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Title: Hematological, biochemical and antioxidant indices variations in regular blood donors among Mediterranean regions

Running Title: Study of clinical status in regular blood donors

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Introduction:

WHO for assessing blood donor suitability supports countries in establishing effective national systems for donor selection, including policies and guidelines in order to ensure the safety of the recipients of blood and blood products and protect donor health safety[1,2]. In the majority of blood banks, haemoglobin has generally been used as a screening test for the suitability to give blood. However, the use of this parameter has been reported to have poor sensitivity in the detection of early stages of iron deficiency. Indeed, an accurate diagnosis of a state of iron deficiency requires several laboratory tests[3]. Screening of iron deficiency by estimating the serum ferritin has been recommended by WHO as the laboratory test of choice[4]. It is well known that iron deficiency appears in blood donors particularly in frequent donors and that this is under-recognized by both donors and their physicians[5]. Several published studies, performed in many different countries (Iran, India, Pakistan) have provided data on the frequency and risk factors for iron deficiency in regular blood donors[6]. The potential for a donor to donate blood without developing iron deficiency anaemia[6] varies with the difference in nutritional iron intake and the prevalence of iron deficiency in the defined population. On the one hand, iron is essential in the human diet and is needed for many important physiologic functions such as bounding to hemoglobin, myoglobin, and several enzymes, and nonheme iron proteins. On the other hand there are several associations of iron's prooxidant properties and the generation of free radicals. Mahmoud Djalali and his team investigated in Iranian population whether blood donation reduce body iron stores, oxidative stress and consequently increase activity of antioxidative enzyme[7]. ~~Taking into consideration that, we investigated the biochemical, hematological and antioxidant status of regular mediterranean volunteer blood donors (VDs) both male and female, from Greece and Italy.~~ In mediterranean population the number of donations is insufficient to cover the demand leading to the importation of blood from abroad almost every year[8]. Greece and Italy an iron deficient population, present high necessities of blood transfusion because of the high frequency of hemoglobin disorders[9]. An overview of European blood donors shows that the distribution of men and women donors isn't similar in Greece and Italy comparing with other European countries in which the percentage (33%) of female donors, is very low. For this reasons in this study we will present the positive for the health hematological and biochemical indices variations of frequent blood donation in order to increase the number of regular VDs in Mediterranean countries. In the present study, we aimed to investigate the effect of regular blood donation on hematological (hematocrit and hemoglobin) and biochemical (serum iron and ferritin, total cholesterol, triglyceride, SGOT, SGPT, total antioxidant status) parameters.

Material and Methods:

Study design

A cross-sectional study was carried out in two mediterranean blood banks in Italy and Greece. The first one carried out on adult males and females, aged 18–60 years in the General Hospital of Naousa, Greece at Blood Bank section from January 2015 to August 2017. The second one carried out on adult males and females, aged 25-65 years in ASL of Sassari, Italy on ~~convenient~~-samples of 100 first-time VDs and 100 regular blood donors in the same period. **Just before blood donation, 10 mL venous blood sample was taken and divided into EDTA and ~~heparin~~ Serum separator tubes. EDTA tubes were used for the measurement of the hematological parameters. The serum ~~by heparin tubes~~ was recovered **from clotted blood and** transferred to a fresh tube for determination of all the biochemical parameters (serum iron, ferritin, cholesterol, triglyceride, Serum glutamic oxaloacetic transaminase, Serum glutamic pyruvic transaminase and total antioxidants). Donors' particulars were obtained from blood bank records. ~~Convenient~~-Samples used in this study were divided in 2 groups: Group 1 comprised of 90 greek and 100 sardinian regular blood donors with 4 donations over the last 2 years or 6 donations in last 3 years and Group 2 comprised of 60 greek and 100 sardinian first-time donors with 1 or 2 donation in the last 3 years. The participants were examined for symptoms of diseases. The data were collected through interview of participants and with the use of a short questionnaire. **For this sample of 350 VDs were taken on the basis of statistical method of sample size determination using the following equation:****

$$n = \left\lceil \frac{(Z_{\alpha/2} + Z_{1-\beta})^2 \times P(1 - P)}{(\text{MOE})^2} \right\rceil$$

Here 95% confidence level and 80% power was taken with 5% as margin of error (MOE).

Participant particulars were obtained from records kept in both Blood Banks. Patients, all adults, provided written, informed consent before entering the study. The study was conducted in accordance with Good Clinical Practice guidelines and the Declaration of Helsinki. Ethical approval to perform the present study was obtained from the Ethical committee of the General Hospital of Naousa. The confidentiality of participants was wholly preserved. The questionnaire covers the sociodemography of donors that included age and residence, reason for and frequency of donation, body mass index (BMI), **nutritional habits**, whether participants had history of chronic diseases and/or hyperlipidemia, **taking iron supplements**, were smokers, and/or consumed alcohol. **Molecular data of the G6PD variants in Sardinian population has been taken from the Blood Bank records.**

Exclusion criteria

Individuals with systemic diseases including hypertension, diabetes, and/or cardiovascular diseases, and those aged below 18 or above 65 years, or with hemoglobin concentration < 12.5 g/L were excluded from the study.

Measurement of parameters

Antioxidant activity status of greek blood donors was assayed spectrophotometrically, using a TAS Kit (Total Antioxidant status colorimetric assay kit, produced by Cayman Chemical Co., Ann Arbor, USA) with the TEAC method in the clinical chemistry laboratory of the National Hellenic University, Thessaloniki, Greece. Hematological indices were measured by a Beckman Coulter counter (Coulter Corporation, USA). All biochemical parameters were measured using Cobas Integra 400 Plus and ferritin with Cobs e411Elecsys automated analyzers.

Statistical analysis

For statistical analysis, SPSS tool version 22.0 was used. Descriptive statistics presented as mean \pm standard deviation and frequencies as percentages. Kolmogorov–Smirnov analysis verified the normality of the data set. Pearson's chi-square test or chi-square test of association was used to discover if there is a relationship between the categorized data, while Fishers exact test was used when expected variable were < 2% of total number of variables. Additionally, independent sample t-test was used to compare between means. In all statistical analysis, level of significance (p-value) was set at $\alpha=0.05$.

Results:

Characteristics of the study population

In figure 1 there is the schematic diagram of our study and in table 1 the socio-demographic characteristics of all study participants. Demographic analysis of the 350 donors showed 56% (196) male and 44% (154) female. The greek regular donors were significantly ($p < 0.001$) older than the greek first-timers (46.1 vs 38 years). More regular donors were 40 years or older while 60% of first time donors were <40 years old. No age difference has been observed in the italian groups. Body mass index (BMI) of italian groups is 5 % lower in compare with the greek groups. All donors had haemoglobin concentrations greater than 12.5 g/L in order to donate and participate to the study. **Blood donors from both Greece and Italy weren't receiving iron supplements.** Table 1 present the prevalence of G6PD deficient volunteers in both countries. The greek national policy of the Blood bank didn't permit Glucose-6-phosphate dehydrogenase deficiency (G6PD) deficient volunteers. On the other hand, we observed in sardinian population a high prevalence (26%) of G6PD deficient (Mediterranean variant 563C→T / 188Ser→Phe)[10] first time volunteers that probably didn't know for their enzyme deficiency. Regarding the regular blood donors only the 8% of them are in the deficient range.

Correlation of Hematological and Biochemical parameters with donation frequency in greek population

In Table 2 (left panel) there are the hematological and biochemical parameters of greek blood donors. Regular blood donors had significantly ($p < 0.05$) higher average hemoglobin (15.3 g/L) and serum ferritin levels (105.3 ng/L) in compare with the first time donors (14.7 g/L and 84.5 ng/L respectively). The differences in iron status comparing the two groups showed lower average iron levels in regular blood donors but were not statistically significant. The lipid profile of the donors was evaluated too. Interestingly the average atheromatic index of the regular blood donors (3.8) was significantly lower in compare with the first time donors (4.3). Moreover, the average total plasma cholesterol, triglyceride of regular donors (207.7 and 123.8) were significantly higher than the levels of the first-time donors (193.5 and 117.1 mg/ml respectively) ($p = 0.04$, $p = 0.03$ respectively). On the other hand, average SGOT and SGPT levels of regular donors (15.5 and 15.3 mg/ml) were significantly lower than the levels of the first-time donors (22 and 21.9 mg/ml respectively) ($p < 0.04$ and $p < 0.05$ respectively). The oxidative stress of the volunteers has been evaluated too and the average total antioxidant status levels of the regular volunteers (256.1) was statistically significant

higher ($p = 0.02$) in compare with the first timers (230.4). In conclusion, high frequency blood donation was associated with evidence of reduction of body iron stores but increase in serum ferritin levels, reduced oxidative stress and improvement of atheromatic index (Table 2).

Correlation of Hematological and Biochemical parameters with donation frequency in Sardinian population

In Table 2 (right panel) there are the hematological and biochemical parameters of sardinian regular and first time blood donors. Regular blood donors had slightly higher average hematocrit levels. In contrast with the greek group, sardinian regular blood donors showed statistically significant higher average iron in regular blood donors in compare with the first timers. **It is worth mentioning that sardinian regular blood donors weren't receiving any iron supplementation.** Looking at the average concentration of serum ferritin in first-time (83.5 ng/L) and regular donors (76.5 ng/L) it was seen that ferritin levels significantly decreased with increasing number of donations. No significant differences have been observed in the atheromatic index of the regular blood donors (2.8) in compare with the first time donors (2.7). In accordance with the lipid profile of the greek groups, the total plasma cholesterol, triglyceride of regular donors (204 and 95) were significantly higher than the levels of the first-time donors (197.7 and 93.2 mg/ml respectively) ($p = 0.05$, $p = 0.04$ respectively). The same trend with the greek groups has been observed in SGOT and SGPT levels of regular donors (19.4 and 24 mg/ml) where were significantly lower than the levels of the first-time donors (20.3 and 27 mg/ml respectively) ($p < 0.05$ and $p < 0.04$ respectively). **Different studies[11,12] demonstrated that iron deficiency was present in regular donors who had four or more blood donations during 1 year. On the contrary the italian group demonstrated a different trend, with increase of iron after frequent blood donation. A preliminary explanation for this results could be based to their dietary intakes. Sardinians are following an iron-enriched diet in iron sources well absorbed by the body than others. Further studies will need to elucidate this hypothesis. A larger study (including also the nutritional status of the VDs) should be carried out to reevaluate the statistical significance of the serum iron results too.**

Gender distribution of blood donors and correlation of their hematological and biochemical parameters with donation frequency

Distribution of hematological and biochemical parameters of blood donors based on gender is shown in Tables 2A and 2B. Hemoglobin concentration of male first time and regular VDs was statistically significant higher ($p < 0.01$) in compare with female VDs of both groups and countries (see tables 2A and 2B). It should be noted that the Sardinian group comprised by high frequent VDs donating over the last decades. Looking at the average concentration of iron stores in Sardinian VDs, it was seen that iron levels of regular male donors statistically significant increased in compare with first-time VDs. The same trend has been followed for the ferritin levels too. That doesn't follow the results of other similar studies and we could attribute this observation to different factors such as, the gender, high frequency over the last decades and high dietary iron intakes of Sardinian male VDs. There were no statistically significant difference for Sardinian female first time and regular VDs. On the other hand in Greek regular female group (menstruating females), it was seen a decrease of their iron stores which is following the observations of other studies[13,14]. There were no statistical difference for Greek male first time and regular VDs. In accordance with other studies[13,14], we have also observed in both female groups that first time donors had higher ferritin contents than high frequency blood donors. The lipid profile of regular VDs in both genders didn't present any notable difference with the general groups of Table 1. Moreover, there was a 25% decrease in atheromatic index in both regular female population while in regular male population a slight increase. Greek male regular VDs presented the highest total antioxidant status in compare with all the tested groups. Gender seems to play an important role in the aspects studied and merit further consideration in relation to strategies to recruit more donors and manage critical events during donation.

Discussion:

There is an increasing demand for blood products and taking into consideration that in the very near future with our current strategies we will not be able to satisfy the need for blood, the health benefits of regular blood donation should be evident to recruit new blood donors[15–17]. On one hand, frequent blood donation is known to be correlated with iron depletion or deficiency[18,19]. Previous studies demonstrated that an increase in the number of donations results in an increase in the frequency of depleted iron stores although the level of haemoglobin remained acceptable for blood donation. It is suggested the need to review the guidelines on acceptance of donors[13,18]. In our study, only the female greek regular VDs had a definite correlation between increase in frequency of donation and decrease in iron levels. The body iron stores of the other study groups (regular greek male VDs and both sardinian VDs) wasn't affected negatively. **The lack of explanation for the difference observed in mean serum iron between these groups could be attributed due to the factor that serum iron measurements lack the prerequisite sensitivity and/or specificity for such low differences confirmed by the weak statistical significance. ($p=0.05$) (89.4 ± 32.2 for first time VDs and 98.5 ± 36.1 for regular donors).** ~~This phenomenon has been observed only in greek female group may be due to low initial serum ferritin values or nutritional factors or due to their menstrual cycle. The last hypothesis is in agreement with the age status of the sardinian female group (normal iron levels) which is comprised by menopausal women.~~

We further confirm the observation of Devi Deepa[19] and his team that there is a definite relationship between low serum ferritin levels and female donors. In addition, in accordance with other studies[13,19,20], we suggest that the knowledge of pre-donation serum ferritin levels will help to decide if a regular donor should be offered iron supplementation[21] or not after frequent donation of blood[9]. In accordance with previous studies[14,22] our findings show health benefits of high-frequency blood donation such as decreased oxidative stress and enhanced activity of antioxidant enzymes when compared with first time donors. These findings suggest that blood donation affected oxidative status and antioxidant capacity in regular donors by removing oxidants and elevating antioxidant enzymes. Uche et al[23] demonstrated that regular blood donation has the potential to decrease the total cholesterol triglycerides and low-density lipoprotein and may be protective against cardiovascular disease. Our study confirm the improvement of the atheromatic index and the lipid profile in female regular VDs groups of both nations but not the decrease of the total cholesterol, triglycerides and atheromatic index in male groups. This phenomenon has been observed probably because of the nutrition intake of the tested participants and the different hormonal profile of the two genders. The question how blood donation improve lipid profile remains unknown although in recent

studies[14] there was found a decrease in lipid peroxidation in regular blood donors when compared with low- frequency donors. This fact could probably explain the increase in antioxidants in regular blood donors. We propose to monitor the nutritional status of regular donors and provide nutritional recommendations by nutritionists for a “healthier” result for the donors and the recipients. Moreover, the importance of G6PD deficiency in transfusion and the modifications of the RBC antioxidant system (of which G6PD is essential) of recipients should be further investigated. In conclusion, blood donation frequency is an alternative method to improve our health but also there are a lot of factors, such as frequency, gender, age, nationality, prooxidant mutations in red blood cells that should be taken into consideration in order to obtain the desired results. Additional comparative studies in different populations are required to better understand the lipid profile of both genders and to confirm the improvement of the antioxidant status of the regular blood donors. **Blood centers in Greece and Italy should consider consultation and iron replacement (if necessary) for regular blood donors in order to retain the volunteer donor base.**

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Tables

Table 1. Baseline characteristics of all study participants (n=350)

	Greek (Macedonian)		Italian (Sardinian)	
	First Time Donors (n= 60) Mean ± SD	Regular Donors (n= 90) Mean ± SD	First Time Donors (n= 100) Mean ± SD	Regular Donors (n= 100) Mean ± SD
Sex, Male/Female	30, 30	50, 40	57,43	59,41
Age	38±9.5*	46.1 ± 10.1*	54± 8.4	54±11
BMI	26.4 ±3.7	26.7 ± 5.5	24.8 ± 2.8	25.7 ± 3.5
Smoking, Yes (%)	30%	27.7%	/	/
G6PD-deficiency	0%	0%	26%	8%

Significant differences between first timers and regular donors group are marked with

* P < 0.05, Data were analyzed using a two-tailed, two-sample Student's t-test.

Table 2. Hematological and Biochemical parameters of north greek and Sardinian first time and regular blood donors.

	<i>Greek (North Greece)</i>			<i>Italian (Sardinian)</i>			<i>p-values</i> ²	<i>p-values</i> ³
	First time Donors Mean ± SD	Regular Donors Mean ± SD	<i>p-values</i> ¹	First time Donors Mean ± SD	Regular Donors Mean ± SD	<i>p-values</i> ¹		
Ht (%)	44.1± 3.7	45.5± 2.7	0.01*	41.5 ± 3.6	42.3 ± 4.2	0.02*	0.01*	0.01*
Hb (g/L)	14.7± 1.2	15.3± 1.1	0.05*	13.7 ± 1.2	13.5± 3.9	-	0.01*	0.18
Iron (ng/L)	118.8± 45.2	110.5± 25.3	0.09	89.4 ± 32.2	98.5± 36.1	0.05*	0.05*	0.05
S. ferritin (ng/L)	84.5± 50.6	105.3± 25.5	0.03*	83.5± 77.1	76.5± 60	0.25	0.3	0.09
T.C (mg/dl)	193.5± 35.4	207.7± 32.1	0.04*	197.7± 41	204±39	0.13	0.3	0.4
T.G (mg/dl)	117.1± 57.3	123.8± 85.3	0.04*	93.2±59	95±51	0.4	0.01*	0.02*
Ath. Index	4.3±1.9	3.8±1.4	0.05*	2.8±1.6	2.7±1	-	0.04*	0.03*
SGOT (U/L)	22± 4.3	15.5± 5.2	0.04*	20.3±8.3	19.4±7	0.05*	0.23	0.1
SGPT (U/L)	21.9± 4.8	15.3± 6.3	0.05*	27.5±18	24±12	0.04*	0.01*	0.03*
Glucose (mg/dl)	116.1± 26.5	116.3± 18.4	-	93.5±7	84.5±11	0.02*	0.21	0.01*
TAS (mmol/L)	230.4± 29.7	256.1± 37.7	0.02*	-	-	-	-	-

Abbreviations: Ht: Hematocrit; Hb: Hemoglobin; SF: Serum Ferritin;; TC: Total cholesterol;TG: Triglyceride; Ath.index: atherogenic index ; SGOT: Serum glutamic oxaloacetic transaminase; SGPT Serum glutamic pyruvic transaminase level; TAS: Total antioxidant status

¹Significant differences between first timers and regular donors group are marked with * P < 0.05,

²Significant differences between first timers greek group and first timer italian group are marked with * P < 0.05,

³Significant differences between regular greek group and regular italian group are marked with * P < 0.05,

Data were analyzed using a two-tailed, two-sample Student's t-test.

Table 2A. Hematological and Biochemical parameters of greek male and female first time and regular blood donors.

Greek (North Greeks)								
Male donors				Female donors				
Parameters	First time donors Mean ± SD	Regular donors Mean ± SD	p-values ¹	First time donors Mean ± SD	Regular donors Mean ± SD	p-values ¹	p-values ²	p-values ³
Ht (%)	46.1± 2.8	46.8± 3.6	0.02*	42.1± 3.2	44.1± 2.4	0.01*	0.002*	<0.001**
Hb (g/L)	15.4± 1.3	15.8± 1.2	0.02*	14.1± 1.1	14.8± 1.6	0.08	>0.001**	0.01*
Iron (ng/L)	114.1± 43.2	123.8± 31.2	0.15	123.4± 55.7	98.2± 33.2	0.02*	0.28	<0.001**
S. ferritin (ng/L)	80.4± 35.2	125.3± 48.1	0.01*	88.5± 25.8	85.3± 24.9	0.1	0.23	0.04*
T.C (mg/dl)	178.5± 25.4	221.5± 23.8	0.01*	208.4± 34.8	193.8± 30.5	0.1	0.004*	0.04*
T.G (mg/dl)	98.1± 48.3	136.8± 46.7	0.02*	136± 67.8	110.7± 56.4	0.05*	0.04*	0.02*
Ath. Index	3.7± 1.8	4± 1.5	0.01*	4.8± 2.1	3.6± 1.3	0.5	0.03*	0.01*
SGOT (U/L)	25.7± 6.2	15.3± 7.2	0.05*	18.2± 2.3	15.8± 3.3	0.05*	0.002**	0.05*
SGPT (U/L)	29.3± 4.9	14.5± 7.5	0.06	14.5± 4.9	16,1± 8.7	0.01*	0.001**	0.02*
Glucose (mg/dl)	96.4± 25.2	135.2± 20.2	0.1	135.8± 28.6	97.5± 19.8	0.05*	0.001**	0.22
TAS (mmol/L)	243± 35.1	280.2± 38.2	0.04*	217.9± 24.3	232± 37.3	0.01*	0.005*	0.05*

¹Significant differences between male or female first timers and regular donors group are marked with * p < 0.05,

²Significant differences between male and female first timers greek group are marked with * p < 0.05,

³Significant differences between male and female regular greek group are marked with * p < 0.05,

Data were analyzed using a two-tailed, two-sample Student's t-test.

Table 2B. Hematological and Biochemical parameters of Sardinian male and female first time and regular blood donors.

Parameters	Italian (Sardinian)							
	Male donors			Female donors			p-values ²	p-values ³
	First time donors Mean ± SD	Regular donors Mean ± SD	p-values ¹	First time donors Mean ± SD	Regular donors Mean ± SD	p-values ¹		
Ht (%)	43.9± 2.9	45± 3.6	0.05*	39.1± 2.6	39.5± 2.5	<0.001**	<0.001**	<0.001**
Hb (g/L)	14.4± 1	14± 2.3	0.05*	12.9± 1	13± 0.8	<0.001**	<0.001**	<0.001**
Iron (ng/L)	91.4± 33.2	105± 37	0.05*	87.3±34.2	92± 29	0.28	0.03*	0.08
S. ferritin (ng/L)	101.3± 64.4	109± 62	0.03*	65.7±43	44± 22	0.05*	0.05*	<0.001**
T.C (mg/dl)	198.7±38.3	212±37	0.03*	196±47	194± 39	0.45	0.38	0.01*
T.G (mg/dl)	118.4±60	117±56	0.45	70±36	73±31	0.3	0.01*	<0.001**
Ath. Index	2.7± 1.1	3.1± 1.1	0.01*	2.8± 2.1	2.1± 1	0.5	0.03*	0.01*
SGOT (U/L)	23.7± 9	21± 6.8	0.04*	17± 4	17.7± 7.2	0.3	>0.001**	0.09
SGPT (U/L)	32± 20	27± 14	0.05*	23± 12	22± 10	0.35	0.01*	0.05*
Glucose (mg/dl)	91± 17	86± 11	0.18	95± 35	83± 10	0.18	0.23	0.16

¹Significant differences between male or female first timers and regular donors group are marked with * P < 0.05, **p < 0.001

²Significant differences between male and female first timers Sardinian group are marked with * P < 0.05, **p < 0.001

³Significant differences between male and female regular Sardinian group are marked with * P < 0.05, **p < 0.001

Data were analyzed using a two-tailed, two-sample Student's t-test.

Figure Legends

Figure 1. Study flow diagram. Number (n) of participants included in the study in regard to group classification.

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Conflict of Interest Disclosures. The authors declare no conflict of interest



Blood donors (n=350)

Italian (Sardinian)

(n=200)

Greek (North Macedonian)

(n=150)

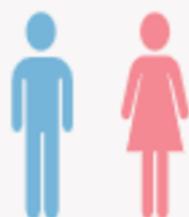


First time donors
(n=100)

Regulal donors
(n=100)

First time donors
(n=60)

Regulal donors
(n=90)



Analysis of
Hematological & Biochemical
parameters



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