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Flow cytometric estimation of 'labile iron pool' in human white blood cells reveals a positive association with ageing

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Abstract

A small part of cellular iron, usually called 'labile iron pool' (LIP), is not securely stored and has the potential to catalyse the formation of highly reactive oxygen species. The present work estimated LIP levels in human white cells by using the analytical power of flow cytometry. The method relies essentially on already established principles but has the added value of monitoring LIP in different subpopulations of human blood cells concurrently in a single sample. Examination of 41 apparently healthy individuals revealed a positive correlation between LIP levels and the age of the donors ($r = 0.656$, 0.572 and 0.702 for granulocytes, lymphocytes and monocytes, respectively, $p < 0.0001$), indicating that cells of older individuals are prone to oxidations in conditions of oxidative stress. It is suggested that LIP estimation may represent a valuable tool in examinations searching for links between iron and a variety of oxidative stress-related pathological conditions.

Keywords: *Ageing, calcein, flow cytometry, human leukocytes, labile iron pool (LIP), oxidative stress*

Introduction

Iron is the most abundant transition metal in the human body and represents an essential element required by every living cell. It is involved in fundamental functions of the organisms, such as oxygen sensing and transport, electron transport, DNA and protein synthesis and the metabolism of xenobiotics [1–3]. However, although essential for life, it has also the potential to catalyse the formation of highly reactive oxygen species [4–6]. Thus, life in general is fundamentally dependent on the delicate balance between the deleterious and the beneficial effects of iron. Consequently, it is not surprising that nature developed sophisticated molecular mechanisms in order to finely regulate iron homeostasis at systemic, tissue and cellular level [2,7–12]. The major part of cellular iron is bound in functional proteins or safely stored in the core of ferritin, which has evolved for this purpose [11,12]. However, the existence of an intermediate and transitory form of intracellular iron that can potentially participate in redox reactions has been proposed and is variably termed as 'labile iron pool' (LIP), 'redox-active iron', 'low molecular weight iron', 'free iron', 'chelatable iron' and more [13–18]. Operationally, this iron comprises an easily chelatable iron pool associated with a diverse range of unidentified populations of intracellular ligands and spatially separated in various cell compartments. It is known that fluctuations of this pool of iron can be sensed by specific cytosolic proteins, namely iron regulated proteins 1 and 2 (IRPs 1 and 2), which consequently regulate the concerted synthesis of

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proteins involved in iron uptake, utilization, storage, transport between cell compartments and export out of the cells [2,19–21]. This is achieved mainly by regulation at the level of mRNA of several iron-related proteins, with transferrin receptor 1, ferritin, divalent metal transporter 1 (DMT1, DCT1, Nramp2) and ferroportin among the most prominent [3,21].

Early attempts to assess the levels of LIP relied on methods involving cell disruption, a measure that contributed to redistribution of iron among different compartments and ligands. A more recent approach employs fluorescent iron chelators that can accumulate into intact living cells and undergo reversible and detectable fluorescence changes due to iron binding [15,22]. Calcein represents such a fluorescent probe that has been used extensively the last decade for assessment of LIP, mainly in cultured cells [17,23].

Several attempts have been made to estimate redox-active iron in human serum and to elucidate its relation with diseases associated with iron overload [23–26]. However, it turned out that under normal conditions the levels of redox-active iron in serum, if existed, was under the detection limit of the methods used and became apparent only in conditions of extreme iron overload [27]. It is plausible to assume that direct estimation of LIP level in human cells should contribute towards the clarification of the relation between iron status perturbations and pathological conditions associated with oxidative stress.

In the work presented here, we used the advantages offered by flow cytometry in order to estimate LIP levels in populations of granulocytes, lymphocytes and monocytes in freshly collected human blood, without previous physical isolation of these cells. By using this methodology, a strong positive correlation was revealed between the LIP values observed and the age of the respective blood donors.

Materials and methods

Materials

DMEM growth medium was supplied from Sigma-Aldrich (St Louis, MO). Foetal calf serum, L-glutamine, Nunc plastic tubes and penicillin/streptomycin were obtained from PAA Laboratories GmbH (Pasching, Austria). Calcein-AM was from Molecular Probes (Eugene, OR) and 12×75 mm plastic tubes were from Becton Dickinson (Mountain View, USA). Salicylaldehyde isonicotinoyl hydrazone (SIH) was a kind gift from Professor Prem Ponka (McGill University, Montreal, Canada). All chemicals used were of analytical grade.

Preparation of leukocytes from human blood

Human peripheral blood leukocytes were isolated from freshly donated heparinated whole blood after hypo-osmotic lysis of red blood cells, as previously described [28]. Briefly, one volume of blood was mixed with six volumes of lysis buffer containing 156 mM NH_4Cl, 10 mM $NaHCO_3$ and 97 μM EDTA (ethylenediaminetetraacetic acid) and placed on ice for 20 min in order to allow the lysis of red blood cells. Leukocytes were then collected by centrifugation at $1.000 \times g$ for 5 min and washed twice in complete DMEM growth medium. Finally, cells were suspended at a density of 1.5×10^6 cells per ml before being proceeded for further analysis.

Estimation of LIP in sub-populations of blood cells by using flow cytometry

Estimation of LIP in freshly prepared human leukocytes was performed by using the metal sensitive probe calcein, as fluorescent molecule, in combination with flow cytometric analysis. Calcein in cell-free systems binds both Fe^{2+} and Fe^{3+} with stability constants of 10^{14} and 10^{24} M^{-1}, respectively [29]. The presence of acetomethoxy esteric groups in the original molecule, calcein-AM, facilitates its diffusion across cell membranes while the intracellular cleavage of ester bonds by non-specific esterases results in intracellular retention of the negatively charged calcein. The binding of iron along with other divalent metals by calcein results in quenching of its fluorescence, while subsequent addition of the strong and specific iron chelator salicylaldehyde isonicotinoyl hydrazone (SIH) leads to the removal of iron from calcein and concomitant increase in fluorescence intensity [22]. In the present study, 1.0 ml leukocyte suspensions (1.5×10^6 cells) were transferred into plastic tubes and analysed by FACScan flow cytometry (Becton Dikinson, Mountain View, USA). Light scattering analysis, i.e. forward and side scattering, allowed the gating of leukocytes into three distinct populations based on differences in size and granularity. Subsequently, cells were loaded with calcein by incubating them with 5 nM calcein-AM for 10 min in the dark at 37°C. The intensity of calcein fluorescence was detected as green fluorescence and expressed in histograms against cell counts (see Figure 1). Finally, 100 μM of the membrane permeable, strong and specific iron chelator SIH was added into the cell suspension and calcein fluorescence was detected again 5 min later. The SIH-mediated shift of mean cell-fluorescence in each cell type was expressed in arbitrary units and apparently reflects the level of LIP in the particular cells.

Laboratory measurements

Hematocrit, haemoglobin, serum ferritin, serum iron and serum total binding capacity (TIBC) were measured using routine laboratory methods. Serum

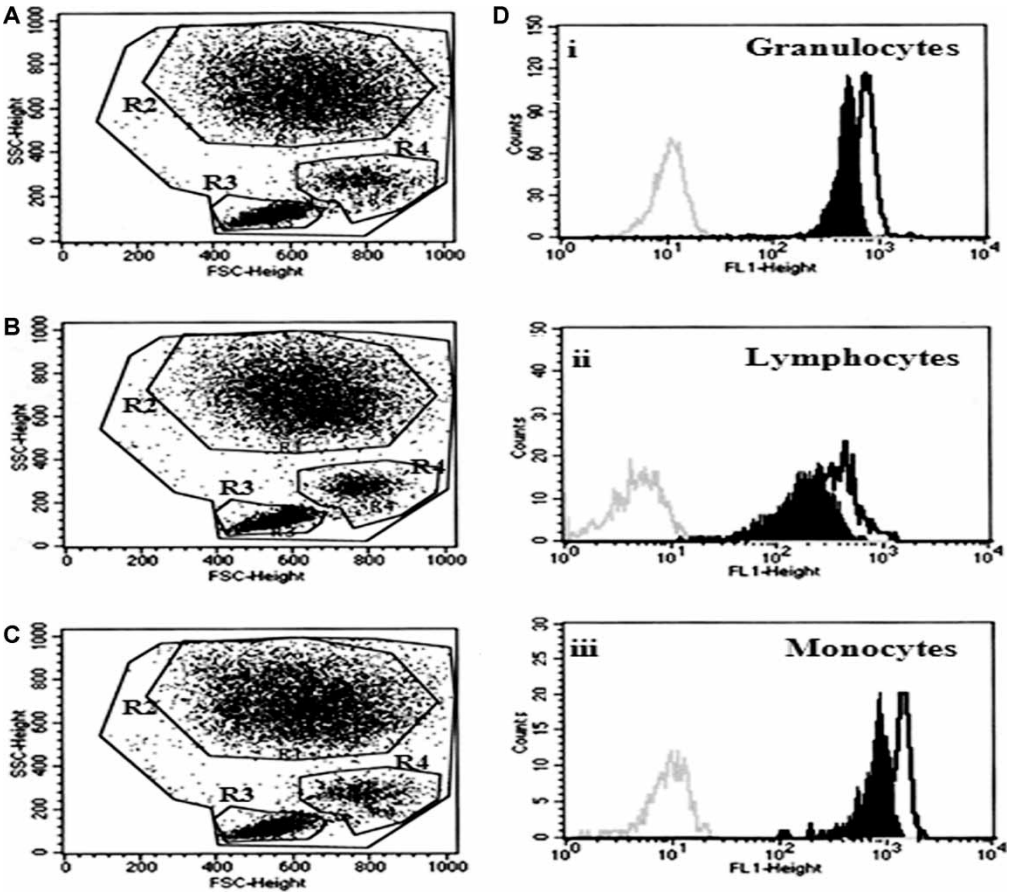

Figure 1. Analysis of peripheral blood leukocytes by flow cytometry. Freshly isolated peripheral blood leukocytes (1.5×10^6 cells/ml) were analysed for light scattering properties (A) and background green fluorescence (grey lined histograms in D). Following, cells were exposed to 5 nM calcein-AM for 10 min and immediately after analysed for the same parameters (B and filled histograms in D). Finally, 100 μM SIH were added to the same cell suspension and 5 min later cells were analysed for light scattering and green fluorescence (C and black lined histograms in D). In dot plots, gate R2 corresponds to granulocytes, R3 to lymphocytes and R4 to monocytes. Increase in mean cell-fluorescence after the addition of SIH is expressed in arbitrary units and reflects a measure for the intracellular calcein chelatable iron pool. This procedure was repeated in all LIP measurements reported in this manuscript.

transferrin saturation was calculated by using the formula

$$\%\ \text{transferrin saturation} = [(\text{serum iron})/(\text{TIBC})] \times 100.$$

Statistical analysis

Correlations between two variables were carried out with SPSS 14.0 software by estimating the Spearmans correlation value.

Results

Human peripheral blood leukocytes were isolated from freshly donated whole blood after hypo-osmotic lysis of red blood cells, as previously described [28]. Flow cytometric light scattering analysis of this leukocyte preparation permitted the gating of sub-populations containing granulocytes, lymphocytes and monocytes based on cell size and granularity (gates R2, R3 and R4, respectively, in Figure 1A).

Loading of these cells with calcein by incubating them with 5 nM calcein-AM for 10 min did not affect their light scattering properties (Figure 1B), while it increased cellular green fluorescence in all three types of cells due to intracellular accumulation of the fluorescent calcein molecule (compare grey lined with filled histograms in Figure 1D). Subsequent addition of the membrane permeable, strong and specific iron chelator SIH did not modulate the light scattering parameters of the cells (Figure 1C) but it resulted in a further increase of fluorescence intensity, indicating de-quenching of calcein fluorescence as a result of removal of iron previously bound on it (Figure 1D). The increase in cellular mean fluorescence (ΔFluorescence) was expressed in arbitrary units and obviously reflected the intracellular calcein-chelatable iron pool or LIP as it is generally termed. It has to be stressed here that ΔFluorescence represents an individual cell value that can be influenced not only by labile iron concentration but also by other parameters, like cell size and compartmentalization.

The reproducibility of the method was evaluated by sequential measurements of the same sample at different times during a day. It was observed that the coefficient of variation (CV) values calculated from the means of quadruplicate determinations of the same sample analysed at two different times were 4.3%, 5.3% and 3.7% for granulocytes, lymphocytes and monocytes, respectively. Remarkably similar values were also obtained when LIP was evaluated in leukocytes from the same donors at time points 7 days apart from each other. As shown in Figure 2, the values obtained were relatively constant for each donor between the two time points regarding all three types of cells (Figure 2A–C), indicating the operation of finely controlled regulatory mechanisms at systemic and cellular level under normal conditions.

Following, the relative values of LIP were estimated in blood cells from 41 apparently healthy individuals. Mean values of 214.4 ± 43.1, 124.0 ± 23.3 and 386.8 ± 66.3 arbitrary units were observed for granulocytes, lymphocytes and monocytes, respectively. Moreover, when these values were plotted against age, a strong positive correlation emerged between these two parameters regarding all three types of cells (Figure 3A–C).

The correlation was strong for all types of cells, with $r = 0.656$, 0.572 and 0.702 for granulocytes, lymphocytes and monocytes, respectively ($p < 0.0001$). Serum iron markers, such as haemoglobin, hematocrit, serum iron, total iron binding capacity, transferrin saturation and serum ferritin were also measured in 31 out of the 41 healthy donors (Table I). None of these parameters was found to be significantly associated with the age of the respective donors.

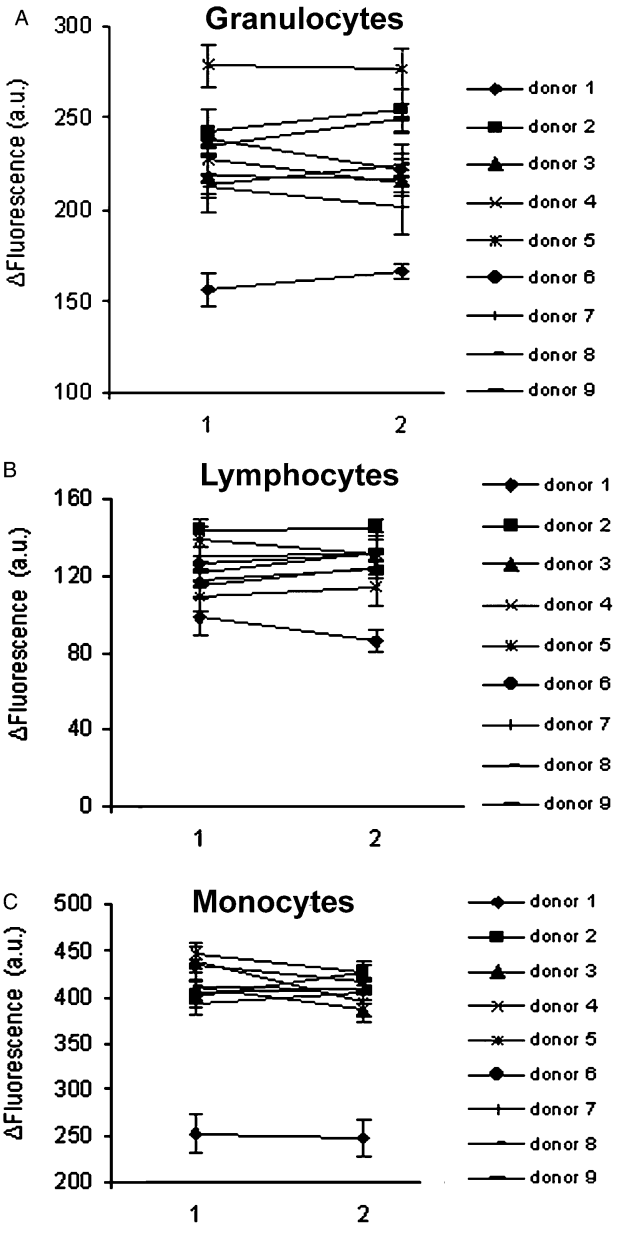

Figure 2. LIP levels are finely regulated in healthy individuals. Freshly isolated peripheral blood leukocytes (1.5×10^6 cells/ml) were analysed by flow cytometry for LIP levels, as described in Figure 1. In A–C, measurements 1 and 2 depict ΔFluorescence values of the same donor at days 1 and 7 for granulocytes, lymphocytes and monocytes, respectively. Each point represents the mean of four measurements in the same sample ± SD. Note that LIP levels for each donor remain relatively stable during this period of time.

Discussion

Epidemiological studies examining the association between body iron stores and the outcome of diseases have yielded conflicting results [30–33]. It is suspected that the main reason for this inconsistency depends on methodological problems regarding the estimation of body iron status [34]. The markers usually used, i.e. ferritin, serum iron, total iron binding capacity and transferrin saturation, do not necessarily represent the fraction of iron that is able to mediate the harmful effects, which ultimately leads to cell and tissue injuries under conditions of oxidative stress. It is plausible to imagine that a marker relevant to the redox-active component of body iron will be valuable in investigations aimed to establish the association of iron with pathological conditions. Indeed, methods for estimation of 'non-transferrin bound iron' in serum have been developed and it has been observed that this form of iron was apparent in conditions of heavy iron overload [13,26,27,35]. However, the level of 'non-transferrin bound iron' in healthy individuals, if existed, was under the detection limit of the methods used, thus limiting the application of this methodology [25–27].

On the other hand, the levels of the intracellular counterpart of 'non-transferrin bound iron', the so called LIP, have been shown to be relatively higher, at least in cells in culture. This probably indicates the high rate of utilization of iron for synthesis of new iron-containing proteins. Based on the above

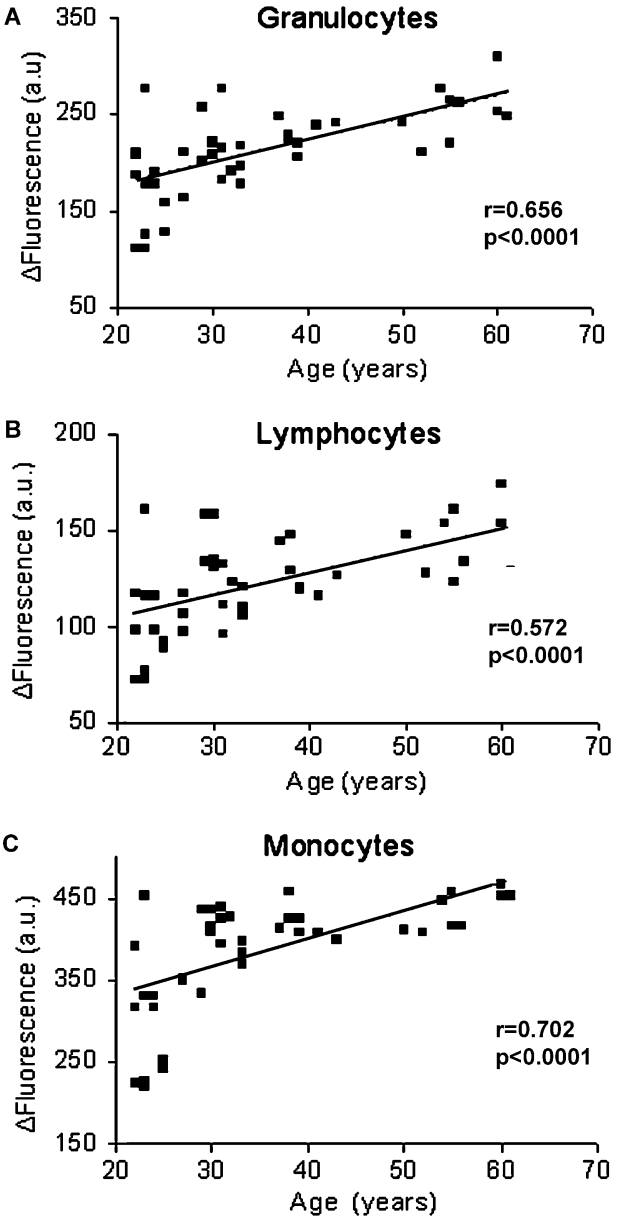

Figure 3. Age-related increase of LIP levels. Peripheral blood leukocytes (1.5×10^6 cells/ml) isolated from healthy donors were analysed by flow cytometry for LIP levels, as described in Figure 1. LIP values (ΔFluorescence, *y*-axis) were next plotted against the age of the respective donors (*x*-axis). Correlation between these parameters was done by evaluating Spearmans *r* values for granulocytes (A), lymphocytes (B) and monocytes (C), respectively.

considerations, we hypothesized that intracellular LIP could be an appropriate marker for the examination of possible associations between iron and oxidative stress-associated pathological conditions. Thus, we exploited the opportunity of using the powerful advantages offered by flow cytometry in order to develop a methodology for comparative estimation of LIP in sub-populations of cells from freshly collected human blood. The method described is simple, fast

Table I. Population characteristics and iron-related markers of healthy individuals.

Median age (years)*	35.7±12.2 (range 22–61)
Sex (Male/Female)	20/21
LIP (arbitrary units)	
Granulocytes	214.4±43.1
Lymphocytes	124.0±23.4
Monocytes	386.8±66.3
Haemoglobin (g/dL)	13.8±1.4
Hematocrit (%)	43.0±3.6
Serum iron (μg/dL)	101.0±67.7
Total iron binding capacity (μmol/L)	332.3±31.0
Transferrin saturation (%)**	30.4±14.6
Serum ferritin (ng/mL)	72.1±76.4

* $M \pm$SD, $n = 41$.
** Serum transferrin saturation was calculated by using the formula; % transferrin saturation = [(serum iron)/(TIBC)] × 100.

and reproducible and can be used for the evaluation of both iron overload as well as iron deficiency, since it can detect either increased or decreased LIP levels. The exact quantification of intracellular iron concentrations is hampered by the inability to correlate the fluorescence differences indicated in the flow cytometer with actual iron concentrations. In addition, we recently reported that the intracellular iron is not homogenously distributed in different cell compartments and that the calcein method was not able to detect iron in all cell compartments [16,36]. Thus, the probability exists that an apparent rise in LIP (as detected in the present work) merely represents a shift of labile iron to subcellular compartments accessible to calcein.

The association of body iron status with ageing has been a controversial area [37–39]. In most of the studies, however, the estimation of the body iron status was based on the levels of serum ferritin. It has to be stressed that ferritin is an acute-phase protein, the serum levels of which do not reflect the functional iron pool. The observation in this investigation that LIP values correlated strongly with the age of the individual donors (Figure 3) indicates an age-related increase of available catalytically active iron, probably as a result of malfunction of homeostatic iron regulatory mechanisms. This observation may offer a mechanistic explanation for the basis of the increased oxidative modifications of main cellular components during ageing, as previously reported [40,41]. The possibility of establishment of LIP as the main catalyst for the oxidation of cell components under conditions of oxidative stress may contribute to far reaching considerations regarding probable interventions intended to modulate the ageing process [42,43].

Contrary to the results presented here, Gackowski et al. [44] recently reported the absence of association between lymphocyte LIP and the age of the donors.

This discrepancy may be derived probably from the fact that, in that work, lymphocytes were physically separated from other blood cell populations by time-requiring and rather complicated procedures, which obviously affected LIP levels. This conclusion is also supported by the high standard deviation values reported compared to values presented in this investigation, where no physical separation of blood cells took place other than the removal of erythrocytes.

Although there are numerous hypotheses trying to explain the ageing process, the best mechanistic explanation is provided by the so called 'oxidative stress hypothesis' initially proposed by Harman [45], which subsequently underwent several modifications. Substantial experimental evidence in support of this theory was accumulated during the following years. Progressive increase in oxidative stress and a generalized decline in antioxidant defenses have been observed in several ageing models [46–48]. These changes lead inevitably to the progressive accumulation of oxidized and aggregated cell components, including proteins, lipids and carbohydrates among others [48–50]. However, the assumption that increased antioxidant consumption should decelerate ageing by scavenging free radicals and preventing oxidations, which was initially raised, never got enough experimental support. This may be due to the fact that extremely reactive radicals, when generated inside the cell, are impossible to be scavenged by any exogenously added compound due to the competition offered by numerous cellular components. On the other hand, it is plausible to assume that the rate of formation of reactive oxygen radicals may be drastically decreased by restricting the availability of catalytically active iron [51–53]. The fact that LIP levels were progressively increased during ageing, as observed for the first time in this study, may represent the basic contributing factor for the parallel accelerated accumulation of oxidized material. The reason for the progressive failure of iron homeostatic mechanisms during ageing is presently unknown. Age-related malfunction of proteins, which regulate intracellular iron homeostasis with subsequent expansion of LIP, represent the most probable explanation.

During the preparation of this manuscript, a new publication appeared using the same methodology for estimation of LIP in human blood and bone marrow cells [54].

In summary, the results of the present investigation indicate the probability of estimating the levels of LIP in individual cell populations of human blood. Since LIP is thought to represent the form of iron that catalyses the oxidation of basic cellular components, strategies aimed to modulate it may have powerful impact on ageing and age-related diseases as well as on other iron- and oxidative stress-related pathological conditions.

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