Anti-inflammatory effect of metallic silver and gold nanoparticles complexed with polyphenolic compounds in human chronic stationary plaque psoriasis

Dissertation zur Erlangung des Doktorgrades der Medizin der Medizinischen Fakultät der Universität Ulm

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Ag-NPs-CM</td>
<td>Silver nanoparticles complexed with <em>Cornus mas</em></td>
</tr>
<tr>
<td>Au-NPs-CM</td>
<td>Gold nanoparticles complexed with <em>Cornus mas</em></td>
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<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CSA</td>
<td>Cyclosporin A</td>
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<tr>
<td>CD18\textsuperscript{hypo}</td>
<td>CD18\textsuperscript{hypomorphic} mutation in (tgb1&lt;tm1Bay&gt;) PL/J mice</td>
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<tr>
<td>DAPI</td>
<td>4′,6-Diamidin-2-phenylindol</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular-signal Regulated Kinase</td>
</tr>
<tr>
<td>FT-IR</td>
<td>Fourier transform infrared spectroscopy</td>
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<tr>
<td>GLP-1</td>
<td>Glucagon-like peptide</td>
</tr>
<tr>
<td>HEP</td>
<td>High echogenic pixels</td>
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<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IκBα</td>
<td>Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactatdehydrogenase</td>
</tr>
<tr>
<td>LEP</td>
<td>Low echogenic pixels</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte chemotactic protein</td>
</tr>
<tr>
<td>MEP</td>
<td>Medium echogenic pixels</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>NALFD</td>
<td>Non-alcoholic fatty liver disease</td>
</tr>
<tr>
<td>µl/ml/µg</td>
<td>Micro liter/milli liter/micro gram</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>NK cells</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>NPC</td>
<td>Neural progenitor cells</td>
</tr>
<tr>
<td>NPs</td>
<td>Nanoparticles</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PASI</td>
<td>Psoriasis Area and Severity Index</td>
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<tr>
<td>PDE</td>
<td>Phosphodiesterase</td>
</tr>
<tr>
<td>PsA</td>
<td>Psoriasis arthritis</td>
</tr>
<tr>
<td>PSORS1</td>
<td>Psoriasis susceptibility locus 1</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
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<tr>
<td>TGF-β</td>
<td>Tissue growth factor beta</td>
</tr>
<tr>
<td>Th cell</td>
<td>T helper cell</td>
</tr>
<tr>
<td>U</td>
<td>Units</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>PUVA</td>
<td>Psoralen and ultraviolet A</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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1. Introduction

1.1 Psoriasis vulgaris

Psoriasis is a chronic, T-cell mediated inflammatory autoimmune disorder of the skin, characterized by keratinocyte hyperproliferation and resulting in indurated, erythematous, scaly plaques. With a prevalence of 1 to 3%, the condition can occur in all age groups, however, it primarily arises in adulthood, with no gender predilection (Parisi et al. 2013). Psoriasis can also affect the joints, leading to the development of a destructive inflammatory arthropathy seen in 25% to 34% of patients with psoriasis (Gladman et al. 2004).

Like other chronic inflammatory dermatoses, psoriasis is characterized by a systemic inflammatory state with increased acute phase reactants and inflammatory biomarkers. Psoriasis is subject to flares and remissions and presents in many different variations and degrees of severity. The condition is associated in its severe form with an increased risk of developing cardiovascular disease, significant psychosocial disability and has a major impact on the patients’ quality of life (Kurd et al. 2010).

The pathogenesis of psoriasis is multifactorial and still not fully understood. The known genetic predisposition is further aggravated by certain stimulating factors. Previous reports have shown that the dominant genetic effect is located on chromosome 6p21.3 within the major histocompatibility complex region (psoriasis susceptibility locus 1 – PSORS1 – encoding the HLA-Cw0602 allele of the MHC class I molecule (Elder et al. 2001). Other additional genes responsible for the skin barrier function, the innate and adaptive immune response have also been identified and associated with the disease by genome-wide association studies (O’Rielly et al. 2015). Several lifestyle factors have also been associated with morbidity in psoriasis. Literature reports have shown that several exogenous and endogenous factors such as alcohol use, smoking, obesity or emotional stress may increase morbidity in psoriasis patients (Wenqing et al. 2012). Furthermore, drugs such as β-blockers, lithium, antimalaria agents, TNFα-blockers or interferon have been known to induce or exacerbate psoriasis (Raut et al. 2013).
1.2 Clinical classification
Psoriasis presents with a large spectrum of clinical features and evolution. According to literature data, about 1/3 of the patients have moderate or severe disease, involving more than 10% of the body surface (Salgo et al. 2009). Psoriasis can be classified by morphology and by the pathogenetic mechanism.

1.2.1 Morphology
The most common form of disease is plaque psoriasis, affecting about 80% of the patients. It is characterized by thick, erythematous plaques with silvery, shiny deposits of scales. In this case the erythematous scaly plaques tend to remain stationary or to progressively enlarge, affecting especially scalp, knees, elbows, or lower back (Christophers 2001).

Inverse psoriasis usually affects the skin folds, especially the regions under the armpits, the abdominal skin fold, the breast area or the gluteal cleft; in this situation the plaques are thinner and there is no or minimal scaling.

Guttate psoriasis usually emerges acutely following a bacterial or viral infection of the upper ways. It usually presents with small round scaly plaques scattered across the entire skin surface; it either resolves with infection or progresses to the development of psoriasis vulgaris (Vence et al. 2015).

Pustular psoriasis is characterized by a disseminated outburst of sterile pustules, accompanied by recurrent episodes of fever. It constitutes a less frequent clinical pattern. If the entire integument is affected, the condition is called “generalized (von Zumbusch) type”. Acrodermatitis continua Hallopeau represents a rare, localized form of palmoplantar pustular psoriasis, often unresponsive to treatment. It usually emerges with pustules on erythematous scaly patches of the distal phalanges of fingers and toes. The frequent involvement of the nail bed and matrix leads to severe nail dystrophy (Oji et al. 2015).

Erythrodermatic psoriasis is the generalized form of the disease, where the entire integument is highly erythematous and covered by superficial scales. Patients usually also develop fatigue, myalgia, fever and chills.

Nail psoriasis emerges in about 61% of cases of cutaneous psoriasis. The classical manifestations of the nail apparatus include pitting, trachyonychia, leuchonichia, Beau’s lines, transverse grooves. The nail bed and hyponychium can present with onycholysis, oil
drops, subungual hyperkeratosis and at the distal nail bed splinter hemorrhages may occur. (Ayala 2007).

1.2.2 Pathogenic mechanism
According to the pathogenetic factors, psoriasis can be classified as:
- **Type 1 psoriasis**: positive family history of psoriasis, onset before the age of 40, association with HLA-Cw6
- **Type 2 psoriasis**: negative family history of psoriasis, onset before the age of 40, no gene association
- **Drug-induced psoriasis**: association with the use of pharmaceutical products (Langley et al. 2004)

1.3 Comorbidities associated with psoriasis
Although psoriasis is usually associated with the development of T-cell driven inflammatory hyperkeratotic, scaly patches of the skin, increasing evidence shows psoriasis to be a multisystem chronic inflammatory disorder with multiple associated comorbidities (Farley et al. 2011). Several extracutaneous disorders have been linked to psoriasis:
- **Psoriasis arthritis**: Psoriasis arthritis represents a chronic inflammatory condition of the joints, tendon sheaths and axial skeleton, which significantly impairs the quality of life and physical function of the patient. A recent review of published epidemiologic studies showed that the reported proportion of psoriatic arthritis among psoriasis patients ranges from 7% to 26.9% (Prey et al. 2010). Radiological identifiable joint damage appears approximately 2 years following diagnosis of psoriasis arthritis. The same study has shown that the appearance of psoriasis arthritis can occur up to 12 years after the diagnosis of skin psoriasis (Gladman et al. 2005).
- **Cardiovascular diseases**: Patients with severe psoriasis or psoriasis arthritis have an increased risk of severe cardiovascular events and related mortality (myocardial infarction, atherosclerosis, ischemic stroke), mostly due to accelerated atherosclerosis. According to Wong et al. the cardiovascular mortality in patients
with psoriasis arthritis is 30% higher than in the general population (Wong et al. 1997).

- **Tumors:** Recent studies show an increased risk for psoriasis patients of developing non-Hodgkin lymphoma, squamous or basal cell carcinoma. The increase in squamous cell carcinoma may however be associated with previous exposure to 8-methoxypsoralen-ultraviolet-A (PUVA) phototherapy and immunosuppressive agents such as cyclosporine and possibly methotrexate. The occurrence of solid tumors in psoriasis patients mostly correlates with alcohol intake and smoking (Pouplard et al. 2013).

- **Crohn’s disease:** Psoriasis and inflammatory bowel disease are tightly related inflammatory diseases, probably sharing immune-pathogenetic mechanisms. Cohen et al. demonstrated on 12502 psoriasis patients and 24287 controls that the prevalence of Crohn’s disease was significantly higher in patients with psoriasis compared to those of the control group (p<0.001) (Cohen et al. 2009).

- **Depression:** Psoriasis and psoriatic arthritis can cause a significant impairment of the quality of life, leading to emotional distress for patients, with increased risk of developing depression.

- **Metabolic Syndrome:** There is a known significant association between psoriatic disease and metabolic syndrome. Danielsen et al. showed in a cross-sectional study including 10521 participants aged 30–79 years that there is a uniformly higher prevalence of metabolic syndrome in men and women with psoriasis compared with those without across all age groups. Furthermore, more women with psoriasis had metabolic syndrome than men (Danielsen et al. 2015).

- **Diabetes:** Several studies indicated an association between type 2 diabetes and psoriasis (Khalid et al. 2013; Armstrong et al. 2013). Armstrong et al. showed in a meta-analysis that the prevalence and incidence of type 2 diabetes are increased among psoriasis patients. Psoriasis patients with an associated diabetes type 2 should therefore be encouraged to improve their metabolic status in order to lower the risk of complications (Hermann et al. 2014). Recent studies have also shown a beneficial anti-inflammatory effect of Glucagon-like peptide-1 (GLP-1) agonists (class of drugs used for the treatment of type 2 diabetes mellitus) in patients with diabetes and psoriasis (Al-Badri et al. 2014).
- **Obesity:** Regardless of etiology, obesity is an independent factor for increased mortality. Naldi et al. pointed out that the severity of psoriasis was directly related to high body mass index (BMI) in a case-control study involving 560 patients with newly diagnosed psoriasis and 690 controls. The same study showed that the prevalence of psoriasis was approximately twice as high in individuals with a BMI of 30 or greater compared with a BMI of less than 26 (Naldi et al. 2005).

- **Non-alcoholic fatty liver disease (NAFLD):** NAFLD is regarded as the hepatic manifestation of the metabolic syndrome. Ganzetti et al. demonstrated that there is a higher prevalence of NAFLD and metabolic syndrome in psoriasis patients compared to the general population and that patients with NAFLD and psoriasis are at higher risk of developing liver fibrosis than patients without psoriasis (Ganzetti et al. 2015).

- **Osteoporosis:** Psoriasis and psoriasis arthritis (PsA) patients have higher risk of developing fragility bone fractures, especially in case of longer disease duration, disability and recurrent falls (Pedreira et al. 2011). Frediani et al. found a significant reduction of bone mineral density in two-thirds of PsA patients when compared to control. This might be due to the fact that psoriasis arthritis patients present higher bone resorption and bone loss related to the inflammatory process (Frediani et al. 2001).

- **Uveitis:** Uveitis tends to develop more frequently in patients with psoriasis arthritis than in patients with other forms of psoriasis. Moreover, HLA-B7 may be associated with more severe uveitis in patients with psoriasis, compared to HLA-B27-negative patients with uveitis. Periodical ophthalmological evaluation should be performed in psoriasis patients with ocular or joint involvement in order to ensure an early diagnosis and appropriate therapy, preventing vision loss in patients with psoriasis and uveitis (Fraga et al. 2012).

### 1.4 Pathophysiology of psoriasis

The pathogenesis of psoriasis involves several dynamic interactions between different cell types and inflammatory cytokines in response to various exogenous or endogenous triggers, leading to the alteration of the skin homeostasis (Mahil et al. 2015). Psoriasis is
regarded as a T-cell mediated inflammatory disease of the skin and joints, characterized by an ongoing inflammation status promoted by activated T cells (Waite et al. 2012). From histological point of view, psoriasis is characterized by acanthosis (thickening of the epidermis), papillomatosis (elongation of the rete ridges) and hypogranulosis (decrease of the granular layer) due to an increase in keratinocyte proliferation in the basal layer (Murphy et al. 2007). Another histological hallmark is parakeratosis (the retention of keratinocytic nuclei in the stratum corneum) which occurs due to the increased epidermal turnover resulting in premature keratinocyte maturation in the upper epidermal layers, and is clinically manifested by the presence of the silvery scales (Figure 1).

**Figure 1. Clinical and histological features of psoriasis plaques.** (a) Clinical aspect of a well demarcated erythematous psoriatic plaque covered with ‘silvery’ scales (University Clinic Ulm, Clinic for Dermatology and Allergic diseases). (b) Schematic structure of psoriasis-affected skin with thickened stratum corneum due to a differentiation disturbance of keratinocytes, elongated rete ridges due to the hyperproliferation of the stratum spinosum, parakeratosis (no loss of nuclei of keratinocytes in the stratum corneum) and acanthosis (thickening of the epidermis).

Furthermore, neutrophils are also found in the epidermis of psoriatic plaques, giving rise to the Munro microabscesses. Psoriasis plaques are also highly vascularized; the new vessel formation being mediated by angiogenic factors such as the vascular endothelial growth factor (VEGF) (Lee et al. 2015; Marina et al. 2015).
Immunohistochemical staining of psoriatic lesions also show a dense dermal immune cell infiltrate of T cells, dendritic cells (DC) and macrophages, known to produce and release a number of pro-inflammatory cytokines such as tumor necrosis factor (TNFα), inducible nitric oxide synthase (iNOS), γ-interferon (IFNγ), interleukin-1β, IL-17, IL-22, IL-23 (Clark et al. 2006). The mechanism underlying the inflammatory events in psoriasis still needs to be elucidated in more detail. It has been suggested that different triggers such as physical injury, streptococcal infections, stress and drugs lead to the release of antimicrobial peptides, such as LL37 (cathelicidin), by keratinocytes, which then bind with pathogen- or self-DNA released by degraded cells, forming a complex which activates Toll-like receptor 9 (TLR9) on dendritic cells (Lande et al. 2007; Gilliet et al. 2008). Activated dendritic cells promote the release of IFNγ, TNFα, IL-1β, IL-6, IL-23 and IL-20, increase the local release of myeloic DC, resulting in further T-cell and macrophage activation (Lee et al. 2004).

Activated macrophages produce TNFα, monocyte chemotactic protein-1 (MCP-1), inducible nitric oxide synthase (iNOS) and promote strong IL-12-mediated Th1 responses (Wang et al. 2009). Recent evidence from genetic mouse models have shown that macrophages play a significant role in T-cell-mediated and epidermis-mediated psoriasiform skin inflammation (Stratis et al. 2006; Wang et al. 2006).

The effector molecules secreted by the T-cells (IFNγ, IL-17, IL-22) further activate keratinocytes, resulting in an increased release of pro-inflammatory cytokines and chemokines which continue to recruit and activate inflammatory cells, amplifying the cutaneous inflammation process (Mahil et al. 2015) (Figure 2).
Figure 2. Pathogenesis of psoriasis vulgaris. Activation of dendritic cells as a result to a stimulus such as trauma or infection leads to further activation of autoreactive T-cells and macrophages within the dermis by production of pro-inflammatory cytokines (IFN, TNFα). Activated macrophages with TNFα, IL-12 release induce activation of epidermal keratinocytes leading to an epidermal remodeling and appearance of the psoriatic plaque.

Conrad et al. showed that in affected psoriasis skin there is a predominance of Th1 type pro-inflammatory cytokine profile comprising IFNγ, TNFα, IL-2, IL-6, IL-12, IL-17 and IL-23, in comparison to Th2 cytokines IL-10 and IL-4, which are poorly represented. This observation supports the idea of psoriasis being a Th1 mediated inflammatory disease, with macrophages playing a crucial role in perpetuating inflammation in psoriatic skin lesions (Wang et al. 2006).

1.5 Therapeutic approaches in psoriasis
Psoriasis therapy comprises three forms:

- External treatment with ointments, creams, emulsions, solutions
- Internal treatment by systemic delivery of immunosuppressive drugs or monoclonal antibodies
- Phototherapy with UVA or UVB light
1.5.1 Topical therapy

About 80% of psoriasis patients have mild to moderate disease severity and can be treated with topical agents alone or, for more resistant lesions, in combination with UV light or systemic medications (Schön et al. 2005). Drugs applied directly to the psoriatic skin lesions are the safest treatment approach but are only practical when treating localized disease. They slow down or normalize excessive cell proliferation and reduce dermal inflammation. The efficacy of a topical therapy is significantly influenced by the choice of the vehicle, since it considerably alters the penetration of the active substance into the skin.

The main topical treatment options in psoriasis include:

- **Corticosteroids** inhibit cytokine production and reduce the amount of other inflammatory mediators such as prostaglandins and leucotrienes (Reich et al. 2011). Clinically, corticosteroids improve local erythema, oedema and diminish keratinocyte hyperproliferation (Vakirlis et al. 2008). However, their local and systemic side effects limit their use to only short intervals. The most commonly side effects following topical corticosteroid therapy include: skin atrophy, hirsutismus, folliculitis, stretch marks and rarely Cushing syndrome.

- **Vitamin D3-derivatives** interact with vitamin D receptors present on keratinocytes and lymphocytes, decreasing epidermal proliferation, excessive keratinisation and angiogenesis. Vitamin D analogues also modulate the inflammatory process in psoriasis by decreasing IL-1 and IL-6 levels and also seem to protect against steroid-induced atrophy and tachyphylaxis (O’Neill et al. 2010). The combination of corticosteroids and Vitamin D analogues is currently the treatment of choice in psoriasis, being superior to monotherapy, since Vitamin D analogues have mainly epidermal anti-proliferative effect and steroids dermal anti-inflammatory effects (Menter et al. 2007).

- **Keratolytics** as salicylic acid are known to reduce keratinocyte-to-keratinocyte binding as well as the pH of the stratum corneum; these effects lead to reduced scaling and softening of psoriatic plaques (Lebwohl et al. 1999). In order to avoid systemic toxicity, they are not to be used in combination with other oral salicylate drugs or applied to more than 20% of the body surface. Keratolytics are often added to corticosteroids or Vitamin D analogues in order to enhance their penetration into the skin (Jacobi et al. 2015).
- **Retinoids** like Tazarotene, a Vitamin A analogue diminishes keratinocyte hyperproliferation in psoriasis plaques and decreases the expression of inflammatory markers. Tazarotene has teratogenic potential and therefore cannot be used in pregnancy (Jeon et al. 2014).

- **Calcineurin inhibitors** *(pimecrolimus, tacrolimus)* block the synthesis of inflammatory cytokines in the psoriatic plaques. These agents may be used alone or in combination with topical corticosteroids as corticosteroid sparing agents for long term maintenance therapy. Several animal studies suggested that the concomitant use of calcineurin inhibitors and UV light might increase the risk of developing epithelial tumors however, no similar observations were made in humans so far (Ring et al. 2005; Margolis et al 2007).

- **Anthracen derivatives** *(Anthralin, Dithranol)* are suggested to prevent T-lymphocyte activation and normalize keratinocyte differentiation by a direct effect on mitochondria (McGill et al. 2005). The substance is most commonly used as short contact therapy (20-30 minutes), starting at 1% concentration with increase over time as tolerated. The most common side effects of anthralin include skin irritation and staining of lesional and adjacent skin, nails and clothing.

- **Coal tar** is a distillation product from coal which suppresses DNA synthesis by diminishing the keratinocytic mitotic activity. Coal tar products are often poorly tolerated by patients because of cosmetic issues.

Current guidelines recommend the short-term use of potent topical agents in acute flares, followed by less potent steroid-sparing agents for long-term management. Patients requiring a continuous topical treatment are instructed to use the least potent agent that allows the control of the disease. The adherence of the patients to topical therapy is a major issue, being generally poor due to delay or lack of response, wrong choice of vehicle, potential side-effects or the use of topical agents in extensive disease. Therefore, the choice of the appropriate topical medication to achieve the desired clinical response, as well as the right vehicle represent important issues which can improve patient adherence to the therapy (American Academy of Dermatology Work Group, 2011).
1.5.2 Systemic therapy

Systemic treatment is usually required for severe or recalcitrant forms of psoriasis. In the past, conventional systemic psoriasis therapies including methotrexate, cyclosporine and acitretin were used only when the psoriasis lesions were too extensive or refractory to topical therapy. In recent years, with the advent of biologics agents the principles of treatment in psoriasis have been revised, as these provide valuable and potent therapeutic options with diminished organ toxicity.

1.5.2.1 Immunsuppressive therapy

The main systemic treatments include:

- **Methotrexate** is an antimetabolite and antifolate drug, which slows down the cell turnover. It is the most commonly prescribed traditional systemic therapy for psoriasis worldwide. It can be used in combination with all of the approved biologic agents for psoriasis, especially with TNFα inhibitors, in order to suppress the occurrence of auto-antibodies against antibodies, especially against adalimumab and infliximab (Atzeni et al. 2008).

- **Cyclosporine (CSA)** is one of the most effective treatments available for psoriasis, slowing down the rapid turnover of the keratinocytes; however, when administered for more than 3 years, CSA induces a significant increase in the incidence of glomerulosclerosis. In patients with severe flares of psoriasis, CSA efficiently induces a rapid remission (Lowe et al. 1996).

- **Acitretin** represents an oral retinoid which in conjunction with UV-therapy represents an alternative for those patients which do not respond to other therapies. Because retinoids are potentially teratogenic, they are not to be used in pregnancy. Of the systemic therapies, acitretin is the least effective as monotherapy and it is therefore often used in combination with UVB or psoralen plus UVA (PUVA) phototherapy. At high doses, it may be associated with significant mucocutaneous effects along with hair loss (Caliskan et al. 2015).

- **Fumaric acid esters** are one of the most commonly used systemic treatments for psoriasis in Germany, having proven to be an extremely effective therapy, with a very good tolerability and no long-term toxicity or immunosuppressive effects. According to literature, fumaric acid esters appear to influence the pro-
inflammatory signal transduction pathway in psoriasis by means of modulating the intracellular redox system. Several studies have shown that about 50-70% of patients treated with fumaric acid esters achieve a PASI 75 improvement after only 4 months of therapy (Roll et al. 2007).

To minimize the toxicity of any therapy, proper patient selection and appropriate monitoring are crucial. The decision to administer methotrexate, CSA, acitretin, or any other traditional therapy must be individualized. Every patient needs to be carefully evaluated with reference to disease severity, quality of life, and general medical and psychological status.

1.5.2.2 Biologic response modifiers and small molecules

The appearance of biologic therapies has revolutionized the therapy of psoriasis. Biologics are agents that can specifically target immune or genetic mediators in a pathophysiologic process. They are antibodies, fusion proteins, recombinant cytokines or small molecules which directly target the pathologic activation of T cells. The mechanism of action of these target therapies involves:

- inhibition of T-cell activation and migration
- elimination of activated T-cells
- inhibition of pro-inflammatory cytokine release including TNFα-blockers, anti-IL-12 antibodies, anti-IL-17 antibodies or PDE4 inhibitors (Lui et al. 2004; Singri et al. 2002).

Biologics are generally safe however, there is modest concern about the risk of developing lymphoma with use of these agents. All anti-TNFα agents have been associated with a variety of serious and opportunistic infections. Therefore, it is crucial to obtain an appropriate history, physical examination and a set of baseline laboratory investigations of the patients prior to treatment initiation. According to the S3 guidelines of the treatment of psoriasis, a chemistry screen with liver function tests, complete blood cell count including platelet count, a hepatitis panel, and tuberculosis (TB) should be performed prior to treatment initiation (Nast et al. 2012).

Treatment with biologics is contraindicated in patients with active, or chronic severe infections. If patients develop severe infections during treatment with a biologic agent, the biologic should be discontinued until the infection has resolved.
The newest psoriasis systemic therapy involves small molecules which target psoriasis inflammation by inhibiting phosphodiesterase 4 (PDE4) (Apremilast). Papp et al. published 2014 the results of a phase III, randomized, controlled trial (Efficacy and Safety Trial Evaluating the Effects of Apremilast in Psoriasis [ESTEEM]), showing that oral administration of Apremilast, a new oral inhibitor, significantly reduced the baseline Psoriasis Area and Severity Index score (PASI 75) by more than 75% in psoriasis patients when compared to placebo (p<0.001) (Papp et al. 2015).

Apremilast appears to work intracellular by regulating the release of inflammatory mediators including the pathways relevant to the pathogenesis of psoriasis (Schafer 2012). According to literature, the inhibition of PDE4 increases the intracellular amount of cyclic adenosine monophosphate, down regulating the inflammatory responses within T helper, Th17 and type 1 interferon pathways, and consequently modulating the release of anti-inflammatory cytokines such as IL-10. By controlling systemic inflammation, Apremilast also improves joint tenderness and swelling in people with active psoriatic arthritis (Rich et al. 2015).

A summary of the current biologic agents and small molecules approved for the treatment of psoriasis along with their main side effects is illustrated in Table 1 (Gordon 2015; Kimball et al. 2008).
Table 1. Summary of the actual biological agents and small molecules approved for the treatment of psoriasis along with their main side effects

<table>
<thead>
<tr>
<th>Biological agent</th>
<th>Pathophysiological pathway</th>
<th>Generic name</th>
<th>Current indications</th>
<th>Route</th>
<th>Frequent adverse effects</th>
<th>Serious adverse effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adalimumab</td>
<td></td>
<td>Moderate to severe plaque psoriasis</td>
<td>sc</td>
<td>Injection site reaction</td>
<td>Possible malignancy Infections</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reumatoid arthritis (RA)</td>
<td></td>
<td></td>
<td>Hepatic dysfunction</td>
<td>Demyelinating disease</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Juvenile RA</td>
<td></td>
<td></td>
<td>Arthralgies</td>
<td>Reactivation of HEP B</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Psoriatic arthritis</td>
<td></td>
<td></td>
<td>Headache</td>
<td>and Latent TB</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ankylosing spondylitis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Crohn’s disease</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Etanercept</td>
<td></td>
<td>Moderate to severe plaque psoriasis</td>
<td>sc</td>
<td>Injection site reactions</td>
<td>Possible malignancy Infections</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reumatoid arthritis</td>
<td></td>
<td></td>
<td>Headaches rhinitis</td>
<td>Demyelinating disease</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Juvenile RA</td>
<td></td>
<td></td>
<td></td>
<td>Reactivation of HEP B</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Psoriatic arthritis</td>
<td></td>
<td></td>
<td></td>
<td>and Latent TB</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ankylosing spondylitis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α inhibition</td>
<td>Infliximab</td>
<td>Moderate to severe plaque psoriasis</td>
<td>iv</td>
<td>Infusion reaction</td>
<td>Possible malignancy Infections</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reumatoid arthritis</td>
<td></td>
<td></td>
<td>Headache</td>
<td>Demyelinating disease</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Psoriatic arthritis</td>
<td></td>
<td></td>
<td>Vertigo</td>
<td>Reactivation of HEP B</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ankylosing spondylitis</td>
<td></td>
<td></td>
<td>Flushing</td>
<td>and Latent TB</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Crohn’s disease</td>
<td></td>
<td></td>
<td>Abnormal liver function tests</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ulcerative colitis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Golimumab</td>
<td></td>
<td>Rheumatoid arthritis</td>
<td>sc</td>
<td>Upper respiratory tract infection</td>
<td>Reaction of HEP B Congestive heart failure</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Psoriatic arthritis</td>
<td></td>
<td></td>
<td>Headache</td>
<td>Multiple sclerosis or</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ankylosing spondylitis</td>
<td></td>
<td></td>
<td>Arthralgia</td>
<td>Guillain-Barré syndrome</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Dizziness</td>
<td>Hepatosplenic T-cell</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lymphoma</td>
</tr>
<tr>
<td>Interleukin 12/23 inhibition</td>
<td>Ustekinumab</td>
<td>Moderate to severe plaque psoriasis</td>
<td>sc</td>
<td>Upper respiratory infection</td>
<td>Blurred or loss of vision</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Psoriatic arthritis</td>
<td></td>
<td></td>
<td>Headache</td>
<td>Disturbed color perception</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Arthralgia</td>
<td>Seizures</td>
</tr>
<tr>
<td>Interleukin 17 inhibition</td>
<td>Sekukinumab</td>
<td>Moderate to severe plaque psoriasis</td>
<td>sc</td>
<td>Diarrhea</td>
<td>Diarrhea</td>
<td>Oral herpes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Upper respiratory</td>
<td>Pharyngitis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>infections</td>
<td>Urticaria</td>
</tr>
<tr>
<td>Small molecules</td>
<td>Apremilast</td>
<td>Psoriasis</td>
<td>oral</td>
<td></td>
<td>Diarrhoea</td>
<td>Upper respiratory tract infection</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Psoriatic arthritis</td>
<td></td>
<td></td>
<td>Headache</td>
<td>Nasopharyngitis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Nausea</td>
<td></td>
</tr>
</tbody>
</table>
1.5.3 Phototherapy and Photochemotherapy

Phototherapy exerts its therapeutic effect by altering the cytokine expression leading to a suppression of the Th1/Th17 inflammatory axis and subsequent up-regulation of the Th2 pathway. This mechanism of action promotes the immunosuppression of the Langerhans cells as well as other key cells that maintain the inflammatory status in psoriasis (Tami Wong et al. 2013).

All patients who are considered for treatment with phototherapy or photochemotherapy must provide a complete medical history and physical examination. Patients with a known history of lupus erythematosus or xeroderma pigmentosum should not be treated with phototherapy or photochemotherapy. Patients with a history of a photosensitivity disorder, taking photosensitizing medications, with a history of melanoma, with atypical nevi, with multiple risk factors for melanoma, with multiple nonmelanoma skin cancers, or who are immunosuppressed as a result of organ transplantation should be screened carefully before initiating phototherapy or photochemotherapy (Anderson et al. 2015).

- **Broadband and narrowband ultraviolet B phototherapy** is indicated for generalized psoriasis (excluding guttate) unresponsive to topicals. A clinical response is observed at 8-10 treatments. Narrowband UVB treatment is superior to broadband UVB (Fitzpatrick 1977).

- **Psoralen and ultraviolet A (UVA) photochemotherapy (PUVA)** is a treatment option that combines oral or topical administration of a photosensitizer (psoralen) with exposure to UVA light. Compared to broadband UVB treatment, PUVA treatment taken two to three times a week clears psoriasis more consistently and in fewer treatment sessions. However, it is associated with more short-term side effects, including nausea, headache, fatigue, burning and itching. Care must be taken to avoid sunlight after ingesting psoralen to avoid severe sunburns. Long-term treatment is associated with an increased risk of squamous-cell and, possibly, melanoma skin cancers (Pai et al. 2015; Szponar-Bojda et al. 2012).

Bathing and climatic therapies and psycho-social therapies are also used as accompanying measures. Some individuals with moderate to severe psoriasis may benefit from counseling or participation in a support group to reduce self-consciousness about their appearance or relieve psychological distress resulting from fear of social rejection.
1.6 Nanomaterials and application of nanotechnology in dermatology

1.6.1 Nanotechnology and nanomedicine
The U.S. National Nanotechnology Initiative defines nanotechnology as “the understanding and control of matter at dimensions between approximately 1 and 100 nanometers, where unique phenomena enable novel applications. Encompassing nanoscale science, engineering and technology, nanotechnology involves imaging, measuring, modeling, and manipulating matter at this length scale” (National Nanotechnology Initiative; http://www.nano.gov/nanotech-101).

The recent development and incorporation of nanotechnologies into the medical field is of great interest due to its therapeutic and diagnostic applications. One of many divisions of nanomedicine is nanodermatology, which focuses on the use of nanomaterials (sized less than 100 nm) on the skin (Abramovits et al. 2010). Nanoparticles (NPs), defined as single particles with a diameter less than 100 nm, are a subset of nanomaterials which, due to their unique physical (small size and their high surface-to-volume ratio) and chemical properties can overcome barriers and interact with biologic systems (Nohynek et al. 2007). Therefore, nanoparticles can be engineered to serve as vehicles that carry various therapeutic agents and may be useful in medical applications, including targeted drug delivery, vaccine delivery, antimicrobials, and immunomodulation (Prow et al. 2011). A summary of the main advantages of nanoparticles is illustrated in Table 2.

Table 2. Main advantages of nanoparticle-based technology

<table>
<thead>
<tr>
<th>Advantages of nanoparticle-based technology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Improvement of drug bioavailability</td>
</tr>
<tr>
<td>2. Reduction of the dosing frequency</td>
</tr>
<tr>
<td>3. Dose proportionality</td>
</tr>
<tr>
<td>4. High stability</td>
</tr>
<tr>
<td>5. High carrier capacity</td>
</tr>
<tr>
<td>6. Increased surface area, resulting in faster dissolution of the active agent in an aqueous environment, thus greater absorption and bioavailability</td>
</tr>
<tr>
<td>7. Less toxicity</td>
</tr>
<tr>
<td>8. Feasibility of variable routes of administration</td>
</tr>
<tr>
<td>9. Control drug release</td>
</tr>
</tbody>
</table>
Over the past few decades, nanoparticles of noble metals such as silver revealed significantly distinct physical, chemical and biological properties. While their use is increasing in many sectors of the economy, there is also growing interest in the biological and environmental safety of their production. The two main methods for nanoparticle production are chemical and physical approaches, which are usually expensive and also harmful to the environment. In the past year a new efficient, inexpensive and environmentally safe method has emerged for the synthesis of nanoparticles by using plant extracts (Makarov et al. 2014).

Several studies have previously shown that many biological systems including plants, algae, diatoms, yeasts, fungi and human cells can transform inorganic metal ions into metal nanoparticles because of the reductive capacities of the proteins and metabolites present in these organisms (Scarano et al. 2002; Anshup et al. 2005). The “green” synthesis of nanoparticles using plant material is of considerable interest especially in the medical field. Studies have shown that silver nanoparticles (AgNPs) obtained using Japanese black pine exhibit a strong antibacterial activity against various Gram-positive and Gram-negative pathogens (Velmurugan et al. 2013). AgNPs synthetized by using the green technology have been also shown to display cytotoxic activity against various tumor cell lines (Valdokar et al. 2012). Of note, the nanoparticles synthesized by using plant extracts already have a functionalized surface that may contain various organic ligands, proteins, polysaccharides and polyatomic alcohols which are absent in conventionally synthesized nanoparticles. This leads to an increased stability of the particles, facilitating the subsequent attachment of functional molecules such as various antibodies or DNA to the nanoparticles (Sintubin et al. 2012).

1.6.2 Application of nanotechnology in dermatology

1.6.2.1 Anti-inflammatory and immunomodulatory properties of nanoparticles

A recent study investigated the anti-inflammatory effects of biomaterials based on silver nanoparticles and polyphenols rich natural extracts in vitro on HaCaT cells exposed to UVB irradiation, in vivo on acute inflammation animal model and in humans on psoriasis lesions. The synthesized nanoparticles presented promising anti-inflammatory properties, in vivo and in vitro; in vitro, the anti-inflammatory effect was demonstrated by the stable
decrease in cytokine production from HaCaT cells upon UVB irradiation (David et al. 2014). Furthermore, silver nanoparticles complexed with *Cornus mas* extract showed broad modulatory effects on cytokines measured in the culture medium of normal human epidermal keratinocytes activated by exposure to UVB radiation, suggesting a potent anti-inflammatory effect (Perde-Schrepler et al. 2014).

Rehman et al. tested the *in vitro* anti-inflammatory effects of Platinum nanoparticles on lipopolysaccharide (LPS)-stimulated RAW 264.7 cells and reported a significantly reduced LPS-induced production of intracellular ROS and inflammatory mediators upon treatment with platinum nanoparticles. This was due to the efficient suppression of ERK1/2 and Akt phosphorylation, as well as of IκBα phosphorylation and subsequent nuclear factor κB (NFκB) degradation, suggesting that the anti-inflammatory effect of platinum nanoparticles is mediated by down-regulation of the NFκB signaling pathway in macrophages, thus supporting the use of the NPs as anti-inflammatory agents (Rehman et al. 2012).

1.6.2.2 Employment of nanoparticles in imaging and targeting anticancer therapeutics

One of the important applications of nanotechnologies in dermatology focuses on diagnosis and treatment of metastatic melanoma (Weiss et al. 2010). Researchers aim at producing nanoparticles capable to selectively deliver chemotherapeutic drugs specifically to melanoma cells. Benezra et al. used 7 nm multimodal silica nanoparticles for targeting M21 melanomas in a xenograft mouse model. Positron emitting silica nanoparticles proved reliable for real-time intraoperative detection and imaging of nodal metastases, tumor burden and lymphatic drainage patterns (Benezra et al. 2011).

Moreover, Huber et al. developed an assay based on microcantilever arrays to detect the BRAF^{V600E} mutation in melanoma patients. This method proved highly sensible and specific in identifying kinase mutations nanomechanically, without amplification, in total RNA samples isolated from melanoma cells, and may replace the current gold standard for mutation screening which uses real-time polymerase chain reaction and sequencing methods (Huber et al. 2013). BRAF is a serine/threonine protein kinase activating the MAP kinase/ERK-signaling pathway. Around 60% of melanoma patients present a BRAF^{V600E} mutation, which is responsible for melanomagenesis, by dysregulated activation of the downstream MEK/ERK effectors (Ascierto et al. 2012). Drugs that target
tumors carrying this mutation such as vemurafenib are successfully used in patients with positive \( \text{BRAF}^{V600E} \) mutation.

### 1.7 Ultrasound in dermatology

The imaging techniques have established as useful, non-invasive methods for skin examination and diagnosis of skin diseases. During the past years the range of applications in the field of clinical dermatology of both conventional and high resolution ultrasonography (US) has continuously broadened (Schmid-Wendtner et al. 2005). Real time sonography (7.5-10 MHz) and color Doppler sonography are important tools for assessing lymph nodes and subcutaneous tumors in a variety of clinical settings, including preoperative staging, relation of tumoral masses to adjacent vessels and follow-up of melanoma patients. On the other hand, high-frequency ultrasound (20 MHz) can be successfully used for the assessment of tumor spreading, as well as of skin thickness or extent of inflammation when assessing inflammatory diseases such as psoriasis or scleroderma (Schmid-Wendtner et al. 2005).

The procedure involving ultrasound is a non-invasive method allowing the \textit{in vivo} and in real time histological assessment of the cutaneous structure as well as specific skin conditions. Several studies have demonstrated a high correlation between sonograms and histologic evaluation (Jasaitiene et al. 2011). Employment of noninvasive ultrasound procedures in the diagnosis of skin diseases may reliably replace many of the currently used invasive procedures, especially the skin biopsy. The motivation for the extensive use of US derives from its ability to reveal in detail the skin components up to 1.5 cm in depth, to assess the axial and lateral tumor extension, the inflammatory and degenerative processes, as well as the efficacy of different topical and general therapies (Table 3).
Table 3. Applications of ultrasound in dermatology according to frequency (MHz)

<table>
<thead>
<tr>
<th>Frequency</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-10 MHz</td>
<td>Abdominal ultrasound</td>
</tr>
<tr>
<td></td>
<td>Obstetric examinations</td>
</tr>
<tr>
<td></td>
<td>Profound vessels</td>
</tr>
<tr>
<td>7.5-15 MHz</td>
<td>Peripheric lymph nodes</td>
</tr>
<tr>
<td></td>
<td>Subcutaneous tumors</td>
</tr>
<tr>
<td>&gt;20 MHz</td>
<td>Skin thickness</td>
</tr>
<tr>
<td></td>
<td>Chronic cutaneous inflammatory diseases</td>
</tr>
<tr>
<td></td>
<td>Wound healing</td>
</tr>
</tbody>
</table>

1.7.1 Conventional ultrasound

The use of conventional US has gained much importance in clinical dermatology since the 70's. It proved to be a valuable diagnostic method for a series of indications, such as: a. identification and description of visible and palpable tumors, including melanoma; b. preoperative and postoperative assessment of peripheral lymph nodes in patients with malignant skin tumors; c. monitoring of cutaneous and lymph node metastases, especially during chemotherapy (Wortsman et al. 2014).

1.7.2 High-frequency ultrasound

High-frequency ultrasound is a new, noninvasive method allowing the *in vivo* assessment of physiologic and pathologic aspects of the integumentary system. It represents an important research tool for the characterization of skin changes with age, allowing the establishment of an imagistic ageing model of the integumentary system (Crisan D et al. 2012). The use of high-frequency ultrasound in dermatology allows the clear identification of the skin layers and thus tissue assessment. At frequencies above 10 MHz, the technology provides the high resolution required to characterize microstructures. High-frequency ultrasound allows the identification of variations both in skin thickness and echogenicity, offering specific ultrasonographic markers for the objective assessment of the skin structure. For example, changes of the extracellular matrix resulting in variations of the dermal density and echogenicity during physiological senescence or various inflammatory diseases can be easily identified with the use of high-frequency ultrasound. The ultrasonographic assessment of the integument with a high frequency transducer (20
MHz high frequency Dermascan device) offers a 80 micrometer axial resolution, a 200 micrometer lateral resolution and a 1-2.5 cm depth.

According to the literature data and our experience, high frequency US is a noninvasive instrument for skin examination having multiple applications both in the clinical and the research setting (Crisan D et al. 2015). The ultrasonographic examination of the skin by using 20 MHz ultrasound allows the distinctive identification of the following structures:

- **Epidermis**: hyperechoic linear band, parallel to the integumentary surface
- **Dermo-epidermal junction**: thin hypoechoic band situated at the limit of the intense hyperechoic epidermis and the subjacent dermis
- **Dermis**: linear structure of variable echogenicity; the inferior limit appears as an undulated line, due to the presence of adipose pannicles at the level of the reticular dermis
- **Hypodermis**: hypoechoic structure with echoic linear patterns corresponding to conjunctive septa; linear or circular hypoechoic (transsonic) structures corresponding to subcutaneous vessels
- **Hair follicles**: hypoechoic linear structures (Crisan D et al. 2012)

We mention some of the most important applications of high-frequency ultrasound in dermatology (Table 4).

**Table 4. Main applications of high-frequency ultrasound in the field of dermatology**

<table>
<thead>
<tr>
<th>Applications of high-frequency ultrasound in dermatology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Histological skin evaluation and identification of each skin component</td>
</tr>
<tr>
<td>2. Measurement of skin thickness (mm)</td>
</tr>
<tr>
<td>3. Assessment and description of pigmented and non-pigmentary tumoral structures: pigmented naevi, melanoma, carcinoma, dermoid cysts (Crisan M et al. 2013)</td>
</tr>
<tr>
<td>4. Assessment of the efficacy of various topical therapies (Crisan D et al. 2012)</td>
</tr>
<tr>
<td>5. Non-invasive monitoring, both qualitative and quantitative, of the cutaneous alterations induced by senescence (Crisan D et al. 2012)</td>
</tr>
<tr>
<td>6. Monitoring of chronic inflammatory conditions (scleroderma, psoriasis)</td>
</tr>
<tr>
<td>7. Evaluation of the degree of cutaneous fibrosis following exposure to ionizing radiation</td>
</tr>
<tr>
<td>8. Assessment of wound healing, scars, lymphoedema, angioedema</td>
</tr>
</tbody>
</table>
In previous studies, we extensively used US method to identify ultrasonographic features specific for benign versus malignant skin tumors, as well as for the assessment of connective tissue and cellular modifications with age and upon treatment with antioxidants. Herein, we asked whether a newly established technology based on metal ions coupled to nanoparticles complexed with *Cornus mas* (NPs-CM) applied topically may improve chronic stationary plaques of psoriasis patients at clinical, ultrasonographic, histologic and molecular level. In detail, the effect of NPs-CM on the disease severity as assessed by the clinical PASI score was investigated. Next, ultrasonographic studies were conducted in order to characterize the changes in different skin compartments upon local application of nanoparticles. Further, immunhistology analysis and *in vitro* cell biology experiments were performed in order to identify the cellular population in psoriasis plaques primarily targeted by the applied nanoparticles. Finally, we tried to decipher the mechanism of action of the nanoparticles at molecular and transcriptional level.
1.8 Aim
Psoriasis vulgaris is a chronic inflammatory skin disease mediated by autoreactive T cells. However, pro-inflammatory molecules released by macrophages, especially TNFα and IL-12, are crucial mediators of the pathogenesis of human psoriasis and, therefore, first-line targets of effective therapeutic strategies.
Systemic antipsoriatic therapies, though very efficient, are associated with immunosuppression-induced, potentially life-threatening side effects. The currently available topical therapies are less efficient than systemic drugs, however, their efficacy may be improved by the use of delivery systems based on new biomaterials. Metallic nanoparticles (Au, Ag) carrying polyphenols-rich natural extracts of *Cornus mas* (Ag-NPs-CM and Au-NPs-CM) recently showed promising anti-inflammatory, anti-tumoral and anti-angiogenic activity.
Therefore, the aim of this study was to assess whether topical application of Ag-NPs-CM and Au-NPs-CM would diminish unrestrained activation of macrophages and thus inflammation in chronic stationary plaques psoriasis. In detail, we investigated whether

- NPs-CM would clinically improve psoriasis plaques upon topical application?
- NPs-CM would dampen unrestrained inflammation in psoriasis plaques and, if so, which cell population would be primarily targeted?
- the effect of NPs-CM can be reproduced with activated inflammatory cells *in vitro*? and whether
- NPs-CM application would result in long-lasting effects by targeting psoriasis-specific pathogenic mechanisms at molecular and transcriptional level?
2. Materials and methods

The current study is conducted on three research directions:

- *In vitro* study of the effect of Au-NPs-CM and Ag-NPs-CM on macrophage-dominated inflammation in psoriasis
- *In situ* study of the anti-inflammatory effect of the nanoparticles in immunflourescent stained human psoriasis skin samples
- *In vivo* study
  - Clinical assessment of the efficacy of topical nanoparticle-based therapy in patients with chronic stationary plaque psoriasis
  - High-frequency ultrasound assessment of human psoriatic plaques prior and after 6 weeks of topical therapy with Au-NPs-CM and Ag-NPs-CM respectively

2.1 Materials

2.1.1 Mice strains

| C57BL/6 mice | Department of Dermatology and Allergology, University of Ulm, Germany |

2.1.2. Antibodies, cytokines, assay kits

| anti-CD68 antibody | clone KP1, eBioscience |
| anti-phospho-IκBα antibody | Cell signaling |
| anti TNF-α human antibody | abcam |
| IL-12p40 | clone 1-1A4; GeneTex |
| IFN-γ | eBioscience |
| IL-12/p40 Ready-Set-Go! ELISA Kit | eBioscience |
| TNFα Ready-Set-Go! ELISA Kit | eBioscience |
| LDH cytotoxicity assay kit | ThermoFisher Scientific |
| DAPI | Sigma Aldrich |

2.1.3 Nanomaterials

| Au-NPs-CM | Frame project no. 147/2012 of the program |
| Ag-NPs-CM | “Partnerships in priority areas – PN II”, developed with the support of ANCS, CNDI – UEFISCDI Romania |
### 2.1.4 Materials for macrophage isolation, culture, harvest, activation, NO assay

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centrifuge</td>
<td>Eppendorf, Hamburg</td>
</tr>
<tr>
<td>Dissection tools</td>
<td>Aesculap, Tuttlingen</td>
</tr>
<tr>
<td>Dulbecco’s phosphate buffered saline (PBS) (1x) (w/o Ca²⁺, Mg²⁺)</td>
<td>Biochrom AG, Berlin</td>
</tr>
<tr>
<td>Microscope (Zeiss Axiophot)</td>
<td>Zeiss, Germany</td>
</tr>
<tr>
<td>Minifuge RF</td>
<td>Heraeus Sepatech GmbH</td>
</tr>
<tr>
<td>Neubauer chamber</td>
<td>Roth, Neu-Ulm</td>
</tr>
<tr>
<td>NUNC 24 wells plates</td>
<td>NUNC, Langenselbold</td>
</tr>
<tr>
<td>NUNC 96 wells plates</td>
<td>NUNC, Langenselbold</td>
</tr>
<tr>
<td>Omnifix-F Syringe (1ml, 5ml and 20ml)</td>
<td>Braun, Tuttlingen</td>
</tr>
<tr>
<td>Pipets</td>
<td>Roth, Neu-Ulm</td>
</tr>
<tr>
<td>Sterile bench</td>
<td>Faster, Italy</td>
</tr>
<tr>
<td>Refridgerator (4°C)</td>
<td>Liebherr, Ochsenhausen</td>
</tr>
</tbody>
</table>

### 2.1.5 Ingredients of buffers and solutions

<table>
<thead>
<tr>
<th>Buffer</th>
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</thead>
<tbody>
<tr>
<td>ACK lysis buffer (pH 7.2)</td>
<td>0.15 M NH₄Cl; 0.1 mM EDTA; 10 mM KHCO₃; H₂O</td>
</tr>
<tr>
<td>PBS</td>
<td>137 mM NaCl; 2.7 mM KCl; 8.4 mM Na₂HPO₄; 1.4 mM KH₂PO₄; pH 7.4</td>
</tr>
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</table>

### 2.1.6 Collection of skin samples, immunfluorescence staining

<table>
<thead>
<tr>
<th>Item</th>
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</thead>
<tbody>
<tr>
<td>Biopsy Punch 5 mm</td>
<td>GlaxoSmithKline GmbH &amp; Co. KG</td>
</tr>
<tr>
<td>Zeiss Axiophot microscope</td>
<td>Zeiss</td>
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</tbody>
</table>

### 2.1.7 Ultrasonographic equipment

<table>
<thead>
<tr>
<th>Item</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Dermascan device</td>
<td>Cortex Technology, Hadsund, Denmark</td>
</tr>
<tr>
<td>Dermavision Software</td>
<td>Cortex Technology, Hadsund, Denmark</td>
</tr>
</tbody>
</table>
2.2 Development of Au-NPs-CM and Ag-NPs-CM
Ag-NPs-CM and Au-NPs-CM nanoparticles were previously synthesized (frame project no. 147/2012 of the program “Partnerships in priority areas – PN II”, developed with the support of ANCS, CNDI – UEFISCDI Romania) and their safety profile was extensively characterized by \textit{in vivo} and \textit{in vitro} toxicity studies (Crisan M et al. 2013).

The nanomaterials based on gold/silver nanoparticles and organic molecules from \textit{Cornus mas} natural extract were prepared using a green method. The gold and silver ions have been reduced in the presence of organic molecules and the obtained metallic nanoparticles were characterized by UV-Vis and fourier transform infrared spectroscopy (FT-IR) and by Transmission Electron Microscopy (TEM). The used nanoparticles displayed diameters between 13-52 nm for Au-NPs-organic molecules and 9-82 nm for Ag-NPs-organic molecules (Figure 3).

The UV-Vis absorption peaks appeared at 532 nm for Au-NPs-organic molecules and 408 nm for Ag-NPs-organic molecules. The obtained solutions used in our experimental setting had the following concentrations: for Au-NPs-CM 0.165 mg/ml and for Ag-NPs-CM 0.19 mg/ml. The ointments used for the topical therapy of the patients had a 2% concentration of nanoparticles. The vehicle (ointment) was prepared as part of a UMF Cluj-Napoca patent (OSIM Nr. A 2012 00699/20.09.2012. by: Crisan M, Mos E, Crisan D, Olenic L).
Figure 3. Synthesis and characterization of silver and gold nanoparticles complexed with *Cornus mas*. Nanomaterials based on gold/silver nanoparticles and organic molecules from *Cornus mas* natural extract were prepared by using a green method (reduction of gold and silver ions in the presence of organic molecules) and were characterized by UV-Vis and FT-IR spectroscopy and by Transmission Electron Microscopy.

2.3 *In vitro* studies

To investigate the effect of nanoparticles on activated macrophages, these were incubated with bone-marrow-derived murine macrophages pro-inflammatory stimulated *in vitro* with LPS and IFNγ, and their cytokine release was measured by ELISA.

2.3.1 Isolation, culture and harvest of bone marrow-derived murine macrophages

In our protocol macrophages were obtained from 6 to 8 weeks old C57Bl/6J mice as previously published (Sindrilaru et al 2011). After administration of ketamine hydrochloride 0,9 mg an 0,4 mg xylasine hydrochloride, the mice were sacrificed by rapid cervical dislocation. Using aseptic technique, the skin from the top of each hind leg was peeled off and down over the foot. After removal of the foot and the skin, the hind legs were cut off at the hip joints with scissors. We then filled a 10 ml syringe with cold sterile wash medium (Dulbecco’s phosphate-buffered saline without calcium and magnesium) and flushed the bone cavity with 1x PBS (w/o Ca²⁺, Mg²⁺), then collected the bone marrow in a sterile tube.
and centrifuged it at 1300 rpm at 4°C for 10 minutes. Depletion of contaminating erythrocytes was performed by suspension in 5mL ACK lysis buffer and left for 3 minutes at room temperature. Falcon tubes were then filled with cold PBS and cells were concentrated again by centrifugation for 10 minutes at 1400 rpm and 4°C. Total bone marrow cells were counted using a haemocytometer and cells were adjusted to the desired concentration in macrophage complete medium. 2 × 10^6 macrophages per sterile plastic culture dish were added in 10 ml macrophage complete medium and incubated in 37°C, 5% CO₂ incubator for 5 days.

At day 5 the culture supernatants were removed and the remaining adherent cells were washed with 5 ml Dulbecco’s phosphate-buffered saline without calcium or magnesium. Thereafter 5 ml Accutase for enzymatic cell dissociation solution were added to each dish, the dishes were incubated 5 min at 37°C, and then the cells were scraped off from the culture dishes. Cells were then placed together with an equal volume of cold DMEM/F12-10 medium and centrifuged for 10 min at 400 × g, 4°C. After removal of the supernatant, cells were resuspended in 5 ml DMEM complete medium and counted using a haemocytometer.

2.3.2 In vitro activation of murine macrophages

For in vitro activation, bone marrow cells were seeded into the wells of 24-well tissue culture plates at a density of 5 × 10^5 cells/well in 1mL complete medium and allowed to attach and differentiate to macrophages at 37°C and 5% CO₂. At day 5, monolayers of macrophages were primed with IFNγ (50 IU/ml) for 24 hours and then re-stimulated with IFNγ (50 IU/ml) and LPS (10 ng/ml) for another 24 hours (Mosser et al. 2008).

In selected wells, naïve and activated macrophages were co-incubated with Ag-NPs-CM and Au-NPs-CM at final dilutions of 1:10 and 1:100, respectively.

2.3.3 Functional Assays

2.3.3.1 Nitric oxide assay

Since LPS-activated murine macrophages are known to produce NO, NO concentration was measured in macrophage culture supernatants for every treatment group. The NO release of the macrophages was determined using a colorimetric assay based on the Griess
reagent, which measures the accumulation of the stable end product of NO degradation, nitrite. For this, macrophage supernatants were transferred to wells of a 96-well microtiter plate. An equal volume (100 μl) of Griess reagent was added to each well. The plate was gently tapped to mix the contents, allowed to rest for 10 min protected from light, and the optical density (OD) was determined at 550 nm using an automated spectrophotometer (Biotek, Winooski, VT). The corresponding nitrite concentration in the analyzed samples was calculated from a standard curve that was generated using given concentrations of sodium nitrite.

2.3.3.2 Measurement of cytokine release
To investigate whether Cornus mas-complexed nanoparticles have any influence on macrophage activation, bone marrow-derived murine macrophages stimulated with LPS and IFNγ towards a pro-inflammatory M1 macrophage phenotype were incubated with NPs at different concentrations (1:10, 1:100). Their IL-12 and TNFα release in culture supernatants from the four study groups was determined by using ELISA kits for murine TNFα and IL-12p40. The IL-12 ELISA was performed with IL-12/p40 Ready-Set-Go! ELISA Kit (eBioscience) following the manufacturer’s protocol. The sensitivity of detection for the ELISA kit was 5 pg/ml. TNFα ELISA was performed by using TNFα Ready-Set-Go! ELISA Kit (eBioscience) following the manufacturer’s protocol. The sensitivity of detection for the ELISA kit was also 5 pg/ml.

2.3.3.3 LDH cytotoxicity assay
The LDH assay was performed to assess the potential cytotoxic effect of the studied nanoparticles. For this, LDH concentration was measured in the supernatants of macrophages using an LDH cytotoxicity assay kit (ThermoFisher Scientific) according to the manufacturer’s protocol.

2.4 In situ study
The effect of nanoparticles complexed with Cornus mas on psoriatic inflammation was assessed by immunohistology of psoriasis plaques treated with Ag-NPs-CM, Au-NPs-CM or cortisone-based creams as standard therapy control.
2.4.1 Collection of human tissue samples

We studied 24 patients with chronic stationary plaque psoriasis with similar disease severity (PASI). After informed consent was obtained, 5 mm biopsies were taken from the psoriasis plaques of the patients. At 6 weeks after topical therapy with Ag-NPs-CM, Au-NPs-CM or cortisone, a second 5 mm biopsy was taken from the same psoriasis plaque. All protocols were approved by the Ethics Committee of the University of Cluj-Napoca, Romania, according to the Declaration of Helsinki Principles.

2.4.2 Immunofluorescence staining

For immunostaining, cryosections (5 μm) were incubated with purified antibodies against human TNFα (abcam), IL-12p40 (clone 1-1A4; GeneTex) and CD68 (clone KP1, eBioscience). Isotype Ig served as negative control, and Alexa Fluor 488 and Alexa Fluor 555 (all Molecular Probes) served as secondary antibodies. Nuclei were counterstained with 2 μg/ml of the nuclear stain DAPI (Sigma Aldrich) for 5 min.Slides were mounted with fluorescence mounting medium containing DAPI (Vectashield, Vector Labs). Photomicrographs were produced. Fluorescence images were captured using a Zeiss Axiophot microscope and corresponding software (Zeiss). Quantitative fluorescence data were further analyzed using Microsoft Excel software.

As TNFα represents the master cytokine which perpetuates macrophage activation in psoriasis in an autocrine manner via activation of the downstream pro-inflammatory NFκB pathway, we studied NFκB activation in macrophages infiltrating human psoriasis plaques treated with NPs-based ointments and cortisone. For this, we performed immunostainings for phosphorylated IκBα as a marker of NFκB activation (Schön et al. 2005; Clark et al. 2006).

Sections were first labelled with anti-human phospho-IκBα antibodies (Cell Signaling) and with anti-human CD68 antibodies (clone KP1, eBioscience). Isotype Ig served as negative control, and Alexa Fluor 488 and Alexa Fluor 555 (all Molecular Probes) served as secondary antibodies. Nuclei were counterstained with 2 μg/ml of the nuclear stain DAPI (Sigma Aldrich) for 5 min. Slides were mounted with fluorescence mounting medium containing DAPI (Vectashield, Vector Labs).
2.5 *In vivo* study in psoriasis patients

The study was performed on 24 subjects with chronic stationary plaque psoriasis (with comparable disease severity), aged 28-70, and divided into 3 study groups (Au-NPs-CM, Ag-NPs-CM, hydrocortisone). The patients were treated once daily for 6 weeks either with Au-NPS-CM, Ag-NPs-CM or cortisone. All patients included in the study signed an informed consent form and the study was approved by the Ethical committee of the University of Medicine and Pharmacy “Iuliu Hatieganu” from Cluj-Napoca, Romania. Clinical pictures and sonograms were acquired from the psoriasis plaques before and after 6 weeks of topical therapy with Au-NPs-CM, Ag-NPs-CM or hydrocortisone. The effect of nanoparticles complexed with *Cornus mas* on psoriatic inflammation was assessed by clinical score and high-frequency ultrasonography.

2.5.1. Clinical assessment

The clinical assessment was performed by determining the PASI score before and after therapy. The PASI score is a well-established clinical objective tool widely used in dermatology to measure the severity and the extent of plaque psoriasis (*Psoriasis Area and Severity Index*) (Fredriksson T et al. 1973). The PASI index assesses the body surface area (BSA) covered with lesions as well as the severity of the affected lesions (by assessment of the erythema, induration and scaling of the lesions), creating a combined score from 0 to 72, where 0 indicates a healthy person without disease and 72 the maximal level of the disease. The PASI score is routinely calculated before, during and after a therapy period in order to assess the therapeutic response (Carlin et al. 2004).

2.5.2 Ultrasonographic assessment

The ultrasonographic evaluation was performed with the Dermascan device (Cortex Technologies, Hadsund, Denmark) equipped with a 20 MHz transducer that allows sectional skin images up to a depth of 1.5 cm. Dermascan has three major components: the transducer, the elaboration system and a database. The ultrasonic wave is partially reflected at the boundary between adjacent structures generating echoes of different amplitudes. The intensity of the echoes is evaluated by a microprocessor and visualized as a colored two-dimensional image. The color scale of echogenicity ranges from white, yellow, red, green to blue and black.
On normal cutaneous sonograms, the epidermis appears as a white hyperechoic band, the dermis as a multi-colored composition (yellow, green, and/or red) and the subcutaneous tissue appears black (hypoechoic) (Figure 4) (Crisan D et al. 2012).

![Figure 4](image)

**Figure 4.** High-frequency ultrasound of normal integument. The epidermis appears as a white hyperechoic band, the dermis as a multi-colored composition (yellow, green, and/or red) and the subcutaneous tissue appears black (hypoechoic)

We applied the transducer perpendicular on the psoriatic plaques, after previously applying ultrasonographic gel. The gain curve was adjusted at a value of 20 dB and at a speed of ultrasound at tissue level of 1,580 m/s. The obtained images were processed with image analysis software (Dermavision, Cortex Technology).

The Dermavision software is endowed with the capacity to associate the pixel amplitude with a numerical scale set ranging between 0 and 255. By selecting a certain interval from the 0–255 scale, we obtained values corresponding to a certain pixel type, present in the analyzed image. Thus, the 0–30 interval corresponds to LEP, the 50–150 intervals to MEP, and the 200–255 intervals to HEP. For every subject, the following parameters were measured on the obtained images before and at 6 weeks of therapy:

- thickness of the epidermis and dermis (mm)
- the number of low echogenic pixels (LEP)
- the number of medium echogenic pixels (MEP)
- the number of high echogenic pixels (HEP)

LEP is an established quantitative parameter for local inflammatory processes, solar elastosis, and collagen degeneration. MEP and HEP quantify the structures of collagen, elastin fibers, and microfibrils.
The thickness of the epidermis and dermis was obtained by calculating the mean of three measurements performed at three different sites of each image (the extremities and the center of the analyzed image).

2.6 Statistical analysis
Quantitative data are presented as mean ± SD. Statistical significance was determined by 2-tailed Student’s $t$ test. $P$ values less than 0.05 were considered statistically significant.
3. Results

3.1 Ag-NPs-CM and Au-NPs-CM significantly inhibit NO release from pro-inflammatory stimulated mouse macrophages in vitro

Pro-inflammatory activated macrophages abundantly accumulate and critically drive the persistent inflammation in human psoriasis plaques. They produce the master cytokine TNFα with pleomorphic effects on cutaneous inflammation, keratinocyte proliferation and angiogenesis, but also other cytokines like IL-12/IL-23 or soluble factors such as reactive oxygen and nitrogen species which further promote psoriasis plaque formation and persistency (Stratis et al. 2006; Wang et al. 2006; Fuentes-Duculan J et al. 2010).

To investigate whether Au-NPs-CM and Ag-NPs-CM have any influence on macrophage activation, bone-marrow-derived murine macrophages were pro-inflammatory stimulated with LPS and IFNγ in absence or presence of NPs at two different concentrations (1:10, 1:100). The release of the inflammatory mediator nitric oxide NO was measured after 24 hours in the culture supernatant. Treatment with LPS and IFNγ resulted in a high release of NO when compared with non-treated macrophages. Interestingly, co-incubation of macrophages with silver or gold nanoparticles significantly inhibited NO release at both concentrations. Especially Au-NPs-CM at 1:100 dilutions efficiently inhibited the NO release at about 65% of the amount released by positive control stimulated macrophages (Figure 5). Thus, both studied nanoparticles proved effective in down-regulating macrophage activation in vitro.
Figure 5. Au-NPs-CM and Ag-NPs-CM significantly decrease NO release from pro-inflammatory stimulated murine macrophages. Nitric oxide (NO) release was measured as nitrite (NO$_2^-$) concentration in the supernatants of murine macrophages activated with 50U/mL IFN$\gamma$ and 10ng/mL LPS alone (as a positive control for maximal NO production) or in presence of Au-NPs-CM or Ag-NPs-CM at 1:10 and 1:100 dilutions, respectively. Ag-NPs-CM and especially Au-NPs-CM efficiently inhibited NO production at both higher (1:10) and lower (1:100) concentrations. Every experimental setting was performed in triplicate. Representative results of one from at least three independent experiments are shown. Results depicted as bars indicate NO concentrations and are given as mean ± SD (n = 3). *p<0.05, ** p<0.001 by Student’s t-test.
3.2 Ag and Au NPs-CM significantly inhibit TNFα and IL-12 release from pro-inflammatory stimulated mouse macrophages

Similarly with NO release, treatment of pro-inflammatory stimulated murine macrophages with metallic nanoparticles showed a dose-dependent significant decrease of the release of the master pro-inflammatory cytokines TNFα and IL-12 as compared with non-treated macrophages. Again, Au-NPs-CM proved to be more effective than Ag-NPs-CM in suppressing cytokine release from macrophages in this setting (Figure 6).

**Figure 6.** Ag and Au-NPs-CM significantly inhibit TNFα and IL-12 release from pro-inflammatory stimulated mouse macrophages. Bone marrow-derived murine macrophages were stimulated with 10ng/mL LPS and 50U/mL IFNγ towards a pro-inflammatory M1 macrophage phenotype. The IL-12 and TNFα release in culture supernatants was measured after activation with LPS and IFNγ (as a positive control for maximal cytokine release) as well as in presence of Au-NPs-CM or Ag-NPs-CM at 1:10 and 1:100 dilutions, by using ELISA kits for murine TNFα and IL-12p40. Ag-NPs-CM and especially Au-NPs-CM efficiently decreased the release of the proinflammatory cytokines TNFα and IL-12 especially at the higher concentration (1:10). One representative of three independent experiments is shown. Results depicted as bars indicating cytokine concentration are given as mean ± SD. *p<0.05, **p< 0.001 by Student’s t-test.
3.3 Ag and Au NPs-CM do not affect macrophage viability in vitro

In order to rule out a potential toxic effect of the investigated nanoparticles on macrophages and consequently a false positive suppressive effect on their cytokine release, the viability of treated macrophages was further investigated using an LDH cytotoxicity assay. The measurement of LDH concentrations in the supernatants of macrophage cultures revealed no significant differences for LDH release between the different culture settings, demonstrating that both nanoparticles were not toxic in the studied in vitro conditions (Figure 7).

![Graph showing Ag NPs-CM and Au NPs-CM viability](image)

**Figure 7. Ag and Au NPs-CM do not affect macrophage viability in vitro.** An LDH cytotoxicity kit was used to assess the potential cytotoxicity of the nanomaterials. Naïve and pro-inflammatory activated macrophage cultures were treated with silver and gold nanoparticles at 1:10 and 1:100 dilutions. The measurement of the LDH concentrations in the supernatants of macrophage cultures 48 hours after treatment did not show any significant differences for LDH release between the different culture settings, and thus no cellular disruption or death due to the treatment with Ag-NPs-CM or Au-NPs-CM. One representative of three independent experiments is shown. Results depicted as bars representing LDH activity as a percentage of control untreated cells are given as mean ± SD.

Taken together, Ag and Au NPs-CM significantly suppressed the pro-inflammatory activation of macrophages to release ROS and inflammatory cytokines TNFα and IL-12, without affecting macrophage viability. As these activated macrophages are abundantly present in psoriasis plaques, the in vivo effect of the Ag and Au-based nanoparticles was next studied in psoriasis patients.
3.4 Ag-NPs-CM and Au-NPs-CM efficiently improve the clinical aspect of psoriasis plaques

For this, 24 patients with comparable disease activity as assessed by the PASI score were treated with either Ag, Au-NPs-CM-based ointments or with cortisone ointment (hydrocortisone) as a standard positive control once daily for six weeks, 8 patients per treatment group. Patients were assessed for subjective clinical improvement and for disease activity using the clinical PASI score before and after 6 weeks of treatment. When interrogated about the tolerability of the applied ointments, the patients reported about a very good skin compatibility and virtually no adverse effects. The daily application of both Ag and Au-NPs-CM-based ointments resulted in a marked clinical improvement of the inflammatory skin lesions (Figure 8).

Figure 8. Significant improvement of the clinical aspect of psoriasis plaques following topical application of Au and Ag-NPs-CM for 6 weeks. 24 patients with comparable disease activity as assessed by the PASI score were treated with either Ag, Au-NPs-CM-based ointments or with cortisone ointment (hydrocortisone) as a standard positive control once daily for six weeks. 8 patients per treatment group. Representative psoriasis skin lesions before and after 6 weeks of topical therapy with Ag-NPs-CM, Au-NPs-CM and cortisone demonstrated in 3 subjects, one from every study group. A significant reduction of plaque thickness, erythema and scaling is noticeable in all three subjects, showing that topical application of Au-NPs-CM and Au-NPs-CM has a comparable anti-inflammatory effect with cortisone.
Remarkably, the objective assessment of the PASI clinical score revealed significantly reduced scaling, erythema and plaque thickness in patients treated with both Ag and Au-NPs-CM-based ointments, even better than lesions treated with the cortisone ointment (Figure 9).

Figure 9. Significant improvement of PASI clinical score following topical therapy with Au-NPs-CM and Ag-NPs-CM. Statistically significant differences of mean PASI scores 6 weeks post-treatment vs. baseline in psoriatic skin lesions of patients (n=8 per study group) treated with Ag-NPs-CM, cortisone and Au-NPs-CM. In patients who were treated with Ag-NPs-CM, the mean PASI score was decreased significantly from 10.5±1.309 at baseline to 7.75 ±1.16 at 6 weeks. Similarly, treatment with hydrocortisone decreased the mean PASI score from 10.6±0.744 to 8.5 ±0.75. The most significant decrease of the mean PASI score was measured in subjects treated with Au-NPs-CM, from 10.75±0.829 to 7±1 after 6 weeks of topical therapy. Bars indicate mean ± SD. *p<0.05, ** p< 0.001 by Student’s t-test.
3.5 Ag and Au NPs-CM significantly reduce skin thickness and inflammation in psoriasis plaques as quantified by high-frequency ultrasound investigation

To strengthen these semi-quantitative clinical observations, I further used a high-frequency ultrasound investigation method to assess the psoriasis plaques before and after treatment. In this method, the skin thickness was obtained by measuring the distance between the upper epidermal layer and the dermo-hypodermic junction at three different sites of the sonograms and by establishing the mean of the three values. By selecting the 0-30 interval from the 0-255 pixel scale, we obtained values corresponding to the low echogenic pixels, known to reflect the density of the dermal inflammatory infiltrate. At the initial time-point, before treatment, the dermal thickness was dramatically increased within the psoriasis plaques of all studied patients. After 6 weeks of treatment, there was an impressive reduction of the plaques thickness in patients treated with silver and gold particles-based ointments, comparable with thickness reduction of cortisone-treated plaques (Figure 10 a).

Remarkably, quantification of low echogenity pixels clearly showed that Ag and Au-NPs-CM primarily exerted their clinical effect by reducing the number of infiltrating inflammatory cells in the psoriatic plaques, in this case even more effectively than cortisone (Figure 10 b).
Figure 10. Ultrasonographic assessment of psoriatic plaques treated with Ag and Au NPs-CM show a significant reduction of the skin thickness and inflammation, comparable with the effects in cortisone-treated control plaques. Assessment of the sonograms by means of Dermavision software (Cortex Technology®) allowed a quantitative assessment of skin thickness (expressed in mm) and amount of inflammatory infiltrate (expressed by the number of low echogenic pixels - LEP) in the psoriasis plaques before and after topical therapy. 

a. Significant reduction of the mean psoriasis plaque thickness following topical application of Au and Ag NPs-CM, results comparable to the cortisone treatment group. Data presented as bars indicating skin thickness at three different sites of the sonograms as mean±SD. 

b. Quantification of low echogenic pixels as a measure of inflammatory infiltrate density showed that Ag and Au NPs-CM primarily exerted their clinical effect by decreasing the inflammatory infiltrate in the psoriatic plaques. Results depicted as bars indicating the numbers of LEP in the different treatment groups are given as mean ± SD *p<0.05, **p<0.001, by two tailed Student’s t-test.

This can be also observed in the clinical sonograms, where a significant increase in dermal echogenicity is seen after therapy (Figure 11).
Figure 11. Ultrasonographic aspect of the psoriasis plaque before and after therapy: comparison between Au-NPs-CM, Ag-NPs-CM and cortisone. High-resolution ultrasound images from psoriasis plaques of the three treatment groups before and after treatment, showing a significant reduction of the plaque thickness and an increase of the dermal echogenicity due to the decrease of the inflammation. In the sonograms, the epidermal echogenicity appears as a white band, the dermis is expressed as a three color composition: yellow green and/or red, and the subcutaneous layer appears black. The dermal hypoechoic (black) areas represent the low echogenic pixels, expressing the inflammatory infiltrate. After therapy, a significant decrease of the hypoechoic areas (LEP) can be noticed, while the dermis increases in echogenicity, demonstrating the decrease of dermal inflammation.

Thus, this set of clinical data indicate that topically applied Ag and Au NPs-CM were highly efficient in dampening inflammation and reducing psoriasis plaque activity, while being very well tolerated.
3.6 Topical treatment with Ag and Au NPs-CM-based ointment results in the significant reduction of TNFα and IL-12 expression in human psoriasis plaques-infiltrating macrophages

To further investigate the mechanism underlying the reduction of inflammation in psoriasis plaques upon treatment with nanoparticles, skin samples from patients in all treated groups were collected before and after 6 weeks of treatment and were assessed for macrophage numbers and cytokine expression using immunohistology. As IL-12 is specifically produced by activated macrophages in the skin, we used this marker to identify macrophages in psoriasis plaques. Immunofluorescence staining of cryosections from psoriasis patients before treatment revealed a high number of macrophages expressing both TNFα and IL-12 infiltrating the psoriasis plaques. As expected, cortisone treatment strongly reduced the number of IL-12 positive macrophages and of IL-12-TNFα-double positive, TNFα-producing macrophages. Interestingly, gross assessment of IL-12 positive and IL-12-TNFα double positive macrophages suggested the significant decrease of the pro-inflammatory stimulated macrophages in nanoparticles-treated psoriasis plaques as well (Figure 12).

Figure 12. Ag and Au NPs-CM significantly reduce TNFα and IL-12 expression in human psoriasis plaques. Immunofluorescence staining of cryosections from psoriatic plaques before therapy as well as after topical therapy with Ag-NPs-CM, Au-NPs-CM and cortisone. Skin samples were collected by punch biopsy. The immunofluorescence staining revealed significantly decreased numbers of TNFα-positive (stained in red), as well as of IL-12-positive CD68-positive macrophages (stained in green) in the inflammatory infiltrate; IL-12 positive TNFα-positive cells are here shown in overlay as yellow cells. Nuclei were counterstained by DAPI. The white dotted lines represent the basal membrane. Scale bar, 100 μm.
Quantification of IL-12 and IL-12-TNFα double positive infiltrating macrophages in psoriasis plaques prior and after topical application of Au and Ag-NPs-CM confirmed the significant reduction of the pro-inflammatory cytokines after treatment with Ag and Au-NPs-CM, Au-NPs-CM being more effective than Ag-NPs-CM (Figure 13).

Figure 13. Quantification of the IL-12 and IL-12-TNFα double positive infiltrating macrophages in psoriasis plaques before and after topical therapy with Au and Ag NPs-CM. Quantification of the IL-12 and IL-12-TNFα double positive infiltrating macrophages in the psoriasis plaques show a significant decrease of TNFα and IL-12 producing infiltrating macrophages after treatment with Ag and Au NPs-CM, Au-NPs-CM being more effective than Ag-NPs-CM. Results depicted as scatter plots representing the percentage of TNFα and IL-12-positive macrophages out of total dermal nuclei are given as mean ± SD. *p<0.05, **p<0.001, ***p<0.0001, **** p<0.00001 by Student’s t-test.
3.7 Ag and Au NPs-CM reduce inflammation by NF\( \kappa \)B inhibition in human psoriasis plaques

As TNF\( \alpha \) and IL-12 are master cytokines driving inflammation in psoriasis, we next studied the effect of gold and silver nanoparticles in chronic stationary plaque psoriasis patients. Since TNF\( \alpha \) is known to perpetuate/maintain macrophage activation in an autocrine manner via activation of the downstream pro-inflammatory NF\( \kappa \)B pathway, I next investigated the modulation of NF\( \kappa \)B activation in human psoriasis plaques upon different treatment approaches. For this, immunostainings were performed for phosphorylated I\( \kappa \)B\( \alpha \), indicative of I\( \kappa \)B\( \alpha \) degradation and subsequent NF\( \kappa \)B activation. In active psoriasis plaques, almost all CD68-positive macrophages expressed phospho-I\( \kappa \)B\( \alpha \), suggesting a highly inflammatory state with marked release of TNF\( \alpha \) and down-stream pro-inflammatory cytokines. Upon treatment with cortisone ointment we noticed a significant reduction of phospho-I\( \kappa \)B\( \alpha \)-positive activated CD68-positive macrophages as expected. Interestingly, we found significantly reduced numbers of phospho-I\( \kappa \)B\( \alpha \)-positive activated CD68-positive macrophages also in psoriasis plaques treated with silver and gold nanoparticles as compared to control-treated lesions, suggesting that silver and gold nanoparticles complexed with *Cornus mas* exert their anti-inflammatory effect by suppressing NF\( \kappa \)B activation and breaking the subsequent autocrine feedback loops which amplify and perpetuate inflammation via TNF\( \alpha \) release in psoriasis skin lesions (Figure 14).
Figure 14. Immunostainings for phosphorylated IκBα as a marker of IκBα degradation and subsequent NFκB activation. Immunostainings of phosphorylated IκBα (stained in red) from psoriasis subjects before and after 6 weeks of daily treatment with topical Au-NPs-CM, Ag-NPs-CM and cortisone. Significantly reduced numbers of phosphorylated IκBα activated CD68+ macrophages are seen in psoriasis plaques treated with Ag and Au-NPs-CM. The dotted white line represents the basal membrane. Scale bar, 100 μm.

Taken together, we here identified a so far undescribed effect of metallic nanoparticles complexed with polyphenolic compounds on psoriatic inflammation. This thesis demonstrates for the first time that, apart from suppressing keratinocytes activation, nanoparticles specifically target macrophage activation in psoriasis. By diminishing their activation and consequent cytokine release, topical treatment with NPs-CM resulted in a significant clinical improvement of the psoriatic plaques. These newly designed metallic nanoparticles may thus represent a future effective anti-inflammatory therapy for various inflammatory skin diseases such as psoriasis.
4. Discussion

The increased accumulation of macrophages, especially at the dermal-epidermal junction in psoriatic skin lesions has been previously reported by several groups (Djemadji-Oudjiel et al. 1996; van den Oord et al. 1994). It becomes increasingly accepted that macrophages play a crucial role in the pathogenesis of psoriatic skin lesions by releasing pro-inflammatory molecules TNFα and IL-12 which perpetuate chronic inflammation and by producing growth factors which induce neoangiogenesis, keratinocyte proliferation and epidermal thickening (Wang et al. 2006; Xaus et al. 2000). Therefore, targeting macrophage activation is a very promising approach in psoriasis treatment.

In this thesis, we demonstrated a so far unreported property of the new silver and gold nanoparticles complexed with polyphenolic compounds from European cranberry bush (Cornus mas) to specifically and selectively inhibit pro-inflammatory activated M1 macrophages in vitro and in human psoriasis skin lesions. Topical application of these novel biomaterials based on metallic nanoparticles (NPs) carrying polyphenols-rich natural extracts of Cornus mas in patients with chronic stationary psoriasis resulted in significant clinical improvement due to the marked reduction in numbers of TNFα- and IL-12-producing pro-inflammatory macrophages infiltrating psoriasis skin lesions.

First, I found that co-incubation of activated murine macrophages with the newly developed silver and gold NPs-CM in vitro caused a significant decrease of NO release from macrophages. In vitro stimulation of macrophages with lipopolysaccharide (LPS) and IFNγ results in the release of high concentrations of nitric oxide (NO) and pro-inflammatory cytokines such as TNFα, IL-12, IL-1β (Willment et al. 2003) and represents one of the most reliable methods to measure classical activation of murine macrophages, closely reflecting M1 type macrophage polarization during inflammation in vivo (Mosser et al. 2008). The inhibition of NO production in these experiments was dependent on the presence of the NPs-CM either in the priming (addition of IFNγ) or in the triggering phase (LPS) of macrophage activation, demonstrating a direct effect of NPs-CM in modulating the inflammatory functions of murine macrophages.

Further, the addition of silver and gold nanoparticles complexed with Cornus mas to IFNγ and LPS-activated macrophages significantly and dose-dependently inhibited the release of pro-inflammatory cytokines. In detail, the addition of Au and Ag-NPS on the activated macrophages lead to a significant decrease of TNFα and IL-12 levels in the culture
supernatants, at both 1:10 and 1:100 studied dilutions. This is remarkable as TNFα represents the key pro-inflammatory cytokine driving inflammation in skin disorders such as psoriasis, contact dermatitis or drug reactions (Teraki et al. 1994), being significantly elevated in the affected skin areas (Kristensen et al. 1993). The studied nanoparticles significantly suppressed TNFα release with about 20% from the full-activation level at both dilutions. This effect, although moderate, may become relevant in vivo, where TNFα was shown to cumulate its secretion via autocrine mechanisms depending on downstream transcriptional NFκB activation (Gupta et al. 2005). Inhibitory abrogation of this autocrine loop may, thus, progressively dampen TNFα secretion in inflamed psoriasis lesions.

Similarly, IL-12, which is selectively released by activated macrophages, has been demonstrated to be highly up-regulated at mRNA and protein level in psoriatic skin (Yawalkar et al. 1998). Our data show nanoparticles to be potent inhibitors of macrophage activation, with gold NPs being even more effective than silver NPs in this setting. This finding is most probably clinically relevant as well, as several studies have shown that the inhibition of macrophage release of IL-12 (Kauffman et al. 2004) also results in significant improvement of psoriasis lesions.

Therapeutical agents which specifically block TNFα have already been successfully implemented in the systemic treatment of psoriasis and currently belong to the standard therapeutic armamentarium for psoriasis vulgaris and psoriasis arthritis, leading to a significant clinical disease improvement or even remission (Gottlieb 2008; Gordon et al. 2015). We were interested to identify whether a novel topical nanoparticle-based anti-inflammatory therapy would achieve a long-term suppression of TNFα-producing macrophages in psoriatic plaques and, if so, what long term side effects would arise.

Metallic nanoparticles have size-, structure- and shape-dependent properties which are of interest for their antibacterial, catalytic or anti-inflammatory activity. Nadworny et al. demonstrated the anti-inflammatory effect of Ag-NPs in a porcine model of contact dermatitis, while Bhol and Schechter utilized Ag-NPs in a rat model of ulcerative colitis. Both groups showed a significant decrease of IL-1 and TNFα in immunohistochemically stained mucosal gut samples following treatment with Ag-NPs, proving a direct anti-inflammatory and healing effect when compared to control (Nadworny et al. 2008; Bohl et al. 2007).
Remarkably, the anti-inflammatory effects of Ag-NPs were mainly due to a reduction of IFN$\gamma$ and TNF$\alpha$ production by macrophages in a murine peritoneal adhesion inflammation model (Wong et al. 2009). Interestingly, silver-based nanoparticles with smaller silver nuclei also seem to exhibit higher antibacterial activity (Yeo et al. 2003; Zhang et al. 2010).

On the other hand, gold nanoparticles have been successfully applied in tumor diagnostics and therapy in the past years, due to their unique properties (non-cytotoxicity, biocompatibility), small size and high surface area to volume ratio (Khan et al. 2014). Due to their small size and large surface area, metal-based nanoparticles promise to be perfect therapeutic agents as they can easily reach target cells and can deliver a high amount of therapeutic drug (Lan et al. 2013).

I herein found that gold and silver nanoparticles conditioned in an appropriate vehicle significantly improved the clinical aspect of psoriasis plaques when topically applied on psoriasis skin. The investigated patients suffering from chronic stationary plaque psoriasis with comparable disease severity were treated with silver or gold nanoparticles, as well as with hydrocortisone ointment as positive control. Treatment of 8 patients per treatment group with Ag and Au-NPs-CM based ointments (nanoparticles in a concentration of 2%) once daily for six weeks resulted in subjective clinical improvement, reduced scaling, erythema and plaque thickness, as assessed by PASI score. This beneficial effect was even higher than in patients treated with the hydrocortisone ointment, possibly due to the better drug delivery capacity of the NPs-CM to the inflammatory cells of the psoriatic plaques. Importantly, the topical treatment was well tolerated during the entire treatment period and there was no report on drug- or treatment-related side-effects from any patient. This observation is consisting with reports from other studies in humans where NP-therapy showed a long-term tolerability (Crisan M et al. 2013).

When comparing the study groups, I interestingly noticed a more efficient anti-inflammatory effect in patients using the Au-NPs-CM ointments when compared with patients treated with Ag-NPs-CM. The exact mechanism underlying this difference in efficacy is not known so far. However, the smaller size of the used gold particles, as compared to the silver-NPs, allowing a better cell penetration and action, may provide one explanation. According to the literature, gold nanoparticles with a diameter of about 1 nm are able to cross the cell membrane and even reach the nucleus to target DNA, whereas
nanoparticles with a diameter of about 18 nm present good cell penetration but no toxicity (Gao et al. 2011). In the current study, the Au-NPs-CM particles were used at 13-52 nm and the Ag-NPs-CM at 9-82 nm. Cheng et al. showed that gold nanoparticles conjugated with doxorubicin represent a promising, compatible drug delivery mechanism, being suitable for targeted chemotherapy and overcoming drug resistance (Cheng et al. 2013). Lan et al. also showed on a nasopharyngeal carcinoma (NPC) cell line and human nasal epithelial cells that Au-NPs with an average diameter of 20.5 nm reduce cell viability of NPC cells in a concentration-dependant manner, while being non-cytotoxic for normal human nasal epithelial cells (Lan et al. 2013).

The clinical findings illustrated in this thesis demonstrate the noticeable potential of nanoparticle-based ointments in reducing local inflammation in psoriasis, with comparable or even better effect than glucocorticoids, however circumventing the known severe side-effects of long-term topical steroid therapy. Several nanoparticle-based drug combinations for the topical treatment of psoriasis have been developed and published in the past years. For instance, Sonavane et al. found a significant delay in keratinocyte turnover of HaCaT cell lines in vitro and a decrease of epidermal thickness in a mouse tail psoriasis model upon application of solid lipid nanoparticles loaded with betamethasone dipropionate and the vitamin D derivative calcipotriol when compared to standard control Daivobet® (calcipotriol, betamethasone dipropionate) application (Sonavane et al. 2014). Furthermore, solid lipid nanoparticles functionalized with capsaicin, a known inhibitor of cutaneous vasodilatation (Bernstein et al. 1986), were also shown to effectively enhance drug accumulation in all skin layers, improving the clinical aspect of psoriatic skin lesions (Agrawal et al. 2015). Recently, David et al. reported for the first time the successful use of silver nanoparticles conditioned with European black elderberry as anti-inflammatory agent in psoriasis. The study group assessed the effect of the nanomaterials in vitro on HaCaT cells exposed to UVB, in vivo in an acute inflammation model and in patients with chronic stationary plaque psoriasis. They reported a significant decrease of cytokine production in HaCaT cell cultures exposed to UVB and nanoparticles, as well as a significant decrease of the cytokine level in paw tissues. Furthermore, topical treatment of psoriasis plaques also resulted in a significant clinical improvement of the lesions, confirming the anti-inflammatory properties of the NPs (David et al. 2014).
The current study on the other hand adds important insight into the mechanism of action of the nanoparticles complexed with polyphenolic compounds in psoriatic inflammation, showing that apart from keratinocytes, nanoparticles also interact with macrophages in psoriatic lesions, thereby diminishing their activation and consequent cytokine release. It also shows that newly designed metallic nanomaterials conditioned with polyphenolic compounds may represent a future, potentially effective anti-inflammatory therapy for chronic inflammatory skin conditions such as psoriasis.

In line with the clinical improvement of psoriatic lesions as assessed by the PASI score, using high-frequency ultrasound investigations, I could further demonstrate a significant reduction of the plaques thickness after 6 weeks of treatment with silver and gold particles-based ointments, an effect comparable with cortisone treatment. As high-frequency ultrasound is nowadays successfully employed for the assessment of skin changes induced by topical therapies, the 20 MHz Dermascan device was used to assess the psoriasis plaques from the different treatment groups before and after treatment (Crisan et al. 2013).

The echogenicity of the skin is given by the extracellular matrix density. Skin echogenicity varies significantly with age, correlates with local degradation or inflammation processes as occurring during senescence or various inflammatory conditions. In high-frequency sonograms, low echogenicity pixels correlate with cell-rich inflammatory processes. Carlsen et al. examined individuals with clinical adverse reactions (contact allergies) in their tattoos and found the presence of prominent low echogenic bands at dermal level, which correlated to the thickness of the cellular infiltration determined by microscopic examination (Carlsen et al. 2014). Furthermore, we could previously show that the degree of cutaneous inflammation can be objectively assessed by quantifying the number of low echogenic pixels using the Dermavision software (Crisan et al. 2013). Therefore, high-frequency ultrasound is suitable as a reliable, non-invasive, complementary method to histopathology to assess the severity and modulation of the inflammatory infiltrate in psoriasis plaques prior and after therapy.

The quantification of low echogenicity pixels in our study groups clearly showed that Ag and Au-NPs-CM primarily exerted their clinical effect by reducing the number of infiltrating inflammatory cells in the psoriatic plaques, in this case even more effectively than cortisone. Again, better effects were observed in the case of Au-NPs-CM when compared with Ag-NPs-CM. I also noticed a significant decrease of the dermal thickness.
in the studied groups, which was primarily due to the decrease of the inflammatory infiltrate at dermal level.

Immunofluorescence staining of cryosections from the studied psoriasis patients before treatment revealed a high number of macrophages expressing both TNFα, and IL-12. As expected, cortisone treatment strongly reduced the number of IL-12 positive and IL-12-TNFα-double positive macrophages. Of notice, consistent with our clinical and ultrasonography findings, the quantification of IL-12 positive and IL-12 TNFα-double positive macrophages confirmed the significant decrease of the pro-inflammatory activated macrophages in nanoparticles-treated psoriasis plaques. These findings indicate Au and Ag-NPs as strong candidates for the topical target therapy against TNFα and IL-12 in psoriasis treatment. Based on our findings and experience with metallic nanoparticles, future studies need to be developed in order to better characterize and improve these potential therapeutic agents to directly deplete activated macrophages in chronic inflammatory skin diseases such as psoriasis. Thus, similar to current systemic “biologics” therapies used for the treatment of psoriasis, the current study showed that silver and gold nanoparticles delivered in a topical manner can also specifically target macrophages in the inflammatory infiltrate of psoriatic plaques by down-regulating the release of two main pro-inflammatory cytokines, TNFα and IL-12.

Our findings on the anti-inflammatory effect of NPs-CM are consistent with previously published data. Howard et al. demonstrated in a murine collagen-induced arthritis model a significant decrease of TNFα after intraperitoneal administration of chitosan/small interfering RNA nanoparticles, resulting in reduced local and systemic inflammation (Howard et al. 2008). In other rat models of ulcerative colitis, nanosilver decreased the expression of metalloproteinases, suppressed the expression of TNFα, IL-12 and IL1β and induced apoptosis of inflammatory cells, thus resolving overall inflammation (Bhol et al. 2007). However, our data corroborate and extend previous reports by demonstrating for the first time that NPs-CM directly inhibit macrophage activation, thereby decreasing the release of the two master cytokines TNFα and IL-12.

As TNFα production and accumulation is known to perpetuate macrophage activation in an autocrine manner depending on the activation of the downstream pro-inflammatory nuclear factor kappa B (NF-κB) pathway, we studied NFκB activation in human psoriasis plaques from different treatment groups. NF-κB is a key transcription factor with an essential role
in the pathogenesis of psoriasis by modulating inflammation, cellular proliferation, differentiation and apoptosis (Goldminz et al. 2013). In immunofluorescence microscopy analyses we interestingly found significantly reduced numbers of CD163-positive macrophages with phospho IκBα expression as a marker of NFκB activation in psoriasis plaques treated with silver and gold nanoparticles as compared to control-treated lesions. This effect was even stronger than the reduction obtained with cortisone treatment. This finding suggests that the consistent reduction of TNFα- and IL-12-expressing macrophages in psoriasis plaques treated with Ag and Au NPs-CM was most likely mediated by NFκB inhibition. To substantiate these highly interesting, but rather descriptive findings, future work will be needed to semi-quantitatively assess NF-κB activation by Western blot analysis of phosphorylated IκBα, total IκBα and NF-κB p50 in lysates from nanoparticles-treated activated macrophages, as well as from treated human skin biopsies. In fact, this mechanism closely reproduces the effect of systemically administered TNFα blockers which have been shown to significantly decrease the active NF-κB levels in psoriatic skin (Tan et al. 2007). Therefore, the development of topical compounds capable of directly targeting the NF-κB signaling pathway may be of great benefit for the therapeutic management of inflammatory disorders such as psoriasis in the near future. However, it was also shown that the chronic inhibition of NF-κB may lead to local immunosuppression with consequent increased susceptibility to bacterial, viral and fungal infections. Therefore, modern therapeutic agents need to maintain a balance between maximizing the therapeutic effect and limiting the potential harmful effect (Goldminz et al. 2012).

To causally address the specific effect of the studied nanoparticles on NFκB-driven psoriatic inflammation a suitable animal model will be employed in a next project. The established CD18 hypomorphic (CD18\textsuperscript{hypo}) murine model of psoriasis may qualify for this purpose. In this model, mice with a severely reduced expression of the membrane-associated adhesion molecules β2 integrins on leukocytes develop a NFκB- and TNFα-dependent inflammatory skin disease very much clinically and pathogenetically resembling human psoriasis (Wang et al. 2006). Topical treatment of CD18\textsuperscript{hypo} mice either with Au-NPs-CM, Ag-NPs-CM or control would allow to analyze samples from skin and skin-draining lymph nodes at different treatment time-points and thoroughly assess inflammation at cellular, molecular and mechanistic level.
The here presented results are novel and highly promising for moving forward the topical therapy in psoriasis. However, there are several critical points which still need to be clarified. The rapidly increasing employment of nanomaterials in biomedical fields has given rise to serious concerns regarding their long-term effects on human health, as it has been shown that nanoparticles accumulate in organs following local and systemic administration, and the skin represents one important organ for the long-term deposition of nanoparticles. Thus, fluorescent gold nanoparticles could be traced in the skin under ambient and UV light when intravenously injected in mice. Furthermore, histopathological findings in excised organs correlated the with the injected dose and the accumulation of nanoparticles in the liver and the spleen (Sykes et al. 2014).

Concerning systemic toxicity, studies have shown that nanoparticles tend to accumulate in the liver and spleen, but there have been limited methods to monitor the exposure of these organs so far. A research group from Singapore used gold nanoparticles to examine their bio distribution in various organs in rats following intravenous administration and found that there was a significant accumulation of Au in the liver and spleen throughout the entire 2 months timeframe of the study, suggesting a significant availability of Au-NPs even after 2 months from therapy initiation, leading to gene expression changes in target organs (Balasubramanian et al. 2009).

Vogt et al. performed a series of skin penetration studies revealing that the highest amount of topically applied solid nanoparticles are retained in the upper layer of the stratum corneum, however at sites of skin barrier dysfunction or inflammation their retention is markedly enhanced up to biologically relevant amounts (Vogt et al. 2014). On the other hand, Adachi et al investigated rat skin exposed to titanium dioxide nanoparticles and showed by electron microscopy that no TiO(2) particles were detected in the viable skin, suggesting that the particles were not able to penetrate into viable cell layers nor induce any cellular changes (Adachi et al. 2010).

It appears that deeper penetration of the keratin layer into the viable epidermis or even deeper does not occur when the skin barrier is intact and if a cellular uptake occurs, the particle size is a major determinant (Rancan et al. 2006). Low penetration rates may however become relevant when large skin surface areas are treated with a particular nanomaterial over prolonged time periods. Skin penetration and cellular uptake of silver nanoparticles (Ag-NP) was studied by the same work group in intact tissue blocks from
porcine skin and showed by means of TEM that HcCaT cells can accumulate Ag-NPs in endosomes (Rancan et al. 2006).

Metal particles alone may indeed display toxic effects mainly due to the production of reactive oxygen species and oxidative stress (Djurisic et al. 2015). Alarifi et al. detected reactive oxygen species-mediated DNA damage and apoptosis in human skin epidermal cells after exposure to nickel nanoparticles (Alarifi et al. 2014). Oxidative stress and skin cell toxicity were also shown for iron oxide nanoparticles (Murray et al. 2013). However, the particular green method of synthesizing nanomaterials by conditioning them with polyphenolic compounds diminishes most of the potential toxic effects of the nanomaterials, making them stable and safe for use.

Prospectively, it will be interesting to use the CD18 hypomorphic (CD18hyp) murine model of psoriasis to trace nanoparticles when topically applied on the skin. Using fluorescence microscopy, we could specifically investigate whether at all and if so, in which organ (skin, lymph nodes, liver, spleen and kidneys) the fluorescence-labeled nanoparticles would accumulate at different time-points. Double staining with different leukocyte markers would allow the identification of the cell type responsible for the systemic delivery of the fluorescent nanoparticles.

Recent data also suggests that hair follicles may be responsible for the longer storage of transcutaneously delivered metal-complexed nanoparticles (Lademann et al. 2011). These accumulated particles may be responsible for latent inflammation and eventually for late skin fibrosis/premature aging/stem cell niche exhaustion, which may represent limiting therapeutic side effects. Therefore, it would be interesting to investigate the faith of topically applied metal-nanoparticles in mouse skin samples obtained 6 months after treatment and examined for the presence and the localization of nanoparticles in the hair follicle compartment. Double immunostaining with keratinocyte markers (K14), hair follicle stem cell markers (K15, CD34) or leukocyte markers will identify the cellular compartment responsible for long time deposition of metallic nanoparticles.

Our study could provide novel insights into the mechanism, the kinetic and the safety profile of Ag and Au-NPs-CM, a modern nanoparticle-based ‘green’ technology which in the near future may help circumventing the immunosuppression-related limiting side effects of biological therapies in psoriasis patients.
The *in vitro* data demonstrated the clear inhibitory effect of nanoparticles on macrophage activation and cytokine release, this effect being also translatable *in vivo*, where clinical and most importantly ultrasonographic examinations confirmed the depletion of the inflammatory infiltrate following topical therapy with nanoparticle-based ointments. Finally, the long-term assessment of the safety profile of Ag and Au-NPs-CM will be of great interest in order to provide patients with a side-effect free therapy.

A series of *in vitro* experiments described in this doctoral thesis highlighted some novel, so far undescribed immunomodulatory effects of Au-NPs-CM and Au-NPs-CM. Especially the property of the nanoparticles to diminish macrophage activation via the NFκB signaling pathway may have important therapeutic implications for inflammatory diseases such as psoriasis.

The inhibition of macrophage activation in psoriatic plaques by means of topical application of nanoparticle-based ointments could result in the future obtainment of potent topical agents that have similar immunomodulatory effect to the current biologic therapies, and may potentially be side-effect free. Nevertheless, these and other so far unidentified effects of metallic nanoparticles complexed with polyphenolic compounds may become relevant with different dosage and therapy regimens and may be successfully exploited as alternative therapeutic tools to specifically modulate pathologic immune responses in psoriasis and other macrophage-dominated disease states.
5. Summary

Activated macrophages releasing pro-inflammatory molecules, especially TNFα and IL-12 play a key role in chronic inflammation of psoriasis skin lesions. Modern systemic therapy with biologics effectively target TNFα and IL-12 in psoriasis, however their use is often limited by immunosuppression-related severe side effects. Selective inhibition of inflammatory macrophages in psoriasis plaques by topical drug delivery has not been reported so far. New biomaterials based on silver and gold nanoparticles carrying polyphenols-rich natural extracts recently showed promising anti-inflammatory activity on activated macrophages and keratinocytes in vitro and in several models of inflammation.

This thesis aims at understanding how topically delivered silver and gold nanoparticles conditioned with polyphenolic compounds of Cornus mas modulate inflammation in psoriasis at cellular and molecular level.

For this purpose, 8 patients with chronic stationary plaque-psoriasis with similar disease activity were treated with nanoparticles-based ointments once daily for 6 weeks and the clinical response was assessed. I have shown that Ag-NPs-CM and Au-NPs-CM conditioned as ointments and applied topically significantly reduced inflammation in vivo in human psoriatic plaques. Nanoparticles complexed with Cornus mas significantly improved the clinical appearance, with reduction of the PASI clinical score and significantly diminished the inflammatory infiltrate in psoriasis plaques as assessed by high-frequency ultrasonography. Quantification of low echogenity pixels as a measure of the inflammatory infiltrate clearly showed that Ag-NPs-CM and Au-NPs-CM primarily exerted their clinical effect by reducing the number of infiltrating inflammatory cells in the psoriatic plaques. Remarkably, the reduction of plaques thickness in silver and gold nanoparticles-treated patients was comparable or even more effective than the reduction achieved with cortisone control treatment.

This important finding was further substantiated by immunofluorescence studies on psoriatic skin before and after treatment which revealed that nanoparticles significantly reduced the numbers of infiltrating CD68-positive macrophages and their IL-12 and TNFα production in the treated human psoriasis plaques.

Moreover, double staining for phosphorylated IκBα, a marker of pro-inflammatory transcriptional NFκB pathway activation, and macrophage markers revealed that NPs-CM significantly reduced the numbers of phosphorylated IκBα-positive, CD68-positive
activated macrophages in psoriasis plaques treated with silver and gold nanoparticles as compared to control-treated lesions. This finding strongly indicates that NPs-CM directly repress NFκB activation in macrophages, thereby inhibiting the production of pro-inflammatory factors, such as TNFα and IL-12, with causal role in psoriasis pathogenesis. As TNFα is one NFκB-dependent master cytokine known to perpetuate macrophage activation in an autocrine manner, the herein identified mechanism very likely underlies the anti-inflammatory effect of topical NPs-CM in human psoriasis plaques. I therefore suggest that the studied nanoparticles induce a specific inhibition of macrophage activation which may be of benefit for the modulation of inflammation as occurring in psoriasis and other autoimmune diseases.

To finally demonstrate the direct effect of the nanomaterials on macrophage activation, as well as on the release of the two master pro-inflammatory cytokines TNFα and IL-12, a series of in vitro experiments were further performed. I found that in vitro treatment of pro-inflammatory activated murine macrophages with both Ag-NPs-CM and Au-NPs-CM significantly inhibited their NO, TNFα and IL-12 release, with Au-NPs-CM being more effective than Ag-NPs-CM. Therefore, NPs-CM specifically and efficiently inhibit macrophage pro-inflammatory activation in vitro, and this effect is also true in vivo, eventually resulting in disease resolution.

In the discussion part, I emphasize the relevance of these findings and place them in the context of the in vivo and in vitro data previously reported by our group and of the most recent knowledge regarding psoriasis pathogenesis and therapeutic employment of nanotechnologies. I integrated the obtained data into the context of currently published findings and tried to open perspectives which should further be addressed to enhance research and implementation of topical psoriasis therapy. Finally, I extensively address positive, but also critical issues regarding nanoparticles, their kinetic and safety profile and try to identify the unique advantages of the medical use of nanoparticles.

The results of this study are important as they highlight the therapeutic potential of an innovative nanoparticle-based ‘green’ technology which may provide a safe and efficient tool for modern psoriasis therapy circumventing the often limiting side effects of systemic biologic therapies.
6. References


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7. Appendix

7.1 Curriculum vitae

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Personal information

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Education and formation
- 07/2013 - present: Residency in Dermatology an Venereology, University Clinic of Ulm, Department of Dermatology and Allergic Diseases, Germany
- 01-06/2013: Residency in Dermatology and Venereology, Dermatology Clinic, University of Medicine and Pharmacy Cluj-Napoca, Romania
- 2009-2010: Universita degli Studi di Milano, Facolta di Medicina e Chirurgia; Instituto Clinico Humanitas, Milano, Italy – Erasmus exchange Program
- 2006-2012: University of Medicine and Pharmacy Iuliu Hatieganu Cluj-Napoca, Romania – Graduation Magna Cum Laude
- 1994-2006: George Cosbuc National College, Cluj-Napoca, Romania – German section

Foreign languages: English, German, Spanish, Italian, French, Romanian
Trainings and Seminars:
- **2015:** Advanced surgical skills, 24th EADV Congress, 7-11th October 2015, Copenhagen, Denmark
- **2014:** Introduction to Dermatohistology – from Biopsy to Diagnosis, 08-09 February, University Clinic Münster, Nordrhein-Westfalen, Germany
- **2013:** 3rd Munich International Summer Academy of Practical Dermatology, 21-26 July, Munich, Germany
- **2013:** EADV Trainee Course Hair and Scalp, 14-16 November, Bologna, Italy
- **2013:** EADV Trainee Course Phlebology, 10-11 May, Bucharest, Romania
- **2010:** Dermatoscopy Seminary– Clinical Applications; 1-3 June, UMF Carol Davila Cluj-Napoca, as part of the project: The development of Dermat- oncology as integrated study line in medical post graduate studies
- **2009:** Training of formation of Internal Evaluators in the field of Superior Education Quality, organized by ARACIS, as part of an European project; 7-8 May, Bucharest, Romania

Awards
- **2015:** Michael Hornstein Memorial Scholarship, 24th EADV Congress, 7-11th October 2015, Copenhagen, Denmark
- **2015:** World Congress of Dermatology Trainee Scholarship, 23rd World Congress of Dermatology, June 8-13, Vancouver, Canada
- **2013:** Global education award of the international Society of Dermatology, December 6th, 2013, New Delhi, India

Scientific activity (see extended list of publications)
- Extensio published papers: 6 first author; 6 (co-author)
- Abstracts: 20 : 8 as first author; 12 – co-author
- Book chapter: 1 (co-author)
- Paper presentations at conferences/meetings: 12
- Prizes: 4
- Co-investigator in 1 research grant
- Principal Investigator of 1 student research grant
- Conference/ International meetings/Congress attendance: 40
Research Grant membership

- **Principal investigator student research grant** UMPh Cluj-Napoca, Romania 2011/2012 – „Imagistic study of the cutaneous aging process and associated tumoral pathology. Histo-imagistic correllations.” Grant number: 22714/7/06.10.2011. Coordinator: Assistant Professor Dr. Monica Lupsor

- **Member of IDEI 2624/2008 research grant** „Noninvasive precocious diagnosis of the cutaneous photoinduced senescence process. Complex histo-clinical-imagistical studies. SERENO”. Principal investigator: Prof. Dr.Maria Crişan
7.2 List of publications

1. Journal articles


2. **Books**

   (a) **Chapters in international books**


3. **Active participation in scientific events**

   (a) **Oral presentation**

   - **Crisan D**. Operative Strategie bei unklarem Pigmentmal im Bauchnabel. *Ulmer Dermatologen Abend*, Ulm, Germany, 06.05.2015 (oral presentation)

   - **Crisan D**. Nekrosen der Kopfhaut. Wie lautet Ihre Diagnose? *Ulmer Dermatologen Abend*, Ulm, Germany, 21.05.2014 (oral presentation)

   - **Crisan D**, Roman I, Olenic I, Crisan M, Scharffetter-Kochanek K, Sindrilaru A. Silver-nanoparticles complexed with natural extract of *Cornus mas* significantly inhibit inflammation in vitro and in human psoriasis plaques. 42. Jahrestagung der Arbeitsgemeinschaft Dermatologische Forschung e. V., ADF. Ulm, Germany 05-07.03.2015 (oral presentation)

   - **Crisan D**, Lupșor M, Cappare G, Crisan M. Can topical anti-aging therapies be objectively assessed by using imagistic methods? *Medicalis International Congress for Medical Students and Young Health Professionals*. Cluj-Napoca, Romania 10-13.05.2012 (oral presentation – 1st prize Medical Sciences section)
- **Crisan D**, Crisan M. Ultrasonographic assessment of the skin ageing process. 21st European Students’ Conference Berlin, Germany 13-17.10.2010 (oral presentation – 2nd prize in Dermatology section)

- **Crisan D**, Kozan A. Preliminary study of apoptotic markers in basal cell carcinoma. Medicalis international congress for Medical students and young doctors. Cluj-Napoca, Romania 14-17.05.2009 (oral presentation)

- **Crisan D**, Kozan A, Olteanu I. Hutchinson-Gilford Syndrome (Progeria). National Congress for students and young doctors. Bucharest, Romania 15-17.03.2007 (oral presentation – special prize)

**b) Poster presentations**

- **Crisan D**, Roman I, Olenic I, Crisan M, Scharffetter-Kochanek K, Sindrilaru A. The anti-inflammatory effect of silver and gold nanoparticles complexed with polyphenolic compound of Cornus mas in plaque psoriasis: in vitro and in vivo studies on human psoriasis plaques. 24th EADV Congress, Copenhagen, Denmark, 7-11.10.2015 (e-poster)

- **Crisan D**, Roman I, Crisan M, Scharffetter-Kochanek K, Badea R. The role of vitamin C in pushing back the boundaries of skin aging: an ultrasonographic approach. 23rd World Congress of Dermatology. Vancouver, Canada, June 8-13, 2015

- **Crisan D**, Roman I, Crisan M, Badea R. Can topical Vitamin C anti-aging therapies be objectively assessed by high-frequency ultrasound (HFU)? 22nd EADV Congress. Istanbul, Turkey, 02-06.10.2013 (e-poster)

- **Crisan D**, Lupser M, Crisan M. Skin Physiology and skin Manifestations according to age: an ultrasonographic approach. European Congress of Radiology ECR. Vienna, Austria, 01-05.03.2012 (e-poster)

- **Crisan D**, Crisan M, Cappare G. Ultrasonographic assessment of the efficacy of Interactive P63 product as antiaging agent. Preliminary study. 18th International Student Congres of (bio)medical Sciences ISCOMS. Groningen, Netherland, 07-10.06.2011 (poster presentation – 1st prize Dermatology Section)
- Lupsor M, Badea R, Crisan M, Stir A, **Crisan D**. Effects of ageing on dermal echogenicity assessed by high-frequency skin ultrasonography. 23rd *European Congress of Radiology*. Vienna, Austria 3-7.03.2011 (e-poster)


- Crisan M, Pop D, Melincovici C, Bosca B, Chindris AM, **Crisan D**. Cell-mediated Immunity in Lower Lip Squamous Cell Carcinomas. *From Hippocrates to Modern Dermatology. 15 EADV Congress*. Rhodes Greece, 4-8.10.2006. (poster presentation)