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ORIGINAL ARTICLE

Inflammatory involvement into phototoxic reaction in erythropoietic protoporphyria (EPP) patients

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Abstract

Phototoxic reaction is a known feature of EPP at least in part triggered by the oxidative status, complement system activation, and mast cell response. The aim of this study was to verify some aspects involved in phototoxic reaction during a season. The complement system was evaluated by C3 assay, alternative pathway by factor-B, and classical pathway by C1q; oxidative status was tested with malondialdehyde (MDA) and mast cell by IL-10 assay. The serum samples were collected in winter and summer from 19 EPP patients and 13 controls. The reaction to sun exposure within each group was monitored without any invasive treatment. In summer, C3 and factor B were higher in patients than in controls ($p = 0.002$ and < 0.0001 respectively), while no change was detected for C1q. The oxidative stress was increased in summer in comparison with the control group ($p = 0.04$), and IL-10 an assay was normal in both seasons. The correlation between the C3 and factor-B in summer was significant. This study shows that the phototoxic reaction is not limited to the dermis but can also exert a systemic response, which could affect the general health of a patient. The knowledge of the pathophysiology of phototoxic reaction is essential for identifying new disease markers useful for improving clinical studies of known and future drugs.

Keywords Erythropoietic protoporphyria · Oxidative status · Complement system · Alternative pathway · Phototoxic reaction

Introduction

Erythropoietic protoporphyria (EPP; MIM 177000) is a rare disorder characterized by severe cutaneous phototoxic reactions due to the accumulation of protoporphyrin-IX (PPIX) in erythrocytes and tissues. The ferrochelatase gene (*FECH*; gene ID 2235) is affected by a loss of function mutation in *trans* with a second low-expression allele pathogenic variant c.315–48T>C, which results in the decreased activity of ferrochelatase enzyme (FECH; EC 4.99.1.1) to less than 30% compared to the normal FECH activity [1–3].

About 2% of EPP patients are affected by X-linked protoporphyria (XLP; MIM 300752) with the same phenotype, but they carry gain-of-function mutations in the erythroid-specific form of 5-aminolevulinate synthase 2 (ALAS2; EC 2.3.1.37) [4].

The symptoms include burning, acute skin pain, and itching causing edema that can worsen at increased sun exposure leading to permanent chronic wounds, as skin lesions, and hyperkeratosis. These symptoms could arise at different time periods and are closely related to the heterogeneity of the weather conditions and affect the quality of life of the patients [5–7].

The biological process that leads to a phototoxic reaction in EPP and XLP patients has not yet completely elucidated. It is widely known that the phototoxic reaction occurs because of the increased level of reactive oxygen species (ROS) in the derma that leads to endothelial cell photo-damaged [8] through complement system activation and mast cell degranulation culminating into exocytosis of vasoactive mediators and acute inflammation [9].

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At the experimental level, dosages on C3 and C5 proteins were performed in vivo or in vitro, after direct UV irradiation of the skin [10] or the plasma samples [11] or cells [12] that caused an acute phototoxic reaction.

However, it has not yet well established if this activation is contributed from any of the three complement system pathways: classical pathway (CP), alternative pathway (AP), or lectin pathway (LP) or by spontaneous hydrolysis. Moreover, there are no C3 values available on EPP patients collected during winter (steady state) and in summer (subacute), when the patients are exposed but not still experiencing the strong classical symptoms linked to the phototoxic reaction.

This study was carried out to assess the three molecular mechanisms that lead to the phototoxic reaction: (a) the assessment of the complement system proteins by different assays: C3 to confirm whether there was or not a lithic activity, C1q specific for CP, and factor B specific for AP to understand which complement system pathways are involved [13]; (b) oxidative status by malondialdehyde assay (MDA) that is shown in the literature as one of most commonly and widely used biomarkers for evaluation of lipid peroxidation (LPO), which is the direct consequence of ROS increase [14–16]; and (c) the evaluation of mast cell contribution through the level of the anti-inflammatory interleukin (IL-10), as an active response against chronic UVB irradiation in the skin [17].

Material and methods

Patients

The study was performed on 19 EPP patients (9 males, 37 ± 10.4 years and 10 females, 38 ± 10.2 years) and 13 control individuals aged matched and randomly collected during the seasons. The blood samples were collected at one point in winter (January and February 2017) and summer (June, July, and the first week of August 2017) to verify the reaction to the light exposure in each group, without any invasive treatment. According to the World Medical Association Declaration of Helsinki for medical research, all subjects involved in this study signed informed consent for the diagnosis and research approved by the ethics committee of our institution, Fondazione IRCCS "Cà Granda" – Ospedale Maggiore Policlinico, and the identity of the study participants was anonymized.

Laboratory testing C3, factor-F, C1q, MDA, and IL-10 assays

Serum was separated from whole blood by centrifuging at 10,000 rpm and refrigerating temperature for 10 min and stored at − 80 °C. The C3 complement system and factor B were determined by quantitative sandwich ELISA kit (MyBioSource, San Diego, USA). C1q serum was evaluated by an ELISA kit (Hycult Biotech, PB Uden, Netherlands). LPO–Malondialdehyde (MDA), as a marker of oxidative stress, was determined colorimetrically in serum using a Bioxytech LPO–586 kit (Oxis International Inc., Portland, OR, USA), and the serum level of IL-10 was determined by the ELISA method using the biotin/streptavidin system (IBL International—Hamburg, Germany).

The tests for each marker were performed in two different samples collected in winter and summer from the EPP patients and compared to values registered in healthy subjects.

Levels of protoporphyrin-IX

Erythrocyte-free protoporphyrin-IX (PPIX) and zinc protoporphyrin (ZPP) were measured after extraction with acetone and 4% aqueous formic acid as previously described (4). The filtered samples (0.45 μm cellulose filters, 30 mm diameter, National Scientific, Rockwood, TN) were analyzed by high-performance liquid chromatography (quaternary pump Agilent Technologies Series 1200, Agilent Technologies, Santa Clara, CA, USA) equipped with a fluorescence spectrophotometer (G1321A Model, Agilent Technologies series 1200) and an 8-μL cell. The separation was achieved using a C18 reversed-phase column (Chromsytems 44100, Chromsystems Instruments and Chemicals GmbH, Gräfelfing, Germany, dimensions 15 cm × 4.6 mm ID, 3 μm) with a Chromolith® guard column. A gradient of methanol and 1% aqueous acetic acid was used as eluent. ZPP was detected at 30.5 min, setting the fluorescence spectrophotometer at 400 nm as the excitation wavelength (λ_{ex}) and 620 nm as the emission wavelength (λ_{em}); PPIX was detected at 33.7 min with λ_{ex} 387 nm and λ_{em} 633. The photomultiplier gain was set at 17. The quantification was performed using a calibration curve and was prepared by the pure chemicals (from Frontier Scientific Porphyrin Product, Logan, UT) dissolved in dimethylformamide and then in a mixture of acetone, water, and formic acid. The concentration of each erythrocyte porphyrin was adjusted by hemoglobin (Hb). The limit of quantification of the assay was 0.5 μg/g Hb for both PPIX and ZPP.

Statistical analysis

The data analysis was performed using GraphPad Prism software version 7. D'Agostino–Pearson's normality test, Shapiro–Wilk normality test, and the KS normality test were applied to confirm the normality of the data from each measurement. In order to identify the outliers, ROUT (1%) test was performed for each experiment. Statistical analyses, between season (winter vs. summer) and controls, were performed using unpaired or paired

parametric *t* tests and Mann–Whitney *U* test. The Pearson correlations were determined between two datasets with two-tailed and a confidence interval of 95%. Linear regression was also calculated at a confidence interval of 95%.

Results

C3 and factor B showed a summer increase compared to winter EPP samples and healthy subjects

C3 in summer (C3-S) showed a significant difference compared to C3 winter (C3-W) (< 0.0002) as well as C3-S vs. C3-CTRL ($p = 0.002$). On the contrary, no difference was detected between C3-W and C3-CTRL ($p = 0.5$) (Fig. 1b). Moreover, a positive correlation ($r = 0.67$, $p = 0.002$) between C3-W and C3-S was observed (Fig. 1c). The alternative pathway of the complement system was evaluated by the factor B level. It is interesting to note that the comparison between factor B level in winter (FB-W) and factor B in summer (FB-S) showed a significant difference ($p = 0.002$). Moreover, the FB-S level was markedly different from that in the healthy subjects (FB-CTRL) ($p = 0.0002$), while marginally significant between FB-W and FB-CTRL ($p = 0.07$) was found, probably linked to the wide biological variability among the subjects and to the small number of samples being a rare disease (Fig. 2b). The factor-B level in the same patients in the two different seasons showed a positive trend of correlation ($r = 0.45$, $p = 0.06$) (Fig. 2c).

The classical pathway of the complement system was evaluated through the C1q level. No difference was observed between the two seasons, and no correlation was detected (Fig. 2e–f).

Moreover, the assays that showed a significant increase between the two seasons, in particular, C3-S vs. FB-S, were found to have a very significant correlation ($r = 0.61$, $p = 0.008$) (Fig. 3c, d).

Correlation of PPIX between the complement system proteins

The correlation between PPIX and the CS proteins in summer samples showed a significant negative tendency for FB-S. At a high level of PPIX, the patients showed a low level of this protein ($r = -0.55$ $p = 0.02$) (Fig. 3b). Unlike FB-S, the correlation between C3-S and PPIX showed a random distribution (data not shown).

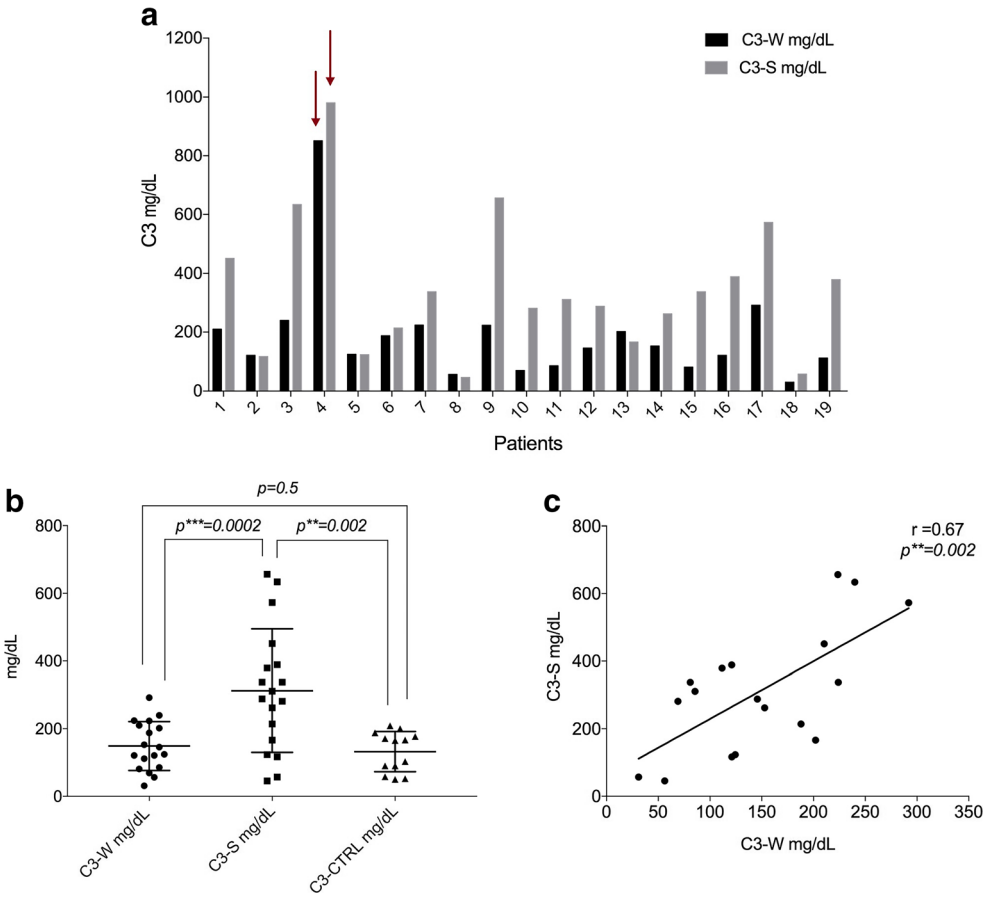

Fig. 1 C3 assay. **a** Raw data for every sample in winter (black) and summer (gray). The arrow points to the value in winter and summer of sample 4 considered outlier. **b** Distribution of the mean levels and ± DS of C3 in winter (C3-W $\mu = 148.9 \pm 72.4$) and summer (C3-S $\mu = 312.5 \pm 182.5$) for EPP patients and the control group (C3-CTRL $\mu = 132.3 \pm 53.5$). The *t* test showed some variations between C3-W and C3-S; moreover, the same significant difference was seen between C3-S and C3-CTRL. The *p* values are shown on the top of the graphic. **c** Positive correlation between the seasonal values in EPP patients ($r = 0.67$, $p = 0.002$)

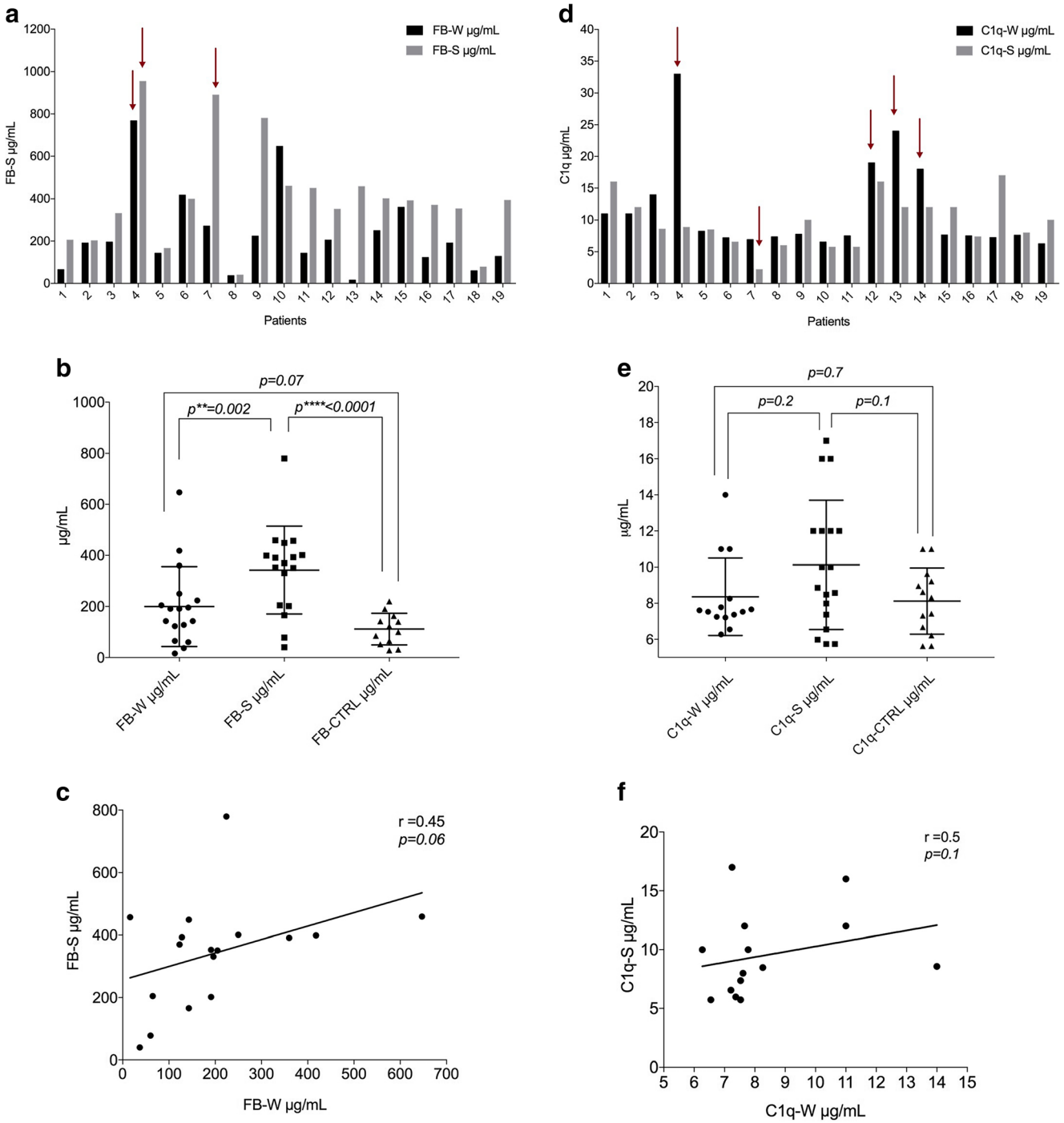

Fig. 2 Alternative pathway factor B assay and classic pathway factor C1q assay. **a** Raw data for every patient in winter (black) and summer (gray). The arrow points to the value considered outlier in winter and summer of sample 4 and summer of sample 7. **b** Distribution of the mean levels and ± DS of factor B in winter (FB-W $\mu = 182 \pm 101$) and summer (FB-S $\mu = 322.9 \pm 120.5$) for EPP patients and the control group (C3-CTRL $\mu = 111.6 \pm 62.5$). The *t* test showed some variations between FB-W and FB-S; the same level of significant difference was seen between FB-S and FB-CTRL. The *p* values are shown on the top of the graphic. **c** Correlation between seasonal values in EPP patients showing an increasing positive trend for the levels in our population. **d** Raw data from every patient in winter (black) and summer (gray). The arrow points to the value considered outlier in winter of samples 4, 12, 13, and 14 and summer of sample 7. **e** No significant difference was demonstrated between C1q-W ($\mu = 8.53 \pm 2.17$) vs. C1q-S ($\mu = 10.4 \pm 3.92$) in EPP patients and control ($\mu = 8.12 \pm 1.8.12$); the *p* values are shown on the top of graphic. **f** Correlation between seasonal values in EPP for C1q values

MDA is increased in EPP patients during summer

The levels of MDA in winter (MDA-W) and summer (MDA-S) EPP samples were compared with those measured in the samples collected from a group of healthy subjects (MDA-CTRL). A significant difference ($p = 0.04$) was observed comparing MDA-S-EPP vs. MDA-CTRL (Fig. 4b). Winter values do not show significant changes compared to control and summer values ($p = 0.07$). Moreover, the correlation between the levels of MDA registered in EPP patients in different seasons showed a positive trend ($r = 0.44$, $p = 0.07$) (Fig. 4c).

IL-10 is not activated in phototoxic reaction

The values of IL-10 in winter (IL10-W) and summer (IL10-S) did not show any difference ($p = 0.5$). IL10-W vs. IL10-CTRL and IL10-S vs. IL10-CTRL gave the same result ($p = 0.7$). Moreover, no correlation between the two seasons was detected (results not shown).

Discussion

The pathophysiological mechanism of phototoxic reaction in EPP is not yet completely studied. Our findings confirm the involvement of complement system (in particular from the alternative pathway) and oxidative stress; these reactions were tested by a non-invasive method designed by us.

Our results show C3 activation of the complement system, as previously described, but tested in a large number of patients compared to other studies [10] (Fig. 1b). In particular, the summer values were higher than winter and control values; this is could be explained by the increase of natural light intensity during the routine life of the patients in summer. This amount of light could be sufficient to affect these metabolites involved in the inflammatory answer without having an acute phototoxic reaction; the positive correlation between the C3 values during the seasons confirm this increase between the same group of patients (Fig. 1c). The complement system includes a network of more than 50 plasma proteins and operates via three different pathways: classical, lectin, and alternative. CP activation occurs after the binding of the first

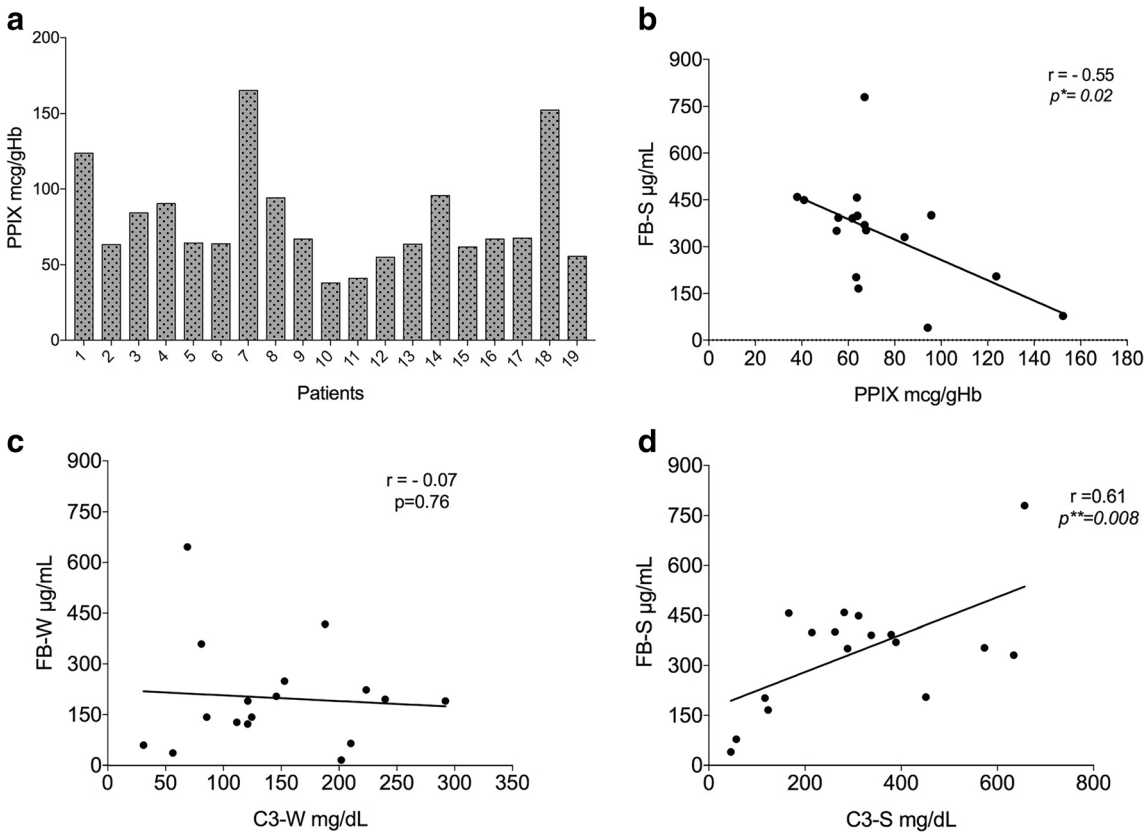

Fig. 3 Correlation between the results of the alternative pathway proteins. **a** Values of PPIX for every patient. **b** Negative tendency of FB-S vs. PPIX concentration ($r = -0.55$ p*=0.02). **c** Correlation between C3-W vs. FB-W. **d** Positive correlation between C3-S and FB-S ($r = 0.61$ p** = 0.008), the principal protein of the alternative pathway

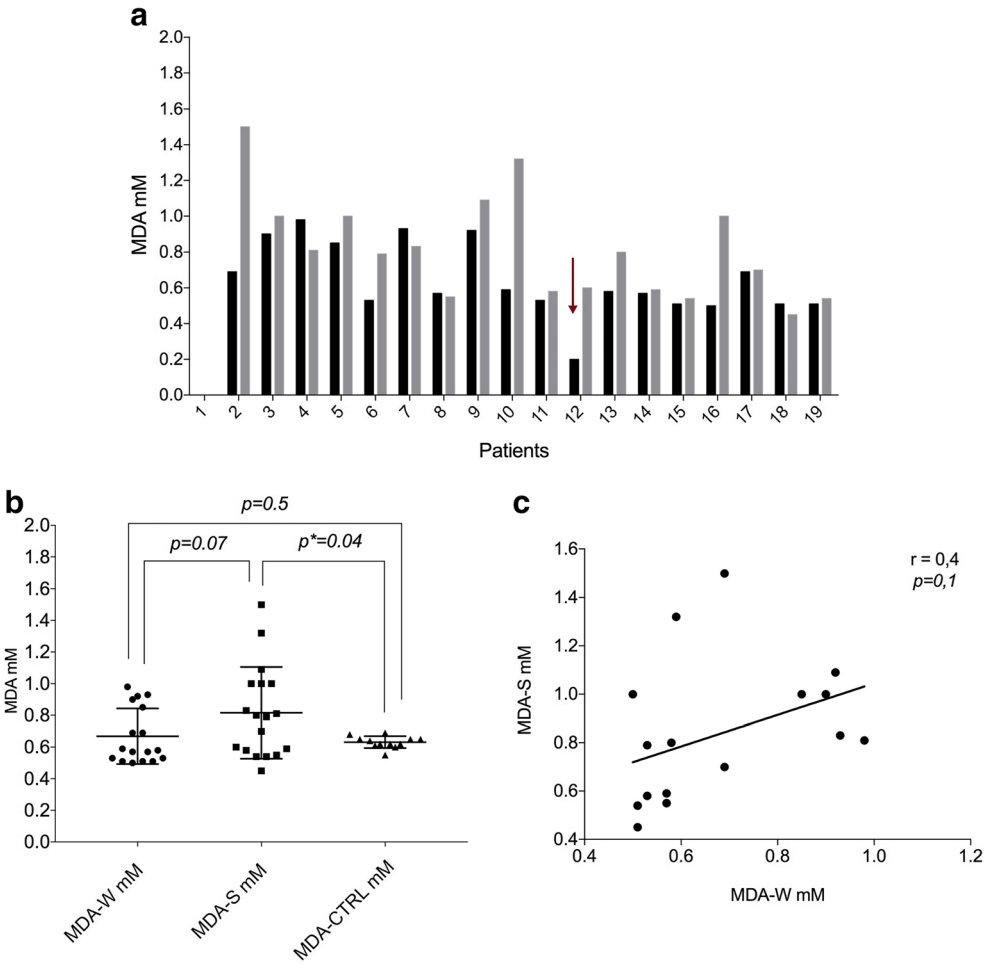

Fig. 4 MDA assay. **a** The raw data in winter (black) and summer (gray), without sample 1 (technical problem). The arrow points to the value considered outlier if compared to the control group. **b** Distribution of the mean levels and ± DS of MDA in winter (MDA-W $\mu = 0.64 \pm 0.20$) and summer (MDA-S $\mu = 0.81 \pm 0.28$) for EPP patients and the control group (MDA-CTRL $\mu = 0.63 \pm 0.03$). The *t* test showed a variation between MDA-S and MDA-CTRL reported by the *p* value on the top of the graphic. **c** Correlation between the seasonal values in EPP patients showing an increasing positive trend for the levels in our population

component C1q with antibody–antigen complexes, cell particles, or certain acute phase proteins. LP is activated during the invasion of pathogens [13]. AP is activated by the low-grade spontaneous hydrolysis of systemic native C3. The activation also depends on other factors such as factor B, factor D, and properdin. The presence of one of these three compounds could indicate the loop activation of C3 which is not related to only spontaneous hydrolysis [18].

Having said that, in order to establish the activation of one of the three pathways, we selected the C1q protein from classical pathway and factor B from alternative pathway, excluding the lectin pathway because it responds only against infections. The result showed positive values for factor B (Fig. 2b), which is a heat-labile serum factor and principal promoter of the activation loop of the AP, while no change was detected for C1q of the CP (Fig. 2e). We focused the statistical analysis on factor B in the summer season, which was higher than that in the winter and control samples.

The presence of a positive correlation between C3 and factor B in summer demonstrates the involvement of alternative pathway in the disease (Fig. 3d), which is not the only effect of the spontaneous hydrolysis. These new observations could lay the pathway for new studies and therapeutic approaches, directly against complement system compounds. There have been some new therapies described, which act on proteins of the complement system such as factor D and factor B to minimize the inflammatory response [18–21].

There is evidence that an increased level of heme stimulates the C3 formation and activation of the alternative pathway in hematological diseases, e.g., as in hemolytic uremic syndrome (aHUS) [22]. PPIX is a tetrapyrrole compound similar to heme without iron in the core. Based on this evidence, we hypothesize that the presence of PPIX could stimulate the AP activation. In order to exclude this hypothesis, we performed statistical analysis. Unexpectedly, no correlation between PPIX and C3 was found (data not shown) and the negative tendency was found between PPIX and factor B (Fig. 3b) suggesting that the concentration of PPIX does not influence the complement system activation. This is the confirmation of our hypothesis that activation of the complement system is due to only at excitation of PPIX and not due to concentration.

It is well known that ROS production occurs in long and repetitive UV exposures in a healthy subject that can lead to an activation of inflammatory cells. This process in the absence

of antioxidant compounds may contribute to increased risk of different diseases [16, 23–25].

Our results show an increase in MDA levels in summer under the natural rise in UV intensity. The phototoxic reaction occurs rapidly; therefore, EPP patients avoid light exposure. The oxidative stress increase is not justified by exposure time, such as in healthy subject, but it is due to a PPIX excitement [26].

Compared to complement system proteins, the MDA shows a different trend in winter; the value was not so different compared to summer ($p = 0.07$). It is important to underline that the EPP is not a seasonal disease and the patients have a problem with some artificial light that can lead to excitation of PPIX [27]. For these reasons, the patients are exposed to light even in winter in a small intensity compared to summer, probably not enough to be significantly different from the healthy controls but sufficient to create an increase between winter and summer. The two systems, MDA and complement, could be not linked or have not the same sensibility of reaction to light.

It is well known that the use of antioxidants is not a valid therapeutic approach to minimize the symptoms caused by the phototoxic reaction in EPP patients [28], but the integration of antioxidants can still be a preventative measure against ROS for the general health condition of the patients [29].

Furthermore, our results regarding the level of the anti-inflammatory interleukin (IL-10) seem to exclude the implication of mast cells in the phototoxic reaction. However, we need to take into consideration that the analyzed patients were not in acute phototoxic reaction because patients usually limit sun exposure with the onset of the symptoms. We can suggest that the involvement of the mast cells occurs after prolonged sun exposure.

In order to confirm and improve these theories, we need to further study and screen a large number of CS proteins. We also need to perform future behavioral studies (through qualitative questionnaires), which will allow us to understand if high-level PPIX patients avoid sun exposure more than those with low levels because they are more photosensitive, explaining the heterogeneity of photosensitivity in these patients. In regard to the mast cells, we need to design a new approach to study the cellular reaction using local blood samples. Even new markers of the disease could also be explored to improve the clinical outcomes of known and future drugs.

In conclusion, this work provides new knowledge about the inflammatory proteins involved in phototoxic reaction on EPP patients. The presence of a positive correlation between C3 and factor B in summer excludes the involvement of spontaneous C3 hydrolysis and simultaneously confirms the involvement of the alternative pathways. Moreover, the finding of altered MDA levels also in winter suggests that the EPP should not be considered a seasonal disease.

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Author contributions FG designed the study, performed statistical analysis, and wrote the manuscript. LD performed the ELISA experiments. GG recruited the patients. VB executed the genetic diagnosis of patients. PM determined the PPIX levels. SF critically revised the manuscript. EDP critically revised the results and manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Informed consent Informed consent for the research was obtained from all individual participants included in the study.

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