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Alterations in the Oxidation Products, Antioxidant Markers, Antioxidant Capacity and Lipid Patterns in Plasma of Patients Affected by Papillon-Lefèvre Syndrome

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Papillon-Lefèvre syndrome (PLS) is an uncommon disease. Less than 300 cases have been described. PLS is characterized by the association between palmar plantar hyperkeratosis (PPK) and severe precocious periodontitis that results in the premature loss of both the primary and secondary dentitions. It is known that periodontitis (PE), the destructive phase of periodontal disease, is a multifactor phenomenon involving a variety of molecular species, among them free radicals and reactive oxygen species (ROS). Antioxidants have been shown to play a critical role in modulating ROS-induced damages during PE. We wondered if patients belonging to a family group with different grades of PLS severity may present altered plasma concentrations of oxidation products as well as of lipophilic antioxidants, like Coenzyme Q or vitamin E, which are molecules that possess well-known antioxidant properties and could play a role in PE processes. We also wondered about the actual plasma total antioxidant capacity of these subjects as well as a complete identification of their plasma fatty acids features, which have been never investigated before. The results we obtained indicate an impairment in the antioxidant capacity of the subjects characterized by abnormally high hydroperoxide levels and, in some cases, by altered CoQ and vitamin E contents. Moreover, an essential fatty acid deficiency (EFAD) was registered on the basis of the peculiar plasma fatty acid patterns found (i.e. low PUFA, high MUFA and high Δ-9 desaturase activity). This finding would support the hypothesis by Gutteridge and co-workers (*Free Radic. Res.* 1998, **28**: 109–114) that conditions exist in which some forms of oxidative stress can lead to changes in fatty acids patterns characteristic of EFAD.

Keywords: Hydroperoxide; Cholesterol; Coenzyme Q; Plasma lipoprotein

INTRODUCTION

Papillon-Lefèvre syndrome (PLS) is a very rare disease first described in 1924.[1] The syndrome is thought to be the homozygous expression of an autosomal recessive trait transmitted with an estimated frequency of 1–2 persons per million. PLS has been reported to occur in a diverse range of ethnic groups and parental consanguinity has been noted in more than half of the cases.[2] Less than 300 cases have been described. Recently, PLS has been grouped with the palmoplantar ectodermal dysplasias. Clinically, PLS (Mendelian Inheritance in Man No. 245000[3] is characterized by the association between palmar plantar hyperkeratosis (PPK) and severe precocious periodontitis resulting in the premature loss of both the primary and secondary

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dentitions. PLS dermatological symptoms begin prior to 2 years of age and continue throughout life. The periodontal disease associated with PLS is particularly aggressive and unresponsive to traditional periodontal therapies. Destruction of the oral tissues in PLS is somewhat unique; the process is characterized by severe inflammation of the gingival/periodontal tissues, destruction of collagen fibers of the periodontal ligament, and alveolar bone loss, resulting in loss of teeth. Onset is reported to begin at the time of eruption of the primary dentition through the periodontium. After exfoliation of the primary dentition, the gingival tissues return to a noninflamed state. However, upon eruption of the permanent dentition, the cycle of severe gingival inflammation, alveolar bone loss, and tooth exfoliation recurs. When teeth are not present, gingival inflammation is unremarkable, and destruction of the periodontium ceases. The genetic defect in PLS appears to profoundly alter host susceptibility to periodontal destruction. The molecular basis for this increased susceptibility for periodontal destruction is unknown.

On the other hand, it is known that periodontitis (PE), the destructive phase of periodontal disease, is a multifactor phenomenon characterized by the presence of a variety of molecular species, among them free radicals and reactive oxygen species (ROS). ROS are essential in a number of metabolic pathways but their excessive production can result in cell damage.[4] Bullon *et al.*[5] found that neutrophil function was impaired in subjects affected by PLS as well as their superoxide anion production. Antioxidants have been shown to play a critical role in modulating ROS-induced damage during PE. We wondered if patients belonging to a same family with different grades of PLS severity may present altered plasma concentrations of oxidation products as well as of lipophilic antioxidants, such as Coenzyme Q or vitamin E, which are molecules that possess well-known antioxidant properties and could play a role in PE processes. We also wondered about the actual plasma total antioxidant capacity of these subjects as well as a complete identification of their plasma fatty acids features, which have never been investigated before.

MATERIALS AND METHODS

Chemicals

6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2′-azinobis-(3-ethylbenz-thiazoline-6-sulfonic) diammonium salt (ABTS), were obtained from Sigma–Aldrich. All other chemicals and solvents used were of analytical or HPLC grade and were purchased from FLUKA.

Clinical Cases

The clinical aspects of the studied family were published previously.[5] The genealogical family tree is indicated in the Fig. 1.

They presented different degree of oral and skin manifestations. The father and the mother were unaffected and revealed no apparent parental consanguinity. Two sisters had dermatological and periodontal manifestations, Two brothers had only dermatological manifestation, one brother and one sister were healthy. The two affected sisters did not respond to the periodontal treatment and lost all their teeth. During the last few years none of the members of the family suffered any kind of variation in their oral and dermatological conditions. Some of them were treated with retinoids, with limited effects on the skin lesions. Some years ago the oldest sister moved to another city (800 km away from her original home),

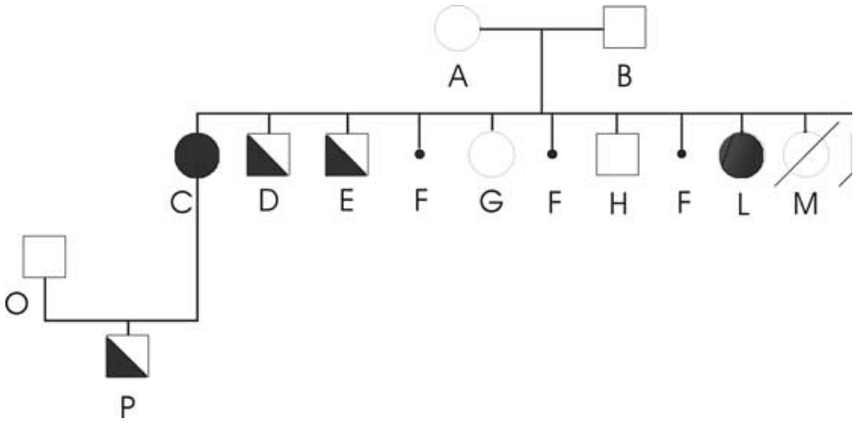

FIGURE 1 Genealogical family tree where circles stand for female and squares for male individuals. Open signs indicate healthy subjects, full signs indicate PLS subjects, ◣ indicates PPK. Two children died stillborn (M and N) and there had been three abortions (F).

married and had a child. The husband is clinically healthy and does not present any oral and skin manifestations.

The child (case P), 3 years old, presents skin disorders without oral symptoms. He has hyperkeratotic lesions in the palmoplantar region and on his knees.

Plasma Samples

Plasma samples were obtained with the informed consent of the individuals. Blood samples were collected by forearm venipuncture after 12 h overnight fasting, without stasis in the sitting position; blood (10 ml) was drawn into EDTA-containing tubes (1 mg/ml). Plasma was isolated by centrifugation at 1000*g*, for 10 min.

Coenzyme Q and Vitamin E Assays

CoQ and vitamin E were assayed by means of HPLC methods assessed as modification of methods we successfully developed in the last years.[6]

The HPLC system consisted of a Beckman Model 126 pump, a Rheodyne model 7125 valve fitted with a 20 μl loop, a stainless steel column 15 cm long 4.6 mm I.D. packed with 3 μm ODS Supelcosil from Supelchem, an ESA Coulochem model 5100 A electrochemical detector and a model 5011 Analytical cell. Chromatograms were integrated with Model 4290 Varian integrator. Mobile phase consisted of lithium 20 mM perchlorate, 10 mM perchloric acid, 20% ethanol, 80% methanol; electrode 1 was set at −0.5 V, electrode 2 was set at +0.35 V. Retention time = 12 min.

Briefly, 50 μl of the sample were precipitated with 150 μl of isopropanol and vortexed for 60 s. After centrifugation at 10,000 rpm for 10 min in a bench top centrifuge for eppendorf vials, 20 μl of supernatant was injected into the HPLC.

Data are the mean values of at least five different extractions and analysis procedures.

Trolox Equivalent Antioxidant Capacity (TEAC)

The plasma antioxidant activity was evaluated according to Re *et al*.[7] This method is based on the ability of antioxidants to quench the long-lived ABTS radical cation, a blue/green chromophore with characteristic absorption at 734 nm, in comparison to that of Trolox, a water-soluble vitamin E analogue. The ABTS radical cation was prepared by reacting a 2 mM aqueous solution of ABTS with 0.7 mM potassium persulfate (final concentration). After addition of 1.0 ml of this solution to aliquots of Trolox or samples, the absorbance reading was taken in a temperature-controlled spectrophotometer cuvette at 30°C exactly 1 min after initial mixing. Appropriate solvent blanks were run in each assay. Addition of antioxidants to the preformed radical cation reduces it to ABTS determining a decolorization. The extent of decolorization as percentage inhibition of ABTS radical cation is determined as a function of concentration and calculated relative to the reactivity of Trolox and were expressed as the Trolox equivalent antioxidant capacity (TEAC), defined as the concentration (mmol/l) of Trolox having the equivalent antioxidant activity to a 1.0 mmol/l solution of the substance under investigation.

Lipid Hydroperoxides (HP)

The ferrous-oxide xylenol orange method (FOX2) was used for determining HP; i.e. HP levels were assayed according to the principle of the rapid peroxide-mediated oxidation of Fe^{2+} to Fe^{3+} under acidic conditions,[8] slightly modified[9] using tryphenylphosphine (TPP), an agent that avoids artifactual color generation in samples which contain substantial quantities of loosely available iron. Briefly, plasma (50 μl) was incubated at 37°C for 30 min with and without 1 mM TPP. FOX2 reagent was then added to each sample and incubated again at 37°C for 30 min in a water shaking bath. After centrifugation (2000*g* for 5 min) the supernatants were spectrophotometrically monitored at 560 nm.

Data are the mean values of three different assays performed in triplicate.

Blood Lipid Assays

Total cholesterol (TC), LDL and HDL cholesterol (LDL-C and HDL-C) and triglycerides (TG) were assayed in plasma samples by a CX-Synchron apparatus (Beckman) using enzymatic kits.

Plasma Fatty Acid Assay

Plasma fatty acid profiles were measured by gas liquid chromatography as described by Lepage and Roy.[10] A gas-liquid chromatograph Model HP-5890 Series II (Hewlett Packard, Palo Alto, CA, USA) equipped with a flame ionization detector was used to analyze fatty acids as methyl esters. Chromatography was performed using a 60 m long capillary column; 32 mm id and 20 mm thickness impregnated with Sp 2330™ FS (Supelco Inc. Bellefonte, Palo Alto, CA, USA). The injector and the detector were maintained at 275 and 275°C, respectively; nitrogen was used as carrier gas, and the split ratio was 29:1. Temperature programming (for a total time of 40 min) was as follows: initial temperature, 160°C for 5 min, 6°C/min to 195°C, 4°C/min to 220°C, 2°C/min to 230°C, hold 12 min, 14°C/min to 160°C.

Plasma fatty acids patterns were calculated on a percentage basis as well as the relative contents of saturated fatty acids (SFA), unsaturated fatty acids (UFA), monounsa2turated fatty acids (MUFA), polyunsaturated fatty acids (PUFA). The unsaturation index (UI) considers all the double bounds existing in 100 fatty acids molecules and the essential fatty acid index (EFA index) is a ratio calculated to estimate possible essential fatty acid deficiency (EFAD).

RESULTS AND DISCUSSION

The first interesting contribution of this investigation concerns, unfortunately, the partial expression, for the time being, of the syndrome in a 3-year-old child of the third generation (case P). PLS is commonly and currently considered an autosomal recessive disorder that usually takes place in case of consanguinity. Taking into account the history of the family, and particularly of the child's parents, it was unlikely that any kind of PLS expression could take place in the child. Possible manifestations of heterozygosity in PLS has already been discussed.[11] Since the PLS locus has been recently mapped to chromosome 11q14–q21,[12,13] an exhaustive genetic investigation of both families of the child's parents should be necessary. However, it has also been pointed out that probably the antioxidant capacity of the subjects was impaired, with specific importance only in the child. It has not been demonstrated that the pathological phenotypic features of PLS patients is a consequence of such impairments but in any case both aspects seem to be associated, at least in the family studied.

Data concerning lipid composition of plasma have been collected in Table I. TC values were normal in all adult individuals with the only exception of case B. HDL-C values were in the normal range for both male and female adult individuals except in case C. Finally, case P (the 3-year-old child) showed low TC levels and very low HDL-C levels. LDL-C and TG were normal in all the subjects. These data seem to indicate that adult individuals were characterized by an acceptable lipid equilibrium whereas the child seems to be affected by some kind of impaired lipid metabolism and his mother also presented some alterations. This idea is confirmed when specific indexes, like the TC/HDL-C ratio or the LDL-C/vitamin E ratio are taken into account. TC/HDL-C ratio is commonly regarded as a risk factor for atherosclerosis and closely related pathologies (e.g. infarctual events and strokes) and is widely considered as a typical index of the plasma lipid imbalance. On the other hand, vitamin E is an essential constituent of LDL where it is the most abundant and active antioxidant.[14] The oxidation of PUFA in LDL occurred after a significant drop in vitamin E[15] or as an effect of its prooxidant activity when specific "co-antioxidants" like CoQ_{10} failed.[16] Therefore, LDL-C/vitamin E ratio might represent another interesting marker in human plasma as has already occurred for the LDL-C/CoQ_{10} ratio which was observed to be higher in subjects affected by coronary heart disease[17] or diabetic patients with signs of increased levels of oxidative stress.[18] In our case, the child (case P) had the highest values for both indexes and his mother (case C) displayed the second highest value for TC/HDL-C ratio. These data are even more interesting when lipophylic antioxidants (CoQ_{10} and vitamin E) and lipid hydroperoxide levels are concomitantly considered (Table II). Again, plasma of cases P and C possessed the lowest CoQ_{10} contents (being both below the reference values: 0.51 and 0.45 μg/ml, respectively) and case P had also the lowest vitamin E, far below normal reference range (4.6 μg/ml). As far as CoQ_{10} is concerned it should be underlined that also case A (0.56 μg/ml) and cases G and O

TABLE I Lipid composition of plasma

Subject	TC	HDL-C	LDL-C	TG
A	177	51	110	77
B	207	54	146	133
C	164	37	108	93
E	125	35	73	82
G	163	58	88	81
H	146	52	69	123
L	165	47	101	82
O	175	47	102	127
P	99	18	57	119
Reference values	60–200	34–58 males, 43–73 females	20–160	35–180

TC, HDL-C, LDL-C and TG values are expressed in mg/dl. The letters indicating the different subjects are as in Fig. 1.

TABLE II Antioxidants, Trolox equivalent antioxidant capacity and lipid hydroperoxides of plasma

Subject	CoQ	Vit. E	TEAC	HP
A	0.56	11.5	1.45	0.132
B	0.99	15.4	1.41	0.179
C	0.45	13.1	1.18	0.299
E	0.99	10.8	1.35	0.177
G	0.65	10.8	1.34	0.081
H	1.04	10.3	1.24	0.102
L	0.78	9.8	1.39	0.192
O	0.65	13.2	1.38	0.185
P	0.51	4.6	1.08	0.283
Reference values	0.7–0.9[30]	7–17[31,32]	1.4–1.8[18,33]	0–0.080*

CoQ, vitamin E values are expressed in μg/ml. TEAC values are expressed in mM Trolox and HP values are expressed in mM. The letters indicating the different subjects are as in Fig. 1.
*Data from authors' laboratory.

(both with 0.65 μg/ml) were characterized by Coenzyme Q concentrations lower than the known reference values. A similar value distribution pattern was identified also for HP content. The highest HP concentrations were detected in the mother of the child of the third generation, case C (0.299 mM), and in her son, case P (0.283 mM). However, it is worthwhile to underline that all other relatives presented extremely high HP contents with the single exception of case G (0.081 mM).

The confirmation that some kind of antioxidant imbalance took place in these PLS subjects comes from TEAC values. Again, cases C and P possessed the lowest values which were far below the reference ones and all other subjects were under reference values or borderline.

In recent years, it has become increasingly apparent that ROS may play a role in deregulating a variety of physiological systems, the impairment of which can lead to a status of disease (rheumatoid arthritis, atherosclerosis, etc.). Several hyperkeratotic syndromes have also been related to altered antioxidant levels in patients' plasma and finally both psoriasis and oral leukoplakia were successfully treated by antioxidant administration.[19–21] The characteristic clinical features of PLS (PPK and PE) are conditions that could be related to, or involved in, modification of the critical balance between endogenous ROS production and antioxidant levels or activities. The results we obtained are sufficiently clear and demonstrated that the subjects we investigated are characterized by abnormally high HP levels and that in some cases CoQ contents could also be altered. HP in PLS subjects resulted up to 10 fold (cases C and P), which are the average values usually detected in a healthy control population. Atherosclerosis, congestive heart failure and other cardiovascular disease as well as drugs or physical exercise could enhance, even drastically, HP in plasma and other blood constituents (i.e. lipoproteins, erythrocytes, etc.). Control values from our laboratory have never exceeded 80 μM (0.032 ± 0.011 μM, $n = 188$). It is interesting that the highest HP level was displayed by case C who has complete manifestations of PLS and is the mother of case P, characterized by the second highest HP level. Moreover, case C possessed the lowest CoQ level and case P the second lowest level. On the other hand, concerning HP levels, cases G and H, who are phenotypically healthy, appeared to be the least jeopardized.

It is possible that some impairment could affect lipoprotein domain. In fact, HP assays evaluate the presence of oxidative modification in the hydrophobic domain of circulating lipoproteins and CoQ is an essential antioxidants of these lipid vehicles as mentioned above. This situation could have dramatic consequences on the quality of life of the subjects as well as be the first step for developing, at older ages, other severe pathologies related to lipoprotein oxidative modification.

The antioxidant imbalance detected seems even more interesting if we consider that the plasma fatty acid patterns found are peculiar in all subjects (Table III). In fact, PUFA n-3 levels were low in all individuals and they were lower than control values when compared with either Spanish,[22] German[23] or US[24] population. Moreover, cases C and P displayed surprisingly low total PUFA content and in case P this fact was associated with concomitantly high MUFA levels. The relationship between SFA and UFA did not vary drastically from a normal population but the rearrangements found in the unsaturated fraction regarding cases C and P can be summarized as follows: (i) decreased PUFA (mainly highly PUFA), (ii) increased MUFA and (iii) Δ9-desaturase activity index also high. An EFAD may be suspect. In fact, if the [n-7 (palmitoleic acid + cis vaccenic acid 16:1 + 18:1)]/[18:2 n-6] ratio is calculated to estimate possible EFAD conditions,[25] the values obtained are higher than the usual upper limit (0.2) considered as reference.[26] In addition, the amount of vitamin E found in case P is very similar to that detected in EFAD children of the same age.[26]

It should be underlined that rats fed fat-free diets which induce EFAD symptoms are more noticeable from the development of dermatitis and that they generally show a loss of hexaenoic and pentaenoic acids in plasma and in tissue and extremely low levels of linoleic acid, whereas 20:3 (ω-9), often called mead acid, oleic, and palmitoleic (ω-7) acids usually increase. Studies carried on humans show similar changes.[27] Therefore, the possible EFAD condition of PLS subjects would accompany (or be part of) the threat constituted by high HP content. These data are in agreement and would support the hypothesis by Gutteridge *et al.* that several conditions exist in which some form of oxidative stress can lead to changes in fatty acid patterns characteristic of EFAD.[28] The suggestion that the production of lipid peroxidation, resulting from tissue damage, may serve as signal molecules for regeneration or repair[29] was extended to desaturase enzymes: depending on the level of oxidative stress, desaturase enzymes might be "activated" or "inactivated" by products of PUFA peroxidation.[28] The loss of ω-3 and ω-6 PUFA by oxidative stress-induced peroxidation would provide the signal for Δ9-desaturase activity which in turn can convert palmitic and stearic acids, respectively, into 16:1 ω-7 and 18:1 ω-9 fatty acids. In fact, the increased synthesis of oleic acid during situations of oxidative stress is often significant and probably represents an adaptive response to loss of PUFA. Thus, it is possible that at least the PLS subjects studied would be an example of partially oxidative stress-dependant EFAD patients (Table III).

TABLE III Fatty acid composition and parameters of plasma (fatty acid amount is expressed in % of total fatty acids)

Subject	SFA	UFA	MUFA	PUFA	PUFA n-6	PUFA n-3	EFA index	PUFA/MUFA	UI
A	39.00	61.00	21.66	39.34	36.44	2.68	0.66	1.82	118.64
B	34.78	65.22	27.74	37.48	35.31	1.88	0.93	1.35	120.61
C	43.97	56.03	24.11	31.92	29.96	1.75	0.91	1.32	102.49
E	33.82	66.18	24.16	42.02	38.09	3.62	0.73	1.74	131.09
G	33.80	66.20	25.85	40.35	37.55	2.48	0.77	1.56	125.51
H	36.51	63.49	27.07	36.42	33.12	2.92	1.01	1.35	122.27
L	36.15	63.85	27.66	36.18	33.07	2.76	0.97	1.31	120.42
O	35.17	64.83	23.19	41.64	38.79	2.52	0.69	1.80	127.06
P	39.80	60.20	31.85	28.35	26.29	1.71	1.33	0.89	100.02

For these reasons, a specific antioxidant therapy, in conjunction with an appropriate essential fatty acid intake, could be a promising approach in treating at least those PLS subjects who are characterized by imbalance in both oxidation product/antioxidant concentrations and essential fatty acid pattern; such approach may also have a role in partially preventing the pathological state.

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