Oxidative stress in stable cystic fibrosis patients: Do we need higher antioxidant plasma levels?

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OXIDATIVE STRESS IN STABLE CF PATIENTS: DO WE NEED HIGHER ANTIOXIDANT PLASMA LEVELS?

ABSTRACT

Oxidative stress plays an important role in cystic fibrosis (CF). However, there is a lack of validated biomarkers of oxidative damage that correlate with the antioxidant needs of patients with CF'. **Objective:** to investigate oxidative stress in stable pediatric CF patients and evaluate if vitamin supplementation may be tailored on individual needs and oxidative status.

Results: Lipid-adducts 4-hydroxynonenal (HNE-L) and malonaldehyde (MDA-L) (chromolipids) were elevated in the majority of patients despite normal plasma vitamin E, A and C. HNE-L and MDA-L increased with age, while plasma vitamins decreased. The most relevant correlation was identified between vitamin C and chromolipids. Patients with pancreatic insufficiency (PI) showed significantly higher plasma chromolipids despite no differences in plasma vitamins.

Conclusions: The majority of patients showed elevated plasma chromolipids that increased with age. Antioxidant vitamins reference ranges provide incomplete information on the redox status. CF patients with PI showed excessive oxidative stress damage.

INTRODUCTION

Cystic fibrosis (CF) is characterized by elevated oxidative stress and impaired antioxidant/oxidant balance (1). Chronic infection and inflammation increase the free radical production. This could modulate the CFTR expression and function having a role in lung disease progression (2). At the same time, antioxidant defenses are dramatically reduced compared to healthy subjects (1,2,3), even early in the life (4).

Respiratory exacerbations lead to impairment of antioxidant defenses that restore after treatment (5). Increased number of exacerbations have been as well correlated to lower plasma vitamin E and A (6). Furthermore, markers of free radical damage have been demonstrated in

plasma of CF patients despite the presence of normal concentrations of circulating antioxidants suggesting inadequate antioxidant defenses to cope with elevated oxidative stress with consequent progressive decline in pulmonary function (4,7).

An important feature in CF patients is the exocrine pancreatic insufficiency (PI), which causes maldigestion and malabsorption of liposoluble vitamins E and A. Unfortunately, pancreatic enzyme replacement therapy (PERT) does not completely restore the absorption of these vitamins. Moreover, it has been demonstrated that vitamin deficiency can be independent of pancreatic function and PERT (8). Therefore, CF patients with PI need to receive daily vitamin supplements. The monitoring of plasma levels and supplementation of these vitamins is part of routine CF care (9,10).

Normal ranges of plasma antioxidants correspond to those regarded as adequate for the non-CF population i.e. healthy subjects. The optimal requirements for CF patients, based on antioxidant needs, may be higher than (these). Discussion on vitamin status has now shifted to the identification of optimal levels that provide better health outcomes than suboptimal ones.

Quantification of oxidative stress can be assessed by the detection of lipid peroxidation end-products deriving from the degradation of polyunsaturated fatty acids, which are susceptible to oxidative attack by free radicals. Several markers for lipid peroxidation both *in vitro* and *in vivo* are available and different detection methods have been described (11). Among these isoprostanes and aldehydes, such as MDA and HNE, are now the most studied products of lipid peroxidation involved in different respiratory pathologies (12,13). In particular, significantly elevated F2α-isoprostane have been found in breath condensate and plasma of CF patients (2,14). Significantly elevated MDA in terms of thiobarbituric acid reactive substances (TBARS):cholesterol ratio, have been described to increase with age in CF patients (3). Compared to isoprostanes, the longer half-life of aldehydes makes them good candidates for

propagation and amplification of effects elicited by free radicals. Among aldehydes, HNE is highly reactive with thiol- and amino-residues of proteins, peptides, lipids and nucleic acids (15).

High MDA concentrations have been found to be negatively correlated with forced expiratory volume in 1 second (FEV1) in different airway diseases (2,3,16,17). Significant reduction and/or normalization of MDA plasma concentrations have been reported after beta-carotene or multivitamin supplementation in CF patients (18,19). However, consideration should be given to the risk of toxicity and side-effects induced by the high dose liposolubile vitamin supplementation. Although there is growing evidence of the potential role of oxidative reactions in the pathogenesis of CF, we still lack validated biomarkers of oxidative damage, which might serve to identify the effective amounts of antioxidants actually needed by CF patients.

The present study was carried out to address this issue. The fluorimetric assay of aldehyde-lipid adducts in plasma has been assessed exploiting the properties of MDA and HNE to be highly reactive forming stable adducts with functional groups of cell macromolecules (20). The aim was to identify the oxidative status in a group of stable CF patients in relation to their antioxidant and clinical status, in presence or absence of pancreatic insufficiency. These results may provide suitable "individual" reference ranges for a proper vitamin supplementation in CF patients.

MATERIALS AND METHODS

Patients' recruitment

We enrolled 70 subjects, 1 – 18 years old, progressively attending the Pediatric Cystic Fibrosis Centre (Regina Margherita Hospital, Turin) from January 2007 to April 2008. All patients were in stable clinical conditions and were evaluated during the routine control visit. Our study included:

Clinical data. Genotype, pancreatic insufficiency (fecal elastase $< 200 \,\mu g/g$), number of respiratory exacerbations during the last year.

Anthropometric measurements. Body weight (kg, percentile, z-score), height/length (cm, percentile, z-score), Body Mass Index (kg/m^2, percentile, z-score) calculated by Epi-Data Nut Stat (Epi Info 2000; developed by the CDC-Center for Disease Control and Prevention-in Atlanta, Georgia; USA).

Respiratory function test (RFT) was performed with a Bio Medine spirometer.

We calculated the mean value of the last year (for patients older than 6 years that were able to perform RFT) for FEV1 expressed as percentage of the predicted value for age and sex.

Vitamin supplementation. Dosages of vitamins E and A and multivitamins were recorded.

Nutritional evaluation. A three-days-food-record was analyzed to calculate the intake of energy, nutrients and antioxidants (software based analysis - Food Meter, Medimatica s.r.l., Martinsicuro, Teramo, Italy, 1990, modified by Turin University according to the Nutrition Tables of Italian National Institute of Nutrition). Patients or parents were required to record prospectively the food consumed through three days. Food quantities were described by using standard household measures (e.g. cups, tablespoons) or weighed if the latter were not available. The cooking method was also described. In the end, during the visit session, a dietitian went through the record with patients/parents to clarify details.

Hematic parameters of antioxidant status: selenium, ascorbic acid, retinol and α-tocopherol in plasma samples, selenium dependent glutathione-peroxidase (GSH-PxSe), reduced glutathione (GSH) in red blood cells were considered as biochemical indexes of antioxidant status.

Selenium (P-Se) was detected by SpectrAA 20 Graphite Furnace Atomic Absorption Spectroscope Varian (Varian Techtron Pty. Ltd., Australia); ascorbic acid was detected in plasma samples by HPLC (High Pressure Liquid Chromatography) according to Harapanalli (21). Retinol and α-tocopherol were simultaneously evaluated by HPLC using a kit of Chromsystems Instruments and Chemical GmbH (Martinsried/Munich, Germany). Selenium-dependent-glutathione peroxidase

activity in Red Blood Cells (RBC) was evaluated by spectrophotometrically (22). Total and reduced glutathione was analyzed by HPLC after derivatization of hemolyzed samples with ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F) (fluorescence detector Varian 9070, 385 nm excitation/ 515 nm emission).

Blood antioxidant were expressed as following: α-tocopherol (mg/l), ascorbic acid (mg/l), retinol (μg/l) and selenium (μg/l), reduced RBC-glutathione (GSH-μmol/gHb), selenium dependent glutathione peroxidase (GSH-PxSe-U/gHb).

Study population was classified in clusters of optimal, suboptimal and deficient plasma vitamin E and C as described by Biesalski (23). For vitamin A we utilized the 25th and 90th percentile of retinol concentrations as cut-off values similar to those proposed by Biesalski that deals with beta carotene.

Oxidative stress markers.

The presence of oxidative damage was assessed in plasma by determining the fluorescent adducts formed between lipid peroxidation-derived aldehydes and plasma lipids, also termed chromolipids. In particular, HNE-lipid adducts (355 nm excitation/460 nm emission) and MDA-lipid adducts (390 nm excitation/460 nm emission) were evaluated. Preparation of samples and fluorimetric measurements were performed according to Biasi et al. (1995) (Kontron SFM 25 spectrofluorimeter, Kontron AG, Zurich, Switzerland). Data were expressed as Arbitrary Fluorescence Units (A.F.U.)/ml plasma and, as required, Arbitrary Fluorescence Units/mg plasma cholesterol. Plasma cholesterol was analyzed by an enzymatic colorimetric test (Olympus analyzer).

Oxidative status was also estimated in RBC as a ratio of reduced glutathione and its oxidized form (GSSG). GSSG was calculated by HPLC as the difference between total and reduced glutathione.

Statistical analysis

Data are reported as median and range for continuous variables and number and percentage for categorical ones.

Differences between groups were evaluated with the Mann-Whitney U test or the Kruskal-Wallis test, whilst correlation between continuous variables was checked with the Spearman correlation coefficient (ρs). All tests are two-sided and significant for p values less than 0.05.

Data were analyzed with SPSS 17 (SPPS Inc., Chicago, IL).

RESULTS

General and clinical characteristics of the study population are reported in Table1.

Red-ox status in CF pediatric patients

Referring to the local reference range the majority of patients had normal concentrations of vitamins E, A, C and selenium (92.8%, 97.1%, 98.6% and 86.6%, respectively). Red blood cell concentrations of reduced glutathione and glutathione peroxidase (97% and 97% respectively) were also recorded (found normal). Interestingly, the GSH/GSSG ratio was low for 89.7% of patients.

Chromolipids HNE-L and MDA-L, either as plasma levels or referred to mg of plasma cholesterol, were significantly higher than those of healthy people (local reference range - l.r.r.). Specifically, only 4.8% and 1.6% of patients showed normal concentrations of HNE-L and MDA-L respectively.

Evaluation of energy and nutrient intakes

Analysis of three day food record revealed adequate energy and macronutrients intakes per kilogram of body weight and in percentage of total energy referring to Italian recommended intakes (LARN - Livelli di Assunzione Raccomandata di energia e Nutrienti) (24), though not as high as expected for CF patients (energy 81kcal/kg, lipids 33.7%, protein 16% and carbohydrates 52.3%). Vitamin A and E intakes were aligned to the upper mentioned recommendations while vitamin C intake was slightly superior (78.4 vs. 40-60 mg/day).

Plasma antioxidant vitamins: relationship to clinical data and oxidative stress parameters.

Applying the cut-off criteria suggested by Biesalsky (23), we obtained a picture that is quite different from the one obtained relying to the local reference range of normality for the vitamins plasma levels. A large group of patients (67.1%, 40.6% and 65.7%) showed sub-optimal plasma levels of vitamin E, C and A respectively, running the risk of oxidative burden (Table 2). Because the sub-optimal levels are within the normal reference range, patients do not usually increase their supplementation doses. However, 21.5% and 22.8% of patients had deficient levels of vitamin E and A despite oral vitamin supplementation.

Age was the most relevant feature across the different clusters of patients: patients with optimal values are significantly younger than those with lower/deficit vitamin levels (p<0.05 for vitamin E and C) (Table 2).

Plasma oxidative stress markers HNE-L and MDA-L as well as the ratio with plasma cholesterol showed an increasing trend with lowering antioxidant vitamin concentrations. Interestingly, the difference is statistically significant between patients with optimal versus suboptimal vitamin C levels (p<0.05) for both chromolipids.

There were no differences among groups for the clinical data.

BMI z-score resulted significantly lower in patients with suboptimal vitamin C concentrations (p<0.05) compared to those with optimal ones (Table 2).

Correlation of age with plasma antioxidant vitamins and oxidative stress markers in CF patients

Plasma antioxidants were inversely correlated with age that exerts an independent significant effect in reducing vitamin E (ρs = -0.25, p = 0.034) and vitamin C (ρs = -0.27, p = 0.025). Although plasma vitamin A also claimed an inverse correlation with age, it was weaker and statistically significance was not reached (ρs = -0.06; p = 0.628). Plasma chromolipids increased significantly with age (MDA-L/cholesterol ρs = 0.36, p= 0.003; HNE-L/cholesterol ρs = 0.33, p= 0.004) (Fig.1). Aging accounted for 10% (MDA-L) and 12% (HNE-L) of increase in plasma chromolipids.

Do antioxidant vitamin plasma levels correlate with oxidative status?

Our data evidenced an inverse relation between plasma concentrations of both chromolipids and those of antioxidant vitamins (Fig. 2). In particular, vitamin C showed a significant inverse correlation with plasma chromolipids (ρs = -0.30, p = 0.018 for MDA-L/cholesterol; ρs = -0.36, p=0.004 for HNE-L/cholesterol).

Effect of pancreatic insufficiency on oxidative stress markers

Patients with or without PI showed similar plasma vitamins and GSH/GSSG ratio in their plasma. Again, plasma chromolipids were significantly higher in patients with PI compared to non-PI ones despite the vitamin supplementation compliant with international recommendations. A clear graphical description of the differences between the two groups is shown in Figure 3.

Discussion

Our data revealed that the majority of our CF patients showed elevated oxidative stress markers even in stable clinical conditions and with plasma antioxidants within the normal range. Aging led to a progressive increase of plasma oxidative stress markers in contrast with plasma antioxidant vitamins, whose availability decreased steadily. A similar inverse correlation was evidenced between plasma vitamins and oxidative stress markers with interesting and consistent results on vitamin C. Patients with pancreatic insufficiency, although well controlled, showed higher oxidative stress markers without significant differences in plasma vitamins. These data suggest the necessity of a careful definition of vitamin needs in CF patients preferably on an individual basis considering oxidative status.

The poor antioxidant status in CF patients versus healthy subjects has been widely demonstrated (1,2,3). Plasma liposoluble vitamins are usually determined once a year and deficient patients are supplemented, compliant to recommendations, in order to comply with the normality range (25,26). Our data evidenced adequate plasma levels of exogenous and endogenous antioxidants and dietetic intakes of antioxidant vitamins in almost all patients. Huang et al. have also documented that, with typical supplementation, very few children had low serum vitamin E status suggesting the necessity of dose and response studies in CF patients (7). Vitamin status is now considered more broadly, in relation to specific health outcomes. When defining the adequacy of vitamin levels as the ones within the normality range, we found some inconsistency: 40.6% of patients with suboptimal vitamin C were not detected by the reference range; with regards to the local reference range we

detected 7.2% of patients with deficient vitamin E versus 21.5% indicated by the "new" classification (23). All of these patients showed significantly higher plasma chromolipids than those with optimal levels (Table 2). The lower level of our local reference range for α-tocopherol corresponds to the 5th percentile of NHANES III (25), the same utilized by Huang as the lower cut-off value for vitamin E (7). Consistently with other studies patients with optimal vitamin E status were significantly younger and showed lower MDA-L/cholesterol (3). We can assume that higher levels of Vitamin E (12.9-18.9 mg/l) provide better protection against lipid peroxidation. None of our patients had deficient vitamin C taking no or low-dose vitamin supplementation as multivitamins (Table 2). Vitamin C levels decreased with age and MDA-L/cholesterol and HNE-L/cholesterol increased with reducing plasma vitamin as documented by previous studies (2,3,26). Reduced BMI z-score in patients with suboptimal versus optimal vitamin C may be, at least in part, attributed to the higher oxidative stress (Table 2). Other studies have demonstrated the role of ascorbic acid as a potent antioxidant (27). It has been suggested to play a key role in controlling CFTR activity modifying its red-ox state (28). There are no recommendations for vitamin C supplementation in CF patients except for subjects with an unbalanced diet (9) or general indication of a standard age-appropriate dose of multivitamins (10) and our data confirmed the importance of vitamin C monitoring and supplementation.

There were not significant differences in age, BMI z-score, number of exacerbations/year and FEV1 between groups of patients with optimal versus suboptimal or deficient vitamin A (Table 2).

We have not observed a significant reduction of vitamin A with age (ρs =-0.06, p=0.628) in contrast with Maqbool et al. (29). Mean serum retinol in that study was much higher than in ours (800μg/l) and 58% of patients had serum retinol above the NHANES reference range.

Our data failed to confirm a correlation between chromolipids and plasma retinol in our CF patients (Fig.2) as identified in other studies. Wood documented an inverse correlation between and 8-iso-PGF2α and β-carotene (2).

8-iso-PGF2α has been considered a good method to measure lipid peroxidation. During this process

a further chain cleavage yields several aldehydes, among them MDA and HNE. These aldehydes are highly reactive, thus the detection of their adducts to macromolecules, i.e. chromolipids, can provide more reliable data than other analytical methods on the extent of oxidative damage.

Consistently with other studies (3,5) our data showed a strong positive correlation of chromolipids with age (Fig.1). An overconsumption of antioxidants was also documented as a consequence of increased oxidative stress with age. We showed that higher vitamin E and C provided a reduction in plasma chromolipids (Fig.2). It has been demonstrated that high doses of fat soluble vitamins carries on the risk of toxicity, whereas the supplementation of vitamin C does not show any important side effects except for very high dosages (30).

Patients with pancreatic insufficiency showed higher plasma chromolipids (Fig.3). Interestingly, the effect persisted even when controlled for plasma vitamin A, E and C. It may not be fully explained with lipid malabsorption because it was well controlled by PERT and vitamin C is not involved. In fact we couldn't evidence any difference in fat soluble vitamins in patients with or without pancreatic insufficiency. We think it could be ascribed to the higher rate of infections (0.64 vs 1.97, p=0.010) and to the genotype (82% ΔF508) of the PI patients. Our data suggest that supplementation strategies might consider these oxidative stress markers in addition to the plasma antioxidant vitamins, at least in patients with pancreatic insufficiency. However, this statement needs to be validated through further research, with a focus in identifying reliable markers of oxidative status to be correlated to the clinical state.

The principal limitation of our study is the limited number of oxidative stress markers considered. In particular, exhaled breath or sputum analysis were not evaluated. Blood antioxidants reflect the overall capacity of the organism to afford oxidative stress burden. Moreover, the airways experienced higher oxidative stress because of the defective glutathione transport through the epithelial cells for the CFTR mutation. We assume that antioxidant vitamins exert different roles on plasma and airways. However, completing data of the present study with further studies focused on antioxidants and oxidative damage markers specific for the airways may be useful to better

understand the complex redox unbalance in CF.

CONCLUSIONS

Oxidative stress characterizes CF patients from the pediatric age and it progressively increases over the years. Reducing its harmful impact is an important clinical goal. This may be achieved by the optimization of antioxidant vitamin status, and reference ranges for healthy people provide incomplete information. The good management of red-ox balance in CF patients requires reference ranges elaborated on the basis of data from adequate control population. Aldehyde-lipid adducts may be used to characterize oxidative status and guide antioxidant supplementation on an individual basis. Definition of optimal levels of fat soluble vitamins require further studies, while obtaining higher plasma levels of vitamin C seems useful and feasible. Patients with pancreatic insufficiency need a careful monitoring of their red-ox balance because at risk of excessive oxidative stress.

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Table 1. Characteristics of study population

	Pts Nr (%)	Median (range)
Age (yrs)	70 (100%)	7.8 (1.1-19)
Sex (M/F)	32/38	
Genotype:		
Homozygous ΔF508	16 (22.9)	
Heterozygous ΔF508	33 (47.1)	
Other/Other	21 (30)	
Pancreatic insufficiency	36/70	
BMI z-score	70 (100)	- 0.03 (-4.2-2.7)
FEV1 (%predicted)	45 (64.3)	90 (33.8-118)
Supplementation		
Vitamin E (mg)	64 (91.4)	100.8 (100-300)
Vitamin C (mg)	45 (64.3)	1249.5 (624- 2499)
Vitamin A (ug)	50 (71.4)	25 (10.4- 60)

Table 2. Clinical characteristics and oxidative stress markers in patient groups with different antioxidant vitamin levels

| Vitamin E-C-A	Vitamin E (mg/l)			Vitamin C (mg/l)		Vitamin A (µg/l)		
	Optimal >12.9	Suboptimal 6.9-12.9	Deficit <6.9	Optimal >8.8	Suboptimal 1.9-8.8	Optimal >676	Suboptimal 373-676	Deficit <373
Pts Nr (%)	8 (11.4)	47 (67.1)	15 (21.5)	41 (59.4)	28 (40.6)	8 (11.4)	46 (65.7)	16 (22.8)
Age (yrs)	4.6 (1.9-7.9)*	8.8 (1.1-19)*	7.0 (1.1-18)*	7.4 (1.1-14.9)*	12.6 (1.1-19)*	7.3 (1.1-19)	7.6 (1.1-18)	8 (3.2-18)
Exacerbations/year	1.1 (0-4)	1.3 (0-12)	1.4 0-8)	1.2 (0-7)	1.1 (0-12)	0 (0-2)	0 (0-7)	1.5 (0-12)
BMI Z-score	0.2 (-1.5-1)	0.01 (-2.7-2.7)	-0.6 (-4.2-1.7)	0.2 (-2.8-1.9)*	-0.5 (-4.2-2.7)*	0.2 (-2.6-1.9)	0.01 (-2.8-2.7)	-0.7 (-4.2-1.7)
FEV1 (%predicted)°	92.6 (91-95)	93.5 (34-118)	82.4 (44-100)	90.6 (40.3-118)	85.1 (33.8-112)	92.0 (85-118)	93.5 (40-112)	82.4 (34-111)
HNE-L/cholesterol (UF/mg chol)	1.6 (1.0-2.3)	1.5 (0.5-3.2)	1.9 (1.1-2.9)	1.4 (0.5-3.2)*	1.9 (0.8-2.9)*	1.2 (0.5-1.8)	1.5 (0.9-3.2)	1.9 (0.8-2.9)
MDA-L/cholesterol (UF/mg chol)	0.8 (0.5-1.5)	0.8 (0.3-3.8)	0.9 (0.5-2.6)	0.8 (0.3-3.8)*	0.9 (0.5-3.3)*	0.8 (2.3-1.1)	0.8 (0.5-3.8)	0.9 (0.5-2.7)
ErGSH/GSSG	3.3 (1.6-6.5)	4.1 (1.3-13.6)	3.2 (2.6-9.7)	3.3 (1.3-13.6)	4.4 (2.3-11.1)	5.5 (1.9-9.7)	3.8 (1.2-13.6)	4.4 (2.1-11.4)

All values expressed as median (range); ° mean value of determinations in last year-data available in 45/70pts; *P<0.05

Figure 1. Effect of age on plasma chromolipids
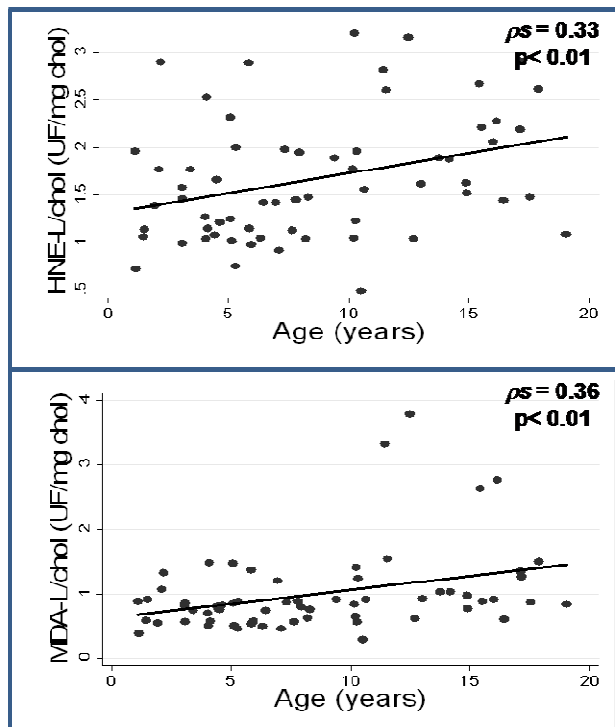
4-hydroxynonenal and malonaldehyde are expressed as HNE-L/mg cholesterol and MDA-L/mg cholesterol respectively. ρs: Spearman rank correlation coefficient;

Figure 2. Correlation between chromolipids and antioxidant vitamins plasma levels
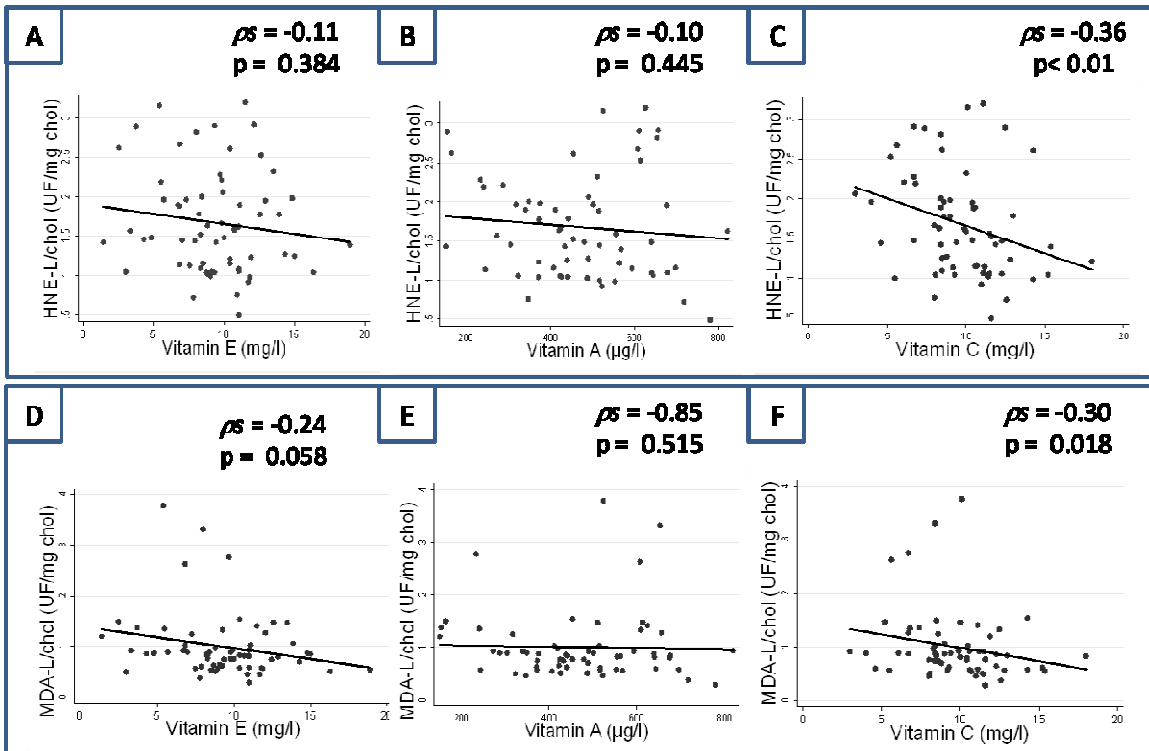
Upper panel: correlation between 4-hydroxynonenal (HNE-L/cholesterol) and plasma levels of vitamin E (A), vitamin A (B), vitamin C (C). Lower panel: correlation between malonaldehyde (MDA-L/cholesterol) and plasma levels of vitamin E (D), vitamin A (E), vitamin C (F). ρs: Spearman rank correlation coefficient

Fig. 3. Antioxidant vitamins and oxidative stress markers in presence and absence of PI

Plasma concentrations	Non-PI patients	PI patients	P value
Nr of patients (%)	34 (48.6)	36 (51.4)	
Vitamin E (mg/l)	9.1 (3.0-18)	9.4 (1.4-15)	0.455
Vitamin C (mg/l)	9.4 (3.0-18)	9.9 (5.2-13)	0.787
Vitamin A (ug/l)	467 (324-784)	449 (151-821)	0.745
MDA-L/cholesterol (UF/mg)	0.68 (0.46-2.63)	0.92 (0.29-3.78)	**0.002**
HNE-L/cholesterol (UF/mg)	1.22 (0.75-2.67)	1.77 (0.49-3.20)	**0.004**
ErGSH/GSSG	2.04 (2.14-11.4)	3.61 (1.25-13.6)	0.501
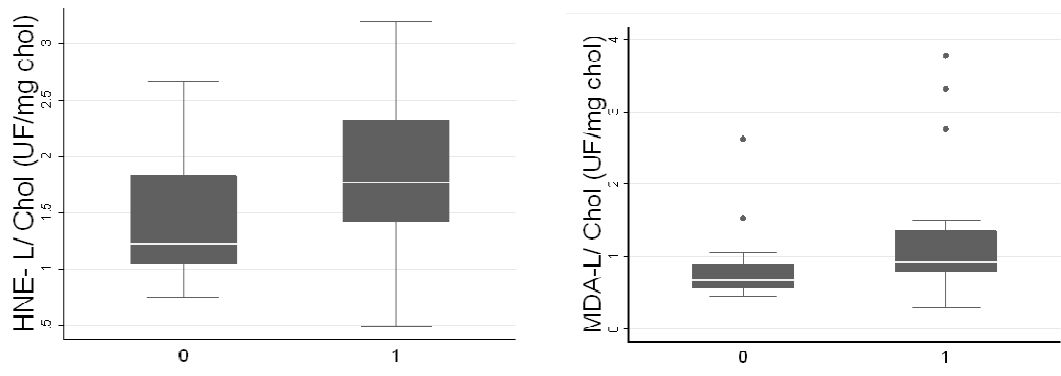
Chromolipids in patients with PI (1) and without PI (0): HNE-L/cholesterol (left panel) and MDA-L/cholesterol (right panel)