

**TISSUE INHIBITOR OF METALLOPROTEINASE 1, MATRIX METALLOPROTEINASE 9, ALPHA-1 ANTITRYPSIN, METALLOTHIONEIN AND UROKINASE TYPE PLASMINOGEN ACTIVATOR RECEPTOR IN SKIN BIOPSIES FROM PATIENTS AFFECTED BY AUTOIMMUNE BLISTERING DISEASES**

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**Abstract**

**Introduction:** Proteinases and proteinase inhibitors have been described to play a role in autoimmune skin blistering diseases. We studied skin lesional biopsies from patients affected by several autoimmune skin blistering diseases for proteinases and proteinase inhibitors.

**Methods:** We utilized immunohistochemistry to evaluate biopsies for  $\alpha$ -1-antitrypsin, human matrix metalloproteinase 9 (MMP9), human tissue inhibitor of metalloproteinases 1 (TIMP-1), metallothionein and urokinase type plasminogen activator receptor (uPAR). We tested 30 patients affected by endemic pemphigus, 30 controls from the endemic area, and 15 normal controls. We also tested 30 biopsies from patients with bullous pemphigoid (BP), 20 with pemphigus vulgaris (PV), 8 with pemphigus foliaceus, and 14 with dermatitis herpetiformis (DH).

**Results:** Contrary to findings in the current literature, most autoimmune skin blistering disease biopsies were negative for uPAR and MMP9. Only some chronic patients with El Bagre-EPF were positive to MMP9 in the dermis, in proximity to telocytes. TIMP-1 and metallothionein were positive in half of the biopsies from BP patients at the basement membrane of the skin, within several skin appendices, in areas of dermal blood vessel inflammation and within dermal mesenchymal-epithelial cell junctions.

**Key words:** endemic pemphigus foliaceus; autoimmune blistering skin diseases; matrix metalloproteinase 9; tissue inhibitor of metalloproteinases 1; urokinase type plasminogen activator receptor;  $\alpha$ -1-antitrypsin

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**Abbreviations and acronyms:** Bullous pemphigoid (BP), immunohistochemistry (IHC), direct and indirect immunofluorescence (DIF, IIF), hematoxylin and eosin (H&E), basement membrane zone (BMZ), intercellular staining between keratinocytes (ICS), pemphigus vulgaris (PV), cicatricial pemphigoid (CP), autoimmune blistering skin disease (ABD), matrix metalloproteinase 9 (MMP9), tissue inhibitor of metalloproteinases 1 (TIMP-1), extracellular matrix (ECM), urokinase type plasminogen activator receptor (u-Par).

## Introduction

Multiple theories have been proposed regarding the pathophysiology of cutaneous autoimmune blistering skin diseases (ABDs). Some involve plasminogen activation, desmoglein compensation, acetylcholine receptor antibodies, and intracellular signal control of autoantibodies [1]. Moreover, human autoantibodies and the presence of complement are primary factors in producing the blisters of human autoimmune skin blistering diseases and are thought to exert their pathogenic effect via proteases [2,3].

Few studies have tested for proteases and protease inhibitors in lesional skin from patients affected by ABDs [4,5]. We decided to investigate enzymes that could be modulated by ions that have been postulated as triggers for ABDs. We also aimed to investigate enzymes that are related to xenobiotics, based on our previous findings of metals and metalloids in skin biopsies of patients with a new variant of endemic pemphigus foliaceus in El Bagre, Colombia (El-Bagre-EPF) that are exposed to significant mercury pollution [5]. Thus, we utilized immunohistochemistry (IHC) to test for anti-human- $\alpha$ -1-antitrypsin, anti-human matrix metalloproteinase 9 (MMP9), anti-human tissue inhibitor of metalloproteinases 1 (TIMP1), urokinase type plasminogen activator receptor (uPAR) and for metallothionein in patients affected by autoimmune skin blistering diseases.

## Methods

### Subjects of study

We tested 30 biopsies from El Bagre-EPF patients and 30 controls from the endemic area. Of the 30 control biopsies, 15 were taken from healthy first degree relatives and 15 from healthy, non-related persons. We also utilized 15 control skin biopsies from cosmetic surgery patients in the USA, taken from the chest and/or abdomen. The Bagre-EPF patients were previously diagnosed by us, fulfilling specific criteria as previously documented [6-10]. We also tested another group of ABD patients, whose skin biopsies were obtained from the archival files of two private dermatopathology laboratories in the USA. Most of the archival sample patients were not taking immunosuppressive therapeutic medications at the time of biopsy. We evaluated 34 biopsies from bullous pemphigoid (BP) patients, 20 from patients with pemphigus vulgaris (PV), 8 from patients with non-endemic pemphigus foliaceus (PF) and 4 from patients with dermatitis herpetiformis (DH). We also tested biopsies from heart, liver, kidney and lung tissue from 4 autopsies of El Bagre-EPF patients. For all of the El Bagre area patients and controls we obtained written consents, as well as Institutional Review Board (IRB) permission. The archival biopsies were IRB exempt due to the lack of patient identifiers.

### Intensity of immunohistochemistry staining

The staining intensity of the immunohistochemistry antibodies was evaluated 1) qualitatively by two independent observers, as well as 2) in a semiquantitative mode by automated computer image analysis (specifically designed to quantify immunohistochemistry staining in hematoxylin-counterstained histologic sections). For the image analysis, slides were scanned with a ScanScope CS scanning system (Aperio Technologies,

Vista, California, USA), utilizing bright field imaging at 20 $\times$  and 40 $\times$  magnifications. The strength of the staining was evaluated on a scale from 0 to 4, where 0 represented negative staining and 4 the strongest staining.

### Immunohistochemistry staining

We performed IHC using antibodies conjugated with horseradish peroxidase (HRP)-labelled secondary antibodies. We utilized multiple monoclonal and polyclonal antibodies, all from Dako (Carpinteria, California, USA). For all our IHC testing, we used a dual endogenous peroxidase blockage, with the addition of an Envision dual link (to assist in chromogen attachment). We applied the chromogen 3,3-diaminobenzidine, and counterstained with hematoxylin. The samples were run in a Dako Autostainer Universal Staining System, as previously described (13-15). Positive and negative controls were consistently performed. For IHC, we utilized Dako antibodies to polyclonal rabbit anti-human  $\alpha$ -1-antitrypsin (cat. No. IR505, flex ready to use, antigen retrieval high pH), rabbit anti-human MMP9, (cat. No. A0150, dilution 1:75, antigen retrieval heat), monoclonal mouse anti-human TIMP-1 (cat. No. M0639, dilution 1:50, antigen retrieval heat), monoclonal mouse anti-human metallothionein (cat. No. M0639, dilution 1:50, antigen retrieval high pH), and monoclonal mouse anti-human uPAR (cat. No M7294, dil, 1:25, antigen retrieval high pH). We also utilized control tissue from 4 non-El Bagre EPF patient autopsies (from the El Bagre EPF endemic area), to rule out false positive and false negative results due to spontaneous autolysis. The organs from the autopsies were taken within 12 hours of patient death. The direct immunofluorescent studies (DIF) were performed as previously described [6-10].

### Indirect immunoelectron microscopy (IEM)

Performed as previously described [10]. In brief, postembedding immunogold labeling was performed on samples of El Bagre-EPF sera and controls. Rat skin was utilized as the substrate antigen; the tissue was dissected, fixed in 4% glutaraldehyde with 0.2% paraformaldehyde, and embedded in Lowicryl® resin. The tissue was then sectioned at 70 nm thickness. The samples were blocked with a solution from Aurion™ (Electron Microcopy Sciences/EMS, Hatfield, Pennsylvania, USA). Our tissue grids were then washed with PBS-BSAC (Aurion™, EMS). The primary antibodies were incubated overnight at 4°C.

The next day the grids were again washed; a secondary antibody solution, specifically 10nm gold-conjugated protein A PBS BSAC (Aurion, EMS™) was applied. The samples were then double-stained with uranyl acetate and lead citrate. The samples were reviewed under a Hitachi H7500 transmission electron microscope. Images of immunogold particles displaying any pattern of positivity were recorded, and converted to TIF format.

### Statistical methods

Differences in staining intensity and positivity were evaluated using a GraphPad Software statistical analysis system, and employing Student's t-test.

We considered a statistical significance to be present with a *p* value of 0.05 or less, assuming a normal distribution of the samples.

## Results

In Table I and Figures 1 through 2 we summarize our primary results. We observed consistent patterns of IHC positivity relative to each autoimmune skin blistering disease. For example,  $\alpha$ -1 antitrypsin was positive in all of the lesions of DH within the subepidermal blisters ( $p < 0.05$ ). Positivity for this marker was also noted in selected acantholytic areas of hair follicles within DH biopsies. In PV, positivity was seen with TIMP-1,  $\alpha$ -1 antitrypsin and metallothionein. Alpha-1 antitrypsin was positive in the majority of active DH active cases in the blisters. El Bagre-EPF patient biopsies and controls stained predominantly positive for TIMP-1, and metallothionein. Contrary to what we expected given the existing literature, most patients with autoimmune skin blistering diseases stained negative for MMP9 ( $p < 0.05$ ). A few patients with BP were positive with MMP9 in the area of the upper dermal blood vessels. Only a few patients with a stable, chronic clinical form of El Bagre-EPF and taking low dosage of prednisone daily (e.g. 10-20 mgs/per day) showed positivity for this marker, especially in telocyte areas. Specifically, the MMP9 positivity was focally noted in the epidermis, but primarily in mesenchymal-epithelial cell junction transitions (METs) in the dermis ( $p < 0.05$ ). Of interest, the same biopsies that stained positive for proteases and/or protease inhibitors demonstrated positive deposits of FITC conjugated IgG in the same areas in the dermis (Fig. 2). In addition, multiple samples from the El Bagre-EPF patients demonstrated positive serum autoantibody deposits (via 10nm Gold particles) on immunoelectron microscopy (IEM)(150 kV) on the METs, highlighted by utilizing using anti-human IgG antibody; these findings correlated with our IHC results, as well as our direct immunofluorescence results for reactivity in the extracellular matrix. Similar positivity was seen in the other autoimmune diseases, demonstrating reactivity to the skin appendices and inflamed dermal blood vessels, and in areas of some type of dermal cell junctions, possibly the METs. IEM only was performed in the El Bagre-EPF patients (Fig. 2).

In 6/10 DH blisters, we found positive staining with  $\alpha$ -1 antitrypsin between epidermal keratinocytes, and in the upper dermis under the blister. El Bagre-EPF patient biopsies stained predominantly positive for TIMP-1 and metallothionein. Healthy relatives of El Bagre-EPF patients also displayed higher positivity for these markers, relative to non-related controls (Tabl. I); this finding demonstrates that although the controls are also exposed to the triggering environmental agents, they do not develop the disease.

Based on the fact that El Bagre-EPF patients have autoantibodies to organs such as heart and kidney (mostly directed against plakins and plakophilins), we decided to test for these enzymes in necropsy tissue. In 4/4 biopsy sets from the heart, liver, kidney and lung from El Bagre-EPF patient autopsies, we found positive staining in cardiac tissue for TIMP1 in the cardiac t-tubule area composita, and to individual renal endothelial cells. In the control biopsies, this marker was consistently negative. TIMP1 was also positive in the renal tubules in 4/4 El Bagre-EPF autopsy tissues (Fig. 1). Further, 4/4 kidney, liver, heart and lung El Bagre EPF autopsy biopsy tissue sets were also positive for  $\alpha$ -1 antitrypsin. MMP9 staining was positive in the endothelial cells of the liver from 4/4 El Bagre-EPF patients. Moreover, in the controls, MMP9 was positive but present

diffusely over the hepatic tissue. Thus, we have evidence of systemic reactivity in El Bagre EPF in that proteases are active in other organs as well as in the skin.

Overall, TIMP-1 was positive in many immunologically active biopsy areas in all the autoimmune skin blistering disease samples studied (Tabl. I). TIMP 1 was positive in most BP biopsies within the blister ( $p < 0.05$ ). The presence of  $\alpha$ -1-antitrypsin within the blisters favored a diagnosis of DH versus BP. Active cases of PV and PF demonstrated a strong presence of  $\alpha$ -1-antitrypsin as well as MMP9 (Tabl. I). Metallothionein was the most common positive marker found in all the autoimmune skin blistering diseases types, as well in controls from genetic relatives of El Bagre-EPF patients (Tabl. I).

## Discussion

Contrary to previously published data regarding positivity of MMP9 and uPAR in human ABDs and respective animal models, our studies of in situ biopsies from active clinical lesions demonstrate significant differences [11-15]. Specifically, uPAR was positive only in one part of a lesional blister from one case of BP, where the blister demonstrated blood deposits. Only a few chronic cases of El Bagre-EPF that were under prednisone treatment of less than 40 milligrams per day for more than 10 years stained positive for MMP9. In our necropsy tissue analysis from controls and patients, we found no differences in regard to MMP9 staining. In few cases of active DH, we observed positivity to MMP9 as shown previously [15]. MMP9 and MMP12 have been previously documented to display positive staining in some patients with clinical enteropathy and DH [16]. Some authors have shown variable collagenase expression in autoimmune skin blistering diseases, including induction during re-epithelialization, and decrease by topical glucocorticoid therapy [17]. One of the pitfalls of our study was the fact that with the exception of the skin biopsies from El Bagre-EPF and the controls, we lack definitive data regarding whether specific patients were taking immunosuppressive therapy at the time of biopsy; this limitation of our study is pertinent.

However, our findings are in agreement with some reported previously [18], since many of these autoimmune blistering diseases stained positive for metallothionein and TIMP1. Our results demonstrated stronger staining in areas of active inflammation in most of the ABDs; significant staining was also noted in inflamed appendices, correlating with major deposits of immunoglobulins and complement as determined by IHC (data not shown). Our study results highlight a previously documented increase in expression of metallothionein, coupled with a simultaneous expression of the TIMP1; TIMP1 may thus be expressed to inhibit damaging effects of the metallothionein [19].

Matrix metalloproteinases (MMPs) are a family of extracellular matrix (ECM) degrading enzymes that are collectively capable of degrading almost all ECM components [20,21]. The extracellular activities of MMPs are regulated by tissue inhibitors of metalloproteinases (TIMPs) [20,21]. Both soluble and membrane-anchored metalloproteinases participate in degradation of the ECM. Metallothioneins have the capacity to bind heavy metals, both 1) physiologic, including zinc, copper, and selenium; and 2) xenobiotic, including cadmium, mercury, silver, and arsenic and pesticides (introduced via food supply and/or other routes). Metallothioneins have a high content of cysteine residues that bind various heavy metals; these proteins are transcriptionally regulated by both heavy metals and glucocorticoids.

Markers	BP n=34	PV n=14	PF n=8	DH n=14	Normal skin controls n=15	El Bagre EPF n=30	Control endemic area group n=15
$\alpha$ -1 anti-trypsin	In 4/34 biopsies, some staining in blister, in dermis and around the eccrine ducts.	Positive in the epidermis in spots, in some blister debris and subcorneal in 8/14.	Positive in the upper vessels and in the inflammatory dermis (3/4).	Upper dermis, blister and under in 8/10. Positive in some fibroblastoid cells.	Mostly negative.	Positive staining in some of the basal keratinocytes hair follicle, neurovascular supplies and subcutaneous fat.	4/15 cases were positive, in neurovascular bundles of the skin and appendices and sweat glands.
MMP-9	Staining in the blister, dermal vessels METS matrix (4/34).	Most cases negative.	Negative.	ICS like, dermis under the blister, neurovascular supplies of appendices (3/10).	Mostly negative.	9/30 of the chronic cases corneal layer, hair follicle some vessels. Positivity in the METS.	Mostly negative.
TIMP1	Weak positive around the blisters, vessels and in the METs and fat (19/34).	Positive in basal keratinocytes, blisters debris, dermal vessels and METs (7/14); linear deposition at the BMZ.	Positive in 2/4 cases around the blisters, upper vessels and METs.	Positive in the blisters in the dermal papillae (3/10).	Mostly negative.	Corneal layer, some epidermis; also, dermal blood vessels and sweat glands.	2/15 Positive sweat and sebaceous glands, vessels and extracellular fibroblastoid cells (8/15).
Metallothionein	Positive in the BMZ of the hair follicles, and some cells of the METs (19/34).	Positive at the BMZ of the hair follicles and sebaceous glands, as well as in some areas in the MTEs matrix (9/14).	Positive in 2/4 cases around the blister and upper vessels and METs.	Positive under the blisters (4/10).	Mostly negative.	Corneal, and cytoplasm of the keratinocytes of the spinous layer. Sebaceous glands in 23/30 patients and some fat tissue.	3/15 positive in the corneal layer. Positive staining to the sweat glands (8/15).
uPAR	Negative	Negative	Negative	Negative	Negative	Negative	Negative

**Table I. Summary of staining patterns of proteases and protease inhibitors in multiple autoimmune skin blistering diseases**

Several cases of DH were positive for  $\alpha$ -1 antitrypsin. We previously described a fatal case of El Bagre-EPF with high levels of  $\alpha$ -1 antitrypsin in a patient superinfected by varicella-zoster virus [23]. The El-Bagre-EPF cases also stained positively for MMP9 only in chronic patients under treatment with prednisone for long periods. The MMP9 reactivity was located in areas of newly reported dermal cell junctions, including dermal METs [24] and telocytes [25]. Our finding may explain dermal histologic sclerodermoid changes occasionally noted in El Bagre EPF, with attendant loss of skin appendices. The fact that the ABDs improve with glucocorticoids may be related to our findings. Other studies of BP have shown altered expression of MMPs and TIMPs within lesional blisters [22].

### Conclusions

We conclude that the observed profile expression of proteases, protease inhibitors and other enzymes in autoimmune skin blistering diseases seems to differ from many published

animal models, thus highlighting significant immunologic differences between these animal models and the in vivo autoimmune skin blistering diseases of human patients. The TIMP1 and metallothionein seem to be expressed in an inverse correlation, suggestive of one enzyme attempting to repair collateral damage from the other enzyme. It is possible that the capacity to bind heavy metals on these enzymes may indicate some exposure to these xenobiotics in ABDs. The presence of enzymes and their inhibitors (not only in the blister areas, but also in the inflamed dermal vessels, skin appendices and METs) merits further testing of autoimmunity in these areas.

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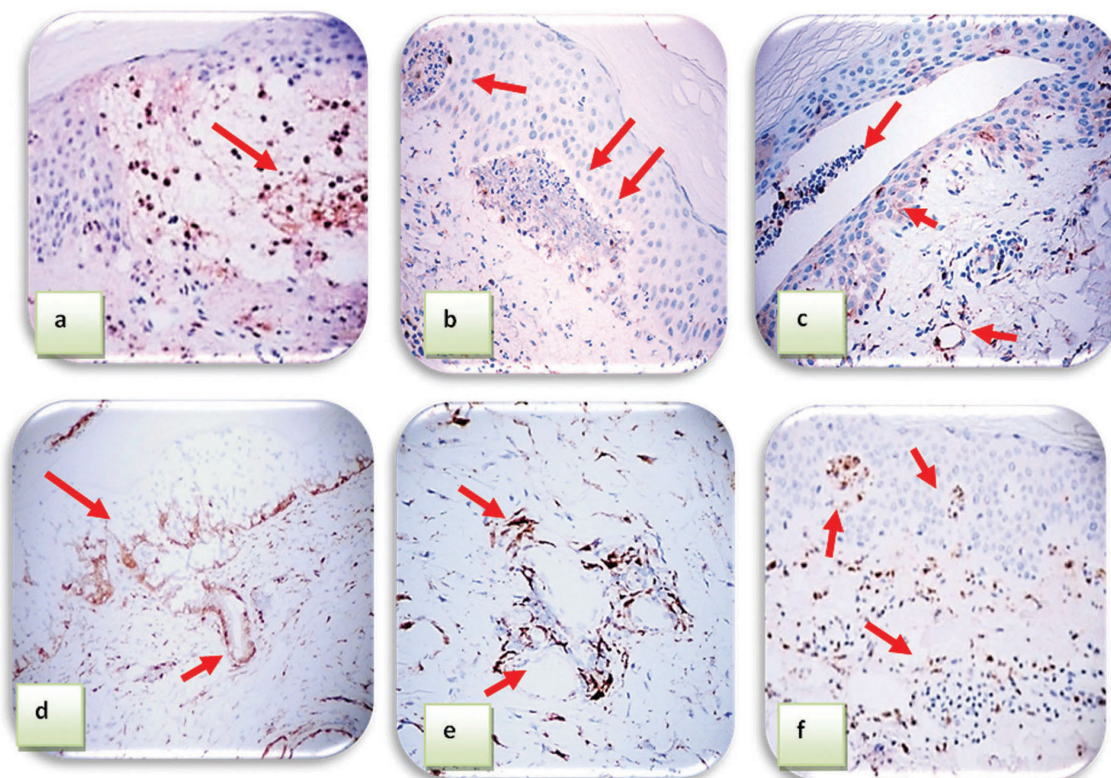
To assisting personnel at the Hospital Nuestra Señora del Carmen in El Bagre Colombia; and also to Jonathan S. Jones HT (ASCP) at Georgia Dermatopathology Associates for excellent technical assistance.

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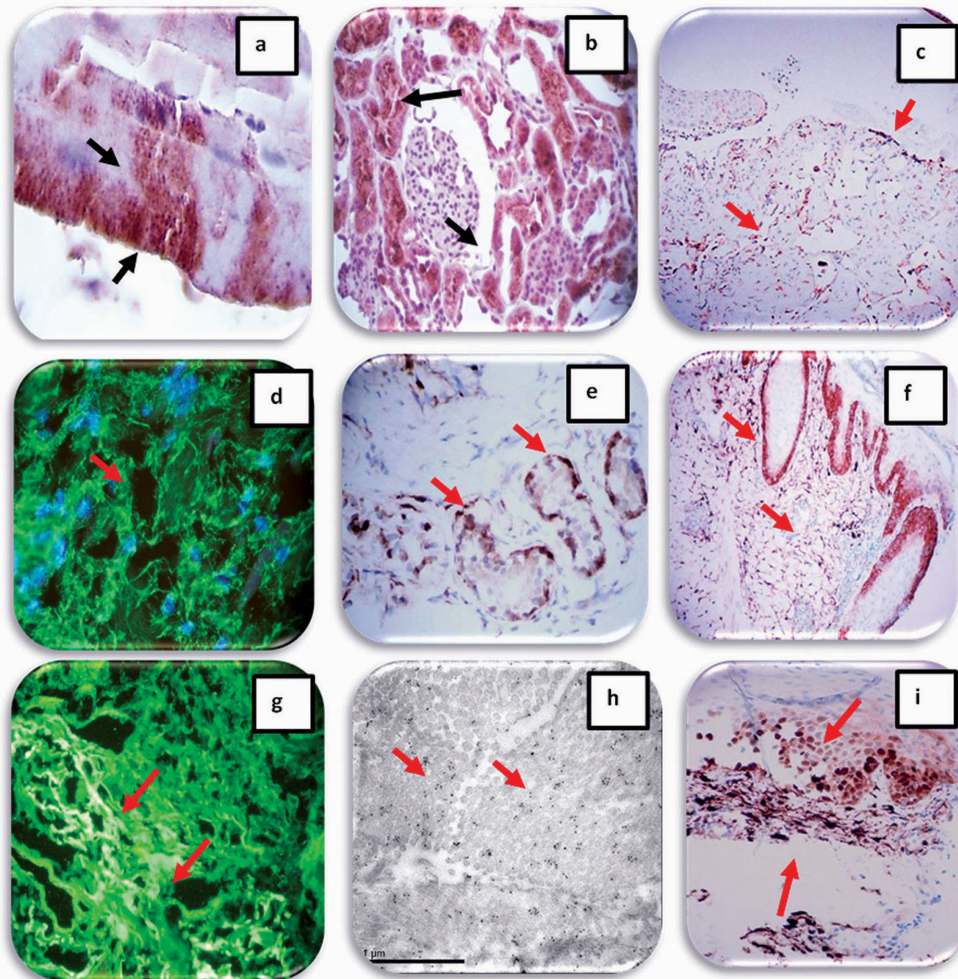
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**Figure 1.** a.  $\alpha$ -1 anti-trypsin DIF positive staining in a case of PV inside the blister (red arrow, dark brown staining) and some punctuate staining in upper dermal blood vessels (200x). b. Case of DH, demonstrating positive TIMP-1 IHC staining in the blister and punctuate staining in upper dermal blood vessels (red arrows, brown staining) (100x). c. Case of PV, with TIMP-1 positive IHC staining in cells within and around the blister, and some in upper dermal blood vessels (red arrows, brown staining). d. BP case with positive IHC staining for metallothionein in the base of the blister and around a dermal sweat gland ductus (red arrows, brown staining). A few positive areas of punctate staining are also noted on upper dermal blood vessels. e. A BP patient with positive metallothionein IHC staining around dermal blood vessels (red arrows, brown staining). f. A DH case, with positive IHC staining in the blister with  $\alpha$ -1 anti-trypsin and on upper dermal blood vessels (red arrows, brown staining).



**Figure 1.** **a** IHC positive TIMP-1 staining of heart necropsy tissue from an El Bagre-EPF patient; and in **b**, the same patient in renal tubule tissue black arrows, red/brown staining). **c**. A BP case, with positive staining with TIMP1 over the blister BMZ and between dermal extracellular matrix fibers (red arrows, brown staining). **d**. The same case of BP as in **c**, using DIF with FITCI conjugated fibrinogen and showing that dermal staining is present, possibly related to dermal reactivity (red arrow, light yellow/green staining). **e**. A BP case, demonstrating IHC positive staining in the sweat glands with metallothionein (red arrows, dark staining). **f**. IHC positive to metallothionein in a patient with PV at the base membrane zone as well as in the dermal cell junctions and or the MET. **g**. DIF from an El Bagre-EPF patient using FITCI conjugated anti-human IgG antibody, and showing positive staining between the dermal fibers (red arrows, yellow/white staining). **h**. IEM, showing positive 10 nm Gold labeled anti-human IgG antibodies, positive to several cell junctions in the dermis (red arrows, black dots). **i**. An El Bagre-EPF patient, demonstrating positive IHC metallothionein staining in epidermal acantholytic cells and in the subjacent inflamed dermis (red arrows, brown staining).

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