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Dietary supplementation with docosahexanoic acid (DHA) increases red blood cell membrane flexibility in mice with sickle cell disease

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Abstract

Humans and mice with sickle cell disease (SCD) have rigid red blood cells (RBCs). Omega-3 fatty acids, such as docosahexanoic acid (DHA), may influence RBC deformability via incorporation into the RBC membrane. In this study, sickle cell (SS) mice were fed natural ingredient rodent diets supplemented with 3% DHA (DHA diet) or a control diet matched in total fat (CTRL diet). After 8 weeks of feeding, we examined the RBCs for: 1) stiffness, as measured by atomic force microscopy; 2) deformability, as measured by ektacytometry; and 3) percent irreversibly sickled RBCs on peripheral blood smears. Using atomic force microscopy, stiffness is increased and deformability decreased in RBCs from SS mice fed CTRL diet compared to wild-type mice. In contrast, RBCs from SS mice fed DHA diet had markedly decreased stiffness and increased deformability compared to RBCs from SS mice fed CTRL diet. Furthermore, examination of peripheral blood smears revealed less irreversibly sickled RBCs in SS mice fed DHA diet as compared to CTRL diet. In summary, our findings indicate that DHA supplementation improves

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RBC flexibility and reduces irreversibly sickled cells by 40% in SS mice. These results point to potential therapeutic benefits of dietary omega-3 fatty acids in SCD.

Keywords

omega-3; animal model; sickle cell anemia; red blood cell disorders; red cell membrane

Introduction

Individuals with sickle cell disease (SCD) exhibit increased red blood cell (RBC) rigidity and adhesion, multiorgan and vascular pathology, and complex pain syndromes [2;6;9;10;14;29;31;33;34;50;60]. In addition, there is evidence of activation of the inflammatory and coagulation pathways [3;4;8;17;61–63]. Berkeley sickle cell (SS) mice exclusively express human sickle hemoglobin and have a phenotype that mimics many features of severe SCD in humans [32;44;53].

Omega-3 fatty acids, such as docosahexanoic acid (DHA), are essential fatty acids that have anti-inflammatory and anti-thrombotic activities [37;38;65]. As dietary supplements, omega-3 fatty acids are beneficial in many cardiovascular diseases [27;37;38;41;49]. In addition, Ren et al [56–58] demonstrated that humans with SCD have decreased omega-3 fatty acids and increased arachidonic acid (an omega-6 fatty acid) in the RBC membrane. To date, trials in humans with SCD have indicated that dietary supplementation with omega-3 fatty acids may reduce severe anemia, vaso-occlusive pain episodes, white blood cell count, and prothrombotic activity [18;52;64]. Of note, these trials did not assess the effect of DHA on RBC structural and functional characteristics.

Several studies have demonstrated that dietary supplementation with omega-3 fatty acids results in increased incorporation of these fatty acids into the RBC membrane, which can influence RBC deformability [15;68]. In the present study, we sought to determine whether dietary supplementation with the omega-3 fatty acid DHA would improve RBC stiffness and other hematologic parameters in SS mice. Our results indicate that 8-week dietary supplementation with DHA increases RBC flexibility and decreases irreversibly sickled RBCs in SS mice.

Methods

Mice

C57BL/6J mice (hereafter, WT mice) were purchased from The Jackson Laboratory (Bar Harbor, ME; stock number 000664). Berkeley SCD mice (Tg(Hu-miniLCR α 1^{G γ} A γ δ β ^S) *Hba*⁰/*Hba*⁰ *Hbb*⁰/*Hbb*⁰; hereafter, SS mice) on the Berkeley mixed genetic background have been previously described [44;53]. Mice were cared for according to AAALAC specifications. Animal experiments were approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin. Experimental groups contained comparable numbers of age- and gender-matched adult mice.

Dietary Feeding

Mice were fed natural ingredient 2016 Teklad Global 16% Protein Rodent Diet supplemented with 3% DHA, an omega-3 fatty acid (DHA diet), or a control diet supplemented with safflower oil, which predominantly contains the omega-6 fatty acid linoleic acid (CTRL diet) (Supplemental Table I). DHA oil was a kind gift from Martek Biosciences (a division of DSM Nutritional Products, Columbia, MD). DHA and CTRL diets were designed in consultation with nutritionists at Harlan-Teklad Laboratories and diets were compounded at Harlan-Teklad Laboratories (Madison, WI). The macronutrient composition of the CTRL and DHA diets is shown in Supplemental Table II. Diets were matched in total fat and had a similar distribution of saturated/monounsaturated versus polyunsaturated fatty acids (Table I). Detailed information on the base 2016 Teklad Global 16% Protein Rodent Diet can be found at http://www.harlan.com/products_and_services/research_models_and_services/laboratory_animal_diets/teklad_natural_ingredient_diets/teklad_global_diets/global_rodent_diets/teklad_global_rodent_diet_16_protein_2016.hl#Macronutrient_Information. Mice were weighed prior to being placed on CTRL or DHA diet, fed their respective diets for 8 weeks, and then weighed again prior to harvest. Mice were treated with only one diet, and were harvested for analyses at the end of 8 weeks of dietary feeding. No significant differences in weight gain were observed between CTRL and DHA diet groups (data not shown).

CBC and reticulocyte measurements

Blood was obtained from deeply anesthetized mice by cardiac puncture and drawn into heparin anticoagulant. Complete blood count (CBC) and reticulocyte analyses were performed as previously described [26].

Flow adhesion assay

RBCs were separated from whole blood and washed twice in citrate/glucose/saline buffer as previously described [31;67]. Washed RBCs were resuspended at a 2% hematocrit in M199 serum free medium (Sigma, St. Louis, MO) containing 0.2% bovine serum albumin (Sigma, St. Louis, MO). RBC adhesion to immobilized thrombospondin (TSP, 1 μ g/75 μ l) was carried out using Vena8 Fluoro Biochips on the VenaFlux Platform (Cellix, Dublin, Ireland). RBCs were perfused through the channel at a wall shear stress of 0.4 dyne/cm², and adherent RBCs manually quantified by analyzing five different images captured from the middle of the channel.

Atomic Force Microscopy (AFM)

Whole blood was collected as described above and stored overnight at 4°C. RBCs were sedimented by centrifugation at 500 x g at 4°C for 10 minutes and the plasma, buffy coat, and uppermost erythrocytes removed by aspiration and discarded. The remaining erythrocytes were washed three times in Cell Wash Buffer (21.0 mM tris (hydroxymethyl)aminomethane, 4.7 mM KCl, 2.0 mM CaCl₂, 140.5 mM NaCl, 1.2 mM MgSO₄, 5.5 mM glucose, 0.5% bovine serum albumin (BSA) fraction V; pH 7.4) specifically formulated to be iso-osmotic for mouse red blood cells to avoid any hydration issues with subsequent measurements [25]. Cells were immobilized on a glass surface

coated with 1 mg/ml poly-L-lysine solution for 10 min in the incubator (37°C, 5% CO₂). Unattached cells were removed by gentle rinsing of the surface with Cell Wash Buffer. We noted no differences in the extent of adhesion of sickle versus normal RBCs to the poly-L-lysine coated surface. Cells were prepared on the day of use and experiments were performed in Cell Wash Buffer maintained at 37°C.

Stiffness measurements were carried out as previously described [43] on non-fixed adhered erythrocytes. Erythrocytes chosen for stiffness measurements were mature RBCs with a biconcave shape, indicating that the membranes were not under tension due to adherence to poly-L-lysine. All force measurements were recorded with a loading rate of 24000 pN/s for small indentation depths up to 150 nm and small indentation forces up to 300 pN [21;28;55]. The Young's modulus was calculated using the Hertz model which describes the elastic deformation of two bodies in contact under load, in this case the contact between the erythrocyte and the AFM probe. Further detail regarding AFM measurements and calculations is found in Supplemental Methods. Five mature RBCs were evaluated for stiffness measurements from each mouse; reticulocytes were excluded based on their larger size [13]. Stiffness results from one RBC were comparable to the stiffness results from the other four RBCs analyzed from each animal (data not shown).

Ektacytometry

Red cell membrane deformability was assessed by ektacytometry as described, previously [16] using a Technicon model 152 Ektacytometer (Spectra Physics, France) at Purdue University. Whole blood was collected as described above and stored overnight at 4°C, followed by centrifugation at 1000 x g for 3 min to pellet RBCs. Packed RBCs were washed twice with Cell Wash Buffer lacking BSA, then resuspended at 0.3% hematocrit in isoosmotic sample buffer (136mM NaCl, 6.3mM Na₂HPO₄, 1.2mM NaH₂PO₄) containing ~4% polyvinylpyrrolidone (PVP). Samples were loaded into the ektacytometer and subjected to increasing shear stresses (0–250 dynes/cm², increased linearly over 125s) at constant osmolarity. The deformation of the cells was quantified by laser diffraction as elongation index (EI) which was recorded as a function of time. RBCs from each mouse were analyzed in duplicate ektacytometer runs, and the overall RBC deformability for each mouse was determined from the average maximum EI of the duplicate trials.

Quantification of Irreversibly Sickled RBCs

Blood smears were prepared on glass slides using anticoagulated whole blood. Unstained smears were examined using a Nikon Eclipse 80i microscope and fields of well-separated RBCs selected for photography using a Nikon Digital DS-Fi1 camera. The total number of RBCs in each field and the number of irreversibly sickled RBCs, defined as having a length greater than 2-fold its width [59], were enumerated by manual counting. Five representative fields were examined and the average percent of irreversibly sickled RBCs was determined for each mouse.

Statistics

GraphPad InStat or GraphPad Prism was used for all statistical analyses. For data that passed tests for normality, unpaired t-tests were used, with Welch's correction applied when

comparing populations with unequal SDs. For data that were not normally distributed, nonparametric Mann-Whitney U-tests were used for comparisons of two groups. Kruskal-Wallis nonparametric ANOVA with Dunn's Multiple Comparisons Post-Hoc Test was used for comparisons of more than two groups. $P < 0.05$, with corrections for multiple comparisons when appropriate, was considered significant.

RESULTS

DHA supplementation decreases RBC stiffness in SCD mice

Atomic force microscopy (AFM) was used to assess RBC stiffness of mature RBCs in wild-type (WT) and SS mice after 8-week dietary feeding on CTRL or DHA diets. RBC stiffness is comparable in WT mice fed either CTRL or DHA diets (Figure 1A, representative WT-CTRL and WT-DHA mice; Figure 1C, group data). RBC stiffness in WT-CTRL mice is similar to that seen in WT mice fed a standard fat diet (not shown), indicating that the increased fat composition of the diets used in this study does not alter normal RBC stiffness.

In comparison to WT RBCs, RBC stiffness is increased in SS mice fed CTRL diet (Figure 1B, filled bars, representative SS-CTRL mouse; Figure 1C, group data, $P < 0.001$ vs. WT-CTRL or WT-DHA). RBC stiffness in SS-CTRL mice is comparable to that obtained from SS mice fed a standard fat diet (not shown), indicating that the increased fat composition of the CTRL diet is not the cause of increased RBC stiffness in CTRL diet-fed SS mice. In addition, since only mature RBCs were assessed for RBC stiffness measurements, the observed difference in RBC stiffness between SS and WT mice is not influenced by the higher presence of reticulocytes in SS as compared to WT blood samples. In marked contrast, RBCs from DHA diet-fed SS mice had decreased stiffness compared to RBCs from CTRL diet-fed SS mice (Figure 1B, open bars, representative SS-DHA mouse; Figure 1C, group data, $P < 0.001$ vs. SS-CTRL). In fact, RBC stiffness in SS-DHA mice was decreased to nearly WT levels (Figure 1C, P value is not significant for SS-DHA vs. WT-CTRL or WT-DHA).

Analysis of hematologic parameters revealed no statistically significant differences in red blood cell parameters in CTRL versus DHA diet groups (Table II). However, there was a trend towards decreased reticulocytes (as a % of total RBCs) in SS-DHA as compared to SS-CTRL mice in the small subset of samples analyzed ($60 \pm 16\%$ in $n=7$ SS-CTRL versus $41 \pm 18\%$ in $n=9$ SS-DHA, mean $\pm$ SD, $P=0.051$).

DHA supplementation improves RBC deformability in SCD mice

To verify the decrease in RBC stiffness seen by AFM, deformability of RBCs from a subset of SS mice fed CTRL or DHA diets was also examined using ektacytometry. Consistent with other studies [5;20;24;35;40;66], RBCs from SS mice fed CTRL diet exhibit low deformability by ektacytometry as compared to RBCs from WT mice (Figure 2; average EI for WT mice is 0.28). Similar to the AFM results, RBCs from SS mice fed DHA diet had improved deformability compared to RBCs from SS mice fed CTRL diet (Figure 2, $P < 0.02$).

DHA supplementation decreases irreversibly sickled RBCs in SCD mice

Both AFM and ektacytometry indicated increased RBC flexibility in SS mice fed DHA diet, suggesting a change in RBC membrane properties. RBC adhesion is known to be elevated in human and murine SCD, and has been attributed to multiple factors including altered membrane properties [6;7;11;12;31;45]. In addition, repeated cycles of sickling of RBCs results in cytoskeletal damage and membrane changes that eventually become permanent, leading to the appearance of “irreversibly sickled RBCs” with altered shape even in the presence of oxygenated sickle hemoglobin [42;59].

Therefore, we next examined whether there was a decrease in: 1) *in vitro* RBC adhesion to thrombospondin; or 2) numbers of irreversibly sickled RBCs in SS-DHA as compared to SS-CTRL mice. While the mean RBC adhesion to thrombospondin *in vitro* was reduced in DHA as compared to CTRL diet fed SS mice (730 RBCs/mm² [CTRL] to 590 RBCs/mm² [DHA]), this difference was not significant in the small number of samples examined (Figure 3A, P=0.098). In contrast, enumeration of irreversibly sickled RBCs from fresh whole blood revealed a ~40% decrease in irreversibly sickled RBCs in SS-DHA as compared to SS-CTRL mice (Figure 3B, P<0.04). These data suggest that the improvement in RBC membrane flexibility after 8 weeks of dietary DHA supplementation decreases the cytoskeletal and membrane damage that leads to irreversibly sickled RBCs.

DISCUSSION

The results of our studies indicate that 8-week dietary supplementation with DHA improves RBC flexibility in sickle cell mice. The complete normalization of RBC stiffness (Figure 1) as compared to only partial improvement in RBC deformability (Figure 2) is not completely surprising. Since deformability reflects not only cell stiffness but also hydration status [16;47], the lack of complete normalization of RBC deformability in SS-DHA mice may reflect the improvement in RBC stiffness without a concomitant improvement in RBC hydration. In addition, only individual mature RBCs were evaluated for stiffness by AFM, while deformability measurements involve the assessment of a population of erythroid cells, which in SS mice includes ~40% reticulocytes. Therefore, it is also possible that the difference in the AFM versus ektacytometry results reflects incorporation of DHA into erythroid membranes with effects that are more pronounced in mature RBCs than in reticulocytes.

In addition to improvements in RBC flexibility, dietary supplementation of SS mice with DHA also significantly reduces irreversibly sickled RBCs. Changes in membrane protein oxidation and permeability are known to play a role in the generation of dense cells in SCD, and irreversibly sickled RBCs are predominantly found in the dense cell population [46]. Therefore, DHA supplementation and/or changes in RBC flexibility resulting from DHA supplementation may modulate membrane protein oxidation and membrane permeability in sickle RBCs.

There are several potential avenues through which DHA supplementation could improve RBC flexibility in SS-DHA mice. Ren et al have demonstrated that humans with SCD have decreased omega-3 fatty acids and increased arachidonic acid (an omega-6 fatty acid) in the

RBC membrane [56;57]. It is known that omega-3 and omega-6 fatty acids compete for incorporation into the phospholipids of the erythrocyte membrane [54], suggesting that DHA is replacing arachidonic acid in the RBC membrane in SS mice. Increasing the degree of unsaturation for a given phospholipid influences membrane fluidity less than increasing phospholipid content itself [39], indicating that the improvement in RBC membrane flexibility in SS-DHA mice is not primarily the result of the increased degree of unsaturation of DHA (22:6, n-3) compared to arachidonic acid (20:4, n-6) or linoleic acid (18:2, n-6; the major fatty acid in the CTRL diet). However, DHA in phospholipid bilayers possesses higher stability toward non-enzymatic lipid peroxidation than arachidonic acid or linoleic acid, which may also contribute to membrane stability [1].

In addition, omega-3 and omega-6 fatty acids can interact and react with the same receptors and enzymes with divergent physiologic effects [39]. For instance, 15-lipoxygenase-1 (15-LOX-1) is activated after erythroblast enucleation and is instrumental in initiating the degradation of mitochondria in maturing reticulocytes [22]. Using arachidonic acid as the substrate, 15-LOX-1 produces the pro-inflammatory, pro-oxidant molecules 15-hydroxyeicosatetraenoic acid (15-HETE) and lipoxins A4 and B4 [39]. In contrast, if DHA is the substrate, 15-LOX produces docosatrienes and protectins, which are anti-inflammatory and protect from oxidative stress [39]. Furthermore, dietary supplementation with omega-3 versus omega-6 fatty acids leads to enhanced transcription of antioxidant enzymes and suppressed transcription of enzymes that produce reactive oxygen species [39]. There is also increased activity of RBC catalase and lowered malondialdehyde levels in rat RBCs after dietary supplementation with omega-3 fatty acids [36]. Taken together, this suggests greater protection from RBC lipid peroxidation and oxidative injury. Finally, supplementation with omega-3 fatty acids influences the acylation of proteins and ion channel function, which can alter intracellular calcium levels, membrane flexibility, protein-protein interactions, and catalytic activity of enzymes [39].

It is surprising that hemoglobin and reticulocyte levels ($p=0.051$, measured in only a subset [$n=7-9$] of animals) were not definitively improved in DHA-treated sickle cell mice, despite a decrease in irreversibly sickled RBCs. There are several reasons why we may not have observed a change in these parameters in DHA-supplemented SS mice. First, it is possible that higher levels of DHA supplementation or a longer supplementation period may be needed to see a significant change in hemoglobin and reticulocyte levels in SS mice. Second, the extremely large spleen may play a significant role in reducing RBC lifespan in SS mice that is not observed in humans with SCD [44;48]. In addition, this large spleen may, to some extent, mask subtle changes in hemolytic rate or overall hemoglobin level. Third, the CBC and reticulocyte parameters in sickle mice are quite variable, and the reticulocyte levels are higher in SS mice than in human SCD [44]. In our study, we did not assess CBC and reticulocyte parameters in the same mouse before and after DHA supplementation. Thus, the higher variability in these parameters (see Table II) may have masked smaller changes that would have been more apparent looking at paired samples from the same animal pre- and post- treatment. Finally, the trend for a decrease in reticulocyte levels in the subset of mice examined suggests that a change in hemolytic rate may be better assessed by other measures. For instance, there is a strong correlation between the percentage of irreversibly sickled RBCs and red cell life span in humans with SCD [48]. For

that reason, an assessment of red cell life span before and after DHA supplementation may be a more sensitive way to detect improvement in red cell survival resulting from DHA supplementation.

In summary, our findings indicate that DHA supplementation improves RBC membrane flexibility and reduces irreversibly sickled cells in SS mice. Initial studies of omega-3 fatty acids in human SCD suggested a decrease in acute painful/vaso-occlusive episodes and prothrombotic activity with dietary omega-3 fatty acids [52;64], but these pilot studies involved small numbers of patients. Recently, a larger study showed verified that omega-3 fatty acid supplementation reduced the frequency of vaso-occlusive episodes, as well as severe anemia, in individuals with SCD [18]. Additionally, this group found that omega-3 fatty acid supplementation did not exacerbate oxidative stress known to exist in SCD [19;30;51], a complication that has been suggested in studies of omega-3 supplementation in non-SCD individuals [23]. However, while these studies have evaluated incorporation of omega-3 fatty acids into the RBC membrane, they have not carefully assessed the effect of omega-3 fatty acids on sickle RBC structural and functional characteristics [18;52;64]. In addition, although individuals with SCD are known to have increased daily pain and allodynia [10], the effects of omega-3 fatty acid supplementation on chronic or neuropathic pain in SCD have not been reported. SS mice are also known to exhibit nociceptive sensitivities at steady state that are exacerbated by acute sickling [32]; it is possible that improvement in sickle RBC flexibility and/or overall oxidative stress could translate to an improved pain phenotype. Additional studies are required to further delineate the potential benefits of dietary supplementation with omega-3 fatty acids in human and murine SCD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

SCD	sickle cell disease
SS	sickle cell mice
DHA	docosahexanoic acid

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Figure 1. Dietary supplementation with DHA decreases RBC stiffness in SS mice

(A, B): Representative results from individual wild-type (WT) (A) and sickle (SS) (B) mice fed either CTRL (filled bars) or DHA (open bars) diet. Data are presented as Gaussian histograms of the frequency of measurements versus the Young's modulus in Pascals (Pa).

(C): Comparison of Young's modulus from the groups indicated on the X axis. Data are presented as median with standard deviation. The n indicated on X-axis indicates the total number of mature RBCs analyzed in each group, obtained from the following numbers of mice: WT-CTRL and WT-DHA: 4 mice; SS-CTRL: 12 mice; SS-DHA: 11 mice. Statistical comparisons by Kruskal-Wallis nonparametric ANOVA with Dunn's Multiple Comparisons Post-Hoc Test: **, $P < 0.001$ SS-CTRL vs. WT-CTRL or WT-DHA; #, $P < 0.001$ SS-DHA vs. SS-CTRL. All other differences (including SS-DHA vs. WT-CTRL or WT-DHA) are not significant.

Figure 2. Dietary supplementation with DHA increases RBC deformability in SS mice
Maximum Elongation Index (EI) for SS mice fed CTRL or DHA diets. EI was determined as described in Methods. Data are presented as mean with standard deviation. n for each group is indicated on the X-axis. *, $P < 0.05$, SS-CTRL vs. SS-DHA, unpaired t-test with Welch correction.

Figure 3A **Figure 3B**

Figure 3. Dietary supplementation with DHA may not affect *in vitro* adhesion to TSP but decreases irreversibly sickled RBCs in SS mice

(A) Adhesion of washed RBCs from SS-CTRL or SS-DHA mice to human thrombospondin (TSP, 1 μ g/75 μ l) at a wall shear stress of 0.4 dyne/cm². Adherent RBCs per unit area were quantified as described in Methods. (B) Percent irreversibly sickled RBCs in SS-CTRL and SS-DHA mice. Irreversibly sickled RBCs were quantified as described in Methods. Data are presented as mean with standard deviation. n for each group is indicated on the X-axis. ns, not significant ($P=0.098$), SS-CTRL vs. SS-DHA, unpaired t-test; *, $P<0.05$, SS-CTRL vs. SS-DHA, unpaired t-test.

Table I**Fatty Acid Composition**

Fatty Acid	CTRL Diet	DHA Diet
Saturated, % of total fatty acids	32	29
Monounsaturated, % of total fatty acids	19	22
Polyunsaturated, % of total fatty acids	48	49
Fatty acid profile, % of total fatty acids		
C8:0 Caprylic	1.0	0.0
C10:0 Capric	1.1	0.9
C12:0 Lauric	5.4	3.6
C14:0 Myristic	4.4	9.9
C16:0 Palmitic	13.8	12.8
C16:1 Palmitoleic	0.4	1.1
C18:0 Stearic	4.7	1.2
C18:1 Oleic	18.1	20.1
C18:2 Linoleic	47.0	18.0
C18:3 Linolenic	1.3	1.2
C22:6 DHA	0.0	30.1
Predominant fatty acid	18:2 linoleic acid	22:6 DHA
Energy Density, kcal/g	3.5	3.5

Table II

Red blood cell measures

Group	Diet	Hgb, g/L	Hct, %	MCV, fl	MCHC, g/L
WT	CTRL (n=10)	133±6	41.2±2.5	45±1	322±16
	DHA (n=11)	126±6	40.5±2.5	45±1	311±10
SS	CTRL (n=18)	50±11	15.0±3.3	45±4	338±44
	DHA (n=22)	50±11	16.1±3.7	48±6	314±29

All values mean±SD. No significant differences between WT-CTRL and WT-DHA or between SS-CTRL and SS-DHA groups for Hgb, Hct, MCV or MCHC after correcting for multiple comparisons.