



High-intensity physical exercise increases serum α -klotho levels in healthy volunteers

Sven-Jean Tan^{1,2}, Melissa M Chu¹, Nigel D Toussaint^{1,2}, Michael MX Cai^{1,2}, Tim D Hewitson^{1,2}, and Stephen G Holt^{1,2}

Abstract

The recently discovered klotho proteins have roles in a diverse range of metabolic processes with the oldest protein, α -klotho, implicated in various cellular pathways in energy, glucose, and phosphate metabolism. Circulating soluble klotho (sKl), derived from membrane α -klotho cleavage, not only has effects on ion channels and insulin signaling pathways, but is inversely associated with mortality. Effects of physical exercise on sKl have not been well studied. The effect of a single high-intensity standardized exercise on sKl and serum phosphate (sPi) levels in healthy adults was investigated. A standard Bruce protocol treadmill exercise was undertaken by 10 fasting healthy volunteers. sKl, sPi, and blood glucose levels were measured in samples collected 1-week prior, immediately pre (T_{pre}), 0 (T_{post}), 30 (T_{30}), 240 (T_{240}) min, and 1-week after exercise. Median (interquartile range) age of participants was 47.5 (44–51) years; five (50%) were male. All study participants achieved at least 90% predicted maximum heart rate (MHR). sKl increased acutely after exercise (T_{pre} median 448 pg/mL vs. T_{post} median 576 pg/mL; $p < 0.01$). There was a nonsignificant sPi decline at T_{30} (T_{pre} 0.94 ± 0.12 mmol/L vs. T_{30} 0.83 ± 0.22 mmol/L). Exercise led to a reduction in blood glucose by T_{240} with median glucose levels at T_{pre} , T_{post} , T_{30} , and T_{240} of 6.0, 6.5, 6.3, and 5.7 mmol/L, respectively. In conclusion, a single high-intensity exercise session is associated with a transient increase in sKl, a delayed reduction in blood glucose, and a nonsignificant decrease in sPi levels in healthy adults. The evaluation of long-term effects of cardiovascular fitness programs on sKl and sPi in healthy individuals and disease cohorts are required to identify potential lifestyle modifications to help improve chronic disease management and long-term outcomes.

Keywords

Exercise, soluble klotho, phosphate

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Introduction

Klotho was originally identified as an antiaging protein almost 20 years ago. Genetic studies identified the klotho (*kl*) gene highlighting that over-expression extended life span and deletion produced complex phenotypes in animals similar to human aging.¹ The *kl* gene encodes a 1012 amino acid long protein, which is expressed in different tissues, although mostly in the kidney especially within the distal convoluted tubule.^{1,2} The gene product is commonly referred to as α -klotho, to differentiate it from two other subsequently discovered members of the klotho family:

β -klotho and γ -klotho. While all three are single-pass transmembrane proteins of different lengths and share a

¹ Department of Nephrology, The Royal Melbourne Hospital, Melbourne, VIC, Australia

² Department of Medicine, The University of Melbourne, Parkville, VIC, Australia

Corresponding Author:

Sven-Jean Tan, Department of Nephrology, The Royal Melbourne Hospital, Grattan Street, Parkville, VIC 3052, Australia.
Email: jean.tan@mh.org.au



substantial degree of homology,³ they appear to have different physiological actions and play a role in a diverse range of metabolic processes.^{1,4–8}

α -Klotho exists in two forms—membrane-bound klotho (mKl) and soluble klotho (sKl). The actions of mKl and sKl differ, with mKl within the kidney predominantly involved in phosphate regulation and renal tubular phosphate reabsorption.⁶ Circulating sKl is the cleaved extracellular domain of mKl (approximately 130 kDa) and can act as a soluble paracrine or endocrine mediator.^{9–12} sKl has also been directly implicated in phosphate regulation.¹³ It displays enzymatic activity that may be important in regulating other ion channels (predominantly involving potassium and calcium transport).^{14–16} sKl has been implicated in peroxisome proliferator-activated receptor- γ (PPAR γ) regulation and insulin antagonism and has been shown to be anti-fibrotic, possess antioxidant, and tumor-suppressive properties.^{17–20} Distinguishing between the various autocrine, paracrine, and endocrine actions of α -klotho has been challenging due to its cell- or organ-specific actions as well as its effects on a wide range of metabolic pathways.²⁰

β -Klotho augments FGF-19 and FGF-21 signaling; is found in the liver, gall bladder, pancreas, colon, and adipose tissue; and participates in bile acid metabolic pathways.^{7,8} γ -Klotho is coupled to FGF19 and is found in the eye, adipose, and kidney, although its function is not clearly understood.³ The cleavage of β -klotho, relative contributions of the three proteins to circulating sKl, the degree of overlap, and mutual influence between the three klotho proteins are unclear.²⁰ Nonetheless, higher sKl levels have been associated with improved survival²¹ and lower levels with increased cardiovascular and all-cause mortality.²²

Physical exercise is known to delay the effects of aging and to protect from cardiovascular disease, diabetes, and cancer.^{23,24} However, the mechanisms through which exercise exerts these beneficial effects are not fully elucidated. It is, therefore, plausible that sKl may mediate some of the benefits of exercise, given the many parallels between physiological effects of exercise and the pleiotropy exhibited by sKl described above. There have been two previous studies demonstrating increased sKl levels in women only, one in response to long-term exercise²⁵ and the other in response to acute, high-intensity exercise.²⁶ We aimed to investigate the effect of an acute high-intensity exercise on serum klotho, glucose, and phosphate in both healthy male and female adults.

Methods

Study population

This single-center study recruited healthy adult volunteers aged >18 years old to participate in a single-intervention study. Exclusion criteria included history of cardiovascular

disease, chronic kidney disease, diabetes mellitus, psychological or medical illness precluding informed consent, and physical handicap impairing ability to undertake a treadmill intervention. Male and females were age-matched with equal numbers of males and females recruited. Informed consent was obtained from all individual participants included in the study. This study was approved by the local human research ethics committee (*HREC 2015.097*) and conducted in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Exercise intervention

All participants were nonsmokers and given routine instructions on how to prepare for a standard exercise test (e.g. instructed to dress with appropriate footwear for the exercise intervention). Participants fasted from midnight, the night prior to the exercise intervention. None of the study participants were on treatment with medications that could affect exercise capacity or exercise responses. A treadmill-based exercise stress test (EST) was performed following a standard Bruce protocol.²⁷ In brief, 12-lead electrocardiogram (ECG) electrodes were placed on the torso with limb leads placed on shoulders and below the level of the umbilicus on respective sides, ensuring good skin contact to reduce skin resistance. All participants started at low workload treadmill speed. A gradual and continuous increase in workload was instituted every 3 min corresponding to the Bruce protocol. An approximately 0.8 mph increase in treadmill speed and 2% ramp incline occurred from stage I (1.7 mph and 10% incline) to stage V (5.0 mph and 18% incline) and a smaller increase in speed occurred for the final stage (VI—5.5 mph and 20% incline). MHR was estimated for each individual participant using the following formula: $MHR = [220 - \text{age}(\text{years})]$. A target of 85% of MHR was applied for the EST. Participants were instructed to advise investigators and EST technician if premature termination of the EST was required. All participants were monitored in the resting phase after the completion of the EST for a minimum of 6 min until blood pressure, heart rate, and ECG tracing approximate baseline values. All participants were provided with a standard meal at 60 min post EST. Participants were also instructed to refrain from strenuous exercise or activity 1-week pre and post the intervention.

Data and sample collection

Baseline measurements including blood pressure, heart rate, and 12-lead ECG were performed on all participants in supine and standing resting positions prior to the EST. ECG recording was continued throughout the EST in accordance with the standard protocol, with standing blood pressure measurements recording at 3-min intervals.

Table 1. Baseline characteristics of study participants ($n = 10$).^a

Clinical parameter	Total ($n = 10$)	Males ($n = 5$)	Females ($n = 5$)
Age (years)	49 (44.75–50.75)	47 (44–50)	50 (48–51)
Gender (male; n , %)	5/10 (50%)	5	5
Resting heart rate (bpm)	84.3 \pm 10.9	86.2 \pm 10.1	82.4 \pm 12.6
Resting systolic blood pressure (mmHg)	131 \pm 9.4	137 \pm 9.1	125 \pm 5*
Resting diastolic blood pressure (mmHg)	83 \pm 7.1	83 \pm 4.5	83 \pm 9.7
Hemoglobin (g/L)	141.1 \pm 16.7	154.6 \pm 4.8	124.3 \pm 5.3
Serum calcium (mmol/L)	2.38 \pm 0.05	2.38 \pm 0.04	2.38 \pm 0.06
sPi (mmol/L)	0.98 \pm 0.12	0.92 \pm 0.12	1.03 \pm 0.11
Serum creatinine (mmol/L)	71.5 \pm 11.1	79.0 \pm 8.6	64.0 \pm 8.0
eGFR (mL/min/1.73 m ²)	98.9 \pm 12.1	99.4 \pm 11.0	98.3 \pm 14.4
Serum sKl (pg/mL)	483 (423–767)	423 (415–614)	669 (448–1095)
Blood glucose reading (optima glucometer; mmol/L)	5.07 \pm 0.42	5.36 \pm 0.15	4.78 \pm 0.41

eGFR: estimated glomerular filtration rate; sPi: serum phosphate; sKl: soluble klotho.

^a t -test or Mann–Whitney U -test performed for continuous variables between gender groups.

* $p < 0.05$ compared to males.

Baseline tests for hemoglobin, blood glucose level, serum biochemistry, and klotho were measured immediately prior to exercise intervention (T_{pre}). Blood samples were also collected 1 week prior to EST (T_{d-7}), 0 (T_{post}), 30 (T_{30}), 60 (T_{60}), 120 (T_{120}), 240 (T_{240}) min; 1 day (T_{d+1}); and 1 week post EST (T_{d+7}) for repeat evaluation of serum phosphate (sPi) and blood glucose levels. sKl was measured at T_{d-7} , T_{pre} , T_{post} , T_{30} , T_{240} , and T_{d+7} .

Biochemical analysis

Routine biochemistry was performed at a central laboratory using standard techniques. sPi was measured via the Olympus AU7000 platform (Beckman Coulter, Inc., Brea, California, USA) using molybdate or the chromogen Arsenazo III, respectively. The analytical range for sPi was 0.32–5.00 mmol/L with analytical coefficient of variation (CV) $< 2.5\%$.

Blood collected in a serum separator tube (SSTTM; BD Biosciences, Franklin Lakes, New Jersey, USA) was allowed to stand at room temperature for 30 min prior to centrifugation (10 min, 4°C, 3000 \times g) and aliquoted for storage at -80°C until batched analysis. Serum sKl concentrations were measured using the IBL sKl ELISA kit (Immuno-Biological Laboratories Co., Ltd, Gunma, Japan) according to the manufacturer's protocol. Based on duplicate measurements, the intra-assay and inter-assay CVs for this study were 3.1 and 7.2%, respectively.

Blood glucose levels were tested at the above time points using a calibrated Freestyle Optium HTM (Abbott Diabetes Care, Doncaster, VIC, Australia) blood glucometer and accompanying blood glucose test strips.

Statistical analysis

Continuous variables have been reported using mean and standard deviation or median and interquartile range (IQR) as appropriate. Student's t -test or Mann–Whitney tests

were used first to evaluate differences between male and females as appropriate. Friedman's test with Dunn's multiple comparison was used to assess changes in sKl and blood glucose levels across all time points measured. Wilcoxon-signed rank test was used to evaluate individual time points against either T_{pre} or T_{post} . Repeated measures ANOVA was used to assess changes in sPi. All statistical analyses were performed with SPSS Statistics Version 24.0 (IBM Corp., Armonk, New York, USA). All graphics have been created with GraphPad Prism 7 for Macintosh (La Jolla, California, USA). A p -value of < 0.05 was considered significant unless otherwise stated.

Results

A total of 10 participants were included in the analysis. All participants were nonsmokers and none were on treatment with medications that could affect exercise capacity or exercise responses (such as beta-blockers or calcium channel blockers). Five participants (50%) were male. Median (IQR) age of participants was 47.5 (44–51) years. Baseline (resting/fasting) characteristics of study participants are presented in Table 1. All participants had baseline blood pressure, heart rate, hemoglobin, glucose, and serum creatinine values that were within the expected range for age and sex. Males exhibited higher resting systolic blood pressures (137 \pm 9.1 vs. 125 \pm 5 mmHg (females); $p = 0.032$). Males demonstrated a trend toward lower baseline sKl levels compared with females (423 (415–614) pg/mL in males vs. 669 (448–1095) pg/mL in females; $p = 0.117$).

All participants achieved at least 90% estimated MHR, and 9/10 reached 100% MHR, with a mean duration of 12.3 min of exercise. All exercise was terminated by the participants with fatigue, and no ECG changes suggesting ischemia were noted at any time during or at least 6 min into recovery. No adverse events were recorded during or following the EST. Mean heart rate and blood pressure readings throughout the EST are shown in Figure 1.

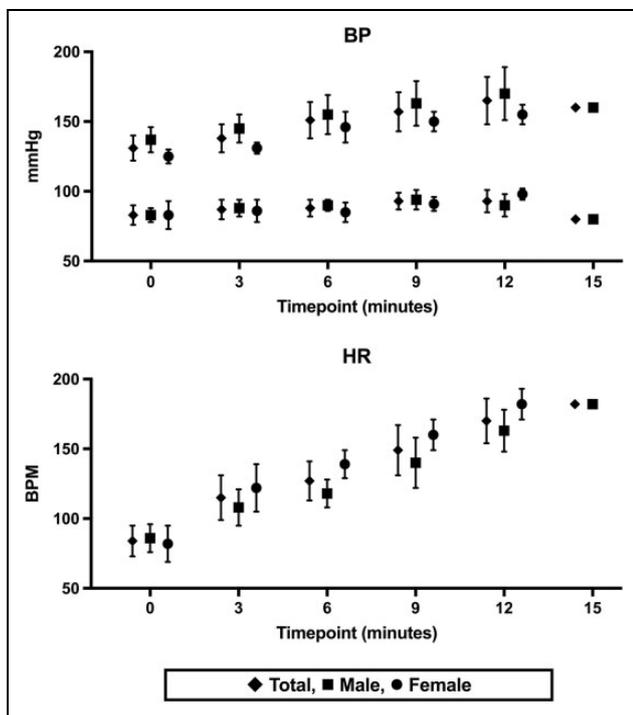


Figure 1. Changes in blood pressure and heart rate with EST intervention. EST: exercise stress test.

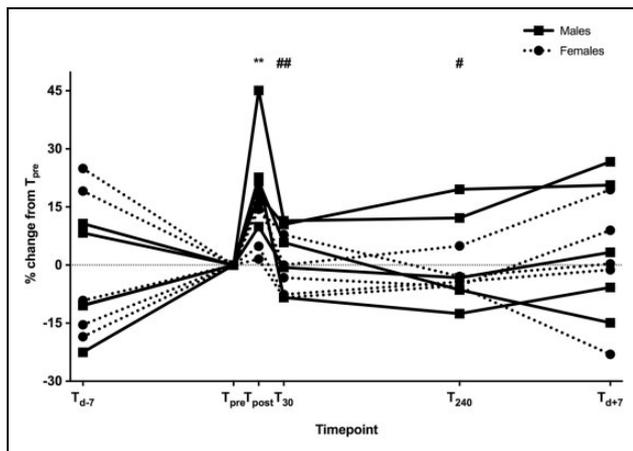


Figure 2. Change in sKl levels in males (black lines) and females (dotted lines) following EST (percentage change from T_{pre}). ** $p < 0.005$ versus T_{pre} . # $p < 0.05$ and ## $p < 0.005$ versus T_{post} . sKl: soluble klotho; EST: exercise stress test.

Changes in sKl for study participants throughout the study period are presented in Figure 2 and Table 2. A significant increase after the EST in sKl was seen (T_{pre} median 483 (423–766) pg/mL vs. T_{post} 602 (514–831) pg/mL; $p < 0.01$) but levels returned to baseline by T_{30} (497 (445–746) pg/mL).

Changes in sPi and blood glucose are presented in Figure 3. A nonsignificant trend toward reduction in sPi post EST was observed, with sPi at T_{pre} compared to T_{30} (T_{pre} 0.94 ± 0.12 mmol/L vs. T_{30} 0.83 ± 0.22 mmol/L;

$p > 0.05$). This reduction was sustained until T_{120} , 0.77 ± 0.17 mmol/L (Figure 3).

Median glucose levels at T_{pre} , T_{post} , T_{30} , and T_{240} were 6.0, 6.5, 6.3, and 5.7 mmol/L, respectively. An initial nonsignificant rise in blood glucose was seen at T_{post} following EST ($p = 0.26$ vs. T_{pre}). This was followed by a reduction in blood glucose observed at the earliest at T_{120} , median glucose level 5.8 mmol/L ($p = 0.65$ vs. T_{post}). This reduction was sustained at T_{240} ($p = 0.013$ vs. T_{post} ; Figure 3).

Discussion

A standardized high-intensity physical exercise in healthy adults causes a transient but immediate increase in sKl levels and a delayed reduction in blood glucose levels. Two other studies have reported an increase in sKl following exercise, and neither of these showed the time course of this rise and were performed in postmenopausal women undergoing a 12-week aerobic exercise program²⁵ or in men and women after a 20-min run.²⁶ In contrast to this study, the latter failed to demonstrate an increase in sKl level in men who were recruited to the same study. However, all participants in this latter study²⁶ were involved in regular physical activity with a minimum of 1-year resistance and aerobic training prior to the study. There are limited studies in this area and the effect of gender has not been widely investigated. It has been suggested though that preexisting fitness level and type of exercise, aerobic versus anaerobic, could impact on the intensity of sKl inflection following the prescribed exercise.^{28,29}

The study presented here included a standardized EST in order to individualize exercise according to age of participants by targeting MHR and included healthy participants who were not allowed to undertake physical activity in the 1 week prior to the intervention. sKl measurements were performed at more than one time point allowing temporal evaluation in sKl change following the intervention. This provides novel additional information compared to the prior two reports,^{25,26} demonstrating that a single episode of high-intensity exercise is not only associated with an increase in sKl in both men and women but is not sustained.

Multiple mechanistic pathways have been considered in the exercise-induced elevation of circulating sKl levels. Physical exercise has been reported to upregulate PPAR γ ,³⁰ downregulate angiotensin-II type-1 receptor (AT1 R),³¹ and oxidative stress³² in animal models. In turn, these pathways have been linked to increased renal klotho mRNA expression and sKl levels,^{33–35} thereby potentially increasing circulating sKl levels. Notably, the time lag in animal models between physical exercise/activity and biochemical evaluation ranged from 6 to 16 weeks,^{30–32} while the follow-on in vitro PPAR γ and oxidative stress effects on klotho mRNA have been reported ranging between 12 h and 48 h^{33,34} and in vivo effects of AT1 R blockade on klotho mRNA between 4 days and 14 days. Correspondingly, the clinical study by Matsubara et al. demonstrated

Table 2. Median sKI levels following EST in males (M) and females (F).^a

Time point	T_{d-7}	T_{pre}	T_{post}	T_{30}	T_{240}	T_{d+7}
sKI (n = 10; pg/mL)	484 (442–748)	483 (423–766)	602 ^{**} (514–831)	497 ^{###} (445–746)	485 [#] (449–738)	517 (419–781)
sKI (n = 5 M; pg/mL)	455 (414–509)	423 (415–614)	596 [*] (508–704)	464 [#] (423–626)	468 (416–603)	507 (416–603)
sKI (n = 5 F; pg/mL)	718 (468–971)	669 (448–1095)	765 [*] (527–126)	722 [#] (440–1006)	649 [#] (446–1041)	671 (436–1148)

sKI: soluble klotho; EST: exercise stress test.

^aFriedman's test with Dunn's multiple comparison was used to assess changes in sKI across all time points measured. Wilcoxon-signed rank test was used to evaluate individual time points against either T_{pre} or T_{post} .

* $p < 0.05$ versus T_{pre} .

** $p < 0.005$ versus T_{pre} .

$p < 0.05$ versus T_{post} .

$p < 0.005$ versus T_{post} .

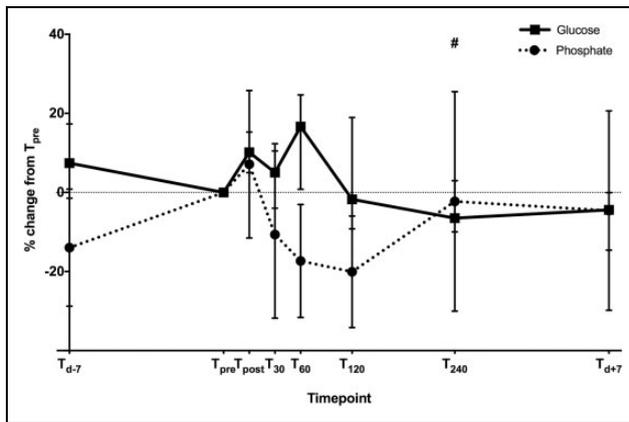


Figure 3. Changes in median blood glucose level (black line) and mean serum phosphate level (dotted line) following EST (percentage change from T_{pre}). # $p < 0.05$ compared to T_{post} for blood glucose level. EST: exercise stress test.

an increase in sKI levels following a 12-week exercise intervention,²⁵ where the aforementioned mechanisms are likely to be implicated. We have shown that acute and transient increases in circulating sKI occur suggesting more acute physiological processes may be involved, such as a rapid activation of cleavage enzymes or a release of preformed sKI within seconds to minutes of strenuous exercise.

A trend toward lower sPi levels post exercise was observed in this study, and while lower sPi levels may be in response to the increased sKI levels mediating phosphate excretion,¹³ the apparent reduction in sPi levels occurs promptly in response to physical activity. Both increase and decrease in sPi levels have been reported previously following physical activity, despite consistently documented increases in parathyroid hormone level.^{36–38} These discrepancies may reflect the different physical exercise programs that the respective study participants were subjected to including a prolonged (5-h) low-intensity bicycle ergometer exercise, a 7-day high-intensity field exercise, and maximal exercise capacity testing prior to and after a 6-week endurance program.^{36–38} However, the reduction in sPi may simply reflect extracellular Pi depletion related to uptake into muscle which outstrips resupply. The wide variation in these prescribed interventions limits the ability to

compare these results and also alludes to the wide variation and day-to-day fluctuations that occur normally when measuring extracellular sPi. Furthermore, no “phosphate sensor” has ever been reported in humans so to invoke changes in sKI levels due to changes in sPi remains highly speculative and this study provides little further insight into such a mechanism.

Lower glucose levels were also detected at T_{240} post EST and this is consistent with well-established evidence showing improvements in insulin sensitivity after a single bout of physical exercise,^{39,40} although the intensity and duration of such activity to achieve maximal beneficial outcome for the healthy population as well as those with insulin resistance continues to be debated.⁴¹ Physical exercise, however, may be one of the key stimulators of skeletal muscle GLUT4 expression.⁴² Taken together, the data presented here might suggest a number of pathways implicated in beneficial changes of physical activity with a central role for glucose and energy metabolism, PPAR γ and sKI.

There are several limitations to this study. The cohort is small in size and, therefore, the findings from this study require validation within a larger cohort. Although there is a lack of representation of patients with chronic disease in this study such as chronic kidney disease, diabetes, and coronary heart disease, the study findings in healthy adults are encouraging. The standardized meal provided to the study participants following the EST in a fasting state was intended to standardize the glucose load, though may have affected the statistical significance of the glucose and phosphate results following T_{60} . Further studies are required to quantify the long-term effects of standardized cardiovascular fitness and high-intensity exercise programs on sKI, sPi, and blood glucose in healthy individuals. Subsequently, studies are needed to evaluate the same programs in disease cohorts in order to identify beneficial lifestyle modifications that could improve chronic disease management and long-term outcomes.

Conclusion

In summary, this study demonstrated that a single short-duration episode of high-intensity physical exercise is

associated with an acute and transient increase in sKl, a delayed glucose reduction, and with sPi levels showing a trend to acute reduction also. Future studies, both larger standardized interventional clinical studies and basic science research, are critical to improve the understanding of the mechanisms involved in benefits of physical exercise and also to enable translation of these concepts to guide clinical practice especially in cohorts with lower sKl or hyperphosphatemia, both of which are associated with impaired glucose tolerance and poorer survival. The relative benefit of exercise in the low baseline fitness group should also be investigated as this may be the group that benefits most from the flow-on effects of sKl rise.

Authors' note

The contents of this article are solely the views of the individual authors and do not reflect the views of NHMRC or the Jacquot Foundation.

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Author contributions

SJT designed and conducted the clinical study, undertook the assays, performed the data analysis, and prepared the manuscript. MMC conducted the clinical study and contributed to manuscript. NDT, MMXC, and SGH aided with the study design and critically appraised the manuscript. TDH provided assistance with the assays and contributed to manuscript.

Declaration of Conflicting Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: SJT has received speaking honoraria from Shire. SGH has received research funding or honoraria from Amgen, Baxter, Gilead, Novartis, and Shire. NDT has received consultancy fees, honoraria and research funding from Amgen, Sanofi, and Shire Pharmaceuticals. MMC, MMXC, and TDH have no conflict of interests to declare.

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Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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