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Original Article

Association between serum hepcidin-25 and primary resistance to erythropoiesis-stimulating agents in chronic kidney disease: a secondary analysis of the HERO trial

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SUMMARY AT A GLANCE

This study aimed to examine and found that pentoxifylline was not associated with a significant decline in serum hepcidin-25 concentration in patients with advanced CKD and primary ESA-resistance. But the extent of the pentoxifylline-associated reduction in hepcidin-25 observed suggests that pentoxifylline may be a clinically and biologically meaningful modulator of hepcidin-25 in patients with ESKD.

ABSTRACT:

Background: **Pentoxifylline has been shown to increase haemoglobin levels in patients with chronic kidney disease (CKD) and erythropoietin-stimulating agent (ESA)-hyporesponsive anaemia in the Handling Erythropoietin Resistance with Oxpentifylline multicentre double-blind, randomized controlled trial. The present sub-study evaluated the effects of pentoxifylline on the iron-regulatory hormone hepcidin in patients with ESA-hyporesponsive CKD.**
Methods: **This sub-study included 13 patients in the pentoxifylline arm (400 mg daily) and 13 in the matched placebo arm. Hepcidin-25 was measured by ultra performance liquid chromatography/quadrupole time-of-flight mass spectrometry following isolation from patient serum. Serum hepcidin-25, serum iron biomarkers, haemoglobin and ESA dosage were compared within and between the two groups.**
Results: **Hepcidin-25 concentration at 4 months adjusted for baseline did not differ significantly in pentoxifylline *versus* placebo treated patients (adjusted mean difference (MD) −7.9 nmol, $P = 0.114$), although the difference between the groups mean translated into a >25% reduction of circulating hepcidin-25 due to pentoxifylline compared with the placebo baseline. In paired analysis, serum hepcidin-25 levels were significantly decreased at 4 months compared with baseline in the pentoxifylline group (−5.47 ± 2.27 nmol/l, $P < 0.05$) but not in the placebo group (2.82 ± 4.29 nmol/l, $P = 0.24$). Pentoxifylline did not significantly alter serum ferritin (MD 55.4 mcg/l), transferrin saturation (MD 4.04%), the dosage of ESA (MD −9.93 U/kg per week) or haemoglobin concentration (MD 5.75 g/l).**
Conclusion: **The reduction of circulating hepcidin-25 due to pentoxifylline did not reach statistical significance; however, the magnitude of the difference suggests that pentoxifylline may be a clinically and biologically meaningful modulator of hepcidin-25 in dialysis of patients with ESA-hyporesponsive anaemia.**

The introduction of erythropoiesis-stimulating agents (ESA) has resulted in a substantial reduction in blood transfusion requirements in patients with chronic kidney disease (CKD).[1] Unfortunately, 7–14% of all patients with end-stage kidney disease show a suboptimal haematologic response to ESA (Hb concentration <100 g/l).[2,3] There are several known causes of

suboptimal response to ESA, including female gender, lower body mass index,[4] inadequate dialysis,[4] older age,[5] diabetes mellitus,[6] cardiovascular disease,[7] inflammation[8] and iron (Fe) deficiency.[4,9] The latter suggests that anaemia of CKD not only results from deficient production of erythropoietin but also from reduced Fe absorption and availability for erythropoiesis. Reduced Fe absorption is likely the consequence of excessive production of the Fe-regulatory hormone hepcidin,[10–12] possibly in response to elevated interleukin-6 (IL-6) or other pro-inflammatory cytokines produced in CKD.

In CKD, hepcidin-25, −22 and −20 levels are elevated and the latter two isoforms of hepcidin increase with declining renal function.[13] Hepcidin levels in CKD are likely to be influenced by a number of additional factors, especially exogenously administered ESAs and Fe therapy. Inflammation (elevated IL-6 and IL1β), Fe therapy and relative erythropoietin deficiency will increase hepcidin levels; however, erythropoietin therapy, reduced Fe stores, hypoxia and anaemia are likely to have a negative effect on hepcidin levels.[14] Interestingly, erythropoietin and Fe are both administered to treat anaemia of CKD but oppose each other's actions on hepcidin production. In a non-randomized, non-placebo-controlled trial, we have previously shown that administration of pentoxifylline to anaemic patients with CKD resulted in significantly reduced serum IL-6 levels, increased haemoglobin (Hb) levels and greater Fe mobilization.[15] It is highly likely that these effects are mediated via a reduction in serum hepcidin levels. Thus, pentoxifylline might be a novel agent for improvement of Fe bioavailability or reduction of total Fe dose requirement in the therapy of anaemia complicating CKD. ESA treatment targeting high haemoglobin levels in people with CKD is associated with increased risks of stroke, vascular access thrombosis and hypertension without any reduction in cardiovascular events,[16] and poor response to ESA treatment is believed to be the major driver of the observed adverse outcomes in CKD.[17,18] Unfortunately, there are no established therapies for primary ESA-hyporesponsive anaemia.[19]

The Handling Erythropoietin Resistance with Oxpentifylline (HERO) trial evaluated the effect of pentoxifylline on erythropoiesis resistance index (ERI) in patients with advanced CKD and primary ESA-hyporesponsive anaemia.[20,21] Its sentinel findings were that pentoxifylline safely increased haemoglobin concentration in patients with ESA-hyporesponsive anaemia but did not significantly modify ESA resistance. In this pre-specified secondary analysis of the HERO study, the role of hepcidin-25 in primary resistance to ESA was evaluated.

METHODS

Details of the HERO study protocol and population are described elsewhere.[20,21] In brief, the HERO study (registration number Australian New Zealand Clinical Trials Registry 12608000199314) was a multicentre, double-blind, randomized placebo-controlled trial to study the effect of pentoxifylline on ERI. The trial included adult patients with Stage 4 or 5 CKD (including dialysis patients) on a stable dose of either erythropoietin or darbepoetin for at least 8 weeks who had ESA-hyporesponsive anaemia for which there was no identifiable cause (such as iron deficiency, bleeding, inadequate dialysis, hyperparathyroidism, malignancy or haematological disorder). ESA-hyporesponsive anaemia was defined as a haemoglobin concentration ≤120 g/l and an ESA resistance index (ERI; calculated as weight-adjusted weekly ESA dose divided by haemoglobin concentration) ≥1 IU/kg per week per gramme per litre for erythropoietin-treated patients and ≥0.005 μg/kg per week per gramme per litre for darbepoetin-treated patients.[22] Participants were randomized in a 1:1 ratio across three variables (study site, CKD stage and ESA class) to pentoxifylline (Trental®, Sanofi-Aventis, Sydney, Australia) 400 mg daily orally, according to manufacturer recommendations in patients with reduced kidney function, or identical matching placebo for a period of 4 months. All other management, including iron supplementation, was provided according to local unit protocols. Of the 53 participants in the HERO trial (26 pentoxifylline, 27 control), 26 consented to participate in the hepcidin-25 sub-study (13 pentoxifylline and 13 control). Plasma concentrations of hepcidin-25 were measured at baseline and 4 months and were compared with changes in the outcome variables (particularly serum iron markers and haemoglobin level). The sampling and handling of specimen for hepcidin-25 levels required collection of a blood sample that had to be rapidly spun and stored at −20°C until shipping of the frozen samples in dry ice was arranged. Not every participating centre was willing to accept this burden, and therefore, not all patients included in the main study participated in the sub-study.

Hepcidin-25 assay

Hepcidin-25 was measured using a highly sensitive and accurate hepcidin-25 assay in humans.[23,24] Hepcidin-25 was isolated from patient serum by solid phase extraction. An isotopically labelled $^{13}C18^{15}N3$-human hepcidin internal standard (Peptides International, Inc., Kentucky, USA) was added to each sample to correct for recovery and quantitation. Analysis was achieved by ultra-performance liquid chromatography (UPLC) / quadrupole time-of-flight mass spectrometry (QTOFMS). Chromatography was by a waters acquity liquid chromatograph (Waters, Milford MA) equipped with an Aeris WIDEPORE 3.6 μ XB C18 column (Phenomenex Inc.) using mobile phases A; 0.2% formic acid and B; acetonitrile (0.2% formic acid) at a flow rate of 500 μl/min. The flow was equilibrated at 10% B, ramped to 15% B over the first minute and 15–40% B over the subsequent 4 min. Mass spectrometry was achieved with a Waters Synapt G2S (Waters, Milford MA). The ion source was operated in positive electrospray ionization mode with a cone voltage of 30 V and temperature of 450°C. The desolvation gas flow and temperature were at 1000 l/h and 450°C, respectively; MS data were collected at a resolution of 18 000. Mass accuracy was maintained by

infusion of leucine encephalin lock mass reference. Quantitation was performed by calculation of peak area, with an ion extraction window of 0.05 Da using Quanlynx (Waters, Milford MA). All samples were measured randomized within a single analytical sequence.

Statistical analysis

This secondary analysis included only the baseline data from the main HERO study to hepcidin-25 sub-study. Results were expressed as frequencies (percentages) for categorical variables, mean ± standard deviation (SD) for continuous normally distributed variables and median (interquartile range) for continuous non-normally distributed variables. All outcomes were analysed in accordance with the intention-to-treat principle. Treatment groups were compared on plasma levels of hepcidin-25 and other outcomes at 4 months, adjusted for baseline values of each outcome of interest, using analysis of covariance. Associations between baseline measurements of hepcidin-25 and changes in haemoglobin and ERI were assessed using a Pearson's correlation test. P values <0.05 were considered statistically significant.

RESULTS

Patient characteristics

The demographics and baseline characteristics of the hepcidin-25 sub-study participants were comparable between the pentoxifylline and control groups (Table 1) and were comparable with those of the main HERO trial study groups. Similarly, ESA dose, ERI, levels of hepcidin-25 and the haematological parameters did not differ between the two groups at baseline (Table 2). Two patients in each treatment group were on parenteral iron, and one from placebo was taking folate. None of the patients were on oral iron or any form of B12 intake. Although it is known that pentoxifylline is capable of modifying the cytokine production,[25] circulating IL-6 and TNF-alpha levels were not measured in this cohort.

Table 1 Demographics and baseline characteristics by treatment group for the hepcidin sub-study. Results are in mean ± SD or numbers (percentage)

	Placebo ($n = 13$)	Pentoxifylline ($n = 13$)
Age at randomization (year)	65.4 ± 16.5	61.8 ± 14.5
Female gender	7 (53.8%)	9 (69.2%)
Ethnicity		
Caucasian	10 (76.9%)	11 (84.6%)
Maori or Pacific Islander	1 (7.7%)	0
Asian	1 (7.7%)	2 (15.4%)
Other	1 (7.7%)	0
Body mass index (kg/m^2)	30.1 ± 6.9	28.9 ± 6.1
Body mass index ≥ 30	6 (46.2%)	4 (30.8%)
Smoking status		
Never	5 (38.5%)	7 (53.8%)
Former	7 (53.8%)	6 (46.2%)
Chronic kidney disease stage		
Predialysis	1 (7.7%)	0
Haemodialysis	12 (92.3%)	13 (100.0%)
Primary cause of end-stage renal failure		
Diabetes	8 (61.5%)	9 (69.2%)
Hypertension	5 (38.5%)	4 (30.8%)

Hepcidin-25 levels

At the end of the 4 month study period, baseline adjusted mean plasma hepcidin-25 tended to be lower in the pentoxifylline group compared with controls (adjusted mean difference −7.92 nmol/l, 95% CI: from −17.9 to 2.04, $P = 0.114$; Table 3). However, this difference was not statistically significant (Table 2). Changes in plasma hepcidin-25 from baseline to month 4 in the pentoxifylline and control groups are shown in Figure 1. There was a significant difference in hepcidin-25 at 4 months compared with baseline in the pentoxifylline group (−5.47 ± 2.27 nmol/l, $P < 0.05$) but not in the placebo group (2.82 ± 4.29 nmol/l, $P = 0.24$). The results did not differ when the one patient with CKD not on dialysis was removed from the placebo group. Three patients showed a significant elevation of hepcidin-25 levels at month 4, none had overt signs of inflammation or infection or elevated circulating iron levels that could explain this increase.

Erythopoietic outcomes

There was no significant difference in haemoglobin concentration at the end of the 4 month study period in the pentoxifylline group compared with controls (adjusted mean difference 5.7 g/l, 95% CI: from −2.51 to 14.0, $P = 0.114$; Table 3). No significant differences were observed between the two groups with respect to ERI, ESA dose, serum ferritin, serum transferrin saturation or reticulocyte count after adjustment for their baseline

Table 2 Baseline biochemical characteristics by treatment group for the hepcidin sub-study. Results are in mean ± SD or numbers (percentage)

	Placebo ($n = 13$)	Pentoxifylline ($n = 13$)
Dosage of ESA (IU/kg per week)	255 ± 87	219 ± 87
ERI (IU/kg per week per gramme Hb)	2.4 ± 0.8	2.1 ± 1.0
Serum hepcidin-25 (nmol/l)	26.9 ± 15.8	28.6 ± 11.5
Haemoglobin (g/l)	106 ± 10	105 ± 8
Serum ferritin (μg/l)	501 ± 350	599 ± 213
Transferrin saturation (%)	26 ± 7	27 ± 11
Reticulocyte count	61 ± 25	57 ± 27
C-reactive protein (mg/l)	23 ± 25	20 ± 19
Vitamin B12 (pmol/l)	425 ± 246	413 ± 157
Folate (nmol/l)	678 ± 1318	1796 ± 1566
Parathyroid hormone (pmol/l)	28 ± 24	31 ± 29
Serum aluminium (mmol/l)	0.38 ± 0.13	0.42 ± 0.08
Haptoglobin (g/l)	1.4 ± 0.6	1.4 ± 0.6

ERI, erythropoiesis resistance index; ESA, erythropoietic stimulatory agents.

Table 3 Primary and secondary outcomes by treatment group for the hepcidin sub-study (mean values and mean differences adjusted for baseline values)

	Placebo ($n = 13$)	Pentoxifylline ($n = 13$)	Difference (pentoxifylline – placebo)	*P*-value
Serum Hepcidin-25 (nmol/l)	30.4 (23.4, 37.4)	22.5 (15.5, 29.5)	−7.9 (−17.9, 2.0)	0.114
Dosage of erythropoietic stimulatory agents (IU/kg per week)	252 (203, 301)	242 (193, 291)	−9.9 (−79.7, 59.8)	0.771
ERI (IU/kg per week per gramme Hb)	2.38 (1.94, 2.83)	2.22 (1.78, 2.66)	−0.17 (−0.46, 0.79)	0.588
Haemoglobin (g/l)	106 (100, 112)	112 (106, 118)	5.7 (−2.5, 14.0)	0.163
Serum ferritin (μg/l)	516 (376, 655)	571 (432, 711)	55.4 (−143, 254.2)	0.570
Transferrin saturation (%)	23 (17, 28)	27 (21, 32)	4.04 (−3.92, 11.99)	0.305
Reticulocyte count	70 (52, 88)	56 (38, 74)	−14.0 (−39.0, 11.0)	0.259

levels (Table 3). Again, the results did not differ when the one patient with CKD not on dialysis was removed from the placebo group.

Although there was substantial intra-individual variability in serum hepcidin-25 (from 4.77 to 67.33 nmol/l), baseline hepcidin-25 levels only showed a positive correlation with baseline ferritin levels (R^2 0.579, $P < 0.005$) but not with baseline transferrin saturation, ESA dose or haemoglobin levels.

DISCUSSION

This pre-specified sub-study of the HERO study showed that oral administration of pentoxifylline in a dose of 400 mg daily for 4 months did not significantly modify plasma concentrations of hepcidin-25 in a selected group of patients with advanced CKD with primary ESA-hyporesponsiveness and who did not have any identifiable cause of ESA-hyporesponsive anaemia.

Anaemia of CKD not only results from deficient production of erythropoietin but also from reduced Fe absorption and availability for erythropoiesis. Fe metabolism is tightly regulated by the Fe-regulatory hormone hepcidin, which is highly expressed by hepatocytes and at lower levels in other tissues including the kidneys. Hepcidin is a negative regulator of Fe absorption by the intestine, and of Fe release from macrophages and hepatic stores. It is secreted into the circulation and binds to the Fe exporter ferroportin, which is expressed on the surface of enterocytes, macrophages and hepatocytes, causing ferroportin internalization and degradation. This limits the absorption and release of Fe and increases retention in the liver and macrophages.[26,27] The balance of a number of positive and negative regulators influences hepcidin expression.[28] Excess Fe and inflammation up-regulate hepcidin expression, which in turn, limits the availability of Fe for erythropoiesis and other Fe-dependent processes. Fe deficiency, anaemia, hypoxia and erythropoietin down-regulate hepcidin expression, which subsequently increases Fe bioavailability.

In the pentoxifylline group, the mean adjusted difference for hepcidin-25 concentration tended to be lower than in the placebo group. While this difference did not reach statistical significance, it is nevertheless noteworthy that the adjusted difference between the pentoxifylline group compared with the placebo baseline mean translated into a greater than 25% reduction of circulating hepcidin-25 due to pentoxifylline, with a lower limit for the 95% CI consistent with a greater than 50%

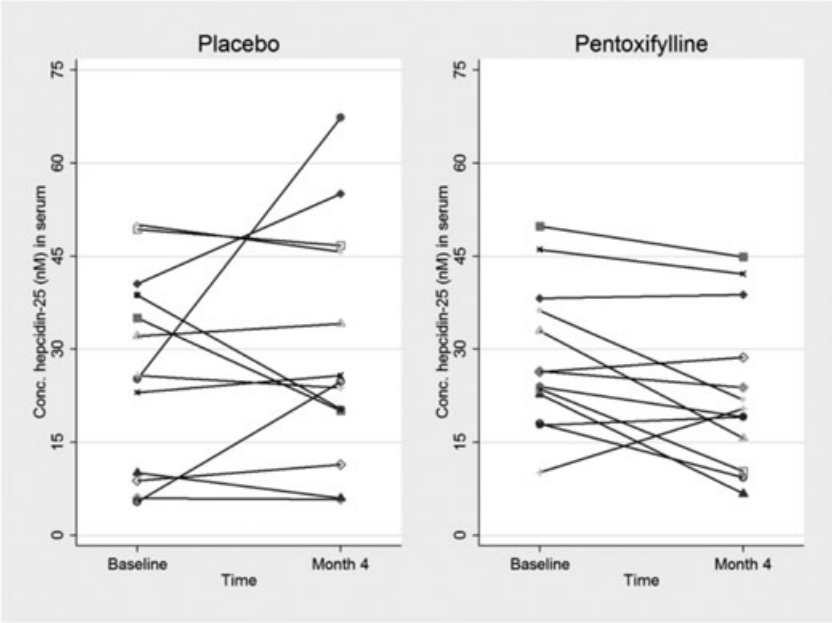

Fig. 1 Serum hepcidin concentration at baseline and 4 months in the pentoxifylline and placebo groups.

reduction. In patients with CKD not on dialysis, hepcidin levels have been found to be approximately 50% lower than in haemodialysis patients and in turn associated with 10% higher haemoglobin levels.[29] Thus, the pentoxifylline-associated relative reduction in hepcidin-25 observed in this study is likely to be clinically or biologically meaningful.

The results of the main HERO study showed a significant increase in haemoglobin in patients with ESA-hyporesponsive anaemia,[21] but in the cohort of the hepcidin-25 sub-study, a similar tendency for higher haemoglobin (pentoxifylline *vs* placebo and 112 *vs* 106 g/l) did not reach statistical significance due to small sample size. The most likely explanation for the lack of a statistically significant difference could be a type 2 statistical error due to the small sample size of patients who consented to be included in this sub-study. A relatively large study in dialysis patients Antunes *et al.* failed to demonstrate an effect of pentoxifylline on either haemoglobin or hepcidin.[30] However, in this study, oral pentoxifylline was given at a dose of 400 mg thrice-weekly only, which is exceedingly low; furthermore, the study was not placebo-controlled and used a commercial hepcidin-25 assay not validated in dialysis patients,[30] not allowing to draw any definitive conclusions despite larger sample size.

An alternative explanation is that there is no relevant biological effect of pentoxifylline on hepcidin-25 secretion. The utility of pentoxifylline for the treatment of anaemia in CKD was recently reviewed in a meta-analysis of seven randomized and four non-randomized studies.[31] The pooled analysis of seven randomized controlled trials of pentoxifylline *versus* placebo or standard therapy (299 participants) did not show any conclusive evidence that pentoxifylline improved anaemia in patients with CKD (mean haemoglobin increase 0.12 g/dl, 95% CI from −0.22 to 0.47), although it was acknowledged that the conclusions that could be drawn from the meta-analysis were limited by an appreciable degree of heterogeneity among studies with respect to CKD stage, anaemia severity, intervention duration and responsiveness to or current therapy with iron or ESAs ($I^2 = 37\%$, $P = 0.14$).[31] A number of the included studies were also limited by small sample size, short follow-up duration and suboptimal methodological quality with either a high or unclear risk of bias.

A third possible explanation for the observed lack of effect of pentoxifylline on serum hepcidin levels in this study may have been that study participants were receiving regular parenteral Fe and their mean serum ferritin levels were quite high. Excess Fe and inflammation up-regulate hepcidin expression, which in turn, limits the availability of Fe for erythropoiesis and other Fe-dependent processes.[28] Therefore, it is possible that excess Fe from parenteral supplementation might have induced up-regulation of hepcidin that could not be offset by the pentoxifylline administered to these patients.

Finally, it is possible that the presumed favourable effect of pentoxifylline on haemoglobin levels may be produced by a mechanism other than hepcidin modulation, for instance, pentoxifylline through improvement of the haemorheological profile and especially its microrheological variables, including plasma and whole blood viscosity, red blood cell aggregation and deformability,[32] which could counteract disturbances in the deformability of the red blood cells that are worsened by the haemodialysis session.[33]

A strength of this study was that it excluded patients if they had evidence of other known causes of erythropoietin-hyporesponsive anaemia, including absolute or functional iron deficiency, vitamin B12 and folate deficiency, elevated aluminium levels, inadequate delivered dialysis dose or hyperparathyroidism.[20,21] The study was also performed within the context of a multinational, multicentre randomized controlled trial, such that the internal and external validity of the findings were high. A key strength of the hepcidin-25 sub-study was the fact that it used an optimized and precise method for quantifying hepcidin-25.[23,24]

Balanced against these strengths, the study was limited by a relatively small sample size, such that the possibility of a type 2 statistical error cannot be discounted. Moreover, the conversion of darbepoetin dose to an erythropoietin-equivalent value using the recommended conversion factor of 200:1 is not exact and potentially introduced variability, although this was mitigated by the inclusion of ESA class in the adaptive randomization allocation algorithm, which balanced erythropoietin and darbepoetin use between each group. Finally, there are some potential causes of erythropoietin-hyporesponsive anaemia that may have occurred throughout the study period and that may have induced up-regulation of hepcidin despite treatment with pentoxifylline. In particular, the occurrence of inflammation, which may have been manifest or occult, in relation to clotted synthetic vascular access, dialysis catheter-related infection, change in the dose of dialysis, periodontal disease,[34–36] or underlying malignancy, was beyond the control of the baseline randomization process.

In conclusion, pentoxifylline was not associated with a significant decline in serum hepcidin-25 concentration in patients with advanced CKD and primary ESA resistance. However, the extent of the pentoxifylline-associated reduction in hepcidin-25 observed in this study suggests that pentoxifylline may be a clinically and biologically meaningful modulator of hepcidin-25 in patients with end-stage kidney disease. Larger prospective studies are required to confirm this association.

COLLABORATORS

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