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ORIGINAL ARTICLE

Sampling and processing of fresh blood samples within a European multicenter nutritional study: evaluation of biomarker stability during transport and storage

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Introduction: Analysis of several biological markers improves the quality and physiologic comprehension of data obtained in epidemiological nutritional studies.
Aim: To develop a methodology that guarantees the centralized analysis and quality assurance of the most relevant blood parameters from fresh blood samples in adolescents in a European multicenter study.
Materials and methods: Stability of selected nutrients and biomarkers (vitamins, fatty acids, iron metabolism and immunological parameters) chosen with respect to time and temperature of sample transport and storage was evaluated as part of the pilot study of the Healthy Lifestyle in Europe by Nutrition in Adolescence (HELENA) project.
Results: Routine biochemistry and iron status parameters included in the HELENA Cross-Sectional Study (CSS) protocol could be analyzed within 24 h from fresh blood samples without any stability problems (coefficient of variation (CV)$<$5%, $P<0.05$). However, stability tests for lymphocyte subpopulations, vitamin C and fatty acids showed that they are very unstable at room temperature without any treatment. Therefore, a special handling for these samples was developed. Vitamin C was stabilized with metaphosphoric acid and transported under cooled conditions (CV 4.4%, recovery rate $>$93%, $P>0.05$). According to the results, a specific methodology and transport system were developed to collect blood samples at schools in 10 European cities and to send them to the centralized laboratory (IEL, Bonn, Germany). To guarantee good clinical practice, the field workers were instructed in a training workshop and a manual of operation was developed.
Conclusion: The handling and transport system for fresh blood samples developed for the European multicenter study HELENA is adequate for the final part of the HELENA-CSS and will provide, for the first time, reference values for several biological markers in European adolescents.
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Keywords: logistic/transport/traceability and quality assurance; stability of fresh blood; vitamins; iron status; immunonutrition; genetics

Introduction

An objective assessment of individual nutritional status should not only be based on body composition and nutrient intake data, but should also consider information concerning specific energy and nutrient depletion as well as

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nutrition-dependent metabolic functions.[1,2] One of the main objectives of the Healthy Lifestyle in Europe by Nutrition in Adolescence (HELENA) Study is to establish the prevalence of overweight and obesity in a selected sample of European adolescents. Although it is hard to conceive of a nutritional deficiency occurring in participants with excessive dietary and caloric intake, obese children may be at risk of micronutrient deficiency. In overweight and obese children and adolescents from Israel[3] and the United States,[4] greater prevalence of low iron blood levels was found than in the normal-weight group.[3] The HELENA data will determine whether it is necessary to routinely screen young people in Europe for iron deficiency, as has been proposed by several authors.[3,4] Overweight children and adolescents also seem to be at higher risk for vitamin B_{12}, α-tocopherol and β-carotene deficiency,[5,6] and reduced total antioxidant status.[7] Inflammation processes, even at the subclinical level, together with other factors can influence micronutrient status.[8] Consequently, analyses of blood samples are part of the nutritional assessment within the European Multicenter Study HELENA.[9–11] In addition to routine hematology and biochemistry, various nutrients and several validated biomarkers such as hormones, cytokines, receptors, adhesion molecules, inflammatory proteins and immunoglobulins will be analyzed in blood, plasma, serum and white blood cells, respectively (Table 1).

Fulfilling the high quality standards of the whole HELENA project and to ensure adequate sample handling, storage and subsequent analysis, logistics of sample transport and major parts of the analytics are centralized at the Analytical Laboratory from the University of Bonn (IEL, Bonn, Germany). Even when using optimal flight facilities between the 10 study centres and the IEL, the time span between sampling and sample processing can be up to 24 h. To analyze stability during this time period is thus a prerequisite for assessing reliable data.[12]

The aim of this part of the study was to evaluate the stability of the nutrients and biomarkers chosen with respect to time and temperature of sample transport and storage within the pilot study of the HELENA project. The results are used to establish a manual of operation for the standardization of sample handling in all study centres.

Materials and methods

For the HELENA pilot study, 30 ml of blood samples were drawn after a 10-h overnight fast at school between 0830 and 0900 hours in 180 adolescents aged 13 ± 1 years in the 10 European cities participating in the HELENA Cross-Sectional Study (CSS) following a standardized blood collection protocol. Serum/plasma was centrifuged directly at the schools at 3500 rpm (for 15 min, at room temperature). Samples for stability tests were tested within the pilot study in two study centres (Rome, Italy and Zaragoza, Spain) as explained below.

To ensure traceability and optimal transport conditions, a novel handling and transport system (SYS) for biological samples (fresh blood, plasma/serum and cell cultures) from study centres to IEL were established within HELENA considering international guidelines such as Good Clinical Practices, Good Laboratory Practices, regulations of International Conference of Harmonization,[13] the International Air Transport Association (IATA)[14] and the European Agreement concerning the International Carriage of Dangerous Goods by Road (class.6.2). Since 2005, blood samples are categorized as diagnostic specimen category B and have to be declared as UN 3373 (IATA Dangerous Goods, 2005). Therefore, special handling is required (specific transport materials, transports only allowed with cargo flights). Within the HELENA-CSS, owing to extra claims from the airline, the transport boxes for the blood samples have to fulfill extra high standard conditions as for diagnostic samples category A (P 620).

In addition, a novel traceability system was developed at the Clinical Investigation Center in Lille on the basis of both

Table 1 Blood parameters included in the HELENA-CSS

IEL/UPM		*INRAN*	*CSIC*	*Institut Pasteur Lille*
Insulin	Albumin	Ferritin	*Adhesion molecules*	Genetic
Vitamins A and E	Creatinine	AGP	s-VCAM, S-ICAM, E-selectin, L-selectin	Phenotypes
Vitamin C	Glucose	C-reactive protein	*Cytokines*	*In situ*
Vitamin D	Uric acid	Soluble transferring receptor (sTfR)	IL-2,4,6,10, IFN-γ, TNF-α, TGF-β1	Hemogram
TEAC	Lipoprotein(a)		*Inflammatory proteins*	
Vitamin B_{12}	ApoA, ApoB		Ceruloplasmin, C3, C4	
Total homocysteine	Cholesterol, HDL, LDL, triacylglycerols			
Plasma and RBC folate	GGT, GOT, GPT		*Immunoglobulins*	
Holo-transcobalamin	Fatty acids (e.g. oleic acid, α-linolenic acid, linoleic acid, 'Mead' acid, arachidonic acid, docosahexenoic acid)		IgA, M, G	
Vitamin B_6			*Lymphocyte subpopulations*	
Adiponectin, leptin			CD3, CD4, CD8, CD16/56 , CD19	
Cortisol				

printed documents and an electronic database. A 'laboratory request form' (Figure 1) is used as the principal document that includes the following information: address of sender/receiver, date of collection, date of sending, code number of participant, type of tube and volume of blood. On the document, the samples are characterized as 'diagnostic specimens, non-restricted human biological sample for laboratory testing only, no commercial value, not for resale.'

All applicable institutional and governmental regulations concerning the ethical use of human volunteers were followed during this research. The protocol was approved by the Human Research Review Committee of the Universities of Bonn (Dortmund), Lille, Rome, Zaragoza, Athens, Heraklion, Pecs, Ghent and Vienna. The study has been performed following the ethical norms of the Declaration of Helsinki 1961 (revision of Edinburgh, 2000), Convention of Oviedo (1997), the Good Clinical Practice of the European Union (EU)[15] and the legislation about clinical research in humans in each of the participating countries. Informed written consent was obtained from participants and both parents.

Stability tests in whole blood and plasma/serum

Serum samples from 14 adolescents participating in the pilot study in Rome were used to test the stability of biomarkers of iron status (ferritin, soluble transferrin receptor, C-reactive protein and α_1-acid glycoprotein) during sample transport. Analyses of these markers were performed at the Istituto Nazionale di Ricerca per gli Alimenti e la Nutrizione (INRAN). The serum samples obtained were divided into two portions: (A) to mimic transport conditions between study centers, one sample was transported without cooling within 24 h to IEL and sent back to INRAN on dry ice; (B) the second portion was directly stored at $-20\,^{\circ}C$ at INRAN (Figure 2). Twenty days after collection at the latest, all serum samples were measured in the same run.

Within HELENA, immunophenotyping was performed by analyzing lymphocyte subpopulations requiring fresh blood samples. Briefly, blood samples are incubated with monoclonal antibodies purchased from BD Biosciences (San José, CA, USA) to differentiate CD3 (T mature cells), CD4 (T helper cells), CD8 (T cytotoxic/suppressor cells), CD19 (B cells) and CD56/16 (natural killer cells) for 30 min at room

Appendix 2 : Laboratory Request Form (LRF)

SENDER :
N° of centre/city : 11 ZARAGOZA
Name : German Vicente-Rodriguez
Address : E.U. CIENCIAS DE LA SALUD UNIVERSIDAD DE ZARAGOZA C Domingo Miral s N E-50009 ZARAGOZA
TEL :
FAX :
Package by :

RECEIVER :
Christina BREIDENASSEL
IEL-Ernahrungsphysiologie
(5. Stock Zi. 503)
Endenicher Alle 11-13
D-53115 BONN
GERMANY
TEL : +49 228 733 767
FAX : +49 228 733 217
Reviewed by :

DIAGNOSIS SPECIMENS, NOT RESTRICTED HUMAN BIOLOGICAL SAMPLES FOR LABORATORY TESTING ONLY, NO COMMERCIAL VALUE, NOT FOR RESALE

To be filled in by the centre/city

Date of blood sample collection : 15.05.2007 (dd mm yyyy)
Date of sending blood collection : 15.05.2007 (dd mm yyyy)

Tubes from EDTA-Monovetten #10 and #20

H211 10 01 20 10 FOR LRF EDTA-Mono 2.7 ml	H211 10 01 20 11 FOR LRF Aliq 0.4ml Blood+0.4ml Cyto	H211 10 01 20 12 FOR LRF Aliq 1 ml of EDTA Blood	H211 10 01 20 20 FOR LRF EDTA-Mono 4 ml

Tubes from Heparin Lithium 2.6 ml #40

H211 10 01 20 41 FOR LRF Aliq 0.4ml Pla+0.4ml MTA	H211 10 01 20 42 FOR LRF Remaining plasma Hep/Lit		

Tubes from 2 Serum-Gel #50

H211 10 01 20 51 FOR LRF Aliquot 1 ml Serum	H211 10 01 20 52 FOR LRF Aliquot 1 ml Serum	H211 10 01 20 53 FOR LRF Rest of Serum Gel	

Before sending Blood samples, Please Fax this form to **CIC-L** +33 320 525 166 and **BL** + 49 228 733 217

Figure 1 Laboratory request form used in the traceability system.

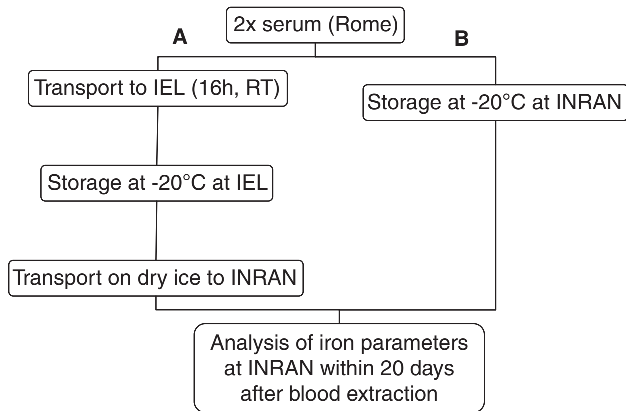

Figure 2 Stability test design for biomarkers of iron status. IEL = Institut für Ernährungs- und Lebensmittelwissenschaften, Universität Bonn. INRAN = Istituto Nazionale di Ricerca per gli Alimenti e la Nutrizione, Rome, Italy.

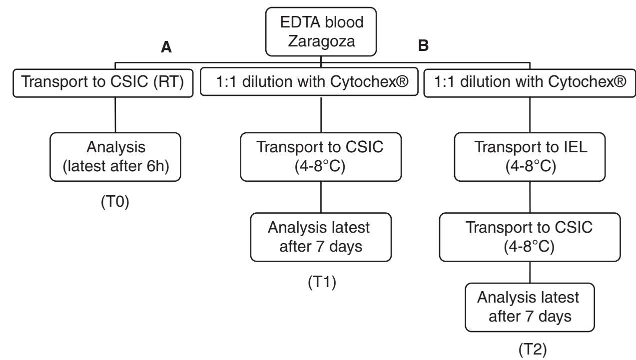

Figure 3 Stability test design for immunophenotyping. IEL = Institut für Ernährungs- und Lebensmittelwissenschaften, Universität Bonn. CSIC = Consejo Superior de Investigaciones Científicas, Madrid, Spain.

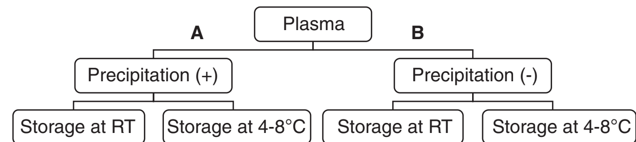

Figure 4 Stability test design for vitamin C.

temperature. After lysis of red cells, lymphocytes are gated by forward and side scatter and pan-leukocyte marker expression (CD45) by four-staining procedure (CD3/CD16 + 56/CD45/CD19 and CD3/CD8/CD45/CD4). To check the influence of transport and storage on the stability of cell surface markers, blood samples (EDTA-tubes) from 16 participants of the pilot study in Zaragoza (14–15 years old, seven females) were aliquoted in three eppendorf vials and treated differently: (A) One sample was transported at room temperature to the immunonutrition lab at the Consejo Superior de Investigaciones Científicas (CSIC) in Madrid and analyzed for 6 h after sampling (T_0) at the latest; (B) The two other whole blood samples were diluted 1:1 with a preservative maintaining (according to the manufacturer) the antigenic site activity of white blood cells up to 7 days (Cytochex Reagent, Streck Laboratories, Omaha, NE, USA). One of the preserved samples was transported at 0–4 °C to CSIC (T_1) and the other one was transported at 0–4 °C from the school to IEL and then back to the CSIC lab (T_2). All samples were analyzed at CSIC by flow cytometry 7 days after blood extraction (Facscan, BD, Sunnyvale, CA) (Figure 3).

To test the stability of vitamins B_{12}, C, A, E, folate and fatty acids (FA), blood was collected from six volunteers (29 ± 3 years, five females), immediately centrifuged and aliquoted. (A) Half of the plasma samples used for vitamin C analysis was precipitated with a 6% (w/w) perchloric acid solution spiked with metaphosphoric acid. The precipitated and non-precipitated samples were then immediately stored cool (4–8 °C) or at room temperature; (B) all other plasma aliquots were stored at room temperature or under cooled conditions over 24 h. All samples were repeatedly analyzed over 24 h at the IEL lab (Figure 4).

Folate and vitamin B_{12} were measured by means of an immunoassay using the Immunolite 2000 analyzer (DPC Biermann GmbH, Bad Nauheim, Germany). Antioxidative nutrients (vitamins A, E and C) were analyzed by high-performance liquid chromatography (Sykam Gilching, Germany) using ultraviolet detection (UV-Vis 205, Merck, Darmstadt, Germany). Serum FA composition was determined by capillary gas chromatography (GC-2010, FID detection, Shimadzu GmbH, Duisburg, Germany) after extraction performed by thin-layer chromatography.

To guarantee the stability of routine biochemistry analyses in fresh serum samples, three samples with high, mean and low baseline values were tested over different time points during 24 h after blood extraction. Serum triglycerides, total cholesterol, high-density lipoproteins and low-density lipoproteins, creatinine, urea, glucose, γ-glutamyl transferase, alanine aminotransferase and aspartate aminotransferase were measured on the Dimension RxL clinical chemistry system (Dade Behring, Schwalbach, Germany) with enzymatic methods using the manufacturer's reagents and instructions. Serum albumin, apolipoprotein A-I and apolipoprotein B concentrations were measured in an immunochemical reaction with a BN II analyzer (Dade Behring, Schwalbach, Germany) according to the manufacturer's instructions. The proteins contained in the serum sample form immune complexes with specific antibodies. These complexes scatter a beam of light passed through the sample. The intensity of the scattered light is proportional to the concentration of the relevant protein in the sample. The result is evaluated by comparison with a standard of known concentration. Serum lipoprotein (a) was measured by means of particle-enhanced immunonephelometry using the BN II analyzer (Dade Behring).

Viability of white blood cells

The influence of transport/storage time of blood samples on the cytokine production of isolated white blood cells (peripheral polymorphonuclear blood cells) in cell cultures was studied using 3 heparinized blood samples from 16 adolescents collected within the pilot study in Zaragoza. Blood was divided into three portions: (A) two tubes were transported at room temperature to CSIC and cell culture procedure was started at 6 h (T_0) and 15 h (T_1) after sampling; (B) one tube was conveyed under similar conditions to IEL (15 h,T_2) (Figure 5).

Lymphocyte isolation techniques as well as *in vitro* cultures were standardized to avoid differences in the analytical methods used between both cities. The lymphocyte coat was isolated by density gradient and incubated with the mitogen phytohemagglutinin (Invitrogen, Karlsruhe, Germany) for 48 h; subsequently, the cell culture media was frozen at −20 °C. All frozen samples (from IEL and CSIC) were analyzed at CSIC at the same time to minimize systematic differences. Tumor necrosis factor-α, γ-interferon, interleukins-2, 4, 6 and 10 (IL-2, IL-4, IL-6 and IL-10) secretion were measured in the cell culture media by cytometric bead array and detected by flow cytometry (Facscan, BD, Sunnyvale, CA).

Statistical analyses

Statistical analyses were performed using the SPSS statistical software release 14 for Windows XP. Data were assessed for normality and homogeneity of variance, and are expressed as mean ± standard deviation (s.d.). Student's *t*-test was used for comparison of means. Variance analysis of repeated measurements was used to compare the different time points to examine the changes over time. For measuring the agreement between IEL and INRAN samples, a confidence interval was built, for each indicator, on the mean of the differences between the two samples of the same participant.[16] Statistical significance was set at $P<0.05$.

Results

Stability tests in whole blood and plasma/serum

The results of the stability tests of biomarkers of iron status performed in 14 Italian participants from the HELENA pilot study are shown in Table 2. No differences were observed between samples (A) and (B). All measured parameters were within the 95% confidence interval for the mean of difference of soluble transferrin receptor, ferritin, C-reactive protein and α_1-acid glycoprotein calculated (−0.25 (95% −2.8 to 2.4); −0.82 (95% −8.5 to 7.5); 0.05 (95% −0.11 to 0.21) and 0.05 (95% −0.45 to 0.56), respectively (Figure 6). According to the results, the shipment at room temperature within 24 h of the serum samples has not influenced the quality of the parameters.

Stability tests for vitamin C (Table 3) and FA confirmed that they are very unstable at room temperature without any treatment. For vitamin C, heparin plasma had to be precipitated after centrifugation with metaphosphoric acid and transported under cooled conditions. After this treatment, vitamin C was stable over 24 h (coefficient of variation (CV) 4.4%, recovery rate >93%, $P>0.05$). Vitamins A and E were stable over 24 h at room temperature (CV: vitamin E: 4.6%, vitamin A: 3.2%, $P>0.05$) (Table 3). Owing to practical reasons, for vitamin B_{12} and folate, stability tests were performed directly only after centrifugation and after 24 h. No significant differences were observed during the 24 h of storage at room temperature.

Table 4 shows the results of the stability tests carried out for each biochemical parameter measured at the central lab of the University Hospital in Bonn. No changes in fresh serum samples were observed over a time span of 24 h either at high or low baseline levels.

Viability of white blood cells

The stability tests for cytokine production capacity between T_0 and T_1 showed significant differences in IL-10 levels

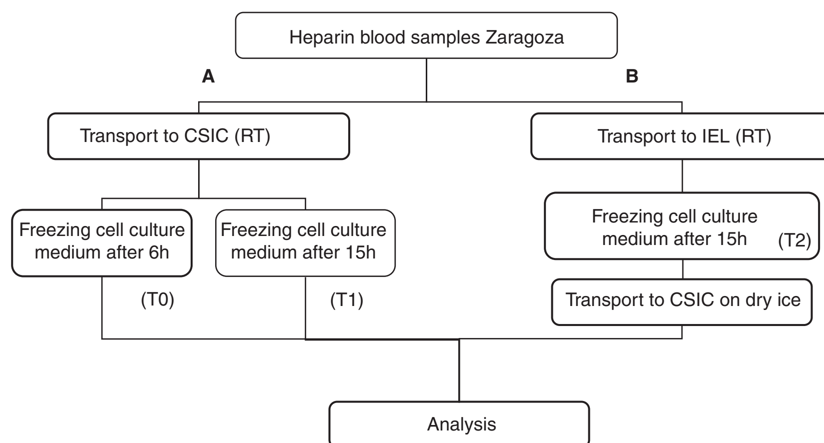

Figure 5 Stability test design for viability of 'whole blood cell/cytokine' production. IEL = Institut für Ernährungs- und Lebensmittelwissenschaften, Universität Bonn, Germany. CSIC = Consejo Superior de Investigaciones Científicas, Madrid, Spain.

Table 2 Results from stability tests of the serum iron parameters in an Italian subpopulation ($n=14$)

	(1) Levels in samples stored directly in Rome (A) (n = *14)*	*(2) Levels in samples after transport Rome-Bonn-Rome (B)* (n = *14)*	*Mean difference*	*CI 95%*
Ferritin ($\mu g\, l^{-1}$)	27.9	27.1	+0.82	+7.5; −8.5
STfR ($mg\, l^{-1}$)	7.37	7.12	+0.25	+2.4; −2.8
CRP ($mg\, l^{-1}$)	1.12	1.17	−0.05	+0.21; −0.11
AGP ($g\, l^{-1}$)	1.26	1.31	−0.05	+0.56; −0.45

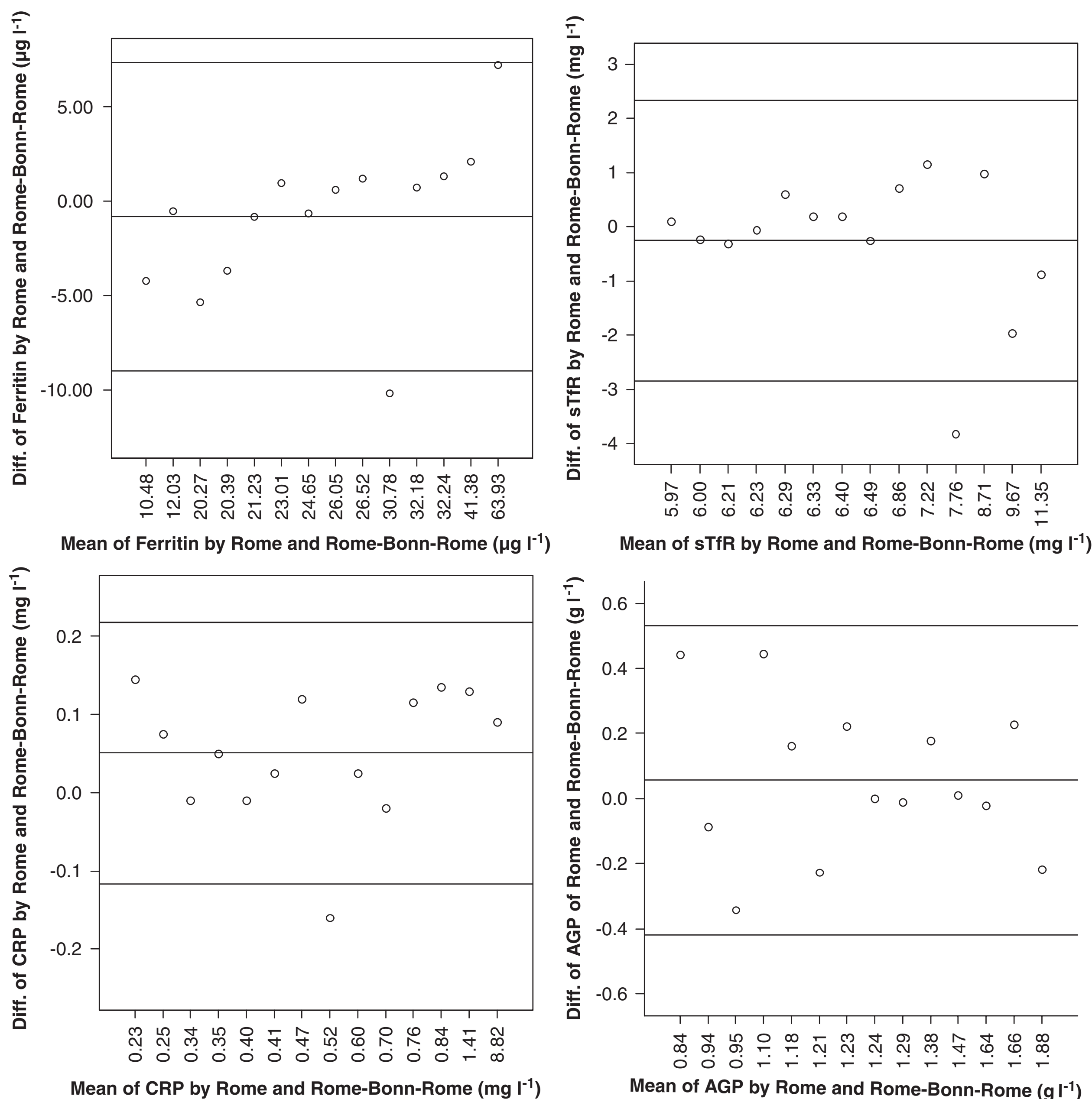

Figure 6 Measuring agreements of the ferritin, sTfR, CRP and AGP values between Bonn serum samples (A) and Bonn Rome Bonn serum samples (B) consequences for shipment.

Table 3 Results from stability tests of vitamin-related parameters (mean ± s.d.)

	Temperature	*0 h*	*2 h*	*4 h*	*8 h*	*24 h*
Vitamin A (μmol l^{-1}) (n=5)	RT	0.59 ± 0.18	—	0.60 ± 0.19	0.56 ± 0.17	0.58 ± 0.19
	4 °C		—	0.58 ± 0.20	0.56 ± 0.17	0.57 ± 0.17
Tocopherol (μmol l^{-1}) (n=6)	RT	12.2 ± 2.2	—	12.9 ± 1.6	12.7 ± 1.6	12.7 ± 1.7
	4 °C		—	12.8+1.7	12.5+1.7	13.4 ± 2.0
Vitamin C (μmol l^{-1}) (n=6)	RT (d.p.)[a]	12.5 ± 3.4	11.6 ± 3.2	11.8 ± 3.3	11.4 ± 3.2	11.1 ± 3.4
	4 °C (d.p.)		11.7 ± 3.4	11.7 ± 3.2	11.8 ± 3.3	11.6 ± 3.6
	RT		11.1 ± 3.1	11.2 ± 2.9	9.9+2.7*	6.1+2.0*
	4 °C		10.8 ± 3.1	11.8 ± 3.0	11.6 ± 3.7	10.2 ± 3.0*
Folate (nmol l^{-1}) (n=7)	RT	11.5 ± 0.6				10.4 ± 0.7
Vitamin B_{12} (pmol l^{-1}) (n=7)	RT	234.1 ± 15.4				226.9 ± 8.7

[a]d.p., direct precipitation with metaphosphoric acid.*P<0.05.

Table 4 Results from stability tests for selected biomarkers

	Sample	*Time after blood extraction (hours)*							*Median of deviation to time 0 (%)*	*Mean of deviation to time 0 (%)*
		0	*2*	*4*	*6*	*8*	*12*	*24*		
Creatinine (mg per 100ml)	1	3.2	3.21	3.27	3.36	3.31	3.31	3.25	2.8	2.7
	2	1.08	1.09	1.1	1.12	1.11	1.07	1.11	2.3	2.2
	3	1.03	1.05	1.05	1.05	1.08	1.04	1.02	1.9	2.1
Urea (mg per 100ml)	1	55.2	54.7	53.9	55.1	56.1	54.7	55.9	1.1	1.2
	2	60.6	60.6	60.5	62.3	60.7	60.5	63.1	0.2	1.2
	3	26.6	26.9	26.4	27.3	26.8	26.8	28	0.9	1.9
GGT (U l^{-1})	1	33	33	33	32	33	34	33	0.0	1.0
	2	48	48	47	49	48	47	47	2.1	1.4
	3	7	7	7	7	7	6	6	0.0	4.8
ALT (U l^{-1})	1	20	18	19	20	19	18	18	7.5	6.7
	2	36	38	35	38	36	37	37	2.8	3.2
	3	15	15	16	16	14	16	15	6.7	4.4
AST (U l^{-1})	1	28	26	26	27	29	29	29	3.6	4.8
	2	45	46	47	45	44	45	47	2.2	2.2
	3	52	53	51	51	52	51	51	1.9	1.6
Triglycerides (mg per 100ml)	1	148	149	148	150	151	150	149	1.0	1.0
	2	48	48	50	50	49	51	50	4.2	3.5
	3	105	103	107	107	108	108	106	1.9	2.1
Cholesterol (mg per 100ml)	1	165	167	166	166	166	168	166	0.6	0.9
	2	125	124	124	127	126	126	127	0.8	1.1
	3	63	62	62	62	63	63	63	0.8	0.8
HDL (mg per 100ml)	1	31	32	31	32	31	31	33	1.6	2.2
	2	33	33	32	34	33	34	34	3.0	2.0
	3	12	12	12	11	12	11	11	4.2	4.2
LDL (mg per 100ml)	1	101	100	103	99	103	102	103	2.0	1.7
	2	77	75	75	74	77	76	77	1.9	1.7
	3	29	29	30	29	30	30	30	3.4	2.3
Lp(a) (mg l^{-1})	1	62	58	64	59	60	60	61	3.2	3.8
	2	62	58	60	59	63	60	62	3.2	3.2
	3	459	480	461	486	474	468	459	2.6	2.7
Apoplipoprotein A-I (g l^{-1})	1	1.37	1.4	1.39	1.39	1.38	1.39	1.4	1.5	1.6
	2	0.82	0.86	0.85	0.8	0.79	0.85	0.84	3.7	3.5
	3	2	2.03	1.99	2.03	1.98	1.99	2.03	1.3	1.1
Apoplipoprotein B (g l^{-1})	1	0.35	0.34	0.35	0.37	0.35	0.35	0.36	1.4	1.9
	2	0.95	0.98	0.97	0.97	0.97	0.96	0.95	2.1	1.8
	3	0.88	0.9	0.89	0.91	0.88	0.87	0.87	1.1	1.5
Albumin (g l^{-1})	1	25.6	25	24.8	25.7	25.9	24.7	24.8	2.7	2.3
	2	38.6	37.6	38.9	39.1	38.9	38.6	39	0.9	1.1
	3	34.5	35.1	35.1	36	34.6	34.8	34.8	1.3	1.6
Glucose (mg per 100ml)	1	99	100	98	101	101	102	101	2.0	1.9
	2	87	87	89	88	89	88	89	1.7	1.5
	3	81	82	82	81	82	83	82	1.2	1.2

Table 5 Results from stability production cytokines after transport from Zaragoza to Madrid through car (T0 and T1) and after transport from Zaragoza to Bonn and frozen cell culture medium to Madrid through plane (T2)

	T0 Levels after 6 h (cell culture performed in Madrid) n = *16*	*T1 Levels after 15 h (cell culture performed in Madrid)* n = *16*	*T2 Levels after 15 h (cell culture performed in Bonn)* n = *16*
IFN-γ (pg ml^{-1})	83710.9 ± 60702.5	93611.9 ± 37041.8	75711.6 ± 57075.6
TNF-α (pg ml^{-1})	2807.7 ± 1692.2^{a}	2713.5 ± 854.7^{a}	1015.7 ± 506.3^{b}
IL-10 (pg ml^{-1})	106.9 ± 49.4^{a}	65.5 ± 30.3 ^{b}	416.3 ± 210.9^{c}
IL-6 (pg ml^{-1})	1199.7 ± 1680.2^{a}	812.3 ± 938.6 ^{a}	39334.3 ± 18520.7^{b}
IL-4 (pg ml^{-1})	235.6 ± 147.9	225.1 ± 98.3	240.8 ± 64.9
IL-2 (pg ml^{-1})	1762.8 ± 1179.1^{a}	2357.6 ± 1538.0^{a}	468.7 ± 307.8^{b}

Mean ± s.e. $^{a, b, c}$ Different superscript letters mean differences between groups ($P < 0.05$).

Table 6 Results from stability of lymphocyte subpopulations (%) without (T = 0) and with stabilization solution Cytochex (T = 1 and T = 2)

	T0 levels after 6 h n = *11*	*T1 Levels after 7 days (Zaragoza-Madrid)* n = *11*	*T2 Levels after 7 days (Zaragoza-Bonn-Madrid)* n = *11*
CD19 (%)	12.4 ± 3.8	12.3 ± 3.4	13.2 ± 4.4
CD3 (%)	70.6 ± 6.4	68.8 ± 5.1	68.8 ± 5.4
CD4 (%)	34.6 ± 5.4	35.3 ± 5.3	35.3 ± 4.8
CD8 (%)	30.7 ± 5.9	31.3 ± 5.4	31.2 ± 5.2
NK (%)	14.4 ± 4.8	13.8 ± 4.3	14.3 ± 3.7

Mean ± s.e.

(106.93 ± 49.4 vs 65.5 ± 30.3 pg ml^{-1}, $P < 0.05$). Differences were also observed between T_0 and T_2 analyses for IL-10, IL-6, IL-2 and tumor necrosis factor-α (Table 5), the most striking one being IL-6 (1199.7 ± 1680.2 vs 39334.3 ± 18520.7 pg ml^{-1}). The stability tests of immunophenotyping of lymphocyte subpopulations (CD3, CD19, CD4, CD8 and natural killer cells) did not show any differences regardless of the transports or the time when the blood was preserved with Cytochex (Table 6).

Discussion

The assessment of iron status was performed by measuring serum ferritin and soluble transferrin receptor. Low serum ferritin indicates low iron stores, whereas iron overload conditions are recognizable by elevated serum ferritin concentrations. However, serum ferritin is also an acute-phase reactant protein that is elevated in response to infection. To avoid false-negative cases, C-reactive protein and α_1-acid glycoprotein were also measured.[17] According to our results, the shipment at room temperature within 24 h has not influenced the quality of the iron parameters in fresh serum samples.

As white blood cells lose their antigenic site activity, immunophenotyping of lymphocyte subpopulations in fresh blood should be carried out within a few hours. However, a short time span is not feasible in a centralized multicenter study such as HELENA. There, the blood for this purpose will be preserved with the reagent Cytochex, which has confirmed the stability of the cells up to 7 days during the pilot study. No modifications in lymphocyte subpopulations were observed because of the transportation, which leads us to continue with this design for immunophenotyping in the HELENA-CSS.

Regarding cell cultures for cytokine production, in the literature it is recommended not to wait more than 8 h to start the procedure.[18] However, our results show statistically stable cytokine levels even after 15 h, except for IL10, when the whole procedure was performed in the same laboratory (CSIC) (Table 5). But when the cell culture was performed at IEL after 15 h of blood extraction and the frozen cell culture medium was sent to CSIC and then analyzed (Figure 5), significant differences were observed. There are two possible reasons for these discrepancies: (1) the transport of *in vivo* cells by plane is not possible, because of stress-inducing processes in the cells, (2) the method of cell culture for cytokine production cannot be harmonized from one laboratory to another, maybe because of personal or technical differences. Nevertheless, samples with a transport duration of 16 h arrived around midnight and night work was not possible for this part of the study. In HELENA-CSS, this *in vitro* analysis of cytokine production by cultured white blood cells, included in the original study proposal, will be changed to the more feasible analysis of systemic cytokine concentrations in frozen serum samples.

Within 24 h, fresh serum samples could be used for the analyses of all routine biochemistry parameters included in the HELENA-CSS without any stability problems. However, the vitamin C and FA stability tests showed that they are very

unstable at room temperature without any treatment. Subsequently, the decision was taken to store the serum for FA determination at origin at −80 °C and to transport it at a later date on dry ice. For the stabilization of vitamins A, E and β-carotene, the serum samples were spiked with butylated hydroxytoluene, a synthetic antioxidant.

Another aim of the pilot study was to test the SYS and traceability system, to analyze the influence of transport time, and to observe variations between the 10 different centres under 'real' conditions. The transport time varied between 6 and 16 h after blood drawing. The influencing factors were distance from the school to the airport, the availability of cargo flights and time schedule of the flights. However, to guarantee an equal time span for all cities, it was decided to perform the routine biochemistry for all samples at the same time (0800 hours). Hemograms had to be analyzed locally to guarantee a maximum of a 3 h time span. The parameters from routine biochemistry (lipid profile, glucose, albumin and so on) showed no significant difference between the centers (data not shown). The SYS and the manual of operation were improved after the pilot study and a Power Point version for a better illustration was developed. The time span until arrival at IEL has been reduced to a maximum of 16 h implying night work for blood management at the end phase of the HELENA project. Serum samples for iron status were sent on dry ice from IEL to INRAN and all serum samples to CSIC and Pasteur Institute arrived safely and in the right volumes. Beside the manual of operation and the field worker training to guarantee a strict standardization of the handling in all centres, it was decided to send a controller for an audit of operation to the first blood sampling of the HELENA-CSS in each city.

Conclusion

Our results indicate that the protocol of the studies has been carried out successfully and also that our volunteers were enthusiastic to participate. The quality control for all parameters was in the range of the recommended levels reported in the literature (for some parameters, they are only available for adults).[19] Finally, the SYS was adequate for the biological samples, as transport had no influence on all the parameters measured. The methodology is adequate for the final part of the HELENA-CSS, which will be performed in 1000 adolescents (100 per city). To the best of our knowledge, for the first time, we aim at giving plasma vitamin reference values, a report on iron anemia deficiency risk, a report on risk for further non-communicable diseases (dyslipidemia and insulin resistance), a report on biological markers for subclinical malnutrition related to eating disorders (obesity/anorexia nervosa), in European adolescents. Furthermore, we will be able to correlate these data with the rest of the data obtained in the HELENA-CSS. The handling, transport and traceability of fresh blood samples within Europe are possible, but require an extremely demanding level of performance with regard to personnel and logistics as well as great economic effort.

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Conflict of interest

The authors state no conflict of interest.

References

1 Kaaks RJ. Biochemical markers as additional measurements in studies of the accuracy of dietary questionnaire measurements: conceptual issues. *Am J Clin Nutr Suppl* 1997; **65**: 1232–1239.
2 Allison S. Malnutrition, disease, and outcome. *Nutrition* 2000; **16**: 590–593.
3 Pinhas-Hamiel O, Newfield RS, Koren I, Agmon A, Lilos P, Philip M. Greater prevalence of iron deficiency in overweight and obese children and adolescents. *Int J Obes* 2003; **27**: 416–418.
4 Nead KG, Halterman JS, Kaczorowski JM, Auinger P, Weitzman M. Overweight children and adolescents: a risk group for iron deficiency. *Pediatrics* 2004; **114**: 104–108.
5 Pinhas-Hamiel O, Doron-Panush N, Reichman B, Nitzan-Kaluski D, Shalitin S, Geva-Lerner L. Obese Children and Adolescents. A risk group for low vitamin B_{12} concentration. *Arch Pediatr Adolesc Med* 2006; **160**: 933–936.
6 Decsi T, Molnar D, Koletzko B. Reduced plasma concentration of alpha-tocopherol and beta-carotene in obese boys. *J Pediatr* 1997; **130**: 653–655.
7 Molnar D, Decsi T, Koletzko B. Reduced antioxidant status in obese children with multimetabolic syndrome. *Int J Obes Relat Metab Disord* 2004; **28**: 1197–1202.
8 Thurnham DI, Mburu ASW, Mwaniki DL, De Wagt A. Micronutrients in childhood and the influence of subclinical inflammation. *Proc Nutr Soc* 2005; **64**: 502–509.
9 Kersting M, Breidenassel C, Sichert-Hellert W, Koeppen E, Gedrich K, Rieken K *et al.*, im Namen der HELENA Studiengruppe. HELENA: healthy lifestyle in Europe by nutrition in adolescence. *Ernährung Wissenschaft und Praxis* 2007; **1**: 17–22.
10 de Henauw S, Gottrand F, De Bourdeaudhuij I, Gonzalez-Gross M, Leclercq C, Kafatos A *et al.*, on behalf of the HELENA Study Group Nutritional status and lifestyle of adolescents in a public health perspective. The HELENA project—healthy lifestyle in Europe by nutrition in adolescence. *J Public Health* 2007; **3**: 187–197.
11 Moreno LA, González-Gross M, Kersting M, Molnár D, de Henauw S, Beghin L *et al.*, on behalf of the HELENA Study Group. Assessing, understanding and modifying nutritional status, eating habits and physical activity in European adolescents. The HELENA Study. *Public Health Nutr* 2007; **6**: 1–12.

12 Al-Delaimy WK, Jansen EH, Peeters PH, van der Laan JD, van Noord PA, Boshuizen HC *et al*. Reliability of biomarkers of iron status, blood lipids, oxidative stress, vitamin D, C-reactive protein and fructosamine in two Dutch cohorts. *Biomarkers* 2006; **11**: 370–382.
13 The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. www.ich.org.Visited 23 November 2006.
14 IATA DANGEROUS GOODS, 2005. www.iata.org. Visited 15 June 2006.
15 European parliament directive 2001/20/CE of the European parliament and council of 4 April 2001. *Official Journal* 2001; **L121**: 34–44.
16 Bland JM, Altman DG. Measuring agreement in method comparison studies. *Stat Methods Med Res* 1999; **8**: 135–160.
17 Erhardt J, Biesalski HK. Diagnosis of nutritional anemia—laboratory assessment of iron status. *The GuideBook—Nutritional Anemia, Sight and Light* (Chapter 4), 2007.
18 Muhl H, Nold M, Chang JH, Frank S, Eberhardt W, Pfeilschifter J. Expression and release of chemokines associated with apoptotic cell death in human promonocytic U937 cells and peripheral blood mononuclear cells. *Eur J Immunol* 1999; **29**: 3225–3235.
19 Al-Tahan J, González-Gross M, Pietrzik K. Vitamin status and intake in European adolescents. A review of the literature. *Nutr Hosp* 2006; **21**: 452–465.