Erythrocyte Hyperchromia Measured by Flow Cytometry: a Marker for Body Iron Overload?

Flow-zytometrische Messung der erythrozytären Hyperchromie: Ein Marker für Eisenüberladung?

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Summary: Using the classical cryolysis test suggested by Rosas and coworkers, it is shown that hyperchromic erythrocytes correspond to spherocytes destined for rapid elimination by lysis in the reticulo-endothelial cells. The percentage of hyperchromioc erythrocytes is determined without further cost by the H*-1, H*-2, and ADVIA 120 haematological analysers. An increased percentage of hyperchromic red blood cells thus emphasises increased haemolysis. It is shown that approximately 80 % of adult men with a percentage of hyperchromic erythrocytes exceeding 9.4 % have serious iron overload evidenced by high ferritin. The percentage in women with hyperchromia is much lower, eventually due to menstrual blood loss. Significant hyperchromia in adult men represents a marker of iron overload, justifying further investigation.

Keywords: hyperchromia; spherocytosis; haemolysis; iron overload.

Zusammenfassung: Unter Verwendung des von Rosas und Kollegen beschriebenen klassischen Kryolyse-Test zeigen wir, dass hyperchrome Erythrozyten Sphärozyten entsprechen, die für die schnelle Elimination in retikulo-endothelialen Zellen bestimmt sind. Der Prozentsatz hyperchromer Erythrozyten wird ohne zusätzliche Kosten auf den H*-1, H*-2 und ADVIA120 Hämatologie-Analysatoren untersucht. Ein erhöhter Anteil hyperchromer Erythrozyten weist auf eine gesteigert Hämolyse. Es wird gezeigt, dass etwa 80 % der erwachsenen Männer mit mehr als 9,4 % hyperchromer Erythrozyten hohe Ferrtin-Spiegel und eine ernstzunehmende Eisenüberladung aufweisen. Der Anteil von Frauen mit Hyperchromie ist deutlich kleiner, möglicherweise in Folge des menstruellen Blutverlustes. Eine signifikante Hyperchromie bei erwachsenen Männern stellt einen Marker für Eisenüberladung dar, welcher eine weitere diagnostische Abklärung rechtfertigt.

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Schlüsselwörter: Hyperchromie; Sphärozytose; Hämolyse; Eisenüberladung.

The need to screen for hereditary haemochromatosis is widely discussed and generally considered to be useful [1, 2, 3], especially as approximately 0.5 % of the northern European population is assumed to carry the typical homozygous C282Y mutation [4]. Studying the penetrance of hereditary haemochromatosis on a very large control population Beutler et al. [5] conclude, however, that less than 1 % of these homozygotes are at risk of developing clinical haemochromatosis and contradict the necessity to screen for this anomaly. Still, the fact remains that iron overload – whatever its origin – is considered toxic for many organs of the human body, leading to damage of liver, heart and immune system, diabetes, and hormonal abnormalities [6, 7, 8, 9]. It has been recognised by many authors as a causal factor in both neoplastic disease and viral and bacterial infection [10, 11, 12, 13]. Even heterozygous haemochromatosis alleles predictably contribute to an iron burden from other causes. Secondary, non genetic haemochromatosis due to other factors is not rare. Identifying and treating these disorders after symptoms occur can arrest their progress but is unable to reverse existing damage. Detection of all types of iron overload at an asymptomatic stage is therefore recommended [14, 15].

HLA-typing appears at first sight the method of choice for overload of genetic origin. Its limitation to this type of overload as well as financial considerations certainly rule out this approach. Ferritin and transferrin saturation are less costly, efficient markers but generally not requested in untargeted health check-ups. Indirect attempts based on the increase of alanine-aminotransferase (ALAT) have been reported [16, 17].

Basic haematology and ALAT are requested in practically every first line exploratory panel. We describe a possible symptom for iron overload obtained by means of a cost-free haematological routine parameter: the percentage of hyperchromic erythrocytes (% hyper). Measuring the diffraction of a laser beam by the red blood cell at two different angles allows determination of both erythrocyte volume and density, in other words haemoglobin concentration for each of approximately

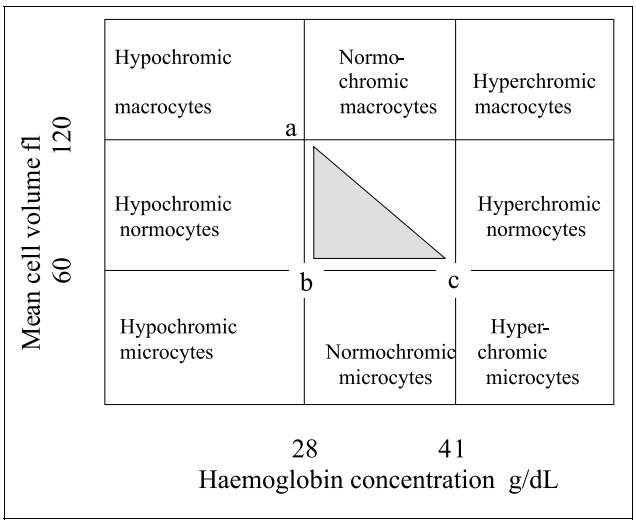

Figure 1 The erythrogram produced by the ADVIA® 120 with the possible pathological aberrations. Normally the erythrocytes are located within the triangle abc.

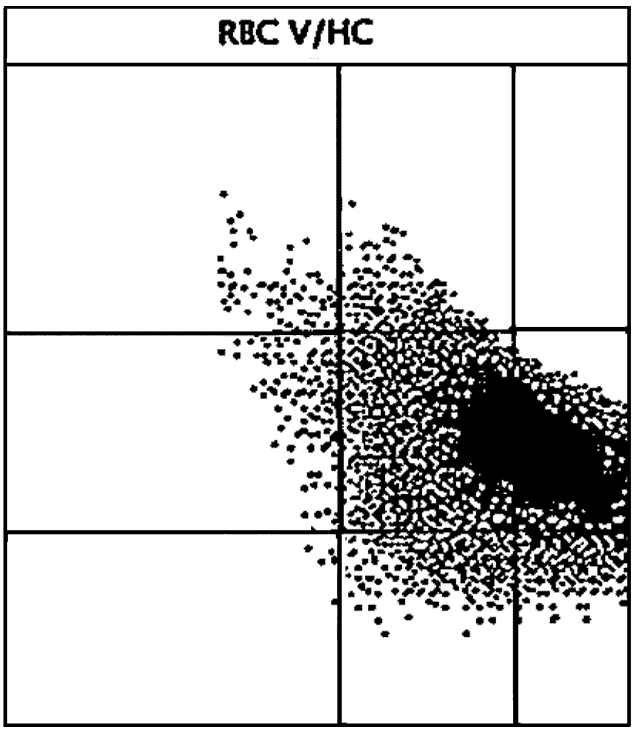

Figure 2 ADVIA-120 erythrogram of a case of hereditary spherocytosis.

50,000 cells. Applying this technique, the ADVIA®120 haematological analyser (Bayer Inc. Terrytown USA) is able to differentiate and quantify the different abnormalities of both volume and concentration for a given cell population in the so-called "erythrogram" (Fig. 1), including the exact percentage of aberrant erythrocytes. Figure 2 shows the typical erythrogram of a case of hereditary spherocytosis. Using the cryolysis test for increased red cell fragility, we were able to conclude that hyperchromic red blood cells are spherocytes and that their increase corresponds to increased haemolysis [18]. While exploring other possible causes of elevated hyperchromia and consequently that of spherocytosis and haemolysis, we came upon a close association with increased ferritin. Thus, the cost-free parameter "increased hyperchromia" seems to allow the selection of a risk group with high prevalence of iron overload. As we consider iron overload to be a consequence and not a cause of increased haemolysis, we understand that these cases represent secondary, non genetic haemochromatosis.

We are aware that an increase of ferritin does not necessarily reflect permanent iron overload and increased haemolysis but may occur temporarily under different circumstances such as infectious and inflammatory syndromes, cytolysis, and ethanol abuse. These possibilities must be considered in every final conclusion. Still, we are using ferritin and transferrin saturation as primary screening parameters to evaluate the diagnostic efficiency of elevated hyperchromia as a cost-free parameter for iron overload. The identification of C282Y and H63D carrier status is unfortunately out of reach for our laboratory.

The aim of this investigation was, on one side, the confirmation of the identity between hyperchromic erythrocytes and spherocytes and, on the other side, quantification of the diagnostic efficiency of significant hyperchromia as marker of iron overload.

As values for ALAT were generally available together with complete blood count, we calculated the efficiency of this parameter for comparison.

Material and Methods

Results from 5162 unselected routine patients were extracted *retrospectively* from the 2002 data bank of the laboratory. It must be kept in mind that our institute is a routine laboratory, executing analysis on request of the local physicians. This means that we do not have free and complete choice of important parameters such as CRP and sTfR. Patients are ambulatory, rarely bedridden, presenting with minor complaints or for general check up. They are never hospitalised. They may, however, not be considered a normal reference population.

For the cryolysis test and the glycerol lysis test studies, we selected 527 and 134 specimens from patients with a % hyper over the entire range.

Routine haematology was performed on an ADVIA®120 analyser. Blood was drawn on K-EDTA and proceeded within 3 h after phlebotomy. The % hyper is automatically edited by the instrument.

The cryolysis test for spherocytes was executed according to the procedure recommended by Rosas-Romero and coworkers [19]. The modification described

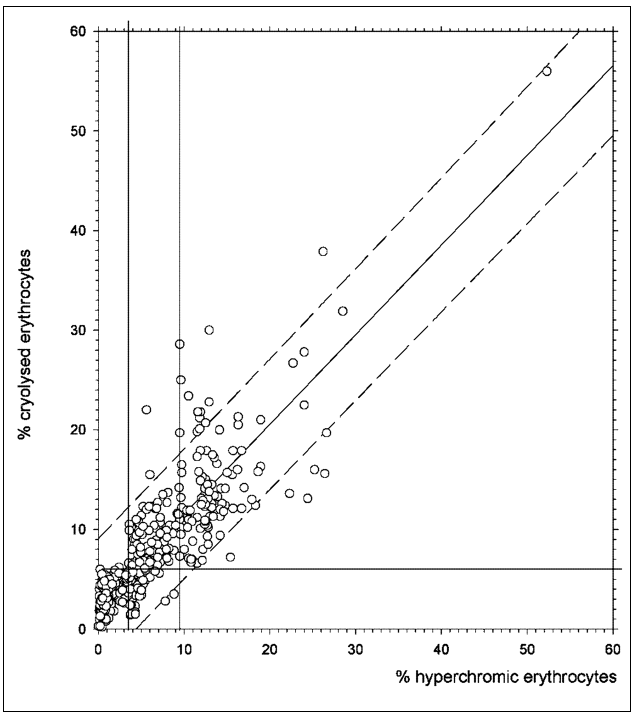

Figure 3 Correlation between the percentage of hyperchromic erythrocytes and percentage of erythrocytes lysed by cryolysis.
y = 2.58 + 0.90 x
r = 0.84
n = 527

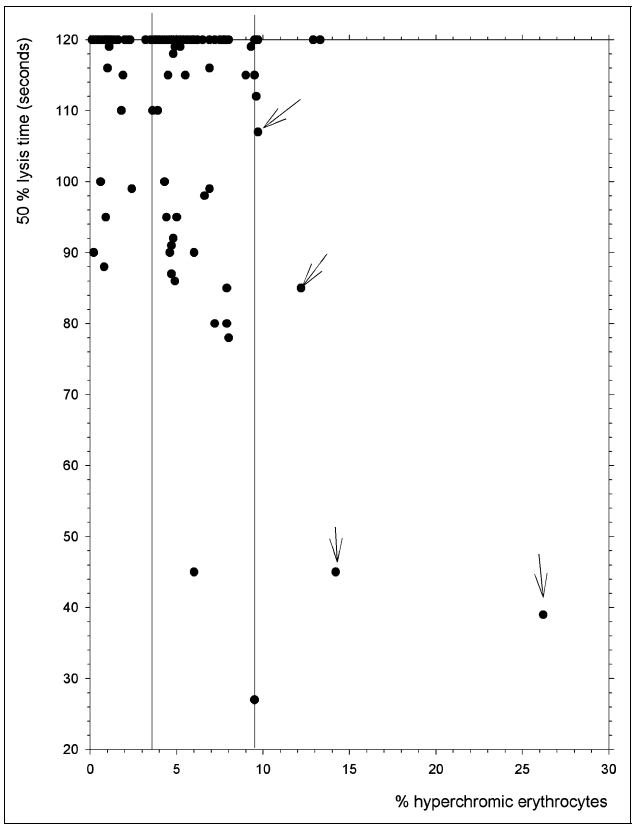

Figure 4 Correlation between the percentage of hyperchromic erythrocytes and the 50 % lysis time in the acid glycerol lysis test (N = 134). The dots marked with an arrow are cases of hereditary spherocytosis.

by Mittler and coworkers [20] was used for the acid glycerol lysis test (AGLT). It was performed at room temperature.

ALAT was performed by the standard kinetic method recommended by the Deutsche Gesellschaft für Klinische Chemie, using a test-kit provided by Roche (Mannheim).

All other parameters were determined by routine methods of the laboratory under permanent internal and external quality control.

The diagnosis of iron overload was mostly based on ferritin values, more rarely on the percentage of transferrin saturation. While for the latter values > 45 % are generally considered as pathological, the normal upper limits for ferritin are highly controversial. While values as low as 150 µg/L are considered suspect by the American Haemochromatosis Society [21], we find upper limits as high as 300 µg/L for adult men and over 50 years old women, 250 µg/L for women of child bearing age on protocols from many laboratories.

We have admitted the following, more or less empirical, upper limits:

Children up to 1 year	up to 100 µg/L
Older children and teens	up to 150 µg/L
Women < 50 years	up to 150 µg/L
Women ≥ 50 years	up to 200 µg/L
Adult men	up to 200 µg/L

Abnormal values up to 500 µg/L are considered moderate iron overload for adults [3], whereas values between 501–1000 µg/L represent serious iron overload. Values > 1000 µg/L correspond to extreme iron overload.

Results

The identity between hyperchromic RBCs as spherocytes has been examined by the classical cryolysis test on 527 specimens (Fig. 3). The acid glycerol lysis test (AGLT) was performed on a smaller series of 134 specimens and compared to % hyper. Results are shown in Figure 4.

Among 1996 routine patients, we encountered 162 with % hyper ≥ 9.5 %. Detailed results including ferritin or transferrin saturation and eventually iron were available for 114 of them. The detailed correlation for different categories of age and gender is shown in Table 1.

In an unselected cohort of 324 children and teens we found only 6 cases with mildly increased ferritin between 150 and 300 µg/L. Three of them had normal RBC indices, 2 had mild hyperchromia, and only one presented % hyper of 11.5.

Table 1 Frequency of iron overload in 114 patients with % hyperchromic RBC ≥ 9.5. Case of hereditary spherocytosis after splenectomy

Category of patients	N	Iron status Cut-off values for ferritin see text					
		Normal	%	Mild iron overload	%	Serious iron overload	%
Children and teens	7	4	57.1	2	28.6	1	14.3
				3 (42 .9%)			
Women 20 – 50 years	11	6	54.6	4	36.4	1	9.0
				5 (45.5%)			
Women > 50 years	18	6	33.3	7	38.9	5	27.8
				12 (66.7 %)			
Men	78	12*	20.0	**38**	**48.7**	**28**	**35.9**
				66 (84.6%)			

An untargeted comparison between % hyper and ferritin was then established for 2842 unselected adult routine patients. It must be stressed that this cohort represents *patients* referred to the laboratory by their treating physicians. It may not be considered a normal population. Table 2 represents a simplified summary.

The comparison between ALAT and ferritin was performed for the same 2842 patients (summary in Table 3).

The overall analytical performance of both symptoms "increased % hyper" and ALAT as marker for serious and extreme increase of ferritin was calculated separately for adult women and men at cut-off values of 9.5 and 3.5 % for % hyper. These cut-off values result from a statistical study on 4688 patients [18]. They delimit 3 subpopulations: values > 3.5 % are considered normal, values between 3.5 and 9.4 % represent mild, values ≥ 9.5 important hyperchromia. The empirical limit of 50 U/L for ALAT has been used by Bhavnani and coworkers [16]. 22 IU/L is the upper limit recommended by Thefeld and coworkers from the former Boehringer Mannheim laboratories [24].We admit it in our institute. Results are summarized in Table 4.

Discussion

Our comparison between % hyper and % of erythrocytes undergoing cryolysis confirms our previous conclusion: hyperchromic red cells are spherocytes. Results of the AGLT are less conclusive. Admitting a normal lysis time of 2 minutes and more, as suggested by Mittler and coworkers [20], we find accelerated lysis in individuals with both normal cryolysis and hyperchromia. On the other hand, the constellation normal AGLT/increased % hyper/increased cryolysis is not uncommon. The 4 cases of hereditary spherocytosis all showed highly decreased AGLT, < 50 seconds. Our results seem to confirm the rather negative views of Zanella and coworkers [22] and of Rutherford et al. [23]. Higher specificity for true hereditary spherocytosis cannot be excluded.

The quasi absence of increase *of both % hyper and increased ferritin* in children and teens has lead us to omit this category from the following calculations.

The ratio women/men among our routine patients is high (1764/1078 = 1.63), against 1.15 in the general adult population. By separate calculation of the percentages of increased ferritin in the different categories, we find that the frequency of iron overload is rather low in women of childbearing age (74 in 870 = 8.5 %), slightly higher in women over 50 years (240 in 894 = 25.1 %),

Table 2 Distribution of ferritin in relation to the % of hyperchromic erythrocytes in 2842 unselected, adult routine patients

Category of patients	Total			Ferritin µg/L			
		% hyperchromic	N % of total	Normal (% of class)	Mild increase % of class	Serious increase % of class	Increase total % of class
Adult men	1078	< 3.5	831 (77.1 %)	362 (43.6 %)	338 (40.7 %)	131 (15.8 %)	469 (56.4 %)
		≥ 3.5	247 (22.9 %)	91 (36.8 %)	107 (43.3 %)	49 (19.8 %)	156 (63.2 %)
		≥ 9.5	22 (2.0 %)	3 (13.6 %)	10 (45.5 %)	9 (40.9 %)	19 (86.4 %)
Women 20–50 years	870	< 3.5	815 (93.7 %)	747 (91.7 %)	62 (7.6 %)	6 (0.7 %)	68 (8.3 %)
		≥ 3.5	55 (6.3 %)	50 (90.9 %)	3 (5.4 %)	2 (3.5 %)	5 (9.1 %)
		≥ 9.5	3 (0.3 %)	2 (66.7 %)	–	1 (33.3 %)	1 (33.3 %)
Women > 50 years	894	< 3.5	832 (93.1 %)	624 (75.0 %)	182 (21.9 %)	26 (3.1 %)	208 (25.0 %)
		≥ 3.5	62 (6.8 %)	32 (51.6 %)	19 (30.7 %)	11 (17.7 %)	30 (48.4 %)
		≥ 9.5	4 (0.5 %)	2 (50.0 %)	1 (25.0 %)	1 (25.0 %)	2 (50.0 %)

Table 3 Distribution of ferritin compared to ALAT in 2842 unselected adult individuals presenting at the laboratory

Category of patients	Total	U/L		Ferritin µg/L			
		ALAT/UL	N % of total	Normal (% of class)	Mild increase % of class	Serious + extreme increase % of class	Increase total % of class
Adult men	1078	< 23	837 (77.6 %)	370 (44.2 %)	353 (42.2 %)	114 (15.8 %)	467 (55.8 %)
		23–49	216 (20.0 %)	52 (24.1 %)	108 (50.0 %)	56 (25.9 %)	164 (76.9 %)
		≥ 50	25 (2.3 %)	4 (16.0 %)	8 (32.0 %)	13 (52.0 %)	21 (84.0 %)
Women 20–50 years	870	< 23	821 (94.4 %)	757 (92.2 %)	61 (7.4 %)	3 (0.4 %)	64 (7.8 %)
		23–49	43 (4.9 %)	32 (74.4 %)	8 (18.6 %)	3 (0.4 %)	11 (25.6 %)
		≥ 50	6 (0.7 %)	2 (33.3 %)	3 (50.0 %)	1 (16.7 %)	4 (66.7 %)
Women > 50 years	894	< 23	833 (93.2 %)	579 (69.5 %)	232 (27.9 %)	22 (2.6 %)	254 (30.5 %)
		23–49	54 (6.0 %)	19 (35.2 %)	21 (38.9 %)	14 (25.9 %)	35 (64.8 %)
		≥ 50	7 (0.8 %)	–	5 (71.4 %)	2 (28.6 %)	7 (100.0 %)

and very high in adult men (644 in 1078 = 59.7 %). The diagnostic efficiency of the % hyper as possible marker of iron overload is calculated in Table 4.

Low sensitivity at both cut-off values for all three categories clearly demonstrates that increased % hyper is *not a screening parameter for iron overload*. This is not astonishing because its pathological outcome is restricted to cases with increased spherocytosis and resulting haemolysis. Iron overload due to other causes remains unnoticed.

Low positive predictive value makes it a poor *symptom in women*. Menstrual blood loss seems to oppose iron accumulation, which is only partly overcome at higher age.

The high positive and negative predictive values at ≥ 9.5 % hyper confirm the quality of first line marker for iron overload in men already predicted in the first group.

Untargeted health screening programs generally include complete blood count, while ferritin and transferrin saturation are often omitted for financial reasons. % hyper determined without additional cost by several haematological analysers draws attention to an eventual iron overload, calling for confirmation only in a limited number of cases.

A recent paper by Delanghe and Langlois [25] demonstrates the association between iron overload and haptoglobin polymorphism, which might explain the rare combination of hyperchromia and iron overload in women and the absence of the same combination in approximately 20 % of the hyperchromic men. Unfortunately, our laboratory is not equipped for the determination of haptoglobin phenotypes.

From the rather poor analytical performance of ALAT shown in Table 4, we conclude that, whatever cut-off chosen, it is neither a screening parameter nor a valid marker for iron overload.

Conclusion

An increased percentage of hyperchromic erythrocytes in men indicates significant, genetic or acquired sphero-

Table 4 Analytical performance of the symptoms 'increased % hyperchromic RBC' and ALAT as marker of seriously and extremely increased ferritin. Joint determination of 2842 unselected specimens from adults presenting to the laboratory

Category of patients	Cut-off value	Sensitivity	Specificity	Positive predictive value	Negative predictive value
Adult women (1764)	≥ 9.5 %hyper	0.036	0.997	0.25	0.976
	≥ 3.5 %hyper	0.29	0.94	0.11	0.98
	> 50 IU/L ALAT	0.067	0.994	0.23	0.976
	> 22 IU/L ALAT	0.44	0.948	0.18	0.985
Adult men (1078)	≥ 9.5 %hyper	0.20	0.999	0.92	0.96
	≥ 3.5 %hyper	0.29	0.81	0.23	0.86
	> 50 IU/L ALAT	0.071	0.987	0.52	0.839
	> 22 IU/L ALAT	0.377	0.807	0.29	0.864

cytosis, entailing increased haemolysis and a shorter life span of erythrocytes, *eventually* leading to iron overload. Values ≥ 9.5 % hyperchromic erythrocytes in adult men thus represent a cost-free marker for haemolysis-induced iron overload, justifying confirmation by an additional determination of ferritin and differential diagnosis by acute phase parameters. The association % hyper ≥9.5 %/increased ferritin is less frequent in women.

References

1. Barton JC, Acton RT. Population screening for hemochromatosis: has the time finally come? Curr Gastroenterol Rep 2000;2:18–26.
2. Dooley JS, Walker AP. Genetic hemochromatosis: detection, management and population screening. Genet Test 2000;4:97–101.
3. Haddow JE, Ledue TB. Preventing manifestations of hereditary haemochromatosis through population based screening. J Med Screen 1994;1:16–21.
4. Barton JC, Bertoli LF. Hemochromatosis: the genetic disorder of the twenty-first century. Nat Med 1996;2:394–395.
5. Beutler E, Felitti VJ, Koziol JA, Ho NJ, Gelbart T. Penetrance of 845G→A(C282Y)HFE hereditary haemochromatosis mutations in the USA. Lancet 2002;359:211–218.
6. Kang JO, Chronic iron overload and toxicity: clinical chemistry perspective. Clin Lab Sci 2001;14:209–219.
7. Bottomley SS. Secondary iron overload disorders. Semin Hematol 1998;35:77–81.
8. Halle M Konig D, Berg A, Keul J, Baumstark MW. Relationship of serum ferritin concentrations with metabolic cardiovascular risk factors in men without evidence for coronary heart disease. Artherosclerosis 1997;128:235–240.
9. Nakao M, Toyozaki T, Nagakawa H, Himi T, Yamada K, Watanabe S, Masuda Y, Asai T. Cardiac dysfunction because of secondary hemochromatosis caused by non-spherocytic hemolytic anemia. Jpn Circ J 2001;65:126–128.
10. Weinberg ED. Development of clinical methods of iron deprivation for suppression of neoplastic and infectious disease. Cancer Invest 1999;17:507–513.
11. Weinberg ED. The role of iron in cancer. European Journal of Cancer Prevention 1996;5:19–36.
12. Weinberg ED, Weinberg GA. The role of iron in infection. Current Opinion in Infectious Diseases 1995;8:164–168.
13. Weinberg ED. Iron withholding: a defense against viral infections. BioMetals 1996;9:393–399.
14. Deugnier Y, Moirand R. Surcharge martiale et santé publique. Bull Acad Natl Med 2000;184:365–373.
15. Deugnier Y, Moirand R, Guyader D, Brissot P. Diagnostic d'une hyperferritinémie. Ann Biol Clin 1998;56:41–43.
16. Bhavnani M, Lloyd D, Bhattacharyya A, Marples J, Elton P, Worwood M. Screening for haemochromatosis in blood samples with raised alanine aminotransferase. Gut 2000;46:707–710.
17. Paterson J, Toolis F. Screening for haemochromatosis in blood samples with raised alanine aminotransferase. Gut 2001;48:441–442.
18. Kutter D, Coulon N, Stirn F, Thoma M. Demonstration and quantification of "hyperchromic" erythrocytes by haematological analysers. Application to screening for hereditary and acquired spherocytosis. Clin Lab. 2001, in print.
19. Rosas-Romero R, Poo JL, Robles JA, Uriostegui A, Vargas F, Majluf-Cruz A. (1997) Usefulness of the Cryohemolysis test in the diagnosis of hereditary spherocytosis. Archives of Medical Research 28:247–251.
20. Mittler U, Radig K, Kluba U, Aumann V, Röppnack R. Erfahrungen mit dem Glyzerol-Lyse-Testim sauren Milieu (AGLT) bei der Diagnostik der hereditären Sphärozytose. Kinderärztl Praxis 1993;61 ;219–222.
21. Educational brochures of the American Hemochromatosis Society. 2001
22. Zanella A, Milani S, Faquani G, Mariani M, Sirchia G. Diagnostic value of the glycerol lysis test. J Lab Clin Med 1983;102:743–750.
23. Rutherford CJ, Postlewaight BF, Hallowes M. An evaluation of the glycerol lysis test. Br J Haematol 1986;63:119–121.
24. Thefeld W, Hoffmeister H, Busch EW, Koller PU, Vollmar J. Referenzwerte für die Bestimmung der Transaminasen GOT und GPT sowie der alkalischen Phosphatase im Serum mit optimierten Standardmethoden. Dtsch med Wschr 1974;99:343–350.
25. Delanghe JR, Langlois MR. Haptoglobin polymorphism and body iron stores. Clin Chem Lab Med 2002;40:212–216.