regression with equal error variances 0.20, and the inverse of the reverse regression 0.58. Slopes between 0.19 and 0.58 therefore fit the data under random measurement error in either Cq. The group difference in NOS3 Cq − slope × GAPDH Cq rose linearly with the assumed slope (panel B of Figure 4). At the upper bound of 0.58 it was 0.58 cycles (Welch p = 0.105; Mann–Whitney p = 0.084). The Mann–Whitney test reached $p < 0.05$ only for assumed slopes of 0.81 or more, and the Welch test did not reach $p < 0.05$ for any slope between 0 and 1.20. In two one-sided tests with a margin of ±1 cycle (a two-fold difference), the ANCOVA group effect was equivalent to zero (90% CI −0.28 to 0.42; $p < 0.001$).

3.5. Sensitivity analyses

Figure 6 compares the fold-change estimates obtained under the different analytic approaches. The ΔCq difference did not reach $p < 0.05$ in any of the four quality-based exclusion sets (Table 6). Excluding samples with GAPDH Cq above 33 removed nine normotensive and two hypertensive samples and reduced the median ΔCq difference to −0.36 cycles (fold change 0.88, 95% CI 0.38–2.03). The other three exclusions left fold changes of 0.51–0.54 with confidence intervals that included 1. Assuming amplification efficiencies between 80% and 100% for either assay changed the fold change only from 0.45 to 0.52, with Welch p between 0.079 and 0.084 and Mann–Whitney p between 0.043 and 0.053. In the leave-one-out analysis, Mann–Whitney p values ranged from 0.025 to 0.069. Removing a single sample gave $p \geq 0.05$ for 29 of the 60 samples (Figure 7).

Table 6. Sensitivity of the ΔCq comparison to quality-based exclusions and to single samples.

Analysis set	n HT/NT	Median ΔCq difference, cycles	Fold change (95% CI)	Welch p	Mann–Whitney p
All samples	30/30	1.88	0.46 (0.19–1.10)	0.081	0.046
Excluding ΔCq < 0	29/27	0.84	0.54 (0.23–1.27)	0.156	0.087
Excluding GAPDH Cq ≥ 35	30/29	1.58	0.51 (0.21–1.21)	0.124	0.069
Excluding GAPDH Cq > 33	28/21	−0.36	0.88 (0.38–2.03)	0.755	0.572
Excluding NOS3 Cq ≥ 35	28/30	1.68	0.51 (0.21–1.25)	0.140	0.079
Leave-one-out (60 analyses)	29–30/29–30	—	—	—	0.025–0.069
Efficiency NOS3 80%, GAPDH 80%	30/30	—	0.51 (0.24–1.09)	0.081	0.046
Efficiency NOS3 80%, GAPDH 90%	30/30	—	0.48 (0.21–1.09)	0.080	0.044
Efficiency NOS3 80%, GAPDH 100%	30/30	—	0.45 (0.18–1.10)	0.079	0.053
Efficiency NOS3 90%, GAPDH 80%	30/30	—	0.52 (0.25–1.09)	0.082	0.043
Efficiency NOS3 90%, GAPDH 90%	30/30	—	0.48 (0.21–1.10)	0.081	0.047
Efficiency NOS3 90%, GAPDH 100%	30/30	—	0.45 (0.19–1.10)	0.080	0.044
Efficiency NOS3 100%, GAPDH 80%	30/30	—	0.52 (0.25–1.09)	0.084	0.044
Efficiency NOS3 100%, GAPDH 90%	30/30	—	0.49 (0.22–1.10)	0.082	0.043

Abbreviations: CI, confidence interval; Cq, quantification cycle; HT, grade 1 hypertension; NT, normotension. Notes: Exclusion thresholds were defined after data collection (post hoc). Efficiency rows give the ratio calculated with assumed amplification efficiencies (Pfaffl form; 100% = doubling per cycle). Fold changes are Livak $2^{-\Delta\Delta Cq}$ estimates with Welch 95% CIs. Mann–Whitney p values are asymptotic with tie correction. In the leave-one-out analysis, removing a single sample gave p ≥ 0.05 for 29 of the 60 samples.

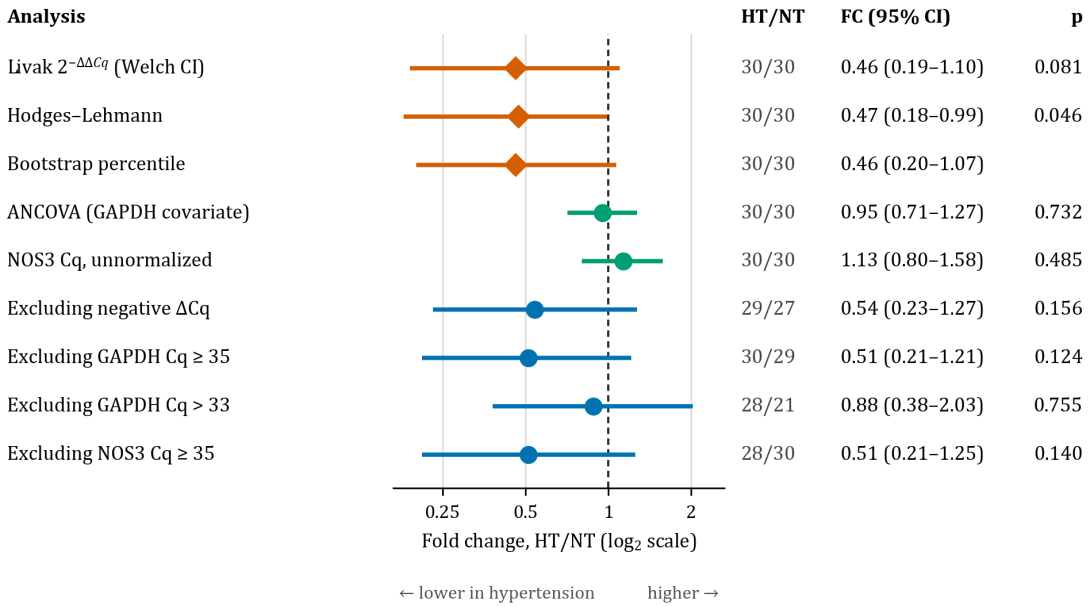


Figure 6. Fold change in whole-blood NOS3 expression (hypertension/normotension) under different analytic approaches. Diamonds show ΔCq-based estimates with all samples, green circles reference-gene-adjusted or unnormalized estimates, and blue circles ΔCq-based estimates after quality-based exclusions; bars are 95% CIs on a $\log_2$ scale. HT, hypertension; NT, normotension; FC, fold change.

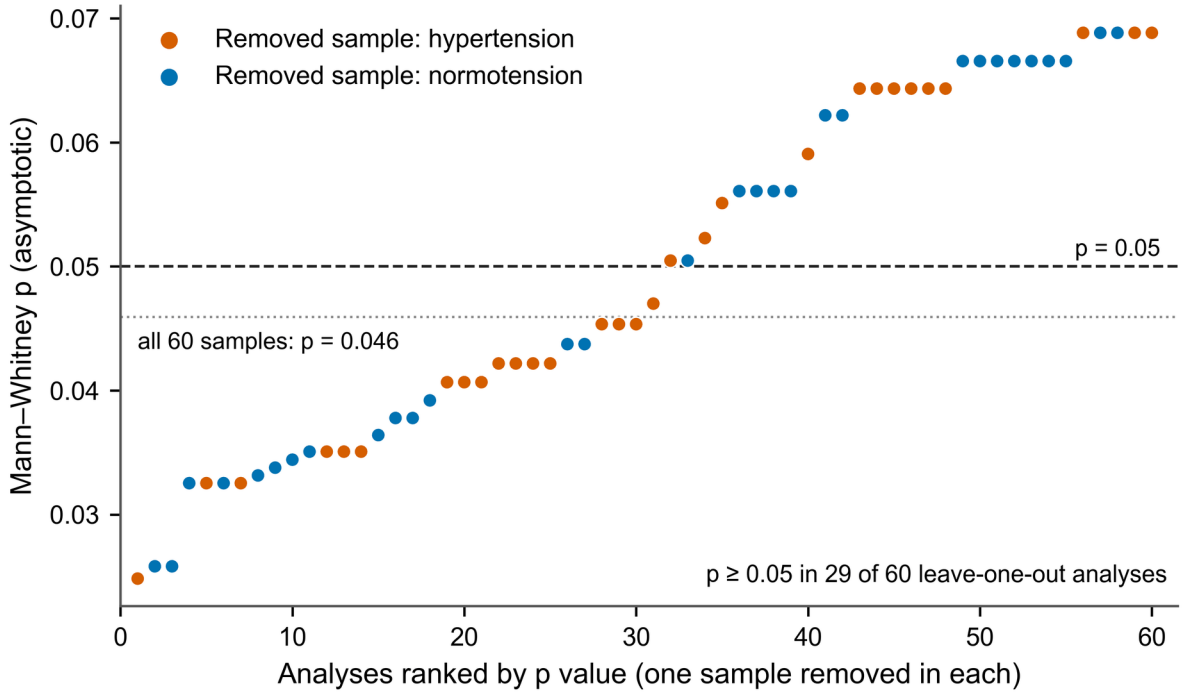


Figure 7. Influence of single samples on the Mann–Whitney comparison. Each point is the p value after removing one sample (colored by the group of the removed sample), ranked from smallest to largest; the dashed line marks p = 0.05 and the dotted line the p value with all 60 samples.

3.6. Age- and sex-adjusted effects: bounds over admissible datasets

The search identified 223 distinct age configurations that reproduced the observed group-level age and sex data exactly (Table 7). Across 200,000 admissible datasets, the age- and sex-adjusted ΔCq difference had a median of 1.13 cycles (2.5th–97.5th percentile 0.75 to 1.50) and reached $p < 0.05$ in 15.0% of datasets. The most extreme admissible values found were −0.98 and 3.20 cycles, corresponding to fold changes from 0.11 to 1.97. This range spans zero, so the observed data cannot identify the direction of the age- and sex-adjusted association. In the model that also adjusted for GAPDH Cq, the group effect had a median of 0.07 cycles (−0.06 to 0.21) and reached $p < 0.05$ in 0.001% of admissible datasets. Extremes were −1.49 and 1.57 cycles (Figure 8).

Table 7. Sensitivity analysis over the space of datasets consistent with the observed age and sex distributions: attainable age- and sex-adjusted group effects.

Model	Unadjusted estimate	Median (2.5th–97.5th percentile)	Most extreme values found	Datasets with $p < 0.05$	Fold-change range
ΔCq ~ group + age + sex	1.13	1.13 (0.75 to 1.50)	−0.98 to 3.20	15.0%	0.11–1.97
NOS3 Cq ~ group + GAPDH Cq + age + sex	0.07	0.07 (−0.06 to 0.21)	−1.49 to 1.57	0.001%	0.34–2.80

Abbreviations: Cq, quantification cycle; ΔCq, difference between the NOS3 and GAPDH Cq. Notes: Admissible datasets reproduce exactly the observed per-sample Cq values, the group age quartiles (41.75/48/51.25 and 42/44/48.25 years; integer ages 40–55), the age comparison p = 0.060 and the numbers of men (4 and 2). 223 distinct age configurations were identified, and 200,000 datasets were sampled by random assignment of ages and sex to participants; percentiles and percentages refer to these. Extremes were sought by hill-climbing from constructed starts and are lower bounds on the attainable range. Effects are in cycles; positive values indicate less NOS3 in hypertension.

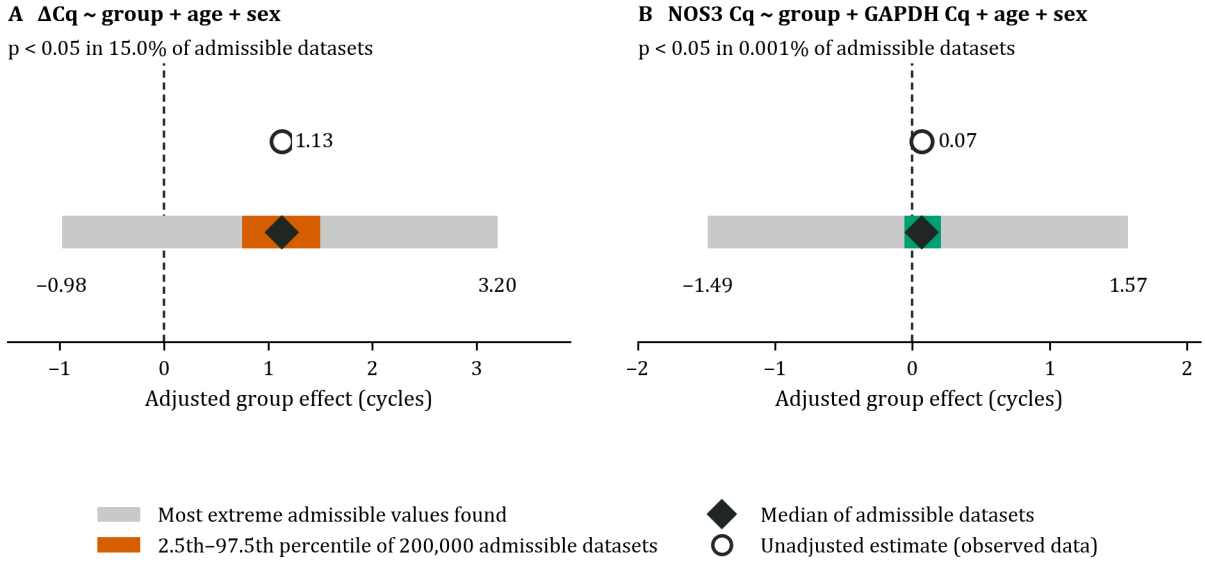


Figure 8. Age- and sex-adjusted group effects over all datasets consistent with the observed group-level age and sex distributions. Panel A: ΔCq model; panel B: NOS3 Cq model with GAPDH Cq as covariate. Colored bars show the central 95% of 200,000 admissible datasets, gray bars the most extreme admissible values found, diamonds the median and open circles the unadjusted estimate; the proportion of datasets with p < 0.05 is given above each panel.

3.7. Power and precision

In a post hoc calculation, with 30 participants per group the study had 41% power to detect the observed standardized difference of 0.45 (Figure 9). Detecting this difference with 80% power would require 78 participants per group, and with 90% power 104. A smaller difference of 0.30 would require 176 per group; Table 8 gives the details. At the observed ΔΔCq, the 95% CI of the fold change would exclude 1 with 60 participants per group (0.25–0.85).

Table 8. Statistical power and precision for whole-blood ΔCq comparisons.

Standardized difference (d)	ΔΔCq, cycles	Fold change	Power with 30 per group	*n* per group, 80% power	*n* per group, 90% power
0.30	0.74	0.60	21%	176	235
0.45 (observed g)	1.12	0.46	41%	78	104
0.60	1.48	0.36	63%	45	60
0.74 (detectable at *n* = 30)	1.81	0.29	80%	30	40
1.00	2.46	0.18	97%	17	23

n per group	95% CI half-width, cycles	Expected fold-change CI at the observed ΔΔCq
30	1.27	0.19–1.10
60	0.89	0.25–0.85
100	0.69	0.28–0.74
150	0.56	0.31–0.67

Abbreviations: CI, confidence interval; SD, standard deviation; ΔΔCq, ΔCq of a participant minus the mean ΔCq of the normotensive group. Notes: Two-sided independent-samples t-test, α = 0.05, pooled SD of ΔCq = 2.46 cycles. Precision rows give the half-width of the 95% CI for the mean ΔΔCq and the corresponding fold-change interval if the observed ΔΔCq (1.13 cycles) were reproduced.

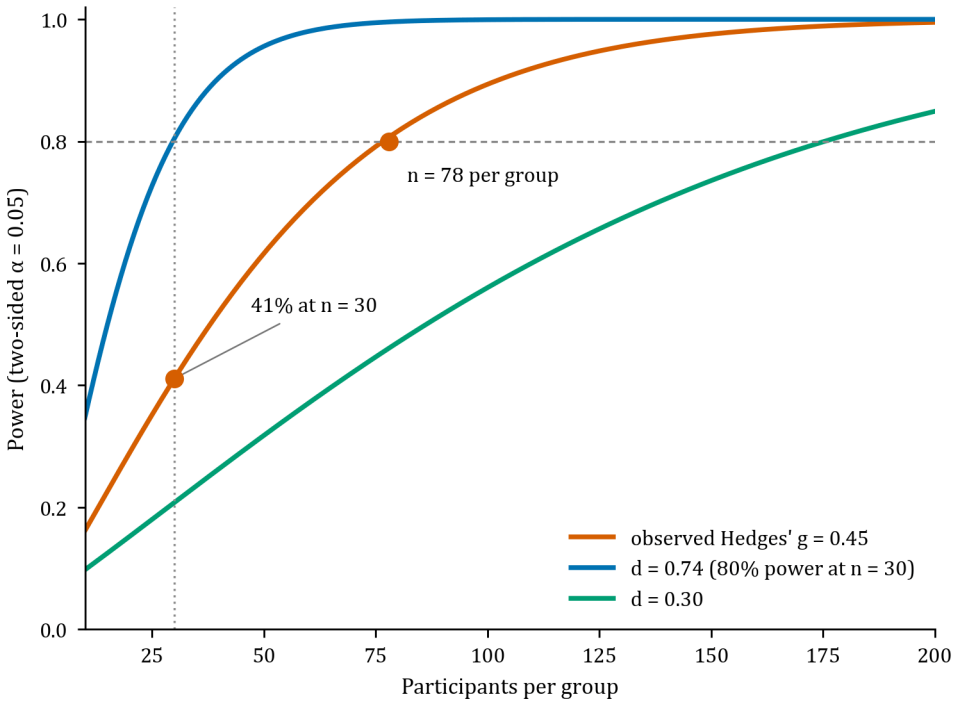


Figure 9. Power of a two-group comparison of ΔCq by sample size. Curves are shown for the observed standardized difference (Hedges' g = 0.45), for the difference detectable with 80% power at 30 per group (d = 0.74) and for d = 0.30 (two-sided α = 0.05).

4. Discussion

The study enrolled adults aged 40–55 years with untreated grade 1 hypertension at RSUD Prof. Dr. M. Ali Hanafiah Batusangkar. In this group, whole-blood NOS3 normalized to GAPDH was lower than in normotensive adults by the protocol-specified rank test, with a fold change of about 0.46. The estimate was imprecise (Livak 95% CI 0.19 to 1.10). Nothing confirmed it. The Welch test, permutation of means, and quality-based exclusion all failed to do so, and the result lost significance when any one of 29 particular samples (of the 60) was removed. The difference was not present in the target gene itself. NOS3 quantification cycles were virtually identical between the groups. GAPDH, by contrast, amplified 1.30 cycles earlier in hypertension and accounted for 90.5% of the variance of ΔCq. Treating GAPDH as a covariate rather than as a fixed one-for-one divisor brought the group effect on NOS3 close to zero (fold change 0.95). The age- and sex-adjusted association could not be identified from the group-level covariate data. The study therefore does not demonstrate reduced NOS3 transcription in early hypertension. It shows something narrower. A single-reference ΔΔCq comparison in whole blood can yield a nominally significant difference that tracks the reference gene and not the target.

4.1. Comparison with previous studies of NOS3 expression

Human data on NOS3 mRNA outside the vessel wall are scarce, and the studies differ widely in material and method. In pre-eclampsia, NOS3 expression was reported to be 8.49-fold lower than in normal pregnancy, although serum NO did not differ significantly (p = 0.20).[30] Placental phosphorylated eNOS protein was also reduced, together with its scaffolding protein striatin, while blood NO again showed no difference.[55] Among peripheral blood leukocytes of patients with early fatty liver disease, NOS2 rather than NOS3 mRNA tracked NO metabolites, TNF-α and IL-6.[33] NOS3 expression in circulating CD34+ progenitor cells rose with hypoxia and with JAK2V617F.[32] Canagliflozin changed the gene-expression profile of these cells in type 2 diabetes.[31] Platelets and erythrocytes add a further and functionally distinct pool. COVID-19 increased the fraction of eNOS-negative platelets in proportion to severity.[37] Erythrocyte eNOS was phosphorylated at Ser1177 by exercise and shear through Piezo1,[35,36] and its activity was increased in diabetes.[38] A disrupted erythrocyte S1P–eNOS axis promoted hypertension in obstructive sleep apnea.[56] Endothelial microvesicles from 12 obese and hypertensive adults reduced eNOS-related NO signaling in recipient cells, compared with those from 12 normal-weight normotensive adults.[57] None of these studies addressed untreated essential hypertension. None reported a reference-gene sensitivity analysis. The one study of blood NOS3 mRNA in hypertension that was identified, in peripheral blood leukocytes of 15 untreated patients with stage I–II hypertension and 25 healthy individuals, found no difference in NOS3 mRNA,[34] so no independent human dataset corroborates the direction of the present point estimate. The 0.46-fold estimate points the same way as the pre-eclampsia data. Unlike those data, it could be traced to the denominator of the ratio.

4.2. Why the reference gene dominated the comparison

Three features of the data may explain the result. First, NOS3 is barely expressed in whole blood. Its Cq values clustered between 32 and 34 however much GAPDH template was present, and the slope of NOS3 Cq on GAPDH Cq was 0.19. A slope well below 1 means that samples with less amplifiable cDNA (late GAPDH) did not show a proportional delay in NOS3. This is the expected behavior of an assay near its limit of detection, where background signal and primer-dimer fluorescence contribute to the threshold crossing. The minor melt-curve shoulder at 72–75 °C agrees with this reading. Relative and absolute methods diverge most when quantification sits at the edges of the dynamic range.[48] MIQE 2.0 requires efficiency, linearity and limit-of-detection data for exactly this reason.[39] Second, GAPDH Cq spanned almost 12 cycles despite a fixed nominal input of 500 ng RNA. That spread suggests variation in RNA integrity, reverse-transcription efficiency or inhibition (none of which was

measured), rather than true differences of more than 3,000-fold in GAPDH expression. Late GAPDH amplification was concentrated in the normotensive group. Removing those samples abolished the ΔCq difference. Pre-analytical handling of EDTA blood, including delays and transport temperature, measurably alters transcript levels.[58] RNA yield and quality from frozen EDTA blood depend on the thawing and extraction protocol.[59] Third, ΔCq normalization forces a slope of 1. Every between-group difference in GAPDH is thereby passed directly into the apparent NOS3 result. Where the reference gene itself behaves differently between groups, ANCOVA-type normalization is more appropriate.[45] The present ANCOVA estimate (fold change 0.95, 95% CI 0.71–1.27) was formally equivalent to no difference within a two-fold margin (90% CI 0.75–1.21; $p < 0.001$). That excludes reductions of the magnitude reported in pre-eclampsia.[30]

Two alternative explanations deserve attention. The first concerns the close correlation between ΔCq and GAPDH Cq, which is partly mathematical coupling. When the reference has about 9.6 times the variance of the target, any difference between them will track the reference, whatever the biology. The evidence that the group contrast belongs to GAPDH therefore does not rest on that correlation. It rests on the separate comparisons (no difference in NOS3 Cq, a 1.30-cycle difference in GAPDH Cq) and on the covariance-adjusted estimate. The second explanation is that measurement error in GAPDH Cq could have attenuated the slope of 0.19. That would bias the ANCOVA towards the unnormalized comparison. Had the true slope been close to 1, ΔCq would be the appropriate normalization and the original result would stand. Under random error in either variable, however, the slope compatible with the data lay between 0.19 and 0.58. This is well below the value of 0.81 needed for the rank test to reach significance. No slope between 0 and 1.20 produced a significant Welch test. Amplification efficiency is a third source of possible bias. The $2^{-\Delta\Delta Cq}$ method assumes doubling per cycle for both assays. Recalculating the ratio for efficiencies of 80–100% left the fold change between 0.45 and 0.52, and the inference did not change.

GAPDH is a glycolytic enzyme. Its expression responds to hypoxia, metabolic state and cell activation, and its suitability varies by tissue and condition. In leukocytes of physically active men, GAPDH was the most stable of six candidates by three of four algorithms, yet the authors still recommended a three-gene panel.[60] In peripheral blood mononuclear cells under hypoxia and activation, commonly used reference genes were among the least stable.[42] Rankings in tuberculosis differed between lesion tissue and blood.[43] A 24-hour sampling study of patients with sleep apnea found ACTB and RPL13A, not GAPDH, to be the most stable in blood.[44] In cord blood of neonates, ACTB with PPIB was the most suitable normalization factor, whereas GAPDH with PPIB or ACTB with GAPDH suited comparisons of neonates with adults.[41] The eight most frequently used housekeeping genes gave opposite conclusions for the same validated target in an experimental hypoxia model. Only an unbiased panel reproduced the true effect.[40] Against this background, a single unvalidated reference gene cannot support a claim about a low-abundance target. The present data are an example of this within a clinical comparison, although only GAPDH was tested.

4.3. What whole-blood NOS3 represents

Whole blood is not endothelium. Its transcriptome is dominated by leukocytes, platelets and reticulocytes. In 138 donors, neutrophil and lymphocyte counts alone were the main determinants of whole-blood levels for up to 75% of protein-coding genes, outweighing age, sex and disease.[46] Shifts in cell proportions can create or mask apparent differential expression.[47] A blood NOS3 signal could therefore change with the leukocyte differential, platelet count or reticulocyte fraction without any change in endothelial transcription. The blood compartments that most directly sample the vessel wall are circulating endothelial cells and endothelial extracellular vesicles.[57] Erythrocyte and platelet eNOS form separate, functionally active pools.[35,37,56] Blood transcriptome studies of hypertension in 497 and 179 Black adults, in 344 African American women, and at single-cell resolution in peripheral blood mononuclear cells identified hypertension-associated genes and long non-coding RNAs. NOS3 was not among the signals emphasized.[49,61,62] Integrated genome-, transcriptome- and expression-quantitative-trait analyses in 60,407 Taiwanese participants identified 14 hypertension loci, including a novel locus at 5p13.1.[50] Salt sensitivity of blood pressure and resistant hypertension have likewise been characterized through other transcripts and microRNAs.[63,64] Blood DNA-methylation studies in Germany, China and Croatia found blood-pressure-associated CpG sites, several of them stable over years. They did not single out NOS3.[51,65,66] Whole-blood NOS3 may therefore be a weak and composition-sensitive proxy for endothelial NOS3.

4.4. Mechanistic interpretation

At the level of the endothelial cell, the biology that links hypertension to eNOS is well established, as shown in Figure 10. NF-κB-driven inflammatory signaling destabilizes NOS3 mRNA.[12] Lipid droplets that build up in endothelium under a high-fat diet do the same and raise blood pressure.[13] Three microRNAs suppress eNOS or its upstream PI3K–Akt signaling: miR-155-5p and miR-214-3p under metabolic stress,[14] miR-155-5p in renovascular hypertension in rats,[15] and miR-24 in spontaneously hypertensive rats and endothelial cells.[67] The hypertension susceptibility gene LSP1 regulates eNOS expression and stability.[68] Angiotensin II and patient-derived microparticles induce endothelial senescence with loss of NO,[69] while endothelial tetrahydrobiopterin deficiency impairs endothelium-dependent vasodilation and produces pregnancy-induced hypertension.[16] Prenatal nicotine programs eNOS uncoupling through RNA methylation.[17] Laminar flow, in contrast, induces eNOS together with KLF2.[10] Every one of these mechanisms predicts lower eNOS output in hypertensive endothelium. None predicts that the change will show up as altered NOS3 mRNA in circulating blood cells. The null ANCOVA result here therefore neither supports nor excludes impaired endothelial eNOS function in the participants, which was not measured. What it shows is narrower: whole-blood NOS3 normalized to a single reference gene is not an adequate readout of that function.

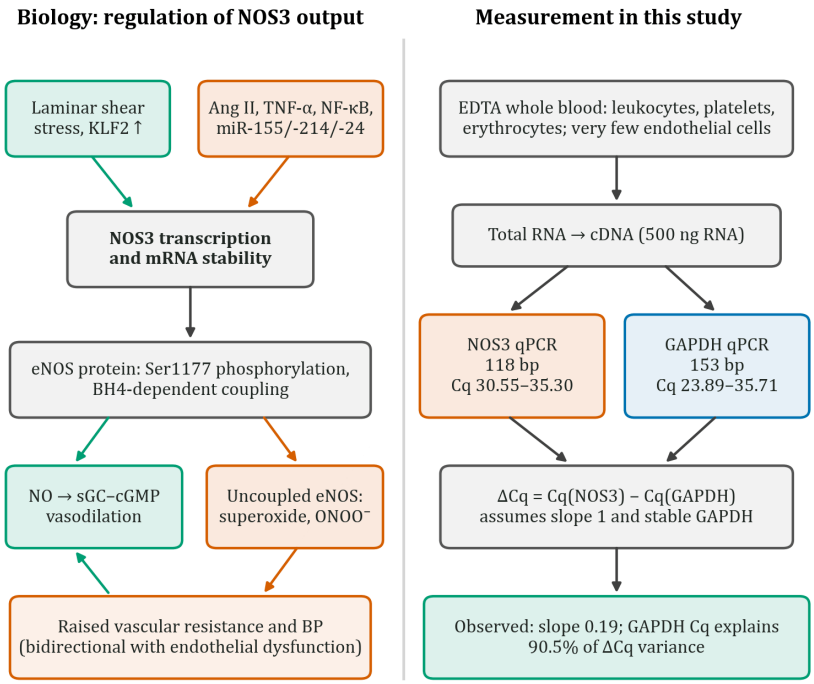


Figure 10. Regulation of NOS3 output in hypertension and the measurement chain of this study. Left: protective (green) and adverse (orange) influences on NOS3 transcription, mRNA stability and enzyme coupling, summarized from published experimental and clinical studies (not derived from the present data). Right: whole-blood NOS3 is measured in circulating cells and expressed relative to GAPDH; the observed slope of 0.19 and the dominance of GAPDH in ΔCq variance limit inference about NOS3 transcription.

Even so, these mechanisms generate testable predictions for blood-based measurement. Suppose inflammatory destabilization of NOS3 mRNA is the dominant process in early hypertension. A reduction should then be detectable in material that carries endothelial transcripts, namely circulating endothelial cells and endothelial extracellular vesicles, and it should come with lower plasma nitrite and nitrate and lower flow-mediated dilation. Suppose instead that the erythrocyte and platelet eNOS pools are affected. The signal should then appear in eNOS protein phosphorylation in those cells and not in whole-blood mRNA. Whole-blood NOS3 mRNA is dominated by leukocyte transcripts and normalized to a single reference gene. Of all the available measurements, it is the one least able to tell these possibilities apart, and this fits the null covariance-adjusted result.

4.5. Clinical and functional context

Functional measures can detect endothelial dysfunction in early hypertension. Brachial flow-mediated dilation averaged 3.5% in 127 untreated middle-aged adults,[18] and values below 6% were associated with worse ventricular indices in newly diagnosed grade 1 hypertension.[19] In de novo hypertension, both flow-mediated dilation and coronary flow reserve were impaired and improved with angiotensin-converting-enzyme inhibition.[20] Lower plasma nitrite and nitrate predicted target-organ damage in 400 hypertensive patients,[21] and a higher L-arginine/asymmetric dimethylarginine ratio went with lower odds of hypertension.[70] In 844 hypertensive patients, endothelial function predicted cardiovascular events with sex-specific thresholds, since women had lower acetylcholine-stimulated vasodilation than men.[71] The NO pathway can also be modified. Exercise increased endogenous NO with a standardized mean difference of 0.89 in a meta-analysis of 37 studies,[72] and across 75 trials dietary nitrate raised plasma nitrate and nitrite and lowered blood pressure in a dose-dependent manner.[73] For clinical practice, these functional and circulating markers, and not whole-blood NOS3 mRNA, remain the evidence-based measures of NO bioavailability in early hypertension.

4.6. Confounding by sex, age and lifestyle

Ninety percent of the participants were women aged 40–55 years, an age range that spans the menopausal transition (menopausal status was not recorded). The hypertensive group was a median of four years older. Menopause lowers flow-mediated dilation, with better preservation after late-onset menopause,[74] and among 10,279 Indonesian women, oral contraceptive use was associated with hypertension (adjusted odds ratio 1.72).[75] Smoking reduced flow-mediated dilation in a dose-dependent manner in 2,209 Japanese men,[76] and Indonesian smokers had serum NO less than half that of non-smokers.[53] Obesity lowered plasma nitrite in young men, although it did not further impair flow-mediated dilation in middle-aged hypertensive patients.[77,78] High sodium intake reduced flow-mediated dilation without changing blood pressure,[79] and aging modified the blood-pressure response to dietary nitrate.[80] None of these variables was linked to the individual expression results in this study. For age and sex, the bounds analysis quantifies what follows from this. The adjusted ΔCq difference could lie anywhere between −0.98 and 3.20 cycles, depending on how age and sex were distributed across the observed Cq values, and it was significant in only 15.0% of the datasets compatible with the data. Small case–control studies often assert group comparability on a non-significant age test. Such a test does not establish that age and sex are irrelevant.

4.7. Regional and national significance

Indonesia faces a rising prevalence of hypertension together with very low control,[4,5] a large undiagnosed fraction,[6] and a disproportionate burden among women with central obesity.[8] National survey analyses led from West Sumatra identified older age and female sex as the strongest demographic correlates.[7] That is precisely the profile of the present sample. Molecular studies of the NO pathway in Indonesia have so far examined NOS3 genotype in rural

populations,[24] serum NO by ethnicity in Aceh[52] and serum NO in smokers in Malang.[53] To our knowledge, this study adds the first whole-blood NOS3 expression data from a regional hospital in Sumatra. A practical lesson follows for regional laboratories that are developing qPCR capacity. Low-abundance targets in whole blood need validated multi-gene normalization, efficiency curves and stabilized blood collection before any biological conclusions are drawn. University biomedical laboratories may be able to meet these requirements within their existing infrastructure, which would help bring Indonesian molecular hypertension research in line with international standards.

4.8. Incidental and technical findings

Four samples had negative ΔCq, which implies that NOS3 was more abundant than GAPDH. For an endothelial transcript in whole blood this is biologically implausible. It suggests that in these samples the GAPDH reaction amplified unusually late. Two hypertensive samples had NOS3 Cq of 35 or more. In that range, stochastic sampling of very few template copies dominates measurement error. Late GAPDH amplification was asymmetrically distributed (9 of 30 normotensive versus 2 of 30 hypertensive samples), and this is the single most influential feature of the dataset. It may reflect differences between the two strata in the timing or handling of sample collection, but the order and timing of collection and extraction by group are unknown, so the idea cannot be tested. The primer design appeared sound on in silico checks (amplification efficiency was not measured). Both NOS3 primers spanned exon–exon junctions, so contamination by genomic DNA would not have produced a product, and the GAPDH amplicon crossed two introns.

4.9. Research agenda

Table 9 details how a confirmatory study should be designed around the failure points identified here. It should enroll at least 78 participants per group to detect the observed standardized difference with 80% power, or 104 for 90% power. Reference genes should be chosen from at least three candidates, such as ACTB, B2M, PPIB and RPL13A, by geNorm or NormFinder in the study's own samples. Normalization should then use the geometric mean of the most stable pair.[40,41,44] Every assay needs a standard curve, no-template and no-reverse-transcriptase controls, and replicate-level reporting. Droplet digital PCR should be considered for NOS3 near the limit of detection.[39,48] Blood should be collected into RNA-stabilizing tubes with a fixed time to processing. A complete blood count with differential should be recorded so that composition can be modeled.[46,58] Participant-level age, sex, menopausal status, contraceptive use, body mass index, smoking, physical activity and sodium intake should be linked to the expression data. Last, whole-blood NOS3 should be anchored to function (plasma nitrite and nitrate, asymmetric dimethylarginine and flow-mediated dilation) and to NOS3 genotype. It should also be compared with endothelial-specific material such as circulating endothelial cells or endothelial extracellular vesicles.

Table 9. Design specification for a confirmatory study of whole-blood NOS3 expression in early hypertension.

Design element	Specification for a confirmatory study	Rationale from the present data
Sample size	≥78 per group (80% power) or ≥104 per group (90% power) for d = 0.45; ≥176 per group for d = 0.30	Observed g = 0.45 had 41% power with 30 per group
Reference genes	≥3 candidates (e.g. ACTB, B2M, PPIB, RPL13A) ranked by geNorm/NormFinder in the study's own samples; normalize to the geometric mean of the most stable pair	GAPDH Cq differed by 1.30 cycles between groups and explained 90.5% of ΔCq variance
Quantification	Standard curves for every assay (efficiency, linear range, limit of detection); NTC and no-RT controls; replicate-level Cq reported; ANCOVA or efficiency-corrected models; droplet digital PCR for NOS3 near the detection limit	NOS3 Cq 30.6–35.3; melt-curve shoulder at 72–75 °C
Specimen	PAXgene or equivalent RNA-stabilizing tubes; fixed time to processing; RNA integrity number	Late GAPDH amplification clustered in controls
Covariates	Patient-level age, sex, menopausal status, hormonal contraception, BMI and waist, smoking, physical activity, sodium intake, complete blood count with differential	Age/sex-adjusted effects not identifiable from group summaries
Biological anchoring	Plasma nitrite/nitrate, ADMA, flow-mediated dilation; NOS3 rs1799983/T-786C genotype; endothelial-specific material (circulating endothelial cells, endothelial extracellular vesicles)	Whole-blood NOS3 reflects circulating cells, not the vessel wall
Exposure	Standardized office BP (≥2 visits) plus home or ambulatory BP to confirm grade; a high-normal BP stratum	Recorded diastolic upper quartile 100 mmHg in cases

Abbreviations: ADMA, asymmetric dimethylarginine; BMI, body mass index; BP, blood pressure; NTC, no-template control; RT, reverse transcription. Notes: Sample sizes from Table 8.

4.10. Strengths and limitations

The study has several strengths. Cases were restricted to untreated grade 1 hypertension. This removes the effect of antihypertensive drugs on endothelial gene expression. Both groups were drawn from the same narrow age band and the same hospital. Quantification cycles were analyzed for every participant, so all expression analyses used individual data and could be decomposed into target and reference components. The primers were verified against reference transcripts. The analysis combined the protocol-specified test with estimation, resampling, covariance-based normalization, influence analysis and an explicit treatment of covariates available only as summaries.

The limitations are considerable. The case–control design cannot establish temporality. Recruitment at a single regional hospital by purposive sampling limits generalizability, and 90% of participants were women. Whole blood was the only material. NOS3 expression in endothelial cells was therefore not measured, and eNOS protein, phosphorylation, coupling and NO production were not assessed. The quality-based exclusion thresholds were defined post hoc. GAPDH was the only reference gene and its stability could not be evaluated, which is the principal reason the biological question remains open. Amplification efficiencies were not determined, although assumed efficiencies of 80–100% did not change the conclusions. Replicate-level Cq values, no-template-control results and RNA integrity numbers were not available either, which prevents correction for efficiency and a formal limit-of-detection analysis. Age, sex and blood pressure were analyzed only as group summaries, so adjusted associations could be

bounded but not estimated. Body mass index, smoking, menopausal status and the leukocyte differential were not available. Recorded diastolic pressure reached 100 mmHg in the upper quartile of cases, so a minority may have exceeded the grade 1 range at the study visit. Finally, the study had 41% power for the effect it observed.

5. Conclusion

Adults aged 40–55 years with untreated grade 1 hypertension showed nominally lower whole-blood NOS3 than normotensive adults when it was normalized to GAPDH (Hodges–Lehmann fold change 0.47, 95% CI 0.18–0.99; Livak fold change 0.46, 95% CI 0.19–1.10). The difference tracked GAPDH and not the target gene. NOS3 quantification cycles did not differ. After adjustment for GAPDH, the fold change was 0.95 (95% CI 0.71–1.27). Group-level data could not identify age- and sex-adjusted associations. Whole-blood NOS3 normalized to a single unvalidated reference gene cannot be interpreted as a marker of endothelial dysfunction in early hypertension unless multi-reference normalization is applied. These findings support the use of MIQE 2.0-compliant multi-reference normalization, efficiency-calibrated or digital quantification and participant-level covariates in future studies of low-abundance blood transcripts. A confirmatory study with at least 78 participants per group, functional NO measures and endothelial-specific material is needed to determine whether NOS3 transcription is altered in early hypertension.

Declarations

Ethics Approval and Consent to Participate

The study was approved by the CMHC Research Center Ethics Committee, Indonesia (approval No. 2026/0398). Written informed consent was obtained from all participants. The study was conducted in accordance with the Declaration of Helsinki.

Consent for Publication

Not applicable; no identifiable individual data are reported.

Availability of Data and Materials

The de-identified dataset and the analysis scripts supporting the findings are available from the corresponding author on reasonable request.

Competing Interests

The authors declare that they have no competing interests.

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Authors' Contributions

D.M.: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – original draft. N.I.L.: Conceptualization, Supervision, Methodology, Writing – review & editing. M.R.: Supervision, Methodology, Validation, Writing – review & editing. H.A., D.R. and F.T.A.: Validation, Writing – review & editing. All authors approved the final manuscript.

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No generative artificial intelligence tools were used in designing the study, collecting or analyzing the data, interpreting the findings, or preparing this manuscript.

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