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Original Article

The value of soluble transferrin receptor and hepcidin in the assessment of iron status in children with cystic fibrosis

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Abstract

Background: The value of ferritin in the diagnosis of iron deficiency is limited in patients with CF since it increases in the presence of inflammation. We hypothesized that the soluble transferrin receptor (sTfR) and hepcidin may provide more information than ferritin in assessing iron status in children with CF.
Methods: We analyzed sTfR and hepcidin in relation to conventional iron status indicators in 49 children with CF.
Results: We found no differences in sTfR concentration between children with and those without ID. sTfR concentrations were within the normal range in all children. Hepcidin concentrations were low, and concentrations below the limit of detection were observed in 25% of the clinically stable children.
Conclusion: The sTfR is not useful to determine the iron status in this population, whereas hepcidin might serve as an early indicator of deficient iron stores in children with CF.
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Keywords: Children; Anemia; Ferritin; Pseudomonas aeruginosa; Hepcidin; Soluble transferrin receptor; Reticulocyte hemoglobin content

1. Introduction

Iron deficiency (ID) as defined by the criteria of the World Health Organization (WHO) (ferritin < 12 μg/L or < 15 μg/L) is common in patients with cystic fibrosis (CF) [1,2]. Although the exact mechanism is unclear, it has been suggested that ID in adult CF patients is primarily functional due to chronic inflammation [3]. In a recent study on iron status in children with CF we showed that low iron stores (ferritin < 12 μg/L) were common in young children, whereas higher ferritin concentrations were observed in older children with CF [2]. These results suggest that unlike in adult CF patients, ID in young CF patients can be solely attributed to an absolute ID. However, ferritin acts as an acute phase reactant, and is therefore no reliable indicator of ID in the presence of infection or inflammation. We suggested that the

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higher ferritin concentrations observed in older children represent an increased state of inflammation, rather than an improved iron status. The results of this study might therefore underestimate the prevalence of absolute ID in children with CF [2].

The clinical implications of ID in children with CF are unclear. In general, ID in children is associated with negative effects on cognitive and behavioral development later in life (4). However, the use of iron supplementation in CF patients is questionable [5] since increased sputum iron levels are thought to contribute to the persistence of Pseudomonas aeruginosa (PA) infections [6]. To our knowledge, there are no data available on the effect of iron supplementation in children with CF. Intravenous (IV) iron supplementation in adult CF patients colonized with PA resulted in an increase in inflammatory markers and worsening of clinical symptoms [7], whereas no clinical deterioration was observed in CF patients with an absolute ID [5]. The potential negative effects of iron supplementation in CF patients likely depend upon the underlying cause; in absolute ID patients might benefit from iron supplementation whereas in functional ID the underlying inflammation should be treated. The accurate assessment of iron status is therefore highly important in the diagnosis and treatment of ID in CF patients.

The soluble transferrin receptor (sTfR) and hepcidin might provide more information than ferritin in assessing iron status in patients with CF. The sTfR is expressed by iron-requiring cells and reflects cellular iron demands and erythropoietic activity. sTfR is less affected by inflammation than ferritin and is therefore suggested as a biomarker that will aid in distinguishing between absolute and functional ID [8].

Hepcidin is an iron regulatory peptide hormone, mainly produced by the liver. Hepcidin decreases intestinal iron absorption, increases iron retention in the cells of the reticulo-endothelial system, and thereby limits the iron availability in the circulation for red blood cell synthesis [9]. Hepcidin is downregulated by absolute ID, hypoxia and erythropoietic activity, and upregulated by inflammation and infection. A recent study in adult CF patients showed that hepcidin concentrations decrease in response to antibiotic treatment of a pulmonary exacerbation [10]. However, no data are available on hepcidin and sTfR in children with CF.

Since CF is characterized by continuous inflammation from an early age [11], we hypothesized that hepcidin concentrations are increased in children with CF, which may contribute to the development of ID. Furthermore, we hypothesized that sTfR may be a more useful indicator of ID than ferritin in children with CF. We therefore assessed sTfR and hepcidin in addition to conventional iron status indicators in children with CF. Furthermore, we analyzed the association between hepcidin and iron status indicators, erythropoietic activity and markers of CF disease progression such as pulmonary function, PA colonization, pancreas insufficiency and liver function.

2. Methods

All 53 children with CF treated in our hospital were included in the study from January 2012 to May 2013. Cystic fibrosis transmembrane conductance regulator (CFTR) genotype was classified as 'severe' if both mutations were class I, II or III, or 'mild' if at least one mutation was from class IV or V, based on previously published classifications [12,13].

A venous blood sample was taken and analyzed for sTfR, hepcidin-25, other iron status indicators (ferritin, hemoglobin (Hb), mean corpuscular volume (MCV), red cell distribution width (RDW), reticulocyte hemoglobin content (Ret-Hb)), C-reactive protein (CRP) as indicator of infection, reticulocyte count and erythropoietin as indicators of erythropoiesis, and albumin as indicator of liver function. Blood samples were drawn between 8 am and 8 pm. The time of blood sampling was categorized as before or after 12 pm, in line with previously reported serum hepcidin concentration patterns throughout the day [14].

Patients (≥12 years) and parents of patients (<12 years) completed a three-day food record to assess dietary iron intake. They received instructions by a dietician before starting the record, and completed records were reviewed with them. We calculated average daily intake of iron from completed records on a personal computer using the Nutricare program (Mc Kesson, Nieuwegein, The Netherlands). We collected data of pulmonary function tests, results of bacterial sputum culture or throat swab, height and body weight. Assessments were preferably done in a stable clinical condition. However, some children were recovering from a pulmonary exacerbation that occurred shortly before the assessment. Assessments were therefore classified as acute or stable, depending on the presence or absence of an increased CRP concentration (>10 mg/L) [15] or a pulmonary exacerbation in the preceding month. A pulmonary exacerbation was defined as clinical deterioration and/or decrease in pulmonary function requiring antibiotic treatment. Children classified as acute were excluded from further analysis. Pulmonary function test was performed and forced expiratory volume in one second (FEV1) expressed as percentage predicted, was chosen as representative parameters for pulmonary function. Bacterial sputum culture or throat swab were obtained and were sent for routine culture in the Department of Microbiology at our institution. PA colonization was defined as the persistent presence of PA in sputum culture despite antibiotic treatment. Patients were defined as pancreas insufficient when fecal elastase-1 concentration was <200 μg/g stool. Height was measured to the nearest millimeter by using a stadiometer. Weight was measured to the nearest 100 g by using a digital weighting scale. Height and weight were expressed as standard deviation scores (SDS). Body mass index (BMI) (weight divided by height^2) and BMI Z-scores were calculated. All children (≥12 years) and parents of children (<12 years) gave written informed consent. The study was approved by the Medical Ethics Committee of South-West Holland.

3. Laboratory analysis

Venous blood was collected and analyzed for sTfR, hepcidin, ferritin, Hb, MCV, RDW, Ret-Hb, CRP, reticulocytes and albumin. Samples were centrifuged and serum was aliquoted and frozen at −80 °C until measurement of erythropoietin and hepcidin-25. Ferritin assays were determined using a Unicel DxI

800 analyzer (Beckman Coulter, Fullerton, CA, USA). Hb, MCV, RDW, Ret-Hb and reticulocytes were performed using Sysmex XE-2100 (Sysmex Corporation, Kobe, Japan) automated hematology analyzers. sTfR was determined using a BN ProSpec system (Siemens, Erlangen, Germany). Soluble transferrin receptor–ferritin ratio (sTfR/logFer) was calculated by dividing sTfR by log10(ferritin). Erythropoietin was determined using an Immulite 1000 (Siemens, Erlangen, Germany). Serum hepcidin-25 measurements were performed by a combination of weak cation exchange chromatography (testing lab: Hepcidinanalysis.com, Nijmegen, The Netherlands) and time-of-flight mass spectrometry (WCX-TOF MS) [16]. Hepcidin measurements were preceded by one freeze–thaw cycle. An internal standard (synthetic heavy hepcidin-25 stable isotope +40 Da; custom made Peptide International Inc.) was used for quantification [17]. Peptide spectra were generated on a Microflex LT matrix-enhanced laser desorption/ionization TOF MS platform (Bruker Daltonics, Bremen, Germany). The lower detection limit of this method was 0.5 nM; average coefficients of variation were 2.8% (intra-run) and 6.4% (inter-run) [17]. For hepcidin concentrations below the limit of detection we used a value of 1/2 times the limit of detection (e.g. 1/2 * 0.5 = 0.25 nM). Hepcidin concentrations are expressed in nanomoles per liter (nM); 1 nM serum hepcidin equals 2.79 μg/L. ID was defined as a ferritin <12 μg/L in children <5 years of age or ferritin <15 μg/L in children >5 years of age, according to the criteria of the World Health Organization [18]. Anemia was defined as Hb > 2 SD below the mean of similarly aged children, as defined by the WHO [18]. Iron deficiency anemia (IDA) was defined as ID in combination with anemia. An increased sTfR and sTfR–Fer ratio were defined as a sTfR concentration and a sTfR–logFer ratio > 2 SD above the mean of similarly aged children as described by Ooi et al. using the same sTFR assay [19]. Pediatric reference ranges for serum hepcidin are only available for a small group of non-anemic, iron replete, young, Kenyan infants with a CRP < 5 mg/L, showing a mean of 2.3 nM (P2.5 P97.5 < 0.5 − 18.1) [20].

4. Statistics

SPSS (version 18.0; SPSS Inc., Chicago, IL) was used for all statistical analysis. Before analysis data were checked on normality using histograms and Kolmogorov–Smirnov test. Distributions of hepcidin and ferritin were positively skewed; logarithmic transformations were applied to normalize the distributions. Data were expressed as means and standard deviations or medians and ranges. A Student *t*-test for continuous variables and a Chi-square test for dichotomous variables were used to compare means between groups. Univariate and multivariate linear regression analyses were used to analyze the association between hepcidin as dependent variable and age, sex, time of blood drawing, iron status indicators, dietary iron intake, CRP, reticulocytes, erythropoietin, FEV1, PA colonization, pancreas insufficiency and albumin as independent variables. A two-tailed P value of <0.05 was considered to be statistically significant.

5. Results

We included 53 children with CF (30 males, 23 females). One child had an unknown genotype and one child had alleles of an unknown functional class. Genotype was classified as severe in the remaining 51 patients. None of the patients used iron supplementation at the time of study. Iron status indicators were available in 49 children (Fig. 1).

Increased CRP concentrations were found in 6 children (range 10–59 mg/L) and assessments were performed during an acute episode in another 7 children (Fig. 1). Children classified as acute (n = 13, Fig. 1) were older compared to stable children, more frequently colonized with PA and had a poorer pulmonary function and (Table 1). We found no differences in time of blood sampling between acute and stable children. Mean ferritin and hepcidin were significantly higher, and sTfR–logFer was significantly lower in acute children compared to stable children

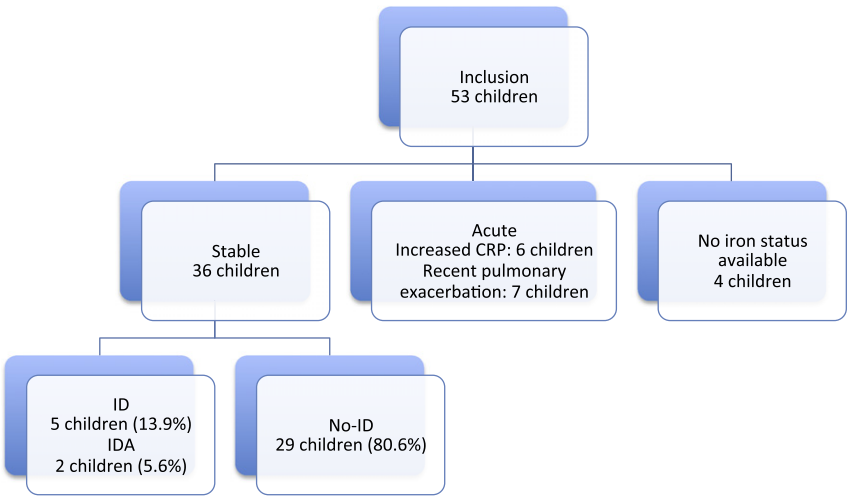

Fig. 1. Flow chart of study population. CRP: C-reactive protein, ID: iron deficiency, IDA: iron deficiency anemia.

Table 1
Characteristics of acute and stable children with CF.
Mean (standard deviation) or number (percentage).

	Acute (n = 13)	Stable (n = 36)	p-Value
Age (years)	12.5 (5.0)	8.9 (4.5)	0.02
Gender	8 (61.5%)	21 (58.3%)	0.84
Blood drawing <12 pm	11 (84.6%)	31 (86.1%)	0.90
BMI (Z-score)	−0.59 (0.89)	−0.13 (0.84)	0.11
Iron intake (mg/day)	6.3 (2.1)	8.4 (4.8)	0.50
FEV1 (% pred)	84.5 (26.5)	100.4 (14.8)	0.02
PA colonization	8 (61.5%)	11 (30.6%)	0.04
Pancreas insufficiency	13 (100.0%)	35 (97.2%)	0.56
Hb (g/L)	129.4 (11.6)	130.7 (9.7)	0.70
MCV (fL)	83.9 (3.0)	82.0 (3.8)	0.11
RDW (%)	13.2 (0.8)	13.3 (0.9)	0.87
Ret-Hb (fmol)	31.3 (1.7)	30.3 (2.4)	0.27
Ferritin (μg/L)	46.8 (27.2)	27.8 (18.8)	0.008
sTfR (mg/L)	1.3 (0.3)	1.5 (0.4)	0.09
sTfR–logFer	0.8 (0.2)	1.1 (0.4)	0.02
Reticulocytes (‰)	10.1 (3.8)	7.9 (2.6)	0.06
Hepcidin (nM)	5.2 (5.8)	1.8 (1.3)	0.001
Albumin (g/L)	37.3 (3.7)	39.6 (3.4)	0.04

(Table 1). Increased sTfR or sTfR/logFer above the cut-off value [19] was not observed in acute children.

In the remaining 36 stable children, ID and IDA were present in 5 (13.9%) and 2 children (5.6%) respectively (Fig. 1). There was no difference in age, gender, dietary iron intake, pulmonary function, PA colonization or pancreas insufficiency, between children in the ID and in the non-ID group (Table 2). No significant difference was observed in mean sTfR concentration between children with and those without ID. sTfR/logFer was significantly higher in children with ID compared to those with no ID (Table 2). However, none of the children with ID had a sTfR or a sTfR/logFer above the cut-off value in similarly aged children [19].

Table 2
Characteristics of stable CF children with ID and those with no ID.
Mean (standard deviation) or number (percentage).

	ID n = 5	No ID n = 31	p-Value
Age (years)	11.3 (4.1)	8.6 (4.0)	0.21
Gender (male)	3 (60.0%)	18 (58.0%)	0.94
Blood drawing <12 pm	5 (100.0%)	26 (83.9%)	0.16
BMI (Z-score)	−0.10 (0.89)	−0.13 (0.85)	0.94
Iron intake (mg/day)	10.6 (5.9)	7.3 (4.1)	0.28
FEV1 (% pred)	102.6 (13.6)	100.0 (15.2)	0.72
PA colonization	2 (60.0%)	9 (29.0%)	0.06
Pancreas insufficiency	5 (100.0%)	30 (96.8%)	0.56
Hb (g/L)	129.8 (17.1)	130.9 (8.3)	0.82
MCV (fL)	80.2 (5.2)	82.3 (3.5)	0.26
RDW (%)	14.1 (1.0)	13.1 (0.8)	0.01
Ret-Hb (fmol)	30.1 (2.4)	30.3 (2.5)	0.87
Ferritin (μg/L)	9.8 (2.8)	30.7 (18.7)	0.02
sTfR (mg/L)	1.7 (0.3)	1.4 (0.3)	0.06
sTfR–logFer	1.8 (0.3)	1.00 (0.3)	<0.001
Reticulocytes (‰)	8.0 (2.6)	7.9 (2.6)	0.96
Erythropoietin	8.8 (7.1)	10.2 (5.3)	0.69
Hepcidin (nM)	0.25 (0.0)	2.0 (1.2)	0.002
Albumin (g/L)	39.3 (2.3)	39.8 (3.8)	0.76

Table 3
Results of linear regression analyses for serum hepcidin concentrations (nM) adjusted for age, gender and time of blood sampling.

	β	95% CI		R^2	p-Value
		Lower limit	Upper limit		
Ferritin (μg/L)	0.027	0.007	0.046	45.1	0.01
Hb (g/L)	0.560	−0.136	1.256	37.9	0.11
MCV (fL)	0.032	−0.085	0.150	33.4	0.58
RDW (%)	−0.294	−0.733	0.146	32.7	0.18
Ret-Hb (fmol)	−0.102	−0.283	0.079	40.5	0.26
sTfR (mg/L)	−0.385	−1.479	0.708	33.8	0.48
sTfR–logFer	−1.081	−2.019	−0.144	42.6	0.03
Reticulocytes (‰)	0.014	−0.157	0.185	35.3	0.87
Erythropoietin (IU/L)	−0.020	−0.085	0.046	33.5	0.55

Hepcidin concentrations below the lower limit of detection (<0.5 nM) were found in 9 children (25.0%), including all children with ID (Table 2). Significantly higher hepcidin concentrations were observed in children with no ID (Table 2). Hepcidin concentrations were lower in blood samples obtained before 12 pm compared to those obtained in the afternoon (1.52 (SD 1.06) and 4.43 (SD 1.36) respectively, p-value 0.001). No association was present between hepcidin and gender, age, dietary iron intake, erythropoietic activity or markers of CF disease progression (data not shown). Results of a multivariate analysis showed that hepcidin was significantly associated with ferritin and sTfR/logFer after adjustment for age, gender and time of blood drawing (Table 3).

6. Discussion

In this study we present for the first time concentrations of hepcidin and sTfR in children with stable CF. ID and IDA, as defined by the WHO criteria [18] were present in 5 (13.9%) and 2 (5.6%) children respectively. sTfR concentrations or sTfR/logFer > 2 SD above the mean of similarly aged children were not observed in any of these children. The mean hepcidin concentration was low, and concentrations below the limit of detection were observed in 9 children (25.0%), including all children with ID. Hepcidin was positively associated with ferritin and sTfR/logFer, but not with erythropoietic activity or markers of CF disease progression.

The use of sTfR and sTfR–logFer ratio did not result in the detection of children with ID as defined by the WHO [18]. Although the sTfR/logFer was significantly higher in children with ID compared to those with no ID, this difference was mainly caused by lower ferritin concentrations in children with ID. Since it has been described that sTfR starts to increase only after severe depletion of iron stores [21,22], we suggest that ID in our population is not severe enough to cause a significant increase in sTfR concentration. We therefore suggest that sTfR and sTfR/logFer are less useful in the diagnosis of ID in our relatively healthy population of children with CF.

Hepcidin concentrations in this study were considerably lower compared to hepcidin concentrations previously reported in 12

adult CF patients [10]. However, these adult CF patients had severe pulmonary function impairment and increased concentrations of the inflammatory marker IL-6 [10,23], whereas children in our study were relatively healthy with good pulmonary function in most patients (mean FEV1 of 100.4% predicted). Even in our acute children, mean hepcidin was lower than concentrations observed in adult CF patients after antibiotic treatment (5.2 nM (SD 5.8) and 8.2 nM, respectively). These data suggest that higher hepcidin concentrations in adult CF patients represent a much more increased state of chronic inflammation than is present in our population of young, relatively healthy CF patients.

The lower hepcidin concentrations in our study might also be caused by different methods of analysis that have been used. The study in adult CF patients used a competitive enzyme-linked immunosorbant assay (c-ELISA) [24] to measure hepcidin. The c-ELISA gives higher hepcidin concentrations compared with the MS-assay used in our study [16]. However, in comparison with studies using the same MS-assay in healthy young Kenyan and non-anemic Malawian infants, hepcidin concentrations in stable CF children without ID in our study were similar, and only in those with ID considerably lower [20,25,26].

Other possible explanations for the low hepcidin concentrations in our study include an increased erythropoiesis due to tissue hypoxia, and impaired hepcidin production due to liver disease. Hypoxia-inducible factor-1 suppresses hepcidin production via erythropoietin [27]. However, mean erythropoietin concentration in our study was lower compared to concentrations observed in another population of stable, young children with CF [28], and similar to those observed in healthy children [29]. Moreover, we found no association between hepcidin and concentrations of erythropoietin or reticulocyte numbers. These results indicate that the low hepcidin concentrations observed in this population of stable, young CF patients, cannot be explained by increased erythropoiesis due to tissue hypoxia. Similarly, impaired hepcidin production due to liver disease is a less plausible explanation since we did not find an association between a low hepcidin and decreased albumin concentration as an indicator of impaired hepatic function.

We found a significant association between hepcidin, ferritin and sTfR/logFer, which suggests that the low hepcidin concentrations in our population reflect deficient iron stores. Since hepcidin was associated with ferritin but not with sTfR, the association between hepcidin and sTfR/logFer was mainly attributed to ferritin. The positive association between hepcidin and ferritin has been reported not only in healthy adult men and premenopausal female blood donors [14,30,31] but also in patients receiving hemodialysis for end-stage renal disease [32]. This association is therefore not unique to CF and perhaps one that is unrelated to systemic inflammation given that direct correlations between these parameters have been described in healthy humans.

Hepcidin concentrations below the lower limit of detection were observed more frequently than ID, as defined by the WHO criteria. Similar results have been reported in anemic, Gambian children with a high prevalence of ID and concomitant infections [33]. It has been reported that regulation of hepcidin synthesis is more dynamic compared with ferritin, which is a specific measure of iron stores and varies more slowly in response to erythropoietic iron demands [34]. Although hepcidin acts as an acute phase reactant similar to ferritin, hepcidin declines more rapidly after an infection or inflammatory signal has been cleared [35]. We hypothesize that in children with ID recovering from inflammation or an infection, hepcidin is already low to maximize iron absorption for erythropoiesis, whereas ferritin is still above the suggested cut-off values for ID. Therefore hepcidin might be a more sensitive indicator than ferritin to detect ID in children with a high burden of inflammation or infections, such as CF patients. However, the clinical use of hepcidin as an indicator of ID is hampered by the limited availability of age-specific reference values for children.

In adults, it has been recently shown that hepcidin could be used as an indicator of dietary iron that is needed for erythropoiesis [33,34] and to monitor the effect of iron supplementation [9] However, the beneficial effect of iron supplementation in CF patients is unclear. The treatment of ID would be desirable considering the negative effects of ID on cognitive and psychomotor development [4] On the other hand PA colonization was observed in 50% of children with undetectable hepcidin concentrations. Since PA biofilm formation requires iron [36], iron supplementation might induce PA colonization in these patients and is therefore questionable.

Our observation of relatively low hepcidin concentrations in children with stable CF warrants further investigation in larger populations. The low number of children with ID is a limitation of our study that possibly explains the absence of a significant difference in sTfR between children with and those without ID. A number of 17 children in both groups would need to be studied in order to detect a possible difference in sTfR concentration between children with and those without ID (power of 0.8, p-value 0.05). Another limitation of our study is that the dietary record used in our study was not validated. Similar to all dietary records, recall bias is a limitation in the use of the three-day food record used in our study and this could have led to inaccurate assessment of iron intake. Inaccurate assessment of dietary iron intake may have contributed to the absence of an association between a low dietary iron intake and ID in our population. However, a 3-day food record is the most accurate method to estimate dietary iron intake in children [37]. More insight on the underlying mechanism of ID in CF patients and the association between PA colonization, serum and sputum iron is warranted in order to identify those patients who might benefit from iron supplementation. Currently, iron supplementation should be considered only in those CF patients with proven absolute ID and no current infection or PA colonization.

7. Conclusion

This study shows that ID was present in 5 children (13.9%) according to the WHO criteria. sTfR concentrations and sTfR/logFer ratios were within the normal range in all children and these biomarkers seem to be less useful to determine the iron status in this population. In contrast to our expectations, hepcidin concentrations were low and concentrations below the lower limit of detection were observed in 9 children (25.0%). Hepcidin was

associated with ferritin and sTfR/logFer, but not with markers of erythropoietic activity or disease severity. We suggest that hepcidin might serve as an early indicator of deficient iron stores in children with CF. However, age-specific reference values for hepcidin in children are urgently needed.

Conflict of interest

DWS is "medical director" of the hepcidinanalysis.com initiative, through which the Radboudumc provides high quality hepcidin measurements for the scientific and medical community on a fee for service basis.

The other authors have no potential, perceived or real conflicts of interest. The authors did not receive any financial assistance to conduct this study or write this manuscript.

Trial registration: http://www.trialregister.nl, Trial registration number: NTR3231.

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