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Short Communication

The association between high sensitivity C-reactive protein and micronutrient levels: A cross-sectional analysis based on a laboratory database

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Background and aims: Low-grade chronic inflammation is a condition underlying many serious diseases but there is no good single biomarker which can estimate and monitor the severity of the inflammation. C-reactive protein (CRP) is the best validated and most extensively used marker. The aims of the study were to investigate the extent to which CRP levels associate with levels of micronutrients.
Methods: We retrieved the levels of S-hsCRP and nutritional variables fB-β-carotenes, fS-Q10 (Ubiquinon), fS-Fe, E-Cu, fS-A vitamin, B–Se, B–Zn, and fB-B12 vitamin from the database of clinical laboratory Mila Oy from the years 1988–2018, a total of nearly 18 800 samples from outpatient clinics, Helsinki and Oulu, Finland. Sample sizes for nutritional variables measured concurrently with S-hsCRP varied between 4356 and 8621. S-hsCRP levels were categorized into five ordered categories. The levels of each micronutrient in those categories were compared using analysis of variance (ANOVA). Males and females were analyzed separately.
Results: It was observed that an increase of S-hsCRP associated with the decrease of fS-Fe ($p < 0.001$ for both genders); fS-A vitamin ($p < 0.001$ for both genders), and fS-β-carotenes ($p < 0.001$ for both genders); these are considered negative acute phase reactants. For both genders there was no significant association between the levels of fS-B12 vitamin ($p = 0.14$ for males; $p = 0.03$ for females), fS-Q10 ($p < 0.001$ for males; $p = 0.06$ for females) and fB-Se ($p < 0.001$ for males; $p = 0.01$ for females) and the categorized S-hsCRP. In contrast, fB-Zn ($p < 0.001$ for both genders) behaved like a positive acute phase reactant whereas copper measured from washed blood cells (E-Cu) did not display any significant associations with S-hsCRP ($p = 0.001$ for males; $p = 0.05$ for females).
Conclusions: A linear association was observed for some micronutrients - the higher the degree of low-grade inflammation (S-hsCRP), the more disturbed were the levels of some micronutrients. For clinicians, this finding means that inflammation needs to be acknowledged when assessing micronutrient deficiency. Substitution therapy should be implemented only after the inflammation has been rectified.

1. Introduction

Low-grade chronic inflammation is a condition underlying many serious diseases, such as cardiovascular, neurodegenerative disorders, autoimmune diseases, transplant rejection and cancer [1]. There is no good single biomarker with which to estimate and monitor low-grade inflammation. Depending on the clinician's experience and the disease in question, indirect markers may be applied for the estimation of insidious chronic inflammation. These approaches may include the measurement of acute phase reactants, e.g. ferritin, serum amyloid A, fibrinogen, α1-acid glycoprotein [2], caeruloplasmin, 1,25-vitamin D (calcitriol), homocysteine,

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The list of abbreviations

B12	vitamin B12
DAD	diode array detection
E	erythrocytes
EFGR	epidermanl growth factor receptor
fS	fasting serum
Hcr	haematocrit
hsCRP	highly sensitive C reactive protein
HPLC	high pressure liquid chromatography
IL-6	interleukine 6
IL-1β	interleukine 1beta
INFγ	interferon gamma
P	plasma
Q10	ubiquinon
Se	selenium
SOD	superoxide dismutase
TNFα	tumour necrosis factor alfa
VEGF	vascular endothelial growth factor
Zn	zinc

measurement of the concentrations of cytokines (INFγ, TNFα, IL-6, IL-1β), chemokines and growth factors or their receptors (VEGF, EFGR), measurement of white blood cell counts or $CD4^{+}/CD8^{+}$ T-lymphocyte ratio, or the detection of activation molecules expressed on their surface (e.g. $CD3^{+}$) [3]. In estimating the extent of inflammation, the measurement of reactive oxygen species (ROS) with various modifications of the Fenton reaction, or indirect assessments of oxidative stress can be also used e.g. by assay of the concentrations of isoprostanes, which are markers of lipid peroxidation [4]. However, of all laboratory approaches adopted in clinical practice, the assay of highly sensitive CRP (hsCRP) is probably the best validated and the most extensively used biomarker for assessing microinflammation.

The assessment of the micronutrient status is crucial for the successful immunonutrition of patients e.g. with cancer [3], inflammatory bowel diseases (Crohn disease and ulcerative colitis [5]), in burn patients [6] and practically in all chronic polymorbid conditions. The increasing importance of micronutrient sufficiency has been recently emphasized for patients with neuropsychiatric diseases [7,8] and other chronic diseases. Supplementation therapy in patients with overt infectious diseases should be performed with some caution because the decrease of some trace elements in times of overt inflammation (CRP over 15 mg/L) may be a compensatory mechanism, and supplementation may lead to increased morbidity [9]. It has been proposed therefore that the assessment of nutritional status should be performed when overt inflammation has been treated and the CRP values are normalized. As far as we are aware, the association of increased CRP with the levels of several micronutrients has been studied mainly in patients with either high or very high CRP levels such as those encountered in systemic infections or SIRS (severe inflammatory response syndrome).

The aim of the present study was to estimate the extent to which small elevations of CRP associate with the levels of measured micronutrients. To achieve this goal, we selected several central micronutrients, routinely measured in our clinical practice. We were especially interested to examine the association of microelement levels with CRP at the so-called sub-clinical levels (below 10 mg/L) because slight increases of CRP have been linked to the higher risk of cardiovascular events and to oxidative stress [10]. The level of the CRP in the range of 0.5–10 mg/L was defined as a low-grade inflammation.

2. Samples and methods

2.1. Samples and nutritional variables

We retrieved S-hsCRP and nutritional variables fB-β-carotenes, fS-Q10 (Ubiquinon), fS-Fe, E-Cu, fS-A vitamin, B–Se, B–Zn, and fB-B12 vitamin from the database of clinical laboratory Mila Oy, during the years 1988–2018, altogether nearly 18 800 samples from an outpatient clinics in Helsinki and in Oulu, Finland. The nutritional measurement was included only if it had been taken concurrently either with S-hsCRP (highly sensitive CRP) or S-CRP. After statistical calculations, the results for S-CRP and S-hsCRP were combined and further presented as S-hsCRP for consistency. Both methods (S-CRP and S-hsCRP) measure the same protein quantitatively, but they differ by the methods' dynamic range. The sample sizes for nutritional variables measured concurrently with S-hsCRP varied between 4356 and 8621. We calculated the association of the S-hsCRP group category with the levels of nutritional variables. The prefaces "fB" refers to a fasting sample, blood; "fS"- the fasting sample, serum; "E" means that the analysis was performed from erythrocytes. The rationale for using of serum, blood or erythrocytes for specific micronutrients has been described in detail elsewhere [11]. Briefly, the analysis of trace elements from blood had been performed to assure an accurate estimation of long-term nutrient sufficiency. Within cells, micronutrients are normally present in storage sites, whereas in the serum their concentrations are less stable. In contrast, long-term iron stores are measured from serum, but not from erythrocytes. The concentration of copper is best measured from washed erythrocytes since this minimizes the influence of acute phase reactants [11]. The concentrations of microelements in the blood were calculated according to the formula B–Zn = (E-Zn - P–Zn)*Hcr + P–Zn, where B–Zn is the concentration of zinc in the whole blood (haematocrit standardized), E-Zn represents the concentration of zinc in red blood cells and P–Zn is the concentration of Zn in plasma, Hcr is haematocrit [11]. The CRP levels were measured by two methods, each with a defined measurement range.

2.2. Analytical methods

The following methods were used in the clinical laboratory: hs-CRP and fS-Fe (Thermo Fisher Scientific); S-β-carotene and fS-A-vitamin are in-house methods (diode array detection (DAD), reverse phase HPLC); fS-Q10 is an in-house method (electrochemical detection, reverse phase HPLC); B–Se, B–Zn are in-house methods (acid digestion, ICP-MS); fS-B12-vit has been analyzed by a subcontractor since 2002 (Yhtyneet Medix Laboratoriot, Microparticle Intrinsic Factor Immuno Assay (CMIA)).

2.3. Statistical analysis

The independent samples t-test was used to compare males and females with respect to nutritional variables and Mann-Whitney U test was used for S-hsCRP. This cross-sectional study aimed to examine the possible association between S-hsCRP and nutritional variables. Due to left-censored observations, S-hsCRP was used as a categorical variable and was divided into five categories: <0.50, 0.50–3.00, 3.01–10.00, 10.01–20.00 and >20.00 mg/L. The nutritional variables fS-Fe, B–Zn, E-Cu, fS-A vitamin, fB-β-carotenes, fS-Q10, B–Se and fS-B12 vitamin were used as continuous variables. Of them, fB-β-carotenes, fS-Q10, B–Se and fS-B12 were skewed to the right and were logarithmically (ln) transformed before analysis.

The first analysis was a multivariable analysis of variance (MANOVA), where each of nutritional variables was separately tested among the five S-hsCRP categories. Gender was included as a categorical covariate. In addition to the main effects of S-hsCRP and gender, the interaction between these variables was tested. Interaction term was significant for four nutritional variables. Thus, the subgroup analyses by gender were performed. The final analysis was one-way ANOVA, in which the S-hsCRP group was the only factor. The one-way ANOVA was conducted separately for males and females for all nutritional variables, regardless of the significance of the main effects or the interactions in the preceding MANOVA. In order to prevent the multiplicity problem, the Dunnett t-test was applied for pair-wise comparisons using the lowest category (S-hsCRP < 0.50) as a reference category. The results are given as means and 95% confidence intervals, and due to logarithmic transformation, fB-β-carotenes, fS-Q10, B–Se and fS-B12 are shown as geometric means and 95% confidence intervals.

All statistical tests were two-sided, and p-values <0.05 were considered to be statistically significant. Analyses were performed using IBM SPSS Statistics for Windows (version 25.0, Armonk, NY, USA, IBM Corp.).

3. Results

The S-hsCRP and nutritional variables for males and females are given in Table 1. There was a significant difference between genders in S-hsCRP and in all nutritional variables, except in B–Se and fS-B12. Males had higher levels of fS-Fe, E-Cu, B–Zn, fS-Q10 and fS-A Vitamin, whereas females had higher levels of S-hsCRP and fB-beta-carotene.

3.1. Trace elements

The levels of serum iron (fS-Fe) were significantly associated with S-hsCRP categories; the mean values started to decline significantly already at very small S-hsCRP elevations (3.01–10.00 mg/L). This trend was significant (ANOVA $p < 0.001$) for both genders and it was consistent without any significant interaction between S-hsCRP and gender ($p = 0.49$) (Fig. 1A).

In contrast, the overall tendency for copper detected from red blood cells (E-Cu) was not as clear as seen in the fS-Fe measurement. E-Cu levels were significantly different between S-hsCRP categories ($p < 0.001$ in males and $p = 0.05$ in females). However, a significant interaction between S-hsCRP and gender was observed ($p = 0.02$) (Fig. 1B).

B–Se levels were also significantly different between S-hsCRP categories ($p < 0.001$ in males and $p = 0.01$ in females). Again, a significant interaction ($p = 0.01$) was observed (Fig. 1C).

B–Zn levels were associated with S-hsCRP ($p < 0.001$ in both genders) without significant interaction ($p = 0.18$) (Fig. 1D).

3.2. Vitamins and ubiquinon

In males, no significant associations were detected between vitamin B12 (fS-B12) and S-hsCRP ($p = 0.14$) and furthermore the interaction was non-significant ($p = 0.18$). However, in males, there was a significant association between fS-B12 and S-hsCRP ($p = 0.03$) (Fig. 2A).

Serum ubiquinon (fS-Q10) levels were different in the various S-hsCRP categories ($p < 0.001$ in males and $p = 0.06$ in females). Again, a significant interaction ($p = 0.01$) was evident, which indicates that there was a different form of this association in males as compared to females (Fig. 2B).

Levels of vitamin A (fS-A vitamin) were significantly associated with S-hsCRP ($p < 0.001$ in both genders). A significant interaction ($p < 0.001$) was observed indicative of a different type of this association in men and women. Pair wise comparisons showed statistically significant difference at S-CRP concentrations over 20 mg/L as compared to S-hsCRP< 0.50 (Fig. 2C).

The level of fS-β-carotene assayed from fasting serum was also significantly associated with S-hsCRP ($p < 0.001$ in both genders) without any significant gender-related interaction ($p = 0.18$). A clear and steady association started from very low S-hsCRP elevations, already in the range of 0.5–3.0 mg/L (Fig. 2D).

4. Discussion

Here, we present data on the association of levels of several important micronutrients: trace elements, water and fat soluble vitamins and ubiquinon with CRP levels. The strength of our study is the large sample size which means that the power of our study is definitely adequate to detect the associations, if they exist. Some of our results have corroborated earlier reports but some are at odds with the published data. Even though we did not examine causality, only the association between the paired laboratories variables, such as S-hsCRP and micronutrients, we would like to make a few comments on the clinical significance of our findings.

Serum iron (fS-Fe) is a negative acute phase reactant [9] that is very sensitive to the presence of inflammation. Even at minor CRP elevations (3.01–10 mg/L), it is trapped in the circulation by acute phase proteins, such as ferritin or lactoferrin that have high affinity for iron. This adaptation of the macro-organism prevents possible bacterial invaders gaining access to nutritional sources. Our results are consistent with the findings of Galloway et al. [9]. One limitation of our study is that we did not analyze simultaneously the

Table 1
High sensitivity C-reactive protein and nutritional variables in males, females and combined for both genders.

	Males		Females		All		P value[a]
	N	Mean (95% CI)	N	Mean (95% CI)	N	Mean (95% CI)	
S-hsCRP (mg/l)[b]	3118	0.90 (0.85–0.97)	6124	0.98 (0.94–1.04)	9242	0.95 (0.92–0.99)	0.04
fS-Fe (mMol)	2221	18.9 (18.7–19.2)	4202	17.4 (17.2–17.6)	6423	18.0 (17.8–18.1)	<0.001
E-Cu (μMol)	2780	10.34 (10.30–10.38)	5282	10.21 (10.18–10.24)	8062	10.25 (10.23–10.28)	<0.001
B–Se (μMol)[c]	2929	2.09 (2.07–2.12)	5692	2.09 (2.08–2.11)	8621	2.09 (2.08–2.11)	0.97
B–Zn (μMol)	2856	90.7 (90.3–91.2)	5473	84.9 (84.7–85.2)	8329	86.9 (86.7–87.2)	<0.001
fS-B12 (pMol)[c]	1585	439 (430–449)	2771	451 (444–459)	4356	447 (441–453)	0.05
fS-Q10 (μMol)[c]	2754	1.41 (1.38–1.44)	5160	1.34 (1.32–1.36)	7914	1.36 (1.35–1.38)	<0.001
fS-A Vitamin (μMol)	2704	2.36 (2.34–2.39)	5189	2.21 (2.19–2.23)	7893	2.26 (2.25–2.28)	<0.001
fS-beta-carotene (μMol)[c]	2493	0.60 (0.58–0.63)	4471	0.83 (0.81–0.85)	6964	0.74 (0.73–0.76)	<0.001

[a] Males vs. females (Mann-Whitney U test for S-hsCRP and independent samples t-test for other variables).
[b] Results are given as median (95% CI).
[c] Results are given as geometric mean (95% CI).

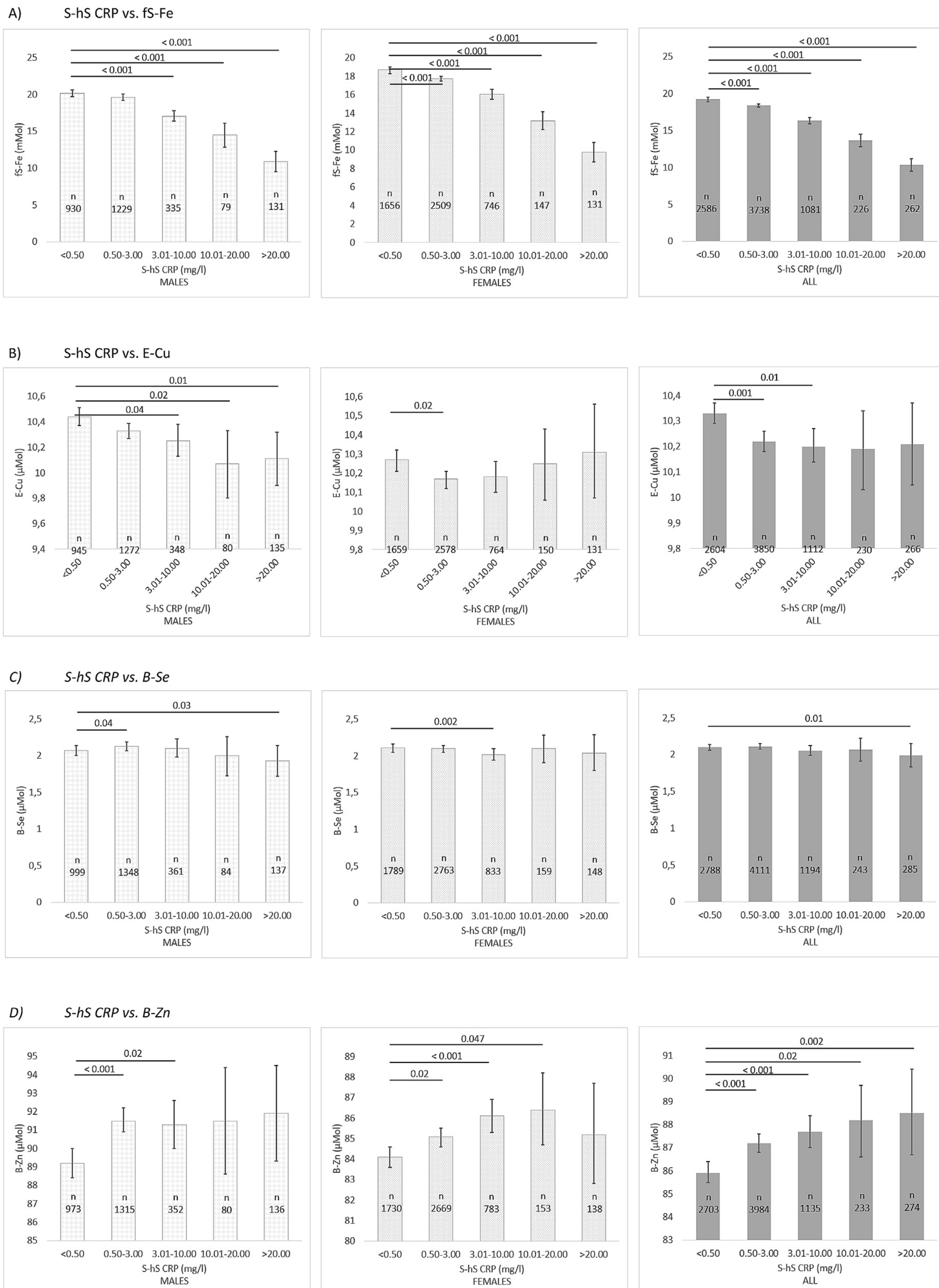

Fig. 1. (A) Mean (95% CI) of serum iron (fS-Fe) in ordered S-hsCRP categories in males, females and in both genders together. (B) Mean (95% CI) of red blood cells copper (E-Cu) in ordered S-hsCRP categories in males, females and in both genders together. (C) Geometric mean (95% CI) of blood selenium (B–Se) in ordered S-hsCRP categories in males, females and in both genders together. (D) Mean (95% CI) of blood zinc (B–Zn) in ordered S-hsCRP categories in males, females and in both genders together. In all figures the p-values are based on the Dunnett t-test which was used for pair-wise comparisons using the lowest category (S-hsCRP < 0.50 mg/L) as the reference category. Only significant ($p < 0.05$) p-values are displayed.

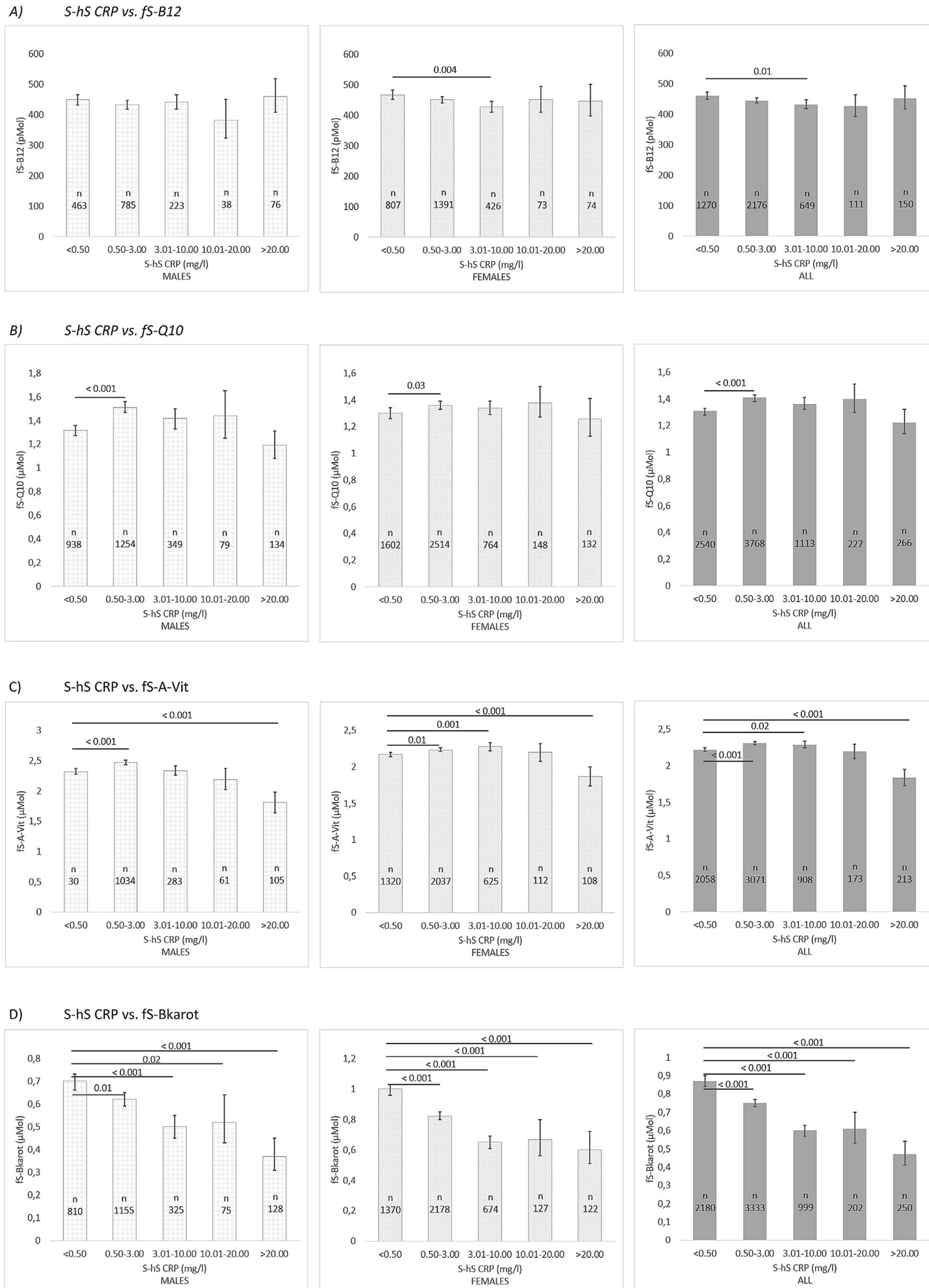

Fig. 2. (A) Geometric mean (95% CI) of vitamin B12 (fS-B12) in ordered S-hsCRP categories in males, females and in both genders together. (B) Geometric mean (95% CI) of serum ubiquinone (fS-Q10) in ordered S-hsCRP categories in males, females and in both genders together. (C) Mean (95% CI) of serum vitamin A (fS-A-Vit) in ordered S-hsCRP categories in males, females and in both genders together. (D) Geometric mean (95% CI) serum beta carotene (fS-β-carot) in ordered S-hsCRP categories in males, females and in both genders together. In all figures, the p-values are based on the Dunnett t-test which was used for pair-wise comparisons using the lowest category (S-hsCRP < 0.50 mg/l) as the reference category. Only significant ($p < 0.05$) p-values are displayed.

levels of ferritin and transferrin saturation as well as hepcidin that would have made it possible to assess the kinetics of iron stores.

Copper is considered as a positive acute phase reactant [9], however this proposal has been based on copper measurements assayed from serum. It is now established that caeruloplasmin is a positive acute phase reactant, i.e. the higher the level of this binding protein in the circulation, the more copper that can be detected from serum. Our method measures the long-term copper stores in washed erythrocytes. As can be observed in Fig. 1B, copper may be washed out from erythrocytes and become bound to caeruloplasmin at lower levels of inflammation but this reaction may reach equilibrium and thus a further increase in the caeruloplasmin concentration will not affect the concentration of copper in the red blood cells (E-Cu). Clearly, the mechanism of self-regulation of copper is important in ensuring red blood cell homeostasis and maintaining cellular functions in the presence of inflammation. Increased caeruloplasmin concentrations will not reduce intracellular copper levels.

Interestingly, blood selenium (B–Se) levels appear to be stable during the inflammation process which is an important adaptation mechanism. It is known that there are no carrier proteins for selenium [9]. Selenium is bound to selenomethionine that is incorporated into peptides and proteins. Since selenoprotein-P and glutathione peroxidase, the Se-containing proteins, are not acute phase proteins, inflammation does not disturb the intra- or extracellular selenium distribution. By contrast, when Se was measured from plasma it appeared to be a negative acute phase reactant [12].

When measured from blood, zinc (B–Zn) behaves like a positive acute phase reactant whereas according to Galloway et al. [9], it can be considered as a negative acute phase reactant. It was speculated that inflammation can result in a surge of cytokines and in this scenario, serum Zn is taken up by the liver where it is complexed to metallothioneins. Zn is also taken up by the tissues experiencing local inflammation. Re-compartmentalization of Zn from the serum is an adaptive mechanism, similarly to the situation for iron. This "escape" from serum prevents microorganisms from acquiring essential microelements. However, we have measured the storage of Zn in the blood. Our results revealed a significant association of B–Zn with low-grade inflammation. This result may indicate that there are mechanisms which ensure that there is a sufficient supply of Zn to maintain cellular function and homeostasis. It is noteworthy that both Zn and Cu are essential metal cofactors of superoxide dismutase (SOD) [13] which is an important intracellular cytoplasmic enzyme involved in the maintenance of redox status within the cell.

As reported earlier, inflammation has no impact on the levels of the water soluble B12 vitamin [9]. Our results, although obtained through a cross-sectional study, are in line with this report [9]. The same applies to the serum concentration of ubiquinon. However, as reported [9], vitamin A and β-carotenes can be viewed as negative acute phase reactants, the latter being more sensitive to the increase of CRP. It has been postulated that during times of inflammation, low vitamin A values do not indicate a true deficiency but rather a redistribution probably to the extra-vascular space to ensure increased tissue bioavailability. An inverse relationship has been reported between the β-carotene concentration and increased CRP concentration in conditions of low-grade inflammation (below 15 mg/L). Similarly, vitamin A and β-carotenes may undergo an extra-vascular redistribution to inflammatory tissues because they have an anti-oxidant activity.

For example, the effects of overt inflammation on the levels of zinc, magnesium, calcium, selenium have been reviewed [9]. It has been found that a mild elevation of CRP (less than 15 mg/L) may decrease the assayed values of these microelements by approximately 10%, whereas overt inflammation (CRP values over 100 mg/L) may result in a decline in the concentrations of these microelements by 40%, even as much as 90% with respect to iron [9]. In situations of overt inflammation, Ca, Mg, Zn can become bound to albumin or to other carrier proteins and therefore are not recovered from plasma (serum) in a free form. As reported by Galloway et al. [9], the effect of inflammation on the serum concentration of vitamin A is quite dramatic. Even rather mild inflammation (CRP less than 15 mg/L) may decrease its concentrations by 40%, whereas there is no further effect as CRP levels increase more. In contrast, the concentrations of vitamin E, B12, and folate remained constant. The effect of inflammation on the measurement of β-carotenes has been reported to be dramatic, with a decline of 90% in situations of severe inflammation and even in mild inflammation, the levels may be halved [9]. These results are in agreement with our findings, although we applied different methodologies. The dramatic decrease in iron levels in the circulation can be explained by several concomitant mechanisms. During inflammation, the concentrations of Liver Expressed Antimicrobial Peptide (LEAP), i.e. hepcidin, is increased and it behaves like a positive acute phase reactant. In the bloodstream, hepcidin is present either in a free form or to a small extent bound to albumin. Hepcidin mediates the transport of iron by proteins, such as ferroportine. By blocking iron transport into and out of enterocytes and macrophages, hepcidin inhibits the absorption of iron from the intestine and prevents iron release from the reticuloendothelial system [14]. It is noteworthy that bacterial SOD enzymes may utilize iron as a cofactor, i.e. in situations of bacterial infection; there is an evident competition between the host and the micro-organism for essential iron.

Any analytical method applied in clinical chemistry needs to be validated appropriately in terms of linearity, reproducibility, sensitivity, specificity and accuracy as well as knowledge of interfering factors. Several factors such as haemolysis or lipemia are known to interfere with the accuracy of the assay, and therefore these samples are often rejected. Haemolysis and lipemia are easily noticed as pre-analytical confounders. The effects of low-grade inflammation on the accuracy of analytical parameters have been less well studied. Due to cellular self-regulation processes, low-grade inflammation may introduce some variability into the assessment of nutritional status. On the basis of our results, it seems more reasonable to measure trace elements from a whole blood specimen that has been standardized for haematocrit rather than from serum. These analytical pitfalls have been discussed, although only for a Finnish speaking audience [11].

In conclusion, decreased levels of some microelements during inflammation might be due to the increased need and turnover or due to an adaptation process that has developed during host-pathogen co-evolution. From the clinical standpoint, when micronutrient supplementation is considered, it is recommended that it is initiated only after acute or chronic inflammation has been cured.

In the future, it would be desirable to unify analytical methods for the assessment of the long-term micronutrient sufficiency. For this, clinical matrixes such as fasting whole blood vs. washed erythrocyte fraction vs. fasting serum (plasma) should be compared. The kinetics of the micronutrient store restoration during deficiency treatment should be recorded for all those matrixes. From this study, better suitability of the whole blood or washed erythrocytes over serum or plasma measurements might turn evident for a large number of clinicians and nutritionists.

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Statement of authorship

TT and EA designed the study, MS retrieved the data and drew the figures, TP performed statistical analysis, KJ was the initiator of the micronutrient measurements in our clinic and set the methods in Mineral Laboratory Mila Oy. TT wrote the first draft; all the authors contributed equally and approved the final version.

Conflict of interests

Kaarlo Jaakkola is the founder of Mineraalilaboratorio Mila Oy, all of the other authors declare no conflict of interests.

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