



## CLINICAL RESEARCH ARTICLE

# Non-invasive measurement of erythrocyte zinc protoporphyrin in children

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**BACKGROUND:** Reliable screening for iron deficiency (ID) has required a blood sample and cost-intensive laboratory measurements. A novel method to non-invasively measure erythrocyte zinc protoporphyrin (ZnPP), an established marker for ID, is evaluated in children.

**METHODS:** ZnPP was determined non-invasively by fluorescence measurements on the wet vermillion of the lower lip in 99 hospitalized children aged 9 months to 5 years. For comparison, conventional ID parameters and ZnPP were determined from blood samples.

**RESULTS:** The non-invasively measured ZnPP values had limits of agreement (LoA) of 14  $\mu\text{mol ZnPP/mol heme}$  (95% confidence interval: 9–20) compared to fluorescence measurements directly in blood. Repeated high-performance liquid chromatography reference determinations had comparable LoA of 14  $\mu\text{mol ZnPP/mol heme}$  (9–17). Non-invasive ZnPP measurements had sensitivity and specificity of 67% (39–88%) and 97% (91–99%), and negative and positive predictive value of 94% (90–97%) and 80% (55–93%), for detecting ID as defined by the soluble transferrin receptor (sTfR). In groups with more severe ID as defined by serum ferritin and sTfR, higher ZnPP values were found, with the highest ZnPP values for the group with ID anemia.

**CONCLUSION:** Non-invasive ZnPP measurements are reliably feasible in children. The simple and fast method has the potential to enable wide-spread screening for ID.

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## INTRODUCTION

Iron deficiency (ID) is the most common nutrient deficiency worldwide.<sup>1</sup> When severe, it can lead to ID anemia (IDA).<sup>2</sup> Among children and adolescents, IDA is the leading cause of years lived with disability, affecting 619 million children and adolescents in 2013.<sup>1</sup> In the United States, ID and IDA have a prevalence of ~7.1 and ~1.1% among children aged 1 to 5 years, respectively.<sup>3</sup> Although ID and IDA can be effectively treated, the prevalence has not decreased over the past decade.<sup>3</sup>

Symptoms of ID include a reduced immune responsiveness<sup>4</sup> and diminished physical performance.<sup>5</sup> In the first years of life, iron is particularly important for the rapidly developing brain.<sup>6</sup> Early ID appears to be associated with persistent central nervous system alterations, which cannot be completely reversed by iron supplementation in later years.<sup>6–11</sup>

Screening for IDA is frequently done by measuring hemoglobin, which can be determined non-invasively and might thus enhance applicability. For the United States, the American Academy of Pediatrics recommends universal screening by hemoglobin for children aged 12 months.<sup>12</sup> Others have come to a similar conclusion,<sup>13,14</sup> while the US Preventive Services Task Force has concluded that there is not enough evidence applicable to the United States to recommend universal IDA screening in children aged 6 to 24 months.<sup>15</sup> In the United States, only about one-third

of children with anemia are iron deficient, while two-third have anemia of other causes, for example, anemia of chronic disease.<sup>3,12,15,16</sup> Consequently, screening for IDA by hemoglobin alone has a positive predictive value (PPV) of only ~35%.<sup>15,16</sup> Other, more reliable parameters to assess the iron status require a blood sample and cost-intensive laboratory measurements.

Erythrocyte zinc protoporphyrin (ZnPP) is a reliable marker for ID, which was shown to be more sensitive and more specific for ID than hemoglobin in children.<sup>17,18</sup> An increased amount of ZnPP is found in erythrocytes when an insufficient amount of iron is present in the last step of the heme synthesis pathway.<sup>19</sup> More pronounced ID leads to higher ZnPP values, with highest values found for severe IDA.<sup>20</sup> ZnPP can be measured with a portable hematofluorometer,<sup>21</sup> but requires a drop of blood. Recently, we presented a method to measure ZnPP non-invasively from the lower lip and established proof of concept in 56 women after childbirth.<sup>22</sup> This method enables non-invasive, fast and specific ID and IDA testing at the point of care, without the need for a blood withdrawal.

The objective of this investigation is<sup>1</sup> to explore if non-invasive ZnPP measurements are feasible in children aged 9 months to 5 years, and<sup>2</sup> to assess if ZnPP is a reliable marker for detecting iron-deficient erythropoiesis (IDE) and IDA in the investigated patient group of hospitalized children.

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## METHODS

### Clinical evaluation

Hospitalized children ( $n = 100$ ) aged between 9 months and 5 years undergoing clinically indicated blood withdrawal were recruited between April and September 2016 at the Dr. von Hauner Children's Hospital, LMU Munich, Germany. Children who received a transfusion of blood products in the 4 months preceding the measurements were excluded. A diverse cohort was chosen to demonstrate the general feasibility and accuracy of the non-invasive method, independent of possible comorbidities. A more detailed description of the cohort can be found in Supplementary Materials. For each subject, the age was recorded and the skin color assessed according to the Fitzpatrick Skin Phototype Classification Score.<sup>23</sup> Ethical approval for the study was given by the Institutional Ethical Board of the Medical Faculty, Ludwig-Maximilians-University of Munich, Germany (study identifier: LFL\_S2/2015, ClinicalTrials.gov identifier: NCT02701309). Written informed consent was obtained from each subject's parent or legal guardian prior to study enrollment.

### Blood sample collection and laboratory analysis

Residual blood samples (ethylenediaminetetraacetic acid (EDTA) and serum tubes) after completed diagnostic analyses were obtained on the day of the non-invasive measurement. The soluble transferrin receptor (sTfR), serum ferritin, c-reactive protein (CRP), and a complete blood count were determined as routine measurements. For fluorescence spectroscopic measurements, a 100  $\mu\text{L}$  aliquot of EDTA blood was stored at  $+4^\circ\text{C}$ ; measurements were carried out within 24 h after the blood withdrawal. For the determination of ZnPP by high-performance liquid chromatography (HPLC), two aliquots of EDTA blood (250  $\mu\text{L}$  each) were stored at  $-80^\circ\text{C}$ . For six subjects, only an incomplete set of parameters could be determined due to an insufficient amount of left-over blood. Threshold values were supplied by the Institute of Laboratory Medicine for ferritin (low:  $<12\ \mu\text{g/L}$  for children  $<12$  months, otherwise  $<24\ \mu\text{g/L}$ ), CRP (elevated:  $>0.5\ \text{mg/dL}$ ), hemoglobin (low:  $<10.7\ \text{g/dL}$ ), and ZnPP (elevated:  $>50\ \mu\text{mol ZnPP/mol heme}$ ). For the sTfR, only a threshold value for adults was available (elevated:  $>1.76\ \text{mg/L}$ ). This threshold does not apply to children, for whom generally higher sTfR values are found.<sup>24</sup> A study in 2153 children aged 1 to 5 years found an 11% higher threshold for sTfR compared to adults.<sup>24</sup> Therefore, in this study, values of sTfR  $>1.95\ \text{mg/L}$  ( $=1.11 \times 1.76\ \text{mg/L}$ ) were considered as elevated.

### Clinical definitions

The stage of ID (iron depletion, IDE, or IDA) was determined for each child from ferritin, sTfR, and hemoglobin values as suggested in the literature:<sup>25</sup> "No ID": normal ferritin, sTfR, and hemoglobin; "Iron depletion": low ferritin, but normal sTfR and hemoglobin; "IDE": low ferritin levels or elevated CRP, elevated sTfR, but normal hemoglobin; "IDA": low ferritin levels or elevated CRP, elevated sTfR, and low hemoglobin.

Normal ferritin values were disregarded if an elevated CRP suggested a falsely elevated ferritin value due to its reaction as acute-phase protein.

### Non-invasive measurement of the ZnPP/heme ratio

All non-invasive fluorescence measurements were conducted by the same physician (FM) during the clinically indicated inpatient stay. The ZnPP/heme ratio was measured non-invasively with a device adapted from the one used in a previous study.<sup>22</sup>

In brief, a 1000  $\mu\text{m}$  optical fiber is placed gently on the red vermillion of the lower lip of the subject. The fiber is embedded into a 12-mm steel handpiece which narrows to 6 mm at the tip. For 13 children an optical fiber probe with a 5-mm handpiece embedded in a sterilized single-use pacifier was used instead to examine the influence on the behavior of the child. To avoid

contamination or transmission of pathogens, the optical fiber probes were disinfected before and after usage.

The optical fiber probe is slowly moved over the lip, until the device indicates that a tissue site is found where the blood content is sufficient to ensure a reliable measurement. The identification is done in real time by analyzing the spectral features of the blood absorption in the tissue background fluorescence. Then, the measurement sequence is started with alternating excitation wavelengths (central wavelengths: 407 and 425 nm). The measurement procedure was repeated until 100 pairs of fluorescence spectra were recorded at tissue sites with sufficient blood content or until the measurement had to be stopped due to the behavior of the child. For the analysis, the fluorescence spectra excited at 407 nm were subtracted from the fluorescence spectra excited at 425 nm, reducing the fluorescence background.<sup>22,26</sup> A spectral fitting procedure determined the amplitude of the ZnPP fluorescence for each spectrum. Averaging over all determined ZnPP amplitudes for each patient yielded the ZnPP value, which is proportional to the ZnPP/heme ratio.

### Fluorescence spectroscopic measurements of the ZnPP/heme ratio in blood

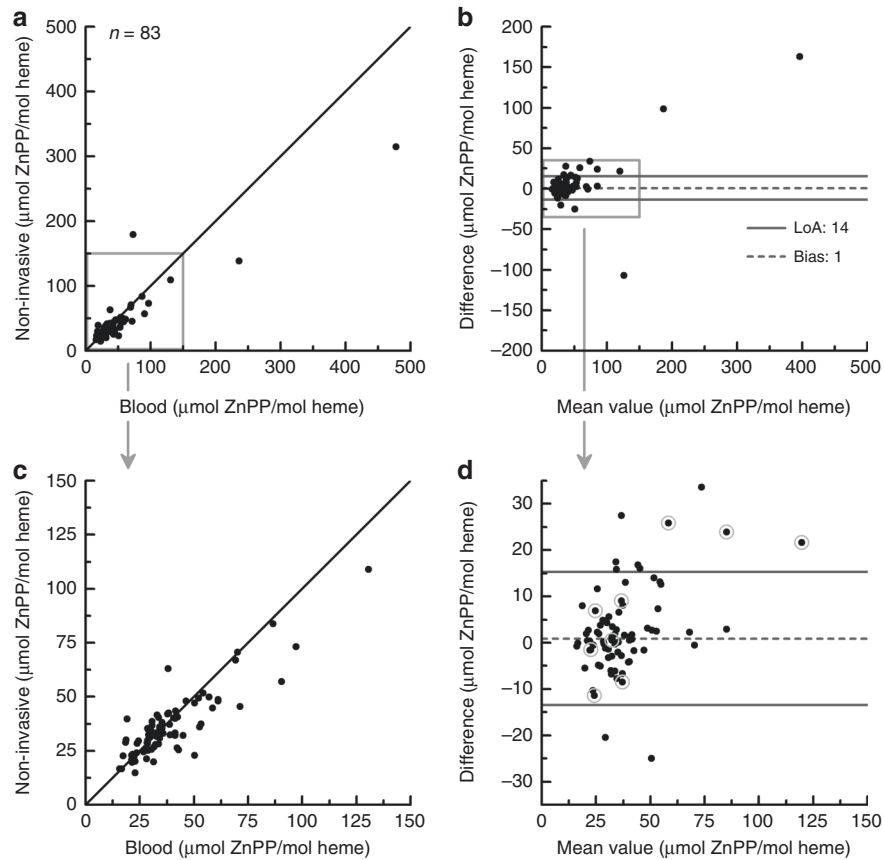
The ZnPP/heme ratio was additionally determined by fluorescence spectroscopic measurements on EDTA blood as a reference determination. A bare 1000  $\mu\text{m}$  optical fiber was connected to the fluorescence measurement device. The distal end was put into an aliquot of 100  $\mu\text{L}$  EDTA blood. To minimize photobleaching, that is, photo-chemical destruction of the ZnPP molecules, the sample tube was rotated axially at 112 rpm during the measurement, yielding a stable fluorescence signal. Fluorescence spectra were acquired for 425-nm excitation. The amplitude of the ZnPP fluorescence was determined for each spectrum by a fit procedure similar to the non-invasive measurements. The resulting amplitudes were averaged, yielding the ZnPP value. The average standard deviation for five repeated measurements in 21 blood samples was 2.2%.

### HPLC determination

HPLC was performed as additional reference determination of the ZnPP/heme ratio at the specialized laboratory MVZ Labor PD Dr. Volkmann und Kollegen GbR, Karlsruhe, Germany, as previously described.<sup>22</sup> For evaluation of the repeatability of the method, a second determination was done for a subgroup of  $n = 58$  children where a sufficient amount of EDTA blood was available. The comparison of these two determinations is shown in Supplementary Figure 1A, B. A bias of  $-10\ \mu\text{mol ZnPP/mol heme}$  was found, and six outliers can be identified. The robust limit of agreement (LoA) equals  $14\ \mu\text{mol ZnPP/mol heme}$  for this comparison. Due to the unexpected bias and high number of outliers, we decided to use the fluorescence spectroscopic measurements in blood as reference measurements in the following. For transparency, all data with HPLC as reference are shown in Supplementary Information (Supplementary Figures S3 and S4 and Supplementary Tables S1 and S4).

### Statistical data analysis

For direct comparison of the ZnPP values measured either non-invasively or directly in blood with the HPLC reference, the values were scaled. The scaling factor was derived by fitting a straight line with zero offset to the comparison of the measured ZnPP values to the reference HPLC determination (software: MATLAB R2014b, The MathWorks Inc., Natick, MA, USA, function: *robustfit*). For the quantitative comparison of two methods, the LoA and the bias were calculated using the robust  $\tau$ -estimate (software: R version 3.2.2; function: *scaleTau2*; package: *robustbase* version 0.92.5) of the differences between the two methods, where the robust standard deviation was multiplied by 1.96. The 95% confidence intervals (CI) were calculated by bootstrapping



**Fig. 1** Comparison of the non-invasive ZnPP measurements with the fluorescence measurements directly in blood. The correlation is shown in **a**, the corresponding Bland–Altman plot in **b**. **c**, **d** show excerpts. In **d**, the  $n = 9$  measurements of children with darker skin types are highlighted by circles

(software: R; functions: *boot* and *boot.ci*; package: *boot* version 1.3.17). Sensitivity, specificity, negative predictive value (NPV), and PPV were calculated from contingency tables (software: R; function: *BDtest*; package: *bdpv* version 1.1).

## RESULTS

### Practical feasibility

The practical feasibility of the non-invasive determination of the ZnPP/heme ratio was investigated in 99 of 100 children, as one child left the hospital prior to the non-invasive measurement. For 16 of 99 children, the non-invasive measurement could not be conducted due to their reluctant behavior and abrupt movements of the head, as well as crying. Most of these children had their pacifier in the mouth prior to the measurement. For the measurement on the lower lip, the pacifier had to be removed, which was often not tolerated by the child. Using the applicator embedded in the single-use pacifier did not result in an appreciable improvement. The percentage of reluctant children was similar among the 66 children aged up to 48 months (see also Supplementary Figure S2). All 33 children aged 4 years and older tolerated the measurement without difficulty. For all children who tolerated the measurement, the average time for the whole measurement procedure was about 3 min (median 169 s, mean 217 s), with a recording time on the lip of about 1 min (median 72 s, mean 85 s).

### Non-invasive detection of ZnPP

The non-invasive measurement results are compared to the fluorescence measurements directly in blood for 83 of 99 children, for which the measurement could be conducted. This comparison

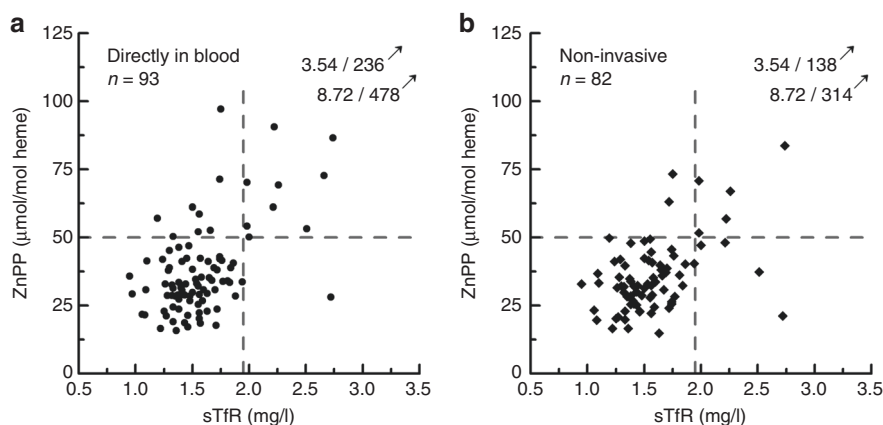
is shown in Fig. 1a, c, with corresponding Bland–Altman plots shown in Fig. 1b, d. The LoA of 14  $\mu\text{mol ZnPP/mol heme}$  and the bias of 1  $\mu\text{mol ZnPP/mol heme}$  are indicated as well.

In addition, both the ZnPP values measured non-invasively and directly in blood were compared with the HPLC reference, with LoAs of 19 and 15  $\mu\text{mol ZnPP/mol heme}$ , respectively (see Supplementary Figure S3 for the correlation). For all comparisons, the LoA, including 95% CI and the bias, are listed in Supplementary Table S1. The non-invasive method approaches the precision of the reference HPLC method, for which, however, a large bias ( $-10 \mu\text{mol ZnPP/mol heme}$ ) was found for the two repeated determinations (see Supplementary Figure 1).

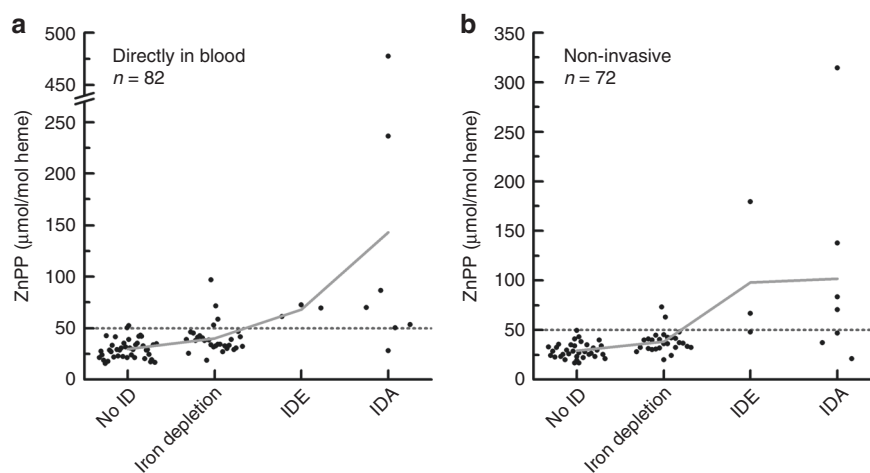
In Fig. 1d, measurement results for the nine children with Fitzpatrick skin types IV, V, or VI are highlighted by circles. No increased variations or systematic deviations dependent on the skin pigmentation are observed for the non-invasive detection of the ZnPP/heme ratio.

### ZnPP measurement for the detection of ID

A secondary aim of this study was the comparison of ZnPP measurements with other ID parameters. In Fig. 2a, b, the ZnPP values are compared with the sTfR. For this comparison, the ZnPP determination by fluorescence measurements in blood is available for 93 children, the non-invasive measurements for 82 children. For the comparison with the ZnPP determination by fluorescence measurements in blood, sensitivity and specificity equaled 92% (95% CI: 66–100%) and 90% (95% CI: 83–95%), respectively. NPV and PPV equaled 99% (95% CI: 94–100%) and 58% (95% CI: 44–71%), respectively. For the comparison with the non-invasive ZnPP measurements, sensitivity and specificity equaled 67% (95% CI: 39–88%) and 97% (95% CI: 91–99%),



**Fig. 2** ZnPP determination (a) by fluorescence measurements directly in blood and (b) non-invasively compared with the soluble transferrin receptor. Threshold values for ZnPP (50 µmol/mol heme) and the soluble transferrin receptor (1.95 mg/l) are indicated by dashed lines



**Fig. 3** ZnPP determination a by fluorescence measurements directly in blood and b non-invasively for different stages of iron deficiency. The mean value is highlighted by the red line

respectively. NPV and PPV equaled 94% (95% CI: 90–97%) and 80% (95% CI: 55–93%), respectively.

In addition to the comparison of ZnPP with the sTfR, ZnPP values were also compared to the stage of ID as defined in the Methods section. For children whose iron status could not be classified according to this scheme, results are detailed below.

Figure 3 shows the distribution of ZnPP values measured directly in blood and non-invasively for the different groups. The corresponding descriptive statistics are given in Supplementary Table S2 and S3. For the measurements directly in blood, the average ZnPP value for the groups increases from 30 µmol ZnPP/mol heme (no ID), over 40 µmol ZnPP/mol heme (ID), over 68 µmol ZnPP/mol heme (IDE) to 143 µmol ZnPP/mol heme (IDA). For the non-invasive measurements, the average ZnPP value for the groups increases from 29 µmol ZnPP/mol heme (no ID), over 38 µmol ZnPP/mol heme (ID), over 98 µmol ZnPP/mol heme (IDE) to 102 µmol ZnPP/mol heme (IDA). Only a low number of subjects are found in the groups IDE and IDA (3 and 7, respectively).

For the children that could not be classified according to the given scheme, one child had borderline normal CRP = 0.49 mg/dL, ferritin = 34 µg/L, elevated sTfR = 1.98 mg/L, and ZnPP = 54 µmol/mol heme (measured directly in blood) and 52 µmol/mol heme (measured non-invasively), indicating IDE. Another four children had elevated CRP or very high ferritin (1531 µg/L) and low hemoglobin, but normal sTfR, indicating anemia of chronic

disease. The mean ZnPP was 49 µmol/mol heme (range: 36–61, measured directly in blood) and 41 µmol/mol heme (range 32–50, measured non-invasively) for this group. The remaining children had low hemoglobin, but all other parameters were normal. The mean ZnPP was 28 µmol/mol heme (range: 23–31, measured directly in blood) and 28 µmol/mol heme (range: 15–39, measured non-invasively) for this group.

## DISCUSSION

The present investigation proves the feasibility of non-invasive measurement of ZnPP in children aged 9 months to 5 years. The precision of the measurement, quantified as LoA between the non-invasive measurements and the reference HPLC determination, was 19 µmol ZnPP/mol heme. This equals the LoA of 19 µmol ZnPP/mol heme found in a study on 56 women after childbirth.<sup>22</sup> When comparing the non-invasive measurements with measurements directly in blood using the same device, a smaller LoA of 14 µmol ZnPP/mol heme was found. This equals the LoA of two repeated HPLC determinations, which had a bias of 10 µmol ZnPP/mol heme and showed six pronounced outliers. We therefore speculate that our fluorescence measurement directly in blood is the most reliable method, outperforming the current standard HPLC. In the non-invasive measurement an additional error is introduced due to variations of the composition of the lip. Though

not systematically investigated, we found no obvious influence of skin pigmentation on the measurement results.

To assess the relevance of the determined LoAs for screening purposes, it can be helpful to compare with the hematofluorometer (Model 206D, AVIV Biomedical Inc., Lakewood, NJ, USA), which is most often used for ZnPP measurements.<sup>27–29</sup> In point-of-care settings, whole blood is often measured with this instrument, because no sample preparation is required, accepting higher readings and lower precision compared to washed erythrocytes due to interfering background fluorescence from the blood plasma.<sup>25,30–32</sup> Under these conditions, measurements of ZnPP by a hematofluorometer had LoA of 22  $\mu\text{mol ZnPP/mol heme}$  when compared to HPLC in our study on 56 women after childbirth.<sup>22</sup> The LoA of the non-invasive measurements are at least comparable and are thus considered sufficiently small for screening purposes.

In 16 of 99 children the non-invasive measurement could not be conducted due to their reluctant behavior. This may be partly due to the investigated inpatients, who may be more stressed and afraid of a medical examination than other children. Also, the measurements were proactively stopped if children were not comfortable with them to avoid study-induced stress to children and their parents. We therefore speculate that the compliance is even better under routine screening conditions. The applicability of the measurement may be further improved by reducing its duration to about 20 s compared to the current median measurement time of 72 s. This can be done by reducing the amount of recorded valid spectra from 100 to 30. In general, a higher number of recorded spectra will lead to a more precise measurement, which is why we a priori chose to aim for the high number of 100 valid spectra. In the present study, the calculated LoA include measurements on 35 children with 30 valid spectra or less, indicating that 30 valid spectra are sufficient for a reliable result. Results from an earlier study confirm this finding.<sup>22</sup>

In addition to the comparison of the non-invasive measurement with other means to assess the ZnPP value, we compared the measured ZnPP values with other ID parameters. For the investigated patient group, ZnPP measured directly in blood had a sensitivity and specificity of 92 and 90%, respectively, and NPV and PPV of 99 and 58%, respectively, for detecting ID as defined by the sTfR. A similar performance was found for the non-invasive ZnPP determination with a sensitivity and specificity of 67 and 97%, respectively, and NPV and PPV of 94 and 80%, respectively. Since only a small number of subjects ( $n = 10$ ) were in the IDE and IDA groups, these parameters are determined only with large CIs. Still, the values show that ZnPP correlates well with the sTfR. This is expected since both parameters are elevated in the same stage of ID, where ID starts to have an impact on the erythropoiesis (IDE).<sup>33,34</sup> For ID, mildly elevated ZnPP values were found only for few subjects, while most subjects had normal ZnPP values. For IDE, all ZnPP values were elevated. For IDA, a wide range of ZnPP values were found, with most ZnPP values elevated and ZnPP values above 300  $\mu\text{mol/mol heme}$ . The group of anemic children without evidence for ID (normal ferritin and sTfR) had also normal ZnPP.

While these findings are in alignment with the literature,<sup>20</sup> and support the usefulness of ZnPP and the non-invasive measurement method to screen for ID, larger studies are desirable for corroboration and to establish appropriate reference ranges for children, especially in risk groups for ID. Furthermore, the hospitalized patients with different diagnoses are not representative of a normal childhood population. For a comprehensive assessment of the iron status, a more complete set of ID parameters including transferrin saturation and hepcidin would be desirable. To estimate the full effect of inflammation, the measurement of both CRP and  $\alpha$ -1-acid glycoprotein are recommended, especially for children.<sup>35</sup> This was not possible in the present study due to an insufficient amount of left-over blood.

All biomarkers for ID are affected by the inflammatory response to infection, and may be influenced by conditions other than ID.<sup>36,37</sup> Consequently, to diagnose ID in the best possible way, an extended set of different biomarkers is necessary. This is not feasible for screening, where time, costs, and ease of application play a major role, typically allowing only the determination of a single parameter. The best single parameter for ID screening may depend on the setting. For use in urban pediatric centers in the United States, ZnPP was suggested as suitable screening parameter for ID.<sup>38</sup> In regions endemic for malaria and other infections, the World Health Organization also recommends measurement of ZnPP as the preferred indicator to screen children for ID.<sup>39</sup> To date, the only other indicator for ID that can be measured non-invasively and at low cost is hemoglobin. However, hemoglobin has only a PPV of ~35% for ID,<sup>15,16</sup> which is significantly lower than the 80% for non-invasive ZnPP measurements in our study. The combination of ZnPP with hemoglobin measurements can further improve the accuracy for ID screening in some settings, as was recently shown for Kenyan pre-school children.<sup>34</sup>

The present prototype device for the measurement is easily portable, robust, and has only a low power consumption below 7W (without tablet computer) with a supply voltage of 5V, making battery operation straightforward. Additionally, it does not need any chemicals or other disposables, except for potentially a single-use plastic cover in front of the measurement fiber to ensure hygienic measurements also without careful disinfection procedures. We therefore see no hurdles for the use of the device in resource-limited settings.

## CONCLUSIONS

The present investigation demonstrates that non-invasive detection of ZnPP at the lower lip is reliably possible for children aged 9 months to 5 years, irrespective of their skin color. This novel method has the potential to improve the screening situation for ID in children by enabling simple and fast non-invasive measurements at the point of care.

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## AUTHOR CONTRIBUTIONS

CH and GH designed and constructed the instrument, helped conceptualizing and designing the study, performed the statistical analyses, drafted the initial manuscript, and reviewed and revised the manuscript. FM conducted the patient measurements, helped drafting the initial manuscript, and reviewed the manuscript. HS designed the instrument, conceptualized and designed the study, and reviewed and revised the manuscript. LMH and MV conceptualized and designed the study, supervised the blood sample measurements and data collection, and reviewed the manuscript. RS helped conceptualizing and designing the study, gave advice for statistical analyses, and reviewed the manuscript. BK conceptualized and designed the study, supervised the patient measurements and data collection, and reviewed the manuscript. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

## ADDITIONAL INFORMATION

The online version of this article (<https://doi.org/10.1038/s41390-018-0247-x>) contains supplementary material, which is available to authorized users.

**Competing interests:** The authors have indicated they have no financial relationships relevant to this article to disclose. CH and GH are co-founders of the company FerroSens GmbH. The other authors declare no competing interests.

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