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Differential diagnosis of hereditary hemolytic anemias in a single multiscreening test by TGA/Chemometrics

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Results and discussion

Hematological characterization

Patients were classified by evaluating the characteristic features for hemolytic anemias due to hemoglobinopathies and membrane defects. Table S1 shows the hematological data expressed as mean ± standard deviation (SD) while the comparison among groups was performed by the analysis of variance (ANOVA) followed by the Tukey test to assess significances among classes. The group of HS/HE patients showed a moderate hemolytic anemia with significantly decrease in RBC, Hb and Hct values, and a significant increase in RDW values, in comparison with healthy subjects. The patients were carriers of anomalies of the main erythrocyte membrane proteins such as spectrin, protein Band 3 and protein 4.1.The thalassemia patient group included patients with Thalassemia intermedia and major both transfusion dependent and not transfused, who had a very heterogeneous degree of clinical severity. The hematological parameters were significantly different from those of healthy individuals and HS/HE with a decrease in RBC, Hb, Hct, MCV, and MCH and a significant increase in RDW.

The group of SCA patients was characterized by a chronic anemia with a decrease in the number of RBC and a corresponding decrease in the levels of Hb and Hct. MCV and MCH were lower in sickle cell patients than healthy donors and the statistical analysis revealed that there are significant differences among classes (significance values of 6.2 E-05 and 1.5 E-04, respectively). The presence of high reticulocytosis and hemolysis, features of sickle cell anemia, made RDW values significantly higher in sickle cell patients than the other groups (significance value of 1.5 E-08).

Experimental

Patient enrollment and characterization

A number of 324 subjects were considered in this study: 120 healthy donors (with mean age ± standard deviation of 40±11 years) were characterized at the National Health Institute of Rome, while 204 patients affected hemolytic disorders were followed for diagnosis, management and therapies at the Thalassemia Unit of S. Eugenio Hospital in Rome. Among these, 37 subjects exhibited defects in the structure of the red cell membrane, such as hereditary spherocytosis (HS) and elliptocytosis (HE). The patients with mean age of 30±20 years, with a male-to-female ratio of 25:12, were eleven pediatric and 26 adults. The main erythrocyte membrane protein defects were reduction in protein Band 3, Spectrin, and protein 4.1 contents as determined by SDS-PAGE electrophoresis. The remaining 167 patients were affected by hereditary hemoglobinopathies as sickle cell anemia (SCA, 103 patients, mean age 39±15 years, 64 males and 42 females) and thalassemia (T, 64 patients, mean age 37±12 years, 40 males and 24 females). In the SCA group there were subjects with HbS homozygosis (HbSS) and with double heterozygosis (HbS/HbD, HbS/HbC, HbS/Thal), either transfusion dependent and not. The thalassemia group included thalassemia major and thalassemia intermedia patients. The diagnosis of SCA and Thalassemia has been performed using the first and second level tests: the analysis of the hemoglobin fractions by HPLC and the molecular analysis of the globin genes. Blood specimens were collected in ethylene diamine tetracetic acid (EDTA) after informed consent of the patient (provided on request) and according to guidelines established by the Ethical Committee for human subject studies (Helsinki Declaration of 1975, revised in 2008).The hematological characterization of patients was performed by the clinical evaluation of the diagnostic features such as red blood cell counts (RBC), hemoglobin (Hb), Hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and red cell distribution width (RDW). An automated hematology analyzer ADVIA 120 (Siemens, USA) was used for the analyses of all the collected samples.

Thermogravimetric Analysis (TGA)

A Perkin Elmer TGA7 Thermobalance (Massachusetts, USA) was used to record the thermogravimetric curves. Non pre-treated whole blood (about 30 µl) was placed into the crucible where temperature was raised from 20°C to 800°C, with a 10°C/min heating rate, as the best resolution rate; the atmosphere was air as carrier gas, at a flow rate of 100 ml/min.

The Curie-point transition of standard metals were involved to calibrate the instrumental response, as reported by the equipment recommendations. Reproducibility was checked by collecting three replicates for each sample. Derivative Thermogravimetric data (DTG) were also calculated to compare samples and represent the derivative of the function TG(T) with respect T. Data were recorded in triplicate and the mean was considered for statistics.

Figures

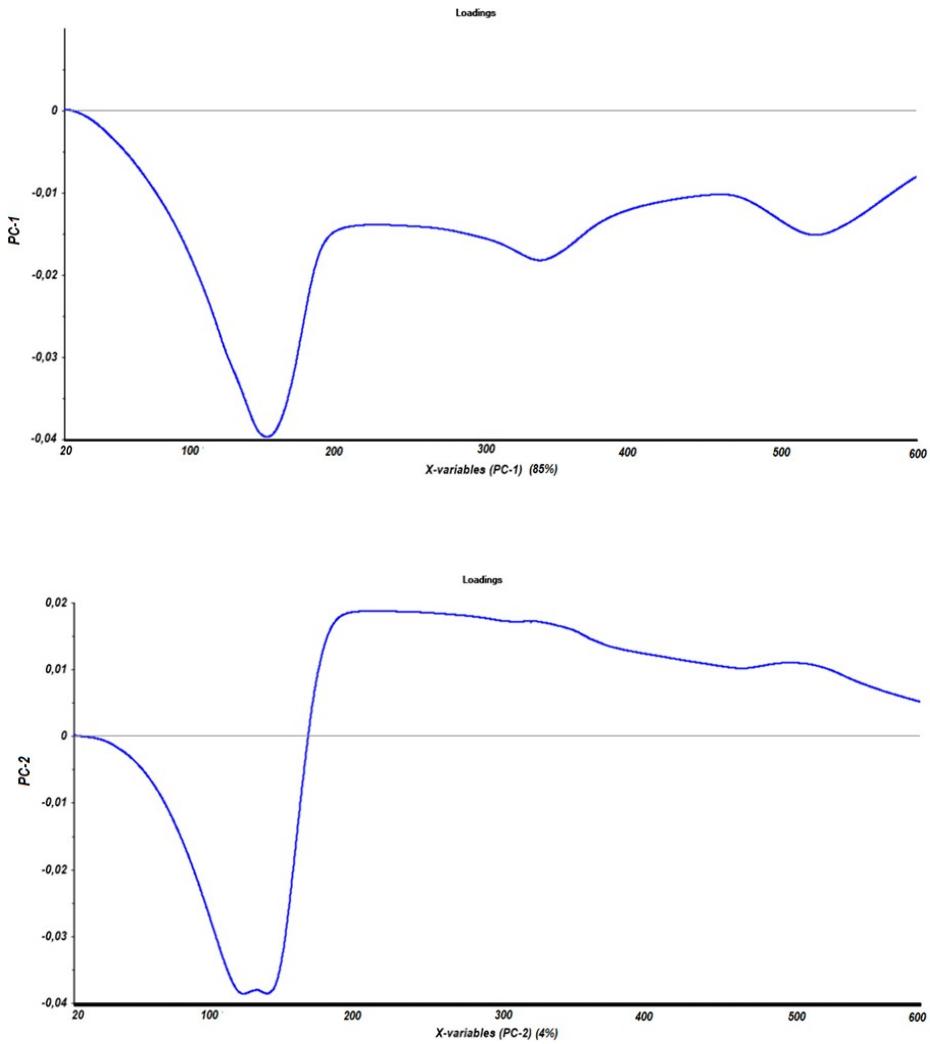

Figure S1. Loadings plots of PC1 and PC2 versus the x-variables (°C).

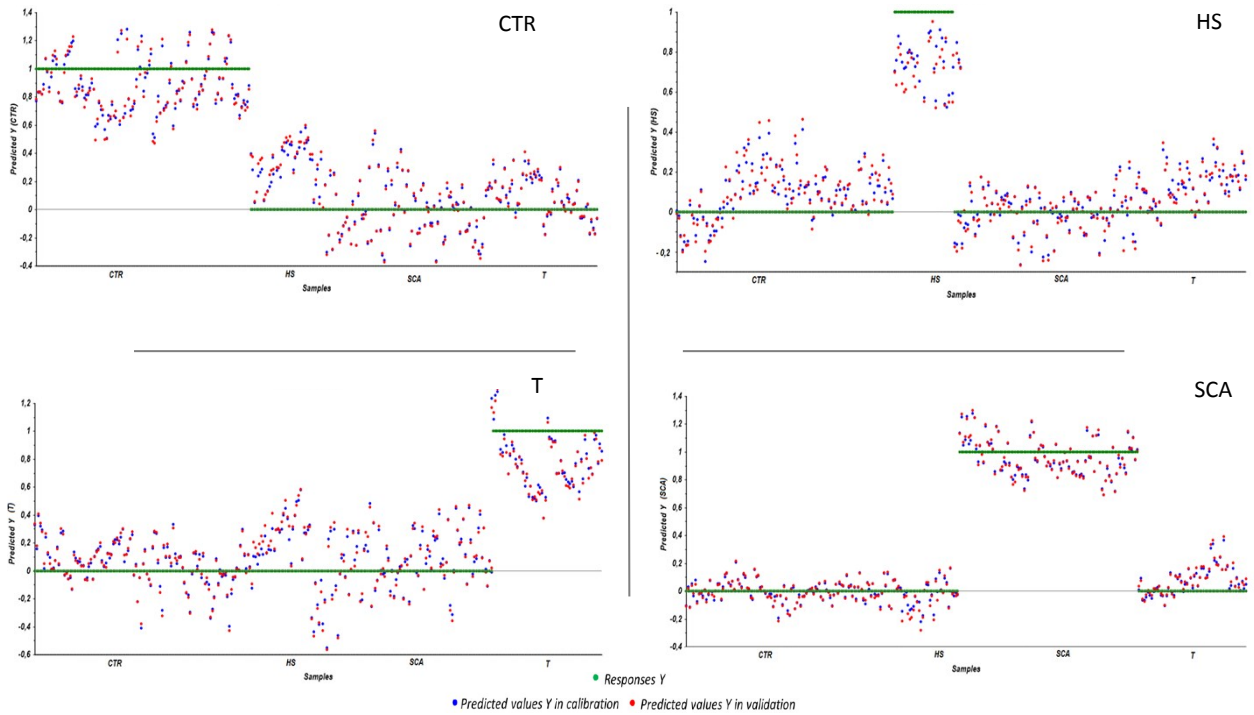

Figure S2. Graphs of class values (y axis) versus the number of samples (x axis) for all the investigated classes.

Tables

Table S1. Hematological characterization of healthy individuals (CTR) and patients with hereditary hemolytic anemia: Hereditary Spherocytosis and Elliptocytosis (HS/HE), Thalassemia (T), and Sickle cell anemia (SCA).

	RBC (106/mL)	HGB (g/dL)	HCT (%)	MCV (fL)	MCH (pg)	RDW (%)
CTR	5.3 ± 0.4	14.8 ± 0.8	46.6 ± 2.8	87.4 ± 3.2	27.8 ± 1.4	13.2 ± 0.7
HS/HE	5.0 ± 1.0	13.4±2.2	41.0±6.6	84.3 ± 7.0	27.8 ± 3.7	15.1 ± 2.7
T	4.1 ± 1.1	10.0 ± 1.7	32.0 ± 6.1	79.9 ± 8.0	25.2 ± 3.5	18.3 ± 4.4
SCA	3.8 ± 0.7	9.9 ± 1.3	30.9 ± 3.8	81.8 ± 8.4	26.4 ± 3.3	20.0 ± 4.2

Table S2. Calculated weight losses from the TG curves for healthy individuals (CTR), Hereditary Spherocytosis and Elliptocytosis (HS/HE), Thalassemia (T), and Sickle cell anemia (SCA).

	Water Content (%)	Bulk Water (%)	Bound Water (%)	Bulk/Bound Water ratio	2nd Weight Loss (%)	3rd Weight Loss (%)
CTR	77.7 ± 0.9	46.3 ± 4.0	31.3 ± 4.8	1.6 ± 0.5	11.7 ± 0.7	9.2 ± 0.5
HS/HE	78.9 ± 2.0	51.3 ± 8.1	27.7 ± 7.4	2.2 ± 1.0	10.8 ± 1.3	8.9 ± 1.1
T	81.8 ± 1.8	59.4 ± 8.0	22.3 ± 7.4	3.3 ± 2.4	9.6 ± 1.1	7.5 ± 0.9
SCA	81.7 ± 1.3	56.6 ± 8.3	25.2 ± 7.8	2.8 ± 2.4	9.4 ± 0.8	7.7 ± 0.9

Table S3. Calculated sensitivity (%) of the model in calibration, cross-validation and prediction, obtained using different chemometric pre-treatments for healthy individuals (CTR), Hereditary Spherocytosis and Elliptocytosis (HS/HE), Thalassemia (T), and Sickle cell anemia (SCA)

	Patient	Mean Centering	SNV	1st derivative + Mean Centering	1st derivative + SNV
Calibration	CTR	98.3	97.4	100.0	91.4
	HS/HE	75.0	68.8	71.9	71.9
	T	86.4	76.3	96.6	74.6
	SCA	100.0	100.0	100.0	100.0
Cross-Validation	CTR	96.6	97.4	99.2	87.9
	HS/HE	75.0	68.8	71.9	68.8
	T	78.0	74.6	93.2	54.2
	SCA	100.0	100.0	100.0	100.0
Prediction	CTR	100.0	100.0	100.0	100.0
	HS/HE	80.0	20.0	80.0	40.0
	T	100.0	100.0	100.0	80.0
	SCA	100.0	100.0	100.0	100.0

Table S4. Calculated specificity (%) of the model in calibration, cross-validation and prediction, obtained using different chemometric pre-treatments for forhealthy individuals (CTR), Hereditary Spherocytosis and Elliptocytosis (HS/HE), Thalassemia (T), and Sickle cell anemia (SCA).

	Patient	Mean Centering	SNV	1st derivative + Mean Centering	1st derivative + SNV
Calibration	CTR	95.0	90.4	94.3	89.1
	HS/HE	100.0	100.0	100.0	100.0
	T	83.6	73.8	100.0	64.7
	SCA	100.0	100.0	100.0	100.0
Cross-Validation	CTR	91.1	89.7	93.5	80.3
	HS/HE	100.0	100.0	100.0	100.0
	T	70.8	72.1	91.7	48.5
	SCA	100.0	100.0	100.0	100.0
Prediction	CTR	83.3	55.6	55.6	71.4
	HS/HE	100.0	100.0	44.4	100.0
	T	100.0	100.0	83.3	80.0
	SCA	100.0	100.0	100.0	100.0

Table S5. Calculated efficiency (%) of the model in calibration, cross-validation and prediction, obtained using different chemometric pre-treatments for healthy individuals (CTR), Hereditary Spherocytosis and Elliptocytosis (HS/HE), Thalassemia (T), and Sickle cell anemia (SCA).

	Patient	Mean Centering	SNV	1st derivative + Mean Centering	1st derivative + SNV
Calibration	CTR	98.1	96.4	96.5	96.3
	HS/HE	86.6	82.9	83.5	83.5
	T	92.6	87.0	89.2	86.7
	SCA	100.0	100.0	100.0	100.0
Cross-Validation	CTR	96.4	96.4	96.4	96.3
	HS/HE	86.6	82.9	83.5	82.9
	T	87.2	86.7	88.9	83.1
	SCA	100.0	100.0	100.0	100.0
Prediction	CTR	96.8	62.5	62.5	62.5
	HS/HE	89.4	30.1	53.4	40.8
	T	100.0	48.2	48.2	48.2
	SCA	100.0	100.0	100.0	100.0