EVALUATION OF THE PRINCIPLES FOR OBTAINING AND USING PLATELET-RICH PLASMA

ALEXANDRU GRIGORIU*, IRINEL NEDELCU*, DACIANA ELENA BRĂNIȘTEANU**, ALIN-CODRUȚ NICOLESCU*

Summary

Platelet-rich plasma (PRP) is by definition an autologous plasma product with a higher concentration of platelets than that of collected blood. It is a modern treatment, which has already earned a place among the most used products in the category of orthobiologics. Platelets are key elements in tissue repair processes. They release numerous growth factors, stimulate fibroblasts and endothelial cells, and can trigger the anagen phase. The principle behind obtaining PRP by centrifugation is that it causes the platelets to settle in the deep layer of the plasma, thus separating them from the superficial layer. The optimal concentration of platelets in separated plasma has not yet been established, despite the many studies performed to date and the variety of fields in which PRP has been used. However, the most important factors that influence cell proliferation have been established, and their observance allows the use of platelet concentrations close to the upper limit of the optimal range: 3.7×10^6 plt/μL. From the point of view of medical ethics, procedures using PRP must be supported by the diagnosis and pathogenic mechanism of the condition to be treated, and the quality of the devices used must be supported by clear evidence of platelet concentration, purity and viability. The interest shown by patients for this type of treatment has caused the global market for PRP to have a significant growth and, starting from the value of 2017 ($195.2 million), with an annual percentage increase estimated at 11.6%, this will reach approximately 715.65 million dollars in 2030.

Key Words: *PRP, Platelet-rich Plasma, Platelet-enriched Plasma, Plasma, Platelets, Orthobiologics, Tissue repair, Growth factors, Treatment, Cell proliferation, Centrifugation, Dermatology, Dermato-cosmetics, Cyto-kines, Hair follicle, Alopecia, Androgenetic alopecia, Alopecia areata, Thixotropic gel, CE mark, Leukocytes.*

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Definition

PRP - Platelet-rich Plasma or *Platelet-enriched plasma* is, by definition, an *autologous plasma product with a higher concentration of platelets than that of collected blood* [1].

The aim is also to drastically reduce "contaminants" - erythrocytes and leukocytes (lymphocytes and monocytes, but especially granulocytes), especially because of their pro-inflammatory role.

It is a modern treatment that has already earned a place among the most widely used products in the category of "orthobiologics" (products found in nature, from 100% biological sources, with a role in increasing the body's innate capacity for repair and regeneration) in various sectors: dermatological treatments and dermato-cosmetic procedures, orthopaedics, general surgery, dentistry, etc [1,2].

* Egoclinic , Bucharest.
** UMF "Grigore T. Popa" and "St. Spiridon" Clinical Hospital, Iași.

Physiology and role of platelets and plasma

Platelets account for about 6% of the figurative elements of blood, together with erythrocytes (93%) and leukocytes (1%). They are small, discoid cells with a lifespan of 7-10 days. Under physiological conditions, they are activated following trauma causing bleeding. Platelets are key elements in tissue repair processes. They release numerous growth factors (FGF, PDGF, TGF-ß, EGF, VEGF, IGF, etc.) and cytokines involved in stem cell migration, differentiation and proliferation. In addition, platelets stimulate fibroblasts and endothelial cells, inducing extracellular matrix synthesis and neovascularization formation, respectively [2,3].

A partial list of the various growth factors and cytokines in PRP, together with their effects, can be found in Table 1 [1,3,4,5].

Growth factors also act at the protrusion of the hair follicle, where they bind to receptors on the surface of stem cells. The dermal papilla contains germ cells of mesenchymal origin. Interactions between these two cell types and growth factors lead to the initiation and maintenance of the anagen phase (growth phase in the hair regeneration cycle). The main factors involved in the growth and differentiation of stem cells appear to be EGF and TGF, and PDGF mediates their interaction with the extracellular matrix, triggering follicular morphogenesis [3,5].

Another pathway present in the dermal papilla is the activation of extracellular signal-regulated kinase (ERK) and protein kinase B (Akt), which promote cell survival and inhibit apoptosis. Numerous recent studies have demonstrated that growth factors in PRP induce hair follicles to enter and maintain the anagen phase by stimulating this pathway. Activation of Akt leads to phosphorylation inhibition of two other signalling pathways – GSK-3 (glycogen synthase kinase-3β) which is involved in β-

Table 1

Growth factors and cytokines	Functions and effects
PDGF	Mesenchymal cell mitosis. Chemotactic and mitogenic for fibroblasts. Regulates collagen secretion. Chemotactic for neutrophils and macrophages.
TGF-ß	Stimulates proliferation of undifferentiated mesenchymal cells. Endothelial cell and fibroblast mitosis. Regulates the mitogenic effect of other growth factors and angiogenesis. Inhibits macrophage and lymphocyte proliferation.
VEGF	Angiogenesis and increased vascular permeability. Endothelial cell mitosis. Expressed in dermal papilla in anagen; increases diameter of perifollicular vessels.
EGF	Proliferation of keratinocytes and fibroblasts. Endothelial cell mitosis. Proliferation and regeneration of hair follicles.
FGF (a - b)	Mesenchymal cell mitosis. Anagen phase inducer.
IGF-1	Chemotactic for fibroblasts and stimulates protein synthesis. Maintains hair follicle growth in vitro.
Ang-1	Angiogenesis by stimulating endothelial cell proliferation. Stabilizes vessel structure by recruiting pericytes.
PF-4	Chemotactic for leukocytes and regulates their activation. Antimicrobial effect on numerous pathogenic bacteria and fungi.
HGF	Regulates epithelial and endothelial cell growth and motility. Supports epithelial repair and neovascularization in tissue healing.

Legend:
PDGF: Platelet-derived growth factor. **TGF:** Transforming growth factor. **VEGF:** Vascular endothelial growth factor **EGF:** Epidermal growth factor. **FGF:** Fibroblast growth factor. **IGF:** Insulin-like growth factor. **Ang-1:** Angiopoietin-1. **PF-4:** Platelet factor 4. **HGF:** Hepatocyte growth factor.

catenin degradation (protein involved in cell adhesion and gene transcription) and the Bcl-2 pathway involved in cell apoptosis [3,6,7].

In turn, plasma contains essential factors for cell survival – nutrients, hormones, electrolytes, vitamins and proteins, for coagulation and the formation of fibrin polymers, which will support cell migration and tissue repair [2,6].

PRP preparation conditions

Numerous preparation modalities have been studied in an attempt to provide a cost-effective method of obtaining PRP, including selective blood filtration and various centrifugation techniques. The classification of these techniques is based on the contact of the collected blood with environmental factors:

- "Closed techniques" involve the use of commercially available CE-marked products (including the equipment used for collection and centrifugation) which allow the blood and derived product to be handled without coming into contact with the air in the medical practice.

- "Open techniques" expose the blood to contact with the air and the various materials required for its processing, so that there is a risk of contamination. The majority of currently commercially available products are based on the closed centrifugation technique due to its ease of use and lower cost [8,9].

Each tube contains a *thixotropic* gel (a special mixture of polymers that can be reversibly liquefied during mechanical action – shaking, ultrasound, etc.) for the separation of the figurative elements. The principle behind obtaining PRP by centrifugation is that it causes the platelets to settle in the deep layer of the plasma, thus separating them from the superficial layer (which has become known as Platelet-Poor Plasma - PPP). In addition, erythrocytes and leukocytes precipitate more rapidly than platelets, which allowed the development of the technique of separating different groups of elements from plasma. This phenomenon is governed by *Stokes' Law* – which states that the sedimentation rate of particles in a liquid medium correlates proportionally to their mass and the sedimentation force to which the particles are subjected [8].

In the preparation of PRP, a higher *centrifugation force* increases the sedimentation force and gives rise to differences in the sedimentation rates of erythrocytes, leukocytes and platelets, while a longer centrifugation *time* ensures better capture and separation of elements due to differences in sedimentation rates. However, in practice, it has been observed that a high centrifugation force applied for a longer time leads to the separation of platelets from the plasma but also to the formation of a "buffer layer" together with part of the leukocytes, making further separation of the elements impossible. Thus, the centrifugation conditions necessary for the preparation of PRP are in a continuous process of optimization, which requires compliance with the conditions established by each individual manufacturer in order to obtain an effective PRP [8,9,10].

The complete removal of erythrocytes from PRP is an ongoing concern for both manufacturers and clinicians, due to the significant adverse effects that their presence can cause upon reinjection. Erythrocyte destruction can occur at the time of blood collection and/or centrifugation – due to shear forces, or after reinjection through various immune-mediated processes. Haemolysis leads to the appearance of free haemoglobin, some porphyrins and iron in the plasma, with the possibility of inducing oxidative stress and pro-inflammatory reactions. In turn, these reactions may trigger *erythroptosis* (apoptosis of erythrocytes due to hyperosmolarity, oxidative stress, energy depletion or exposure to xenobiotics), with exposure of phosphatidylserine to the membrane surface and release of Platelet-Activating Factor (PAF) and Macrophage Migration Inhibitory Factor (MIF) from the cytoplasm. The release of PAF can lead to disruption of local microcirculation, and MIF has been identified as a very potent pro-inflammatory cytokine. Furthermore, these degradation products cannot be easily removed by natural protective mechanisms in the extracellular matrix [11]. These physio-pathologic observations may also underlie cases of skin hyperpigmentation after the use of PRP for facial rejuvenation, or in an attempt to reduce hyperpigmentation post laser procedures. Some authors draw attention to these cases and emphasize that PRP injections

should not be used in the face as a treatment for post-inflammatory hyperpigmentation [12].

Platelet concentration in PRP

The optimal concentration of platelets in separated plasma has not yet been established, despite the very large number of studies performed to date (both in vitro and in vivo) and the variety of fields in which PRP has been used (dermatology, BMF surgery, orthopaedics, etc). Thus, platelet concentrations reported as 'effective' vary, being 2 to 5 times higher than that in collected blood. Most initial studies considered that a platelet concentration of less than 106/microliter of plasma is not effective in triggering natural healing mechanisms, and a concentration higher than this does not appear to be associated with significant benefit. In recent years, as knowledge about the use of this product has increased, observations on the optimal amount of growth factors released by platelets from PRP have emerged, paving the way for new studies with widely varying results, both positive and negative in terms of clinical efficacy [10,13]. There are significant correlations between the personal characteristics of the patients (age, platelet concentration in the blood collected, etc.) and the amount of growth factors obtained, which largely account for the variations observed, both between different commercial products and between consecutive uses of the same product [5]. Also, the technique used to obtain PRP has a significant impact on the concentrations of growth factors obtained, with some very recent studies claiming that open techniques (double centrifugation) have better and more consistent results than some kits available on the market [14].

A recent meta-analysis on optimal platelet concentration studies (O.K. Straum, 2020) identified two main causes for the large diversity of results reported by these studies. *Too high* a concentration of platelets in the solutions used (in the case of in vitro studies) resulted in a commensurate decrease in nutrients available for cell growth, which created an environment that was unfavourable for tissue repair. The *number of leukocytes* in the PRP was determinant for the observed rate of tissue proliferation, due to proinflammatory factors resulting from their degranulation. Thus, a high concentration of remaining leukocytes significantly decreased proliferation, irrespective of the number of platelets in solution. Conversely, a low leukocyte concentration allowed the use of PRP with higher platelet counts with better tissue proliferation rates. The conclusions of this meta-analysis are as follows: although an optimal "universal" platelet concentration of PRP cannot be established, there are two particularly important factors that influence cell proliferation – 1) the volume of PRP used should be $\leq$ 10% of the volume of the medium into which it is injected and 2) the amount of leukocytes in the PRP should be kept as low as possible, preferably < 0.1/μL. Compliance with these two conditions would allow the use of platelet concentrations in PRP towards the upper limit of the range considered optimal: **3.7×106 plt/μL** (with a mean value in the most representative studies calculated to be 2.94 μ 106 plt/μL) [15].

Factors Affecting platelet viability

There are numerous protocols for PRP preparation in the literature. These relate to the force and duration of centrifugation, as well as the number of centrifugations. Changing these parameters may result in different platelet concentrations. Very few studies, however, have sought to provide a clear picture of the processes and transformations that platelets undergo after blood collection until reuse.

Blood collection – The clotting process can be triggered at the time of blood collection. To avoid unintentional platelet activation, the use of large gauge needles (from 22 G upwards - 0.71 mm) [16] is indicated. It has also been shown that platelet survival decreases as the duration of blood collection increases [17], leading to the universally accepted recommendation that the required blood volume should be obtained as quickly as possible.

Centrifugation – The Earth's gravitational force is sufficient to separate the figurative elements from plasma. Anticoagulated blood stored in a vertical tube will naturally separate

into plasma, leukocytes and erythrocytes. However, the time required for separation makes it impossible to use this technique in practice. Taking also into account the degradation of biological compounds over time, the need for faster separation techniques becomes evident [16]. This has led to numerous attempts to optimize the technique, i.e. numerous protocols for the preparation of PRP. As mentioned above, the technique used may be an open technique, with one or two successive centrifugations of the collected blood, or a closed technique, usually with a single centrifugation – due to the use of thixotropic gels in the collection tubes.

A meta-analysis of protocols used between 2007-2018 highlights the difficulty in comparing results in the absence of a common protocol. All single centrifugation techniques appear to achieve a clinically effective platelet concentration. Also, assessing efficiency strictly by the concentration of growth factors in PRP can be misleading. VEGF concentration increases with increasing centrifugation time. But the platelets are very fragile, and too high a centrifugation force can lead to their disruption with degranulation. In conclusion, a closed technique with simple centrifugation seems to be a good compromise [18].

Temperature – Temperature is one of the critical factors in the process of obtaining PRP. To avoid platelet activation, the American Association of Blood Banks (AABB) guidelines recommend that blood should be centrifuged and handled at a temperature between 22 and 24°C [16].

Anticoagulant used – The use of an anticoagulant that does not affect platelet morphology, integrity and ultimately platelet function is particularly important. In addition, the anticoagulant must be safe for in vivo use, i.e., not have adverse effects on the tissues in which the PRP is administered [16,19]. The main molecules used are EDTA (ethylenediaminetetraacetic acid), ACD-A (acid citrate dextrose solution A), CTAD (citrate theophylline adenosine dipyridamole). Studies also mention heparin and sodium citrate, but these are less commonly used in practice [20].

Although a longer survival of platelets was observed when EDTA was used, their morphology was altered after centrifugation (ballooning), which raised the question of whether their function could be maintained after reinjection [19,21].

The majority of authors agree that *citrate*-based solutions are the most suitable for PRP production, in particular due to the observed better proliferation of mesenchymal stromal cell (MSC) cultures (compared to EDTA) and the release of more growth factors. CTAD appears to be superior to other anticoagulants in terms of maintaining platelet structural integrity, preventing spontaneous platelet activation and TGF-β1 release [19,20,21].

Of note, the morphology of MSCs in culture was not altered by the anticoagulants used and the rate of MSC proliferation was proportional only to the platelet concentration of the added plasma [21].

PRP activation – PRP activation prior to reinjection is another parameter that is constantly under discussion. Exogenously, PRP can be activated by the addition of thrombin, calcium chloride (CaCl2), or exposure to mechanical forces. Collagen, on the other hand, is a natural activator of PRP, which has led some authors to argue that it is unnecessary to activate PRP prior to tissue injection [22].

The term "activation" refers to two key processes that are initiated at the time of PRP preparation: (1) degranulation of platelets, with the release of growth factors from α-granules, and (2) fibrinogen breakdown with the initiation of coagulation, a process that contributes to the retention of these factors at the injection site[23]. The mode of activation has been shown to have a net influence on fibrin network formation, leading to differences in both the amounts of growth factors released and the kinetics of their release. Collagen (type I) was shown *in vitro* to be a weaker activator of platelets compared to thrombin and CaCl2. Thrombin and type I collagen produced immediate PDGF and TGF-β1 release – which remained constant for 24 hours. During the same interval, VEGF showed an

increasing trend, starting 15 minutes after injection. CaCl2 induced a progressive release of all growth factors starting 15 minutes after injection [24]. As growth factors are molecules with short half-lives (minutes or hours), if they are not utilized immediately after release from platelets, they may be degraded before new tissue receptors become available [25]. Some authors also draw attention to the fact that too much growth factor released too quickly into the tissue may have an inhibitory effect on cellular functions and healing processes, with increased inflammatory response and fibrosis. This raises the question of choosing an activator that offers the possibility of releasing bioactive molecules according to the needs of the target tissue [24,25].

Patient selection

The use of PRP is an invasive technique, and discussions on its safety, efficacy, ethicality and even legality are common both in the medical sectors of which it is part of the therapeutic arsenal, and in forensic medicine, i.e. ethics and bioethics committees [26,27].

As autologous products, included in the category of orthobiologics, PRP and derived products are much safer to use than any other homologous or allogeneic products and do not carry the risk of transmitting diseases such as hepatitis, HIV, West Nile fever, etc. They also exclude the problem of antibody formation and the risk of graft-versus-host disease. This particular safety profile makes PRP a product that is easily accepted and even requested by patients, with very few reported cases of adverse reactions [26]. However, possible adverse reactions related either to the quality of the product itself (post-injection inflammation in case of a large number of leukocytes remaining in the plasma, hyperpigmentation due to tissue degradation of the remaining erythrocytes, possible foreign body reactions if micro-particles of the separating gel are trapped in the buffer layer together with platelets) or to the safety of administration (infections in case of non-compliant kits) cannot be ignored. Although very rare, allergic reactions triggered by the anticoagulant used by manufacturers in PRP kits have been described [27,28]. Last but not least, local reactions due to the injection itself should be mentioned, such as pinpoint bleeding, ecchymosis, transient oedema, pain at the injection site – which may last up to three days, erythema, moderate pruritus and a feeling of tension in the scalp [29].

Numerous clinical trials have evaluated the effectiveness of PRP use in various sectors, from sports medicine to dermatology, with both positive and negative results. Large prospective placebo-controlled studies have not yet been conducted, but most practitioners do consider that the use of PRP can benefit patients. In this context, the question of the *ethicality* and *legality* of PRP use has also been raised, as patients cannot be guaranteed that the treatment will work. Codorean et al. argue that the use of PRP is legal and ethical for a patient who is correctly and fully informed about the technology used and the performed procedure, the possible outcomes of the treatment and the information available in the literature, possible side effects and contraindications to the procedure [27].

Contraindications for PRP use are as follows:

Absolute contraindications: platelet dysfunction syndrome, critical thrombocytopenia, hemodynamic instability, sepsis and injection site infections, anticoagulant therapy (warfarin, dabigatran, heparin).

Relative contraindications: HGB < 10 g/dl, Platelets < 105 /μl, use of NSAIDs in the last 48 hours before the procedure, use of systemic corticosteroids in the last 2 weeks, or local corticosteroid injection in the last month before the procedure, neoplasms – especially haematologic, hypofibrinogenemia, chronic liver disease, pregnancy and breastfeeding [26,27,30].

Many studies, both clinical and laboratory, have attempted to clarify the role of drugs with direct or indirect effects on platelet function in the PRP setting. Of these, NSAIDs and antihistamines have been of particular interest.

Aspirin, as an exponent of its class, is very commonly used in the general population precisely due to its effect of inhibiting platelet aggregation. It blocks the conversion of arachidonic acid to proinflammatory prostaglandins by irreversible inhibition of cyclooxygenase 1

(COX-1). The release of growth factors from PRP has been shown to be dependent on platelet aggregation[31]. This raised the question of whether the use of aspirin can influence the amount of growth factors in freshly prepared PRP [31,32]. As observed by Jayaram et al., the use of low-dose aspirin (81mg) for 14 days prior to PRP preparation did not alter the number of platelets or leukocytes in whole blood or plasma. There was, however, a significant decrease in the growth factors that were expressed (VEGF, PDGF and TGF-β1), which was also correlated with the chosen mode of PRP activation. The authors emphasize the need for clinical studies to determine the significance of these observations *in vivo* [32].

In contrast to NSAIDs, H1 antihistamines, in particular generation I antihistamines, inhibit *in vitro* platelet aggregation by inhibiting systems within platelets (probably phospholipase A2). The effect is much lower for second-generation molecules (with Loratadine as representative), and when this effect is studied in plasma or whole blood, i.e., when the extra-platelet medium contains proteins, it becomes almost insignificant at usual therapeutic doses. The observation has been attributed to the binding of loratadine to plasma proteins [33,34].

Discussions

Out of a desire to offer the most clinically-effective products at the most attractive price, many manufacturers have developed and patented complete PRP preparation systems. Thus, we are faced with a great variability in the equipment required, different centrifugation parameters from one manufacturer to another, all of which have a direct influence on platelet degranulation and therefore on the clinical results obtained.

The lack of standardization of PRP products means that the success observed for a particular product in a specific pathology cannot be automatically extended to the whole range.

At the same time, there continue to be differences of opinion among specialists about the indications for using PRP in different conditions. A striking example is the use of PRP in alopecia areata (AA). This belongs to the group of immune-mediated alopecias and continues to represent a therapeutic challenge, as many treatments underperform, while side effects can be significant [35]. In addition, they have high rates of relapse and spontaneous remission respectively, which has made it even more difficult to objectively assess the efficacy of treatment. The majority of publications supporting the use of PRP in AA represent case studies, or studies in small groups of patients without control groups, which also raises problems in performing meta-analyses. The most recent of these have failed to prove the efficacy of PRP in this condition and emphasize the need for standardization of protocols as a precondition for establishing recommendations [35,36,37]

Taking into account the pathophysiological mechanisms, the role of IL-6 in AA proven by numerous studies - its serum level being correlated with disease duration(38,39), and the fact that PRP administration significantly increases the level of IL-6 and other pro-inflammatory molecules, respectively[40], the authors of this article are of the opinion that PRP should be used with restraint as a therapeutic agent for alopecia areata, possibly after the inflammatory phase has subsided.

Last but not least, the concern in this area also derives directly from the global interest shown by increasingly informed patients. Both manufacturers, through the media and social media platforms, and doctors who offer such procedures have played a very important role in disseminating information. As proof of this, if the global PRP market in 2009 was valued at 45 million dollars, in 2017 its value reached 195.2 million dollars, well above the estimates made in the initial stages of the launch of this type of products. Starting from the balance of 2019 (211.96 million dollars) and considering the growing interest in the most technologically advanced medical means, a *compound annual growth rate* (*CAGR*) of 11.6% is evaluated, so that in 2026 the global market for PRP is estimated to reach approximately 543.5 million dollars, and in 2030 715.65 million dollars [41,42].

In terms of medical ethics, procedures using PRP must be supported by the diagnosis and pathogenic mechanism of the condition to be treated, and the quality of the devices used to obtain PRP must be sustained by clear evidence of the concentration, purity, and viability of the platelets obtained.

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Conflict of interest
NONE DECLARED

Correspondance address: Egoclinic, Bucharest
office@egoclinic.ro

AN ERRATUM

RESULTS AND CHARACTERISTICS OF NON-MELANOCYTIC SKIN CANCERS AND OF ACTINIC KERATOSIS IN A PATIENT WITH MYELOFIBROSIS UNDER TREATMENT WITH FEDRATINIB

1. Page 16

– and basal cell carcinoma on the left shoulder.

2. Page 18

– On histopathological examination were identified lesions of actinic keratosis surrounding the SCC (Figure 10 and Figure 11), and on the left infraorbital region of ulcerated Bowenoid actinic keratosis was diagnosed (Figure 12).

Skin lesion location	Biopsy	Excision	TNM staging
Right preauricular	Well differentiated squamous cell carcinoma, with superficial invasion	Well differentiated squamous cell carcinoma, keratoacantoma-like, with in situ lesion and multifocal, bowenoid and proliferative actinie keratosis	pTl
Nasolabial fold - superior	benign	Intradermal nevus	benign
Nasolabial fold - inferior	Basal cell carcinoma	Multifocal nodular basal cell carcinoma	pTl
Presternal	Well differentiated squamous cell carcinoma	Well differentiated squamous cell carcinoma with in situ lesion and proliferative actinie keratosis. Lympho-vascular invasion + (LVI 1) Perineural invasion + (Pnl)	pTl
Left shoulder	Basal cell carcinoma	Ulcerated nodular basal cell carcinoma	pTl
Left infraorbital	Actinie keratosis with plasmocitosis	Ulcerated bowenoid actinie keratosis	premalignant

Page 19

– Fig 6. Well differentiated squamous cell carcinoma, H.E x 20.

– Fig 7. Well differentiated squamous cell carcinoma, H.E x 10.

– Fig.8. Adipose tissue invasion of squamous cell carcinoma.

– Fig. 9. Small nerve bundle with perineural invasion of squamous cell carcinoma (nerve bundle diameter < 0.1 mm)

– Fig.11. Proliferative actinic keratosis, H.E x 10.

Page 20

– Fig.12. Ulcerated bowenoid actinic keratosis, H.E x 10.