Available online at www.sciencedirect.com
SCIENCE DIRECT®
Nuclear Instruments and Methods in Physics Research B 249 (2006) 697–701
NIM B
Beam Interactions with Materials & Atoms
www.elsevier.com/locate/nimb

Using skin to assess iron accumulation in human metabolic disorders

I. Guinote [a], R. Fleming [b], R. Silva [c], P. Filipe [c], J.N. Silva [c], A. Veríssimo [a], P. Napoleão [a,d], L.C. Alves [a,d], T. Pinheiro [a,d,*]

[a] *Laboratório de Feixes de Iões, Instituto Tecnológico e Nuclear, E.N. 10, 2685-953 Sacavém, Portugal*
[b] *Imunohaemotherapy Department, Hospital de St. Maria, Lisboa, Portugal*
[c] *Dermatology Department, Hospital de St. Maria, Lisboa, Portugal*
[d] *Centro de Física Nuclear, Universidade de Lisboa, Portugal*

Available online 16 May 2006

Abstract

The distribution of Fe in skin was assessed to monitor body Fe status in human hereditary hemochromatosis. The paper reports on data from nine patients with hemochromatosis that were studied along the therapeutic programme. Systemic evaluation of Fe metabolism was carried out by measuring with PIXE technique the Fe concentration in plasma and blood cells, and by determining with biochemical methods the indicators of Fe transport in serum (ferritin and transferrin). The Fe distribution and concentration in skin was assessed by nuclear microscopy and Fe deposits in liver estimated through nuclear magnetic resonance. Elevated Fe concentrations in skin were related to increased plasma Fe ($p < 0.004$), serum ferritin content ($p < 0.01$) and Fe deposits in liver ($p < 0.004$). The relationship of Fe deposits in organs and metabolism markers may help to better understand Fe pools mobilisation and to establish the quality of skin as a marker for the disease progression and therapy efficacy.

PACS: 07.77.Ka; 87.57.−s; 87.64.−t; 87.59.Jq; 82.80.−d; 87.19.Xx

Keywords: Nuclear microprobe; Skin; Blood; Iron; Hemochromatosis

1. Introduction

Human hereditary or secondary hemochromatosis, are related to abnormal Fe intestinal absorption that induces Fe overload in organs facilitating the occurrence of diseases, such as, cirrhosis, congestive heart failure, diabetes, and hepatocellular carcinoma [1]. The hereditary hemochromatosis is better characterised and can derive from hetero- or homo-zygoty for at least one HFE gene mutation (C282Y or H63D), a gene encoding a protein related to the transferrin receptor in cell membranes. Although, there is a broad spectrum of clinical manifestations, from no clinical or biochemical evidence of Fe overload to major alterations in adulthood. The clinical significance of some HFE polymorphisms found so far, the control mechanisms of intestinal Fe absorption, circulating Fe levels, and its accumulation in tissues are still not clarified issues. Therapy has not changed significantly in the past 50 years. It consists of periodic phlebotomies to maintain the Fe levels in circulation low, trying to prevent excessive Fe accumulation in organs [2]. The clinical status evaluation is based on the determination of levels of proteins that transport Fe in serum such as ferritin and transferrin, which can give an indication of the rate of Fe intestinal absorption and of Fe mobilisation, although they cannot grant the magnitude of Fe deposition in organs [2,3]. Organ Fe deposits are difficult to measure usually requiring invasive methods with a significant risk for the patient, such as the case of liver biopsy. Skin is an accessible organ affected by this disease that can be obtained without risks, and can therefore be used to monitor body Fe status.

* Corresponding author. Address: Laboratório de Feixes de Iões, Instituto Tecnológico e Nuclear, E.N. 10, 2685-953 Sacavém, Portugal. Tel.: +351 21 994 6250; fax: +351 21 994 1525.
E-mail address: murmur@itn.pt (T. Pinheiro).

0168-583X/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.nimb.2006.03.120

Nuclear techniques based on accelerated particle beams enabling elemental concentrations determination as well as elemental distribution images in tissues, proved to be powerful methodologies to investigate essential elements imbalance such as occurs in human hemochromatosis [4]. Also, recent methods in nuclear magnetic resonance based on the properties of superparamagnetic Fe oxide particles can provide a useful indication of Fe deposits in the liver although the method consistency seem to depend on the extend of tissue damage [5]. Combining different techniques that make possible to relate systemic Fe indicators of metabolism, to the distribution and concentration of Fe at different organs and tissues, a contribution to the understanding of Fe pools mobilisation in this pathology can be provided. In particular, studying the cutaneous Fe deposition and how it can reflect body Fe pools may pave the way to establish alternative markers to the disease progression and therapy efficacy.

The paper reports on data from nine patients with hereditary hemochromatosis that were studied since the disease was diagnosed, along the therapeutic programme. Cutaneous, hepatic, and serological evaluation of Fe levels and of Fe metabolism was made before and after the initial phase of therapy and six months after stopping the initial phase of the phlebotomy programme. The relationship of the parameters measured for Fe metabolism and organic Fe deposition along the therapy phases was assessed in what concerns the quality of skin to reflect body Fe pools.

2. Material and methods

2.1. Study groups

The pathology group consists of nine patients with a diagnosis of hereditary hemochromatosis (HC), based on clinical signs and symptoms, and on the determination of HFE gene mutations (C282Y or H63D). The subjects included in the study were homo- or hetero-zygotes for both mutations.

Patients were characterised through liver and systemic indicators of Fe metabolism (see Table 1) at an initial phase of the disease diagnosis, prior to therapy – Phase 1, at the end of the phlebotomy therapy programme – Phase 2, and six months later, after stopping therapy programme – Phase 3.

For blood elemental contents determination purposes a cohort of 49 healthy, without hypertension, normo-lipemic, and non-diabetic Portuguese individuals, was considered as a reference group [6]. Skin reference values for Fe and other elemental concentrations were obtained from a different group of donors constituted by 15 healthy Portuguese individuals.

The study was carried out according to ethical local regulations and all individuals gave informed consent.

2.2. Sample collection and preparation

Blood and skin samples were collected for elemental concentrations determination. Blood was drawn by venopuncture and plasma, serum, and blood cells fractions obtained by centrifugation using standard procedures.

Skin was collected by punch biopsy of 3 mm diameter. Skin biopsies were immediately frozen in 2-methylpentane cooled in LN_2 and stored at -70 °C until sectioning in a cryostat at -25 °C. The obtained sections of 14 μm thickness were dried overnight inside the cryostat, and subsequently selected and mounted in self-supported mode, held by the edges with carbon tape (Agar™) for nuclear microprobe analysis purposes [7]. Thinner sections of 5–7 μm thick were also produced and stained to inspect sample quality and identify eventual artefacts resulting from sample handling, freezing, and preparation.

2.3. Iron assessment in blood, liver and skin

Ferritin concentration and free and bound (to transferrin) Fe contents in blood serum were determined using standard enzyme linked immunoassay (ELISA) procedures and spectrophotometric methods. The total Fe concentration in plasma and blood cells fractions were determined by particle induced X-ray emission (PIXE) as described elsewhere [8] using the Van de Graaff accelerator facilities at ITN. Hepatic Fe deposits were assessed by nuclear magnetic resonance (NMR) non-invasive methods. Fe concen-

Table 1
Patients characterisation along the three phases of therapy programme

	HC – Phase 1 ($N = 9$)	HC – Phase 2 ($N = 5$)	HC – Phase 3 ($N = 4$)	Reference values[a]
Liver function				
ALT (U/l)	76 ± 36	31 ± 18	25 ± 10	0–41
AST (U/l)	56 ± 46	24 ± 7	20 ± 4	0–37
γGT (U/l)	47 ± 46	41 ± 48	34 ± 8	11–50
Glucose (mmol/l)	8.4 ± 5.5	6.8 ± 2.6	5.4 ± 1.1	3.9–6.1
Systemic iron				
Ferritin (ng/ml)	1380 ± 500	43 ± 66	30 ± 2	24–336
Transferrin saturation (%)	76 ± 20	65 ± 37	57 ± 30	< 50

[a] Laboratory of Pathology, Sta. Maria Hospital, Lisbon, Portugal.

trations in blood were expressed as mg/L and in liver as μmol/g on a wet weight basis.

Skin morphology and elemental distributions in self-supported sections were assessed by nuclear microscopy methods using the ITN nuclear microprobe [9]. High-resolution images were obtained by scanning transmission ion microscopy (HR-STIM) and Fe concentrations, as well as other minor and trace elemental contents, were determined by PIXE technique from point analysis performed along regions of interest. Rutherford backscattering spectrometry technique was used to obtain matrix density and composition needed for mass normalisation of the PIXE spectra [10]. As skin is a stratified tissue, Fe deposits along epidermal strata and dermal regions were validated by HR-STIM which provided images capable of accurately differentiating epidermis strata, dermal regions and skin appendages [7].

2.4. Statistical analysis

The data were expressed as mean ±SD. Significant differences between subject groups were determined using parametric *t*-test and nonparametric Kruskal–Wallis approaches and considered significant when the two-tailed confidence interval of 95% was exceeded at a probability error (p level) inferior to 0.05 in all tests applied [11].

To assess the dependence of Fe concentration in skin and Fe concentration in other organs or tissues, or levels of Fe metabolism markers (independent variables), a linear regression was performed considering the standard deviation of the Fe skin content as weights ($1/SD^2$) for linear fit and confidence intervals calculation. In addition to intercept and slope values and respective standard errors, the correlation coefficient, R-square estimation, t-value statistics, ANOVA, and F distribution (yielding a p-value) for the estimation of goodness of fit, were calculated.

3. Results and discussion

The Fe contents in blood serum and plasma of naïve hemochromatosis patients (Phase 1) were significantly higher than those obtained for the reference group. For subsequent phases, i.e. after stopping the phlebotomy therapy programme (Phase 2) the Fe concentrations decreased to normal values in serum and plasma and below the reference interval in blood cells (Table 2). At Phase 3 of evaluation, an increase in serum and plasma Fe concentrations was observed despite ferritin concentration and transferrin saturation values remain within normal values (see Table 1). So far, only data of two patients at Phase 3 was available for plasma; therefore the tendency observed for the Fe increase should be taken cautiously. Liver Fe deposits also decrease along therapy programme assessed phases, although values are still above the reported values of non-significant Fe overload (Table 2) being significantly high for patients studied in Phase 1 and Phase 2.

Analysing skin sections of patients and healthy individuals Fe concentration levels were more significant in epidermis in particular at regions close to the basal layer. The limits of different strata can be established (Figs. 1 and 2) using HR-STIM imaging after PIXE analysis for normalisation of map coordinates. As shown in Fig. 1, the HR-STIM images enable to define frontiers of major epidermal strata (stratum corneum and granulosum, and strata spinosum and basale, the lower epidermal region), and dermis, which enable to determine elemental concentration ranges within and between them. This aspect is of the utmost importance as the preferential localisation of Fe, in the case of hemochromatosis, may have a specific biological significance. A similar distribution in minor and trace elements, such as P, S, Cl, K, Ca and Zn was observed in skin of patients when compared to healthy individuals, although some of the elemental concentrations vary significantly along therapy. Specifically, P, Cl and K can give a good indication of epidermal–dermal boundary and of the limits of stratum corneum, the external layer of skin.

As referred above, patients with hemochromatosis assessed in Phase 1, have a systematic and significant increase in Fe contents at epidermal ($p < 0.01$) and dermal ($p < 0.02$) regions when compared with the reference cohort. In particular, Fe deposits are significantly higher at lower epidermal areas, close to the basal epidermis layer (Fig. 3). A sharp decrease in skin Fe concentrations to control levels was observed in patients in Phase 2, if we exclude the upper epidermal region, where Fe contents still show increased levels relative to controls. For the two patients at Phase 3 analysed so far, Fe levels do not differ from reference values for all regions studied. Alterations in S, Cl, K and Zn concentrations were also observed in epidermal and

Table 2
Fe concentration in blood and hepatic Fe levels in patients with hemochromatosis

	HC – Phase 1 ($N = 9$)	HC – Phase 2 ($N = 5$)	HC – Phase 3 ($N = 4$)	Reference intervals
Serum Fe (mg/L)	2.39 ± 0.44 (1.57–2.87)	0.86 ± 0.83 (0.15–2.76)	1.22 ± 1.51 (0.15–2.28)	0.65–1.75[a]
Plasma Fe (mg/L)	2.52 ± 0.94 (1.15–4.15)	0.78 ± 0.42 (0.48–1.07)	– (2.23–2.50)	0.42–1.80
Blood cells Fe (mg/L)	726 ± 106 (673–851)	648 ± 35 (588–661)	– (626–692)	720–986
Hepatic Fe (μmol/g ww)	161 ± 91 (55–260)	93 ± 85 (35–240)	50 ± 14 (40–60)	< 40[a]

Values are means and the associated standard deviations for N analysed individuals. Minimum and maximum values (between brackets) as well as reference intervals obtained for the control group are also listed.

[a] Laboratory of Pathology, Sta. Maria Hospital, Lisbon, Portugal.

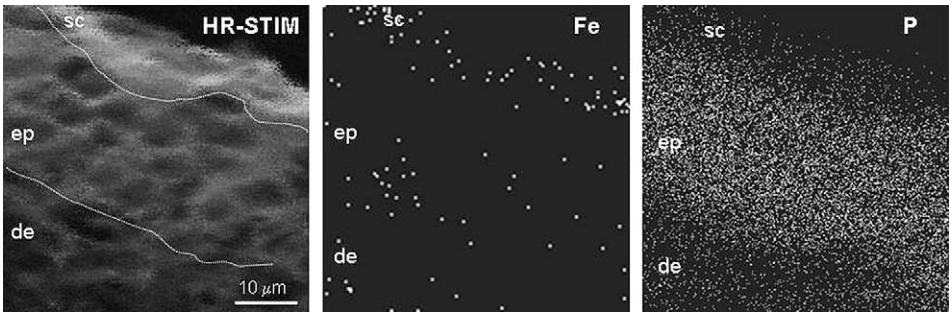

Fig. 1. Nuclear microscopy analysis of skin morphology (HR-STIM), Fe and P distribution (PIXE) in health condition. The boundaries of stratum corneum (sc), epidermis (ep) and dermis (de) are indicated schematically by a dashed line in HR-STIM image. Increasing signal intensity represented by a gradient from dark to white.

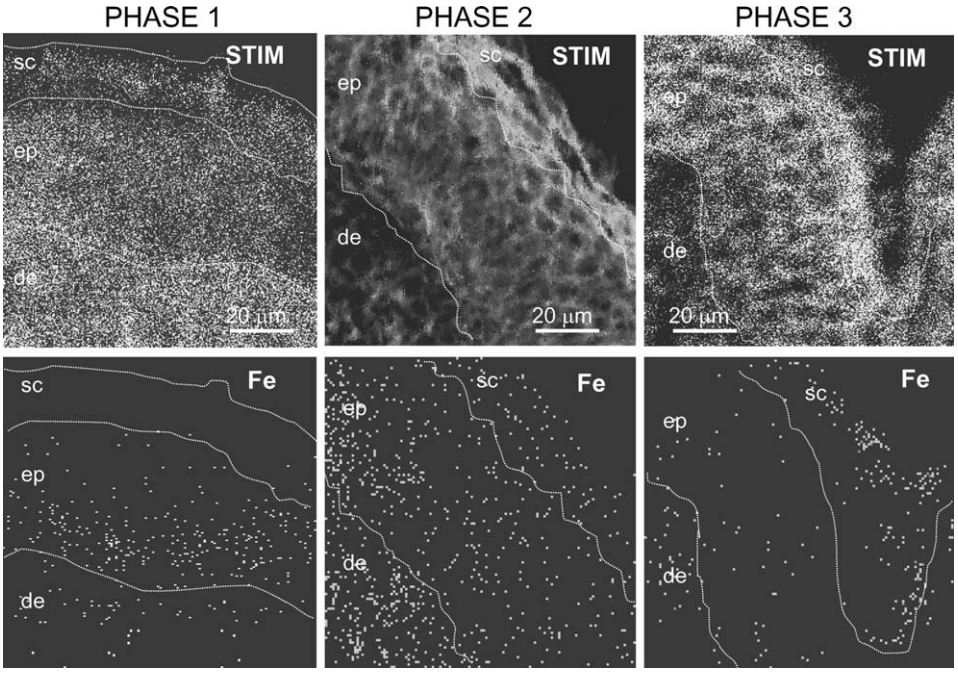

Fig. 2. Fe distribution in skin of one patient at the three phases of therapy. The boundaries of stratum corneum (sc), epidermis (ep) and dermis (de) are indicated schematically by a dashed line in HR-STIM image. Increasing signal intensity represented by a gradient from dark to white.

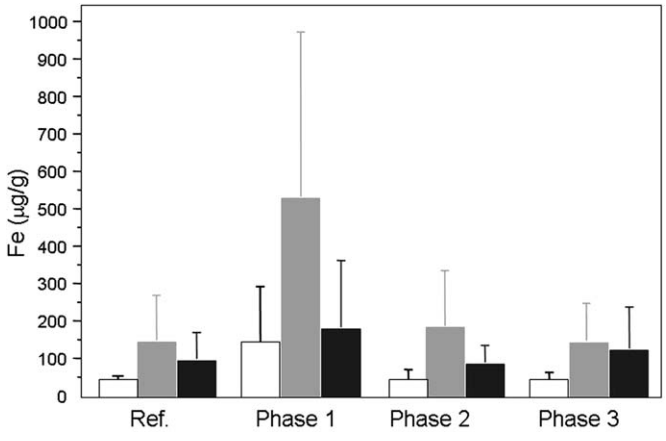

Fig. 3. Average concentration of Fe in different skin layers for the reference group and for patients at the three phases of therapy. White bars – upper epidermal region (granulosum and spinosum strata); light grey bars – lower epidermal region (low spinosum and basale strata); dark grey bars – dermis.

dermal regions at Phases 1 and 2 of therapy relative to controls, although not always significant. Assessing the relationship of Fe content levels in different tissues along therapy programme a good linear regression function was observed between epidermal Fe concentration, hepatic Fe deposits ($r = 0.85$, $p < 0.004$), plasma iron concentration ($r = 0.77$, $p < 0.004$), and ferritin concentration ($r = 0.6$, $p < 0.01$).

Epidermis receives nutrients from blood and lymphatic capillaries through the dermal–epidermal barrier at the basal layer. Therefore, as Fe concentrations in blood increase, its deposits in tissues and cells occur whether free or bound to ferritin, haemosiderin, albumin, or other transport proteins, affecting cell electrolytic balance [12], which can be expressed by changes in K (significantly increased) as observed in this work (results not shown). As keratinocytes that may incorporate Fe evolve and migrate to upper epidermal regions, Fe is carried along

epidermis until keratinocytes suffer apoptosis and become packed together in stratum corneum, what explain the increased Fe contents in upper epidermal regions at Phase 2. At this stage, the significant alterations observed (results not shown) in the cutaneous concentrations of S, K and Zn (reduced) and Cl (raised) may reflect, in different ways, a decrease of metabolism that might be caused by erythropoietic depression following phlebotomies [12,13].

4. Conclusions

Skin Fe deposits seem to reflect systemic and organic Fe deposition in patients with hemochromatosis, however, results cannot be fully conclusive as still few patients got to Phase 3 and reduced number of individuals was surveyed. The excellent correlation between Fe concentrations in skin and those found in blood and liver pools along therapy, position skin as a good indicator for Fe levels in other organs. In addition, the gathered data suggests that measuring Fe deposits in skin in combination with hepatic Fe estimation and systemic markers of Fe metabolism may constitute a good approach to detect alterations induced by the disease at early phases and along therapy. Therefore, skin Fe evaluation can become a potential surrogate marker for assessing therapy efficacy and progression.

Acknowledgements

Authors are indebted to Dr. Helena Proença of the Laboratory of Pathology of Santa Maria Hospital in Lisbon for the collaboration on ferritin and transferrin determination and to Rute Pinheiro, Filomena Baptista and Jorge Rocha for their help on laboratory protocols carried out and accelerator operation. The work was mainly subsidised by a SPDV (Portuguese Society of Dermatology and Venereology) research contract. The reference groups for skin and blood were established under the research contracts, EC/QLK4-CT-2002-02678-NANODERM and FCT-POCTI/ESP/41008/2001, respectively.

References

[1] N.C. Andrews, New Eng. J. Med. 341 (1989) 1986.
[2] E. Beutler, A.V. Hoffbrand, J.D. Cook, Hematology 20003 (2003) 40.
[3] P. Nielsen, R. Engelhardt, J. Dullmann, R. Fischer, Blood Cell. Mol. Dis. 29 (2002) 451.
[4] T. Pinheiro, J.N. Silva, L.C. Alves, P. Filipe, Nucl. Instr. and Meth. B 210 (2003) 373.
[5] J.H. Jensen, R. Chandra, Magnet. Reson. Med. 47 (2002) 1131.
[6] T. Pinheiro, P. Napoleão, P. Lopes, A.M. Viegas-Crespo, L. Pavão, M.C. Santos, P. Monteiro, A. Bugalho de Almeida, in: R. Cesareo, A. Castellado, G. Gigante (Eds.), Proceedings of the European Conference on X-ray Spectrometry, EXRS, Alghero, Italy, 2004, p. 181.
[7] P. Aguer, L.C. Alves, Ph. Barberet, E. Gontier, S. Incerti, C. Michelet-Habchi, Zs. Kertész, A.Z. Kiss, P. Moretto, J. Pallon, T. Pinheiro, J.E. Surlève-Bazeille, Z. Szikszai, A. Veríssimo, M.D. Ynsa, Nucl. Instr. and Meth. B 231 (2005) 292.
[8] M.A. Barreiros, T. Pinheiro, M.F. Araújo, M.M. Costa, M. Palha, R.C. Silva, Spectrochim. Acta Part B 5 (11) (2001) 2095.
[9] L.C. Alves, M.B.H. Breese, E. Alves, A. Paul, M.R. da Silva, J.C. Soares, Nucl. Instr. and Meth. B 161–163 (2000) 334.
[10] G.W. Grime, M. Dawson, Nucl. Instr. and Meth. B 104 (1995) 107.
[11] M.J. Norusis, SPSS for Windows Release 11.0, User's Guide, SPSS Inc, Chicago, 2001.
[12] A.C. Guyton, J.E. Hall, Textbook of Medical Physiology, 10th ed., W.B. Saunders Company, Philadelphia, 2000.
[13] G. Papanikolaoua, K. Pantopoulos, Toxicol. Appl. Pharmacol. 202 (2005) 199.