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Vidar Rao

Extracorporeal photochemotherapy in patients with Cutaneous T Cell Lymphoma or Graft-vs-Host Disease

Doctoral thesis

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NTNU
Norwegian University of
Science and Technology
Thesis for the degree of
Philosophiae Doctor
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Fotoferesebehandling av pasienter med CTCL eller GVHD

Fotoferese *(extracorporeal photochemotherapy, ECP)* har blitt en akseptert behandling av enkelte T celle medierte tilstander, som kutant T celle lymfom (CTCL) og Graft-vs-Host disease (GVHD). Blant CTCL pasientene så har omtrent halvparten en positiv effekt av behandlingen. Det finnes imidlertid ingen gode prediktive markører for respons. I **Paper I** har vi evaluert responsraten ved ECP hos pasienter med CTCL som har blitt behandlet på St. Olavs Hospital, i tillegg til å undersøke flere potensielle prediktive markører. 7 av 16 pasienter ble klassifisert som respondere på ECP-behandling. Hverken T celle klonalitet eller noen av serummarkørene som ble undersøkt kunne benyttes som prediktiv markør før behandlingsstart, men det kan se ut til at forandringer i enkelte av markørene etter 6 måneders behandling korrelerer med klinisk respons evaluert etter 12 måneders behandling.

Virkningsmekanismen til ECP er fortsatt ikke fullt ut kjent. Det man vet er at behandlingen induserer apoptose av lymfocytter. Inntil nylig har man trodd at monocytter ikke går i apoptose av behandlingen, men heller aktiveres og differensieres til umodne dendrittceller (DC), som effektivt fagocytterer og presenterer antigen til immunforsvaret. Ved CTCL har man derfor trodd at en cytolytisk, tumorspesifikk immunrespons settes i gang. I **Paper II** har vi undersøkt hvordan ECP påvirker lymfocytter, monocytter og umodne DC. Vi fant at alle cellene går i apoptose kort tid etter ECP-behandling, samt har redusert evne til å differensiere. Vi mener derfor at det er sannsynlig at den terapeutiske effekten av ECP heller skyldes regulatoriske mekanismer *in vivo* som følge av infusjon av apoptotiske celler. Vi viser videre at 30 Gy bestråling induserer apoptose av lymfocytter, men påvirker tilsynelatende ikke monocytter og DC. Dette kan muligens utnyttes til å øke tumor fagocytose *ex vivo* og dermed effektivisere antigen presentasjon *in vivo*.

GVHD oppstår når T celler fra en donor angriper mottakeren. Det er foreslått at ECP trigger visse toleransemekanismer, som hemming av pro-inflammatoriske cytokiner, samt økt produksjon av anti-inflammatoriske cytokiner og regulatoriske T celler (Treg). Det kan imidlertid virke paradoksalt at den samme behandlingen skal sette i gang en tumorspesifikk, cytolytisk immunrespons mot lymfomceller hos CTCL pasienter, mens den fører til økt regulatorisk aktivitet hos GVHD pasienter. I **Paper III** har vi sammenlignet andelen av sirkulerende Treg celler hos pasienter med CTCL eller GVHD. Ved CTCL var det dobbelt så høy forekomst av Treg celler i sirkulasjon, sammenlignet med GVHD og normal kontroller, muligens en respons som er involvert i nedregulering av lymfomceller. I tillegg undersøkte vi også om det var en målbar forandring av et utvalg cytokiner i serum før og etter ECP. Det anti-inflammatoriske cytokinet TGF-β var nær tre ganger høyere hos GVHD pasientene og kan indikere høy Treg aktivering. Begge pasientgruppene hadde en økning av TGF-β under behandlingen. Dette kan tolkes som en forbedret Treg funksjon hos begge pasientgruppene.

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Hovedveileder: Prof. Torolf Moen
Finansieringskilde: Institutt for LBK, DMF, NTNU

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List of abbreviations

8-MOP	8-methoxypsoralen
β_2-M	Beta2-microglobulin
CD	Cluster of differentiation
CTCL	Cutaneous T cell lymphoma
CR	Complete Remission
DC	Dendritic cells
ECP	Extracorporeal photochemotherapy
Foxp3	Forkhead box p3
GM-CSF	Granulocyte macrophage colony-stimulating factor
GVHD	Graft vs Host Disease
HC	Healthy Controls
HCT	Haematopoietic cell transplant
HLA	Human leukocyte antigen
IL	Interleukin
IFN-γ	Interferon gamma
LD	Lactate Dehydrogenase
LPS	Lipopolysaccharide
MF	Mycosis fungoides
MR	Minor Response
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase Chain Reaction
PD	Progressive Disease
PI	Propidium iodide
PR	Partial Remission
PUVA	Psoralen + total body UVA
RM	Red Man Syndrome
SS	Sezary Syndrome
sIL-2Rα	Soluble Interleukin-2 Receptor alpha
TGF-β	Transforming Growth Factor beta
TNF-α	Tumour Necrosis Factor alpha
Treg	Regulatory T cells
UVA	Ultraviolet-light A
UVB	Ultraviolet-light B

Acknowledgements

This thesis is based on work done between 2003-2009 at the Faculty of Medicine (DMF), Department of Laboratory Medicine, Children's and Women's Health (LBK), Norwegian University of Science and Technology (NTNU), Trondheim. In 2003, after my second year of medical school, I was accepted in the Medical Student Research Programme (Forskerlinjen) with a project about extracorporeal photochemotherapy. When I completed my cand.med.-degree in June 2008, I was fortunate to be offered a one-year ph.d.-scholarship from DMF, NTNU to finalize the project.

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Secondly, I want to acknowledge my co-supervisors ***Dr. Kristin Ryggen,*** and later on ***Dr. Marit Saunes*** at the Department of Dermatology, as well as ***Prof. Størker Jørstad*** at the Department of Nephrology, for exceptional counselling, frequent meetings and helpful discussions during the performance of the thesis. Their effort and willingness to guide me through the process has been more than one can ask for.

I'm also in debt to all employees, doctors and bioengineers at the Department of Immunology and Transfusion Medicine, St. Olavs Hospital, for proving a unique willingness to help me solve the day-to-day challenges, as well as creating a pleasant work environment. ***Bioengineers Marit Aarhaug, Toril Holien, Lene Kjøsnes and Oddrun Storrø*** have all been involved in the Photopheresis project, and I couldn't have done this with out their guidance and expertise. Oddrun, who was the last to be involved in the project has been an exceptional co-worker. I'm truly grateful for all your help, and I admire your laboratory skills and positive attitude. I'm also grateful for the opportunity to work with ***bioengineer Nina Sandberg***. Thank you for helping me find the things I had misplaced in the lab, and for showing interest in my day-to-day activities. Thanks to ***Hong Yan Dai***, at the Department of Medical Genetics, for helping me with the evaluation of T cell clonality.

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List of papers

I. **Rao V, Ryggen K, Aarhaug M, Dai H, Jørstad S, Moen T.** *Extracorporeal photochemotherapy in patients with cutaneous T-cell lymphoma: is clinical response predictable?* J.Eur.Acad.Dermatol.Venereol. 2006;20:1100-7.

II. **Rao V, Saunes M, Jørstad S and Moen T.** *In vitro experiments demonstrate that monocytes and dendritic cells are rendered apoptotic by extracorporeal photochemotherapy, but exhibit unaffected surviving and maturing capacity after 30Gy gamma irradiation.* Scand J Immunol. 2008;68:645-651.

III. **Rao V, Saunes M, Jørstad S and Moen T.** *Cutaneous T cell lymphoma and Graft-vs-Host disease: a comparison of in vivo effects of extracorporeal photochemotherapy on Foxp3+ regulatory T cells.* Clinical Immunology. Accepted for publication, July 2009.

Summary in Norwegian

Extracorporeal photokjemoterapi (ECP) har blitt akseptert som behandling av enkelte T celle medierte tilstander, som kutant T celle lymfom (CTCL) og Graft-vs-Host disease (GVHD). Ved CTCL responderer omtrent halvparten av pasientene på behandlingen. Det finnes imidlertid ingen gode prediktive markører for respons, og en slik markør ville naturlig nok være av stor nytte. I **Paper I** har vi evaluert responsraten ved ECP hos pasienter med CTCL som har blitt behandlet på St. Olavs Hospital, i tillegg til å undersøke flere andre potensielle prediktive markører. 7 av 16 pasienter ble klassifisert som respondere på ECP-behandling. Hverken T celle klonalitet eller noen av serummarkørene som ble undersøkt kunne benyttes som prediktiv markør før behandlingsstart, men det kan se ut til at forandringer i enkelte av markørene etter 6 måneders behandling korrelerer med klinisk respons evaluert etter 12 måneders behandling.

Virkningsmekanismen til ECP ved CTCL er fortsatt ikke kjent. ECP induserer apoptose av lymfocytter. Inntil nylig har man trodd at monocytter ikke går i apoptose av behandlingen, men heller aktiveres og differensieres til umodne dendrittceller (DC). Disse er effektive fagocytter og presenterer fagocytert tumor antigen til immunforsvaret. Slik har man trodd at en cytolytisk tumorspesifikk immunrespons settes i gang. I **Paper II** har vi undersøkt hvordan ECP påvirker monocytter og umodne DC når det gjelder indusering av apoptose samt evne til å differensiere etter behandling. Vi fant at både lymfocytter, monocytter og umodne DC går i apoptose innen 72 timer etter ECP-behandling. Siden de også har en redusert evne til å differensiere etter ECP er det sannsynlig at den terapeutiske effekten av ECP heller skyldes in vivo mekanismer som følge av infusjon av apoptotiske celler, fremfor infusjon av monocytter som er indusert til å differensiere til umodne DC. Monocytter og DC som ble bestrålt med 30Gy gamma bestråling, dvs doser som induserer apoptose av lymfocytter, ser ut til å være uaffisert av denne behandlingen når det gjelder overlevelse og evne til differensiering.

GVHD oppstår når allogene effektor T celler fra en donor angriper mottakeren. Det er foreslått at ECP trigger visse toleransemekanismer, som hemming av pro-inflammatoriske cytokiner, stimulering av regulatoriske T celler (Treg) og produksjon av anti-inflammatoriske cytokiner. Det kan imidlertid virke paradoksalt at den samme behandlingen skal sette i gang en tumorspesifikk, cytolytisk immunrespons mot lymfomceller hos den ene pasientgruppen, mens den fører til økt regulatorisk aktivitet hos GVHD pasienter. I **Paper III** har vi

sammenlignet den relative andelen av sirkulerende Treg celler hos pasienter med CTCL eller GVHD. Vi fant her at CTCL pasientene har nær dobbelt så høy forekomst av Treg celler i sirkulasjon, sammenlignet med GVHD og normal kontroller. I tillegg undersøkte vi også om det var en målbar forandring av cytokiner i serum før og etter ECP. Før behandling var det anti-inflammatoriske cytokinet TGF-β i gjennomsnitt tre ganger høyere hos GVHD pasientene, sammenlignet med CTCL. Begge pasientgruppene hadde en liten, men signifikant økning av TGF-β etter behandling. Disse resultatene kan tolkes som en forbedret Treg funksjon som resultat av behandlingen. Økt TGF-β kan indikere høy Treg aktivering hos GVHD pasientene, mens en forhøyet relativ andel Treg celler hos CTCL pasientene kan tolkes som en respons som muligens er involvert i nedregulering av lymfom cellene.

Summary in English

Extracorporeal photochemotherapy (ECP) has been accepted as a standard therapy in certain T cell mediated conditions, such as cutaneous T cell lymphomas (CTCL) and Graft-vs-Host Disease (GVHD). In CTCL approximately one half of these patients respond to the treatment. However, predictive criteria for selecting patients who will respond to ECP are mainly lacking. In **Paper I**, we have evaluated the response rate to ECP in CTCL patients treated at St. Olavs Hospital, as well as investigated several potential predictive markers. Seven out of 16 patients evaluated were classified as responders to ECP. Neither T cell clonality nor any of the serum markers assessed pre-treatment could reliably predict the response to ECP treatment. However, the individual relative changes in some of the serum markers assessed during 6 months of ECP treatment coherently displayed correlation to the clinical response as evaluated after 12 months of ECP treatment.

The mechanism of effect in ECP therapy is somewhat unclear. ECP has been shown to induce apoptosis in lymphocytes. Until recently the prevailing opinion has been that the monocytes were mainly not affected by this treatment, but rather were activated and differentiated into immature dendritic cells (DC), capable to phagocytize apoptotic CTCL-cells, and initiate a cytolytic tumour specific immune response. In **Paper II** we have tried to elucidate the effect of ECP on monocytes and immature DC, and followed the ability of the cells to differentiate and survive post treatment. We found that lymphocytes, monocytes and immature DC become apoptotic within 72 hours when treated with ECP. Since monocytes and immature DC seem to have a reduced ability to differentiate after ECP treatment, it is likely that the therapeutic effect of ECP is caused by in vivo effects of reinfused apoptotic cells, rather than by infusion of monocytes induced to differentiate into immature DC. Monocytes and DC treated with 30Gy gamma irradiation, i.e. doses that induce apoptosis in lymphocytes, were unaffected regarding survival and ability to differentiate post-treatment.

GVHD is mainly mediated by allogeneic effector T cells recognizing the transplant recipient as foreign. ECP is reported to be triggering certain tolerance mechanisms, such as inhibition of pro-inflammatory cytokines, stimulation of regulatory T cells (Treg) and production of anti-inflammatory cytokines. It may, however, seem like a paradox that ECP initiates a tumour specific, cytolytic response against lymphoma cells in one condition, whereas it increases the regulatory function in another. In **Paper III** we have compared the relative

levels of circulating Treg cells in patients with CTCL or GVHD. We found that the relative amount of Treg cells was twice as high in CTCL compared to GVHD and healthy controls. In addition, we investigated whether there were any measurable changes in serum cytokine levels during ECP therapy. Pre-treatment, the anti-inflammatory cytokine TGF-β was on average 3 times higher in GVHD than in CTCL. Both patient groups had a small, but significant increase in TGF-β after treatment. Our results indicate a strengthened Treg function as a result of ECP. Elevated TGF-β may indicate high Treg activation in GVHD, whereas an increased number of Treg cells in CTCL could be interpreted as a response that possibly is involved in down-regulating the lymphoma cells.

“Live as if you were to die tomorrow. Learn as if you were to live forever.”
Mohandas Karamchand Gandhi (1869 – 1948)

Introduction

Historical aspects

Therapy for dermatological diseases based on non-ionizing electromagnetic radiation (especially long-wave UV radiation) is a concept developed in a subspeciality of medicine that has become known as *photomedicine*, and the combined use of electromagnetic energy and a photoactive drug has been termed *photochemotherapy*. The combination of psoralens and UVA-light (PUVA) was first reported as a treatment for psoriasis in 1974 [1], but the practice is in fact almost 4000 years old. In the ancient world, especially in Egypt and India, it has been used since 1200-2000BC as a therapy for the common dermatological disease vitiligo, also known as leukoderma or "white leprosy", causing depigmentation in patches of the skin. An extract made from the leaves, seeds or roots of psoralen-containing plants such as *Ammi majus* (Fig. 1), originating in the Nile River Valley in Egypt, and *Psoralea corylifolia* in India, was either ingested or applied to the affected skin areas before exposing the skin to the bright sunlight [2].

Fig. 1 Ammi majus, *originating in the Nile River Valley in Egypt. (Also known as Bishop's weed, Bullwort, Greater ammi, Lady's lace and Laceflower. "Kongeskjerm" in Norwegian).*

Niels Finsen received the Nobel Prize in Medicine in 1903 for his successful treatment of lupus vulgaris with phototherapy, which marked the beginning of modern phototherapy. Almost 50 years later, the active ingredient of the *ammi majus* plant was isolated by an Egyptian pharmacologist and characterized as 8-methoxypsoralen (8-MOP), which was later shown to be photoactivated by exposure to UV-light [3]. In addition to having an effect on

psoriasis [1], PUVA was reported by Gilchrest to be effective in Cutaneous T cell lymphoma (CTCL) [4]. Soon it was demonstrated that photoactivation of 8-MOP induced T cell apoptosis by forming interstrand crosslinks in DNA [5]. A study from Yale University published in 1987 by Edelson and colleagues reported a favourable effect of extracorporeal photochemotherapy (ECP or photopheresis) in CTCL[6]. This formed the basis for approval by the Food and Drug Administration (FDA) of ECP as the first selective immunotherapy for the treatment of cancer. ECP was introduced as a treatment of CTCL, but has later been applied on a variety of T cell mediated diseases, such as Graft-vs-Host Disease (GVHD)[7-9], transplant rejection[10-12] and selected autoimmune disease[13-15]. Since 1992, St. Olavs Hospital has been authorized as a national centre for ECP treatment in Norway, as a part of the Department of Nephrology, until the responsibility was transferred to the Department of Dermatology in 2005. The treatment procedure, however, is still being performed at the dialysis ward.

The ECP procedure

ECP is a medical therapy based on leucapheresis, followed by extracorporeal treatment of the leucocytes with 8-methoxypsoralen (8-MOP) and illumination with ultraviolet light A (UVA) before being reinfused into the patient (Fig. 2). 8-MOP is a DNA-intercalating drug and acts by binding covalently to DNA only when illuminated with UVA, which results in induction of apoptosis in the treated leucocytes[16]. A procedure much used is to perform ECP of the patient on two consecutive days, once or twice a month. However, according to the severity of the disease, it may be performed more frequently. ECP is considered to be an extremely safe treatment and serious toxicity and complications such as catheter related infections are infrequent. In a multicenter study of ECP in patients with chronic GVHD, no serious adverse effects were reported that could be related to ECP. Additionally, there was no evidence that ECP induced a generalized immunosuppression, as the incidence of infections was the same as in the controls[9]. This is a great advantage especially in GVHD patients, in whom therapy with high doses of immunosuppressives would be the alternative.

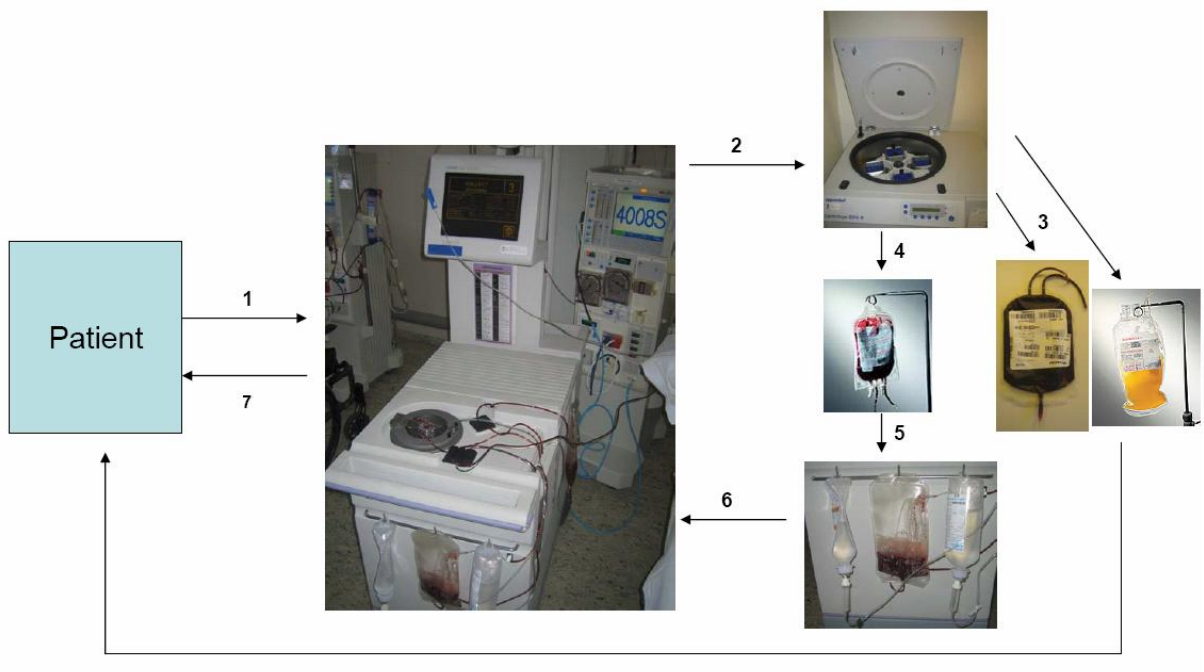

Fig. 2 The ECP procedure (schematic). *Whole blood is drawn from the patient (1), and cells separated from plasma in the photopheresis apparatus by centrifugation (2). Red cells and plasma are returned to the patient (3), while leukocytes (buffy coat) (4) are processed in the apparatus by mixing with 8-methoxypsoralen (5), which is then photoactivated by illumination with UVA-light (6) and returned to the patient (7) in a closed circuit.*

Graft vs Host Disease

GVHD is a condition that evolves when immuno*competent* cells, especially T cells, are introduced into an immuno*incompetent* host, and was first described by Billingham in 1959 [17]. The main cause is haematopoietic cell transplant (HCT), but solid organ transplants[18] and blood transfusions[19] are also reported to cause GVHD. The acute form of GVHD arises within the first 3 months after transplant, and clinically presents with the triad: dermatitis, enteritis and hepatitis[20]. The chronic variant of GVHD often presents more like a lichen planus-like eruption or as scleroderma, by definition more than 3 months after transplant. The chronic form may occur either as a continuation of acute GVHD, or as a distinct entity[21].

In chronic GVHD, the skin is often the first organ affected, and the patients may present with erythematous macules and papules. This may resemble several other similar skin conditions that also occur after transplantations, such as viral exanthemas, drug eruptions and engraftment syndrome, as well as more benign dermatitis, making the diagnosis difficult[22]. In spite of immunosuppressive treatment before, during and after transplant, GVHD is still the leading cause of morbidity and mortality after HCT[20, 21].

The pathophysiology of GVHD is the recognition of target tissues as being foreign, with a subsequent induction of an inflammatory response, leading to death of the targeted tissue. Regulatory CD4+CD25+Foxp3+ T cells are reported to protect against chronic cutaneous GVHD[23].

The best treatment for GVHD is prophylaxis, and usually consists of immunosuppressive drugs, such as methotrexate, prednisone, cyclosporine etc. Once the diagnosis is made, the treatment consists of increasing the immunosuppressive agent, and possibly adding other modifying agents[24]. Immunomodulation by phototherapeutic agents has been proven beneficial for some patients, especially with chronic GVHD. PUVA is most widely used, but success has also been reported with ECP[9, 25], which is thought to trigger certain tolerance mechanisms [26, 27].

Cutaneous T cell lymphoma

Primary cutaneous T cell lymphoma (CTCL) is a rare entity. It is subdivided and classified according to the WHO-EORTC 2005 classification [28], and Mycosis Fungoides (MF) is the most common subtype, accounting for about 75% of the CTCLs. The incidence varies across the world. In the US, the incidence of MF from 1973-1992 was 0,36 per 100.000 person years [29], whereas the incidence rate in Norway is somewhat lower [30].

MF is an indolent type of lymphoma. The disease starts with patches, which after years or even decades develop into thin and thick plaques. In a minority of patients, the disease results eventually in tumours and in dissemination to lymph nodes, blood, bone marrow, and internal organs. Involvement of mucous membranes is an exception. The disease does not develop continuously, but instead shows a stepwise progression[31]. Sezary Syndrome (SS) is the erythrodermic, leukemic subtype of CTCL and accounts for only about 3%. This leukemic form of CTCL is defined by erythroderma, lymphadenopathy and the presence of neoplastic T cells (Sezary cells) in skin, lymph nodes and peripheral blood, whith an absolute Sezary cell count of at least 1000 cells/ml. Clinically, edema, hyperkeratosis of palms and soles, and therapy-resistant pruritus are typically present[31].

Table 1. World Health Organization-European Organization for Research and Treatment of Cancer (WHO-EORTC) classification of cutaneous lymphomas with primary cutaneous manifestations.

Cutanous T cell and NK cell lymphomas
Mycosis fungoides
Mycosis fungoides variants and subtypes
Folliculotropic mycosis fungoides
Pagetoid reticulosis
Granulomatous slack skin
Sezary syndrome
Adult T-cell leukaemia/lymphoma
Primary cutanous CD30+ lymphoproliferative disorders
Primary cutaneous anaplastic large cell lymphoma
Lymphomatoid papulosis
Subcutaneous panniculitis-like T-cell lymphoma
Extranodal NK/T-cell lymphoma, nasal type
Primary cutaneous peripheral T-cell lymphoma, unspecified
Primary cutanous aggressive epidermotropic CD8+ T-cell lymphoma (provisional)
Cutaneous γ/δ T-cell lymphoma (provisional)
Primary cutaneous CD4+ small/medium-sized pleomorphic T-cell lymphoma (provisional)

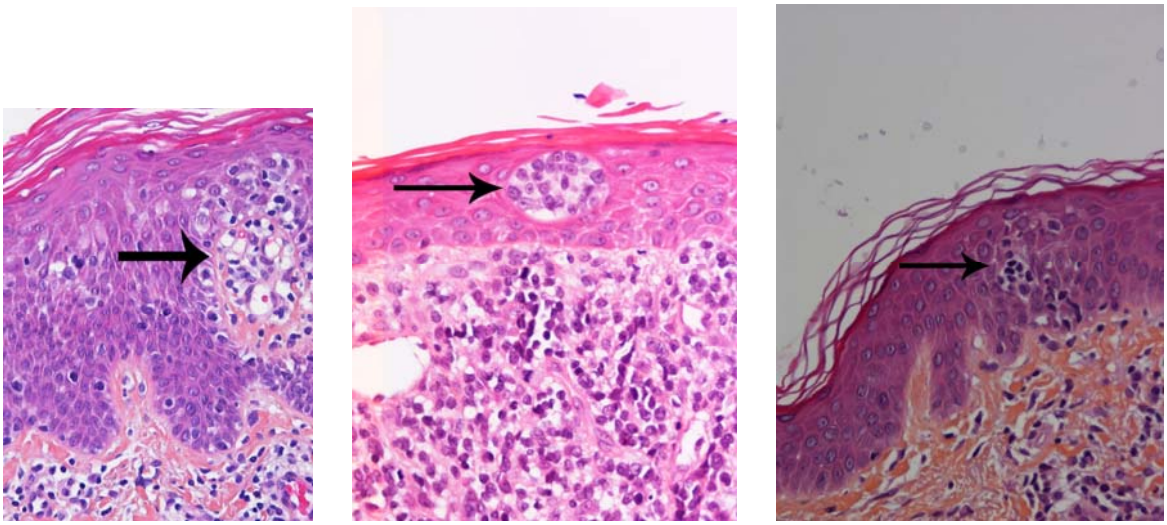

Fig. 3 Histological hallmark: *Lymphocytes infiltrate the epidermis, and create the characteristic Pautrier's micro-abscess (black arrows), which in these three examples are situated just below the stratum corneum in mycosis fungoides. Thanks to Dr. Harald Aarset (Dept. of Pathology, St. Olavs Hospital) for giving permission to use his illustrative photos.*

Histologically, in MF there is an infiltrate of CD30- T cells in the skin, and as they move throughout the epidermis they tend to create Pautrier's micro-abscesses (Fig. 3), which are a hallmark of the disease. As the condition progress, cells with a large, cerebriform nucleus (Sezary cells) can be seen in a blood smear[31].

The treatment is usually palliative only. An exception is the early Stage Ia, where treatment is initiated with a curative intention. Early detection and aggressive intervention is not shown to cure nor delay the progression of the disease. The prognosis of MF is dependent on stage and extent of the skin lesions, as well as the presence of extracutaneous manifestations. Patients with limited patch/plaque stage has a 10-year survival similar to a matched control population (98-99%), compared to approx. 40% in the tumour stage, and 20% if there is confirmed lymph node involvement[32-34]. When it comes to palliative treatment, there are several possibilities[35]. In the early stages, local steroids may limit the symptoms, as well as narrowband ultra violet light-B (UVB), whereas later stages could respond to oral psoralen + total body UVA (PUVA) or local chemotherapy in the combination with interferon. In the tumour-stage, systemic chemotherapy or radiation therapy may ease the symptoms. ECP is usually performed on erythrodermic stages of MF and SS resistant to conventional treatment, and sometimes given in combination with interferon. The effect of ECP is, however, variable. Approximately one half of the patients with CTCL demonstrates a reduction in skin score by at least 50% within 12 months of treatment, and is categorized as responders to ECP[36]. Prognosis in CTCL is much related to clinical and histopathologic classification[32-34, 37], but criteria for predicting clinical response to ECP are mainly lacking.

In search of predictive markers

The mechanism of therapeutic effect of ECP is still uncertain, but is likely to be explained by 8-MOP binding covalently to DNA in circulating lymphocytes. This ultimately leads to cell proliferation arrest and induction of apoptosis in the treated cells[16]. In the case of CTCL, as the apoptotic cell debris is removed by DC which are then presenting tumour antigens on their surface, a tumour specific immune response against the lymphoma cells has been thought to occur. Based on this, one might expect to find circulating monoclonal T cells in peripheral blood of CTCL patients responding to this treatment, and stored patient samples were analyzed to retrospectively investigate the potential usefulness of determining T cell clonality as presented in **Paper I**.

Interleukin-2 (IL-2) is a cytokine synthesized and secreted by activated T lymphocytes, and leads to proliferation of cells carrying the IL-2 receptor (IL-2R). When activated, T cells start shedding their IL-2Rα, which is then recovered in serum as a soluble form (sIL-2R). Activation and proliferation of T cells is thus reflected by increased serum levels of sIL-2R, an indirect measure of IL-2 production[38]. The sIL-2R is exploited to monitor several inflammatory, infectious and neoplastic diseases associated with B- or T cell activation. sIL-2Rα can thus be measured in blood or other body fluids of both healthy and diseased individuals such as in viral infections and arthritis, as well as in lymphomas[39]. The level of lactate dehydrogenase (LD) is also used clinically as a prognostic factor to characterize the growth and invasive potential of tumors[40].

Neopterin is produced and secreted by activated monocytes, i.e. macrophages and DC, and is a sensitive marker for cellular immunity. The serum concentration is reported to be elevated in several T cell associated diseases, such as CTCL, transplant rejections and viral infections such as HIV[41]. Beta2-microglobulin (β_2-M), which otherwise constitute the invariant β-chain of the HLA class I molecules, are together with sIL-2R as well as LD reported to be of prognostic value in CTCL, as high serum levels correlate to a poor therapy response and prognosis[42-45].

Cytolytic T cells contain preformed granules containing enzymes. After binding of the cytolytic cell to a target cell, the granules are released into the intercellular space. The granules contain a pore-forming protein (perforin), allowing released exogenous serine proteases (granzymes) to enter the cytosol of the target cells through the transmembrane

pores. Granzyme B activates an intracellular cascade of caspases, finally resulting in killing of the target cells[46]. Not all of the granzyme B will enter the cell, as some of it may leak out and enter the blood stream and thus be reflecting the cytolytic activity *in vivo*[37, 47].

In Paper I, we have reanalysed in stored samples the occurrence of T cell monoclonality in skin biopsies and peripheral blood of CTCL patients at the time of starting ECP treatment.
We have also investigated the serum levels of sIL-2R, LD, neopterin, β_2-M and granzyme B in CTCL patients during the course of treatment. The intention was to look for factors correlating to the therapeutic effect of ECP and thus possibly be of value in predicting which patients who will benefit from ECP treatment and in monitoring disease activity during the therapy.

Apoptosis

Apoptosis is a normal process in living organisms, and between 50 – 70 billion cells undergo apoptosis each day in the average human adult. The process of apoptosis was initially discovered for over 100 years ago, but described in detail by Kerr et al. in 1972 while he was studying tissues using electron microscopy at the University of Queensland in Brisbane. He had originally called it "programmed cell necrosis"[48]. He credited Prof. Cormack, a Professor in Greek, for suggesting the term "apoptosis", which translates to "dropping of" petals or leaves from plants or trees. Hippocrates had used the term to mean "falling of the bones", and Galen extended its meaning to "the dropping of the scabs". The term 'apoptosis' is now synonymous with programmed cell death, and is an active, and controlled process requiring energy. This is in contrast to necrosis, which is a passive, degenerative process, caused by injury or compromised metabolism. Apoptosis, despite a variety of inductive signals, follows the same pathway and ultimately leads to fragmentation of cells into apoptotic bodies with a still intact plasma membrane. A cell undergoing apoptosis shows a characteristic morphology that can be observed with a microscope: Cell shrinkage and rounding due to the breakdown of the cytoskeleton. The cytoplasm appears dense, and the organelles appear tightly packed. Chromatin undergoes condensation into compact patches against the nuclear envelope in a process known as pyknosis, a hallmark of apoptosis. The nuclear envelope becomes discontinuous and the DNA inside it is fragmented in a process referred to as karyorrhexis. The nucleus breaks into several discrete chromatin bodies due to the degradation of DNA. The cell membrane shows irregular buds. The cell breaks apart into several vesicles called apoptotic bodies, which are then finally phagocytized by macrophages

or DC[49]. One of the many common events of apoptosis is the externalization onto the cell membrane of phosphatidylserine, which can be detected with flow cytometry, by the binding of Annexin V.

What happens to monocytes and dendritic cells after ECP?

8-MOP is a DNA-intercalating drug and acts by binding covalently to DNA only when illuminated with UVA, hence inducing apoptosis in the treated lymphocytes. 8-MOP alone, without UVA, has no apparent effect[50]. ECP is known to induce apoptosis in lymphocytes[16] whereas until recently a common opinion has been that the monocytes were mainly not rendered apoptotic, but rather activated to differentiate into immature dendritic cells (DC). This theory is however under debate[50-58]. After the ECP treatment, the apoptotic cells are phagocytized in vivo and peptide antigens from phagocytized cells are expected to be presented on class II HLA molecules and even on class I (transimmunization)[51]. The exact mechanisms explaining the clinical response to ECP has yet to be fully revealed.

Ionizing radiation is another method to induce apoptosis of lymphocytes, but the effect on monocytes and immature DC is less known. However, even though being remarkably resistant to radiation-induced apoptosis, it has recently been suggested that irradiation may affect some of the functions of the DC, such as reduced IL-12 production and impaired T cell priming[59].

DC can be generated from blood monocytes during 5 days of cultivation with the cytokines interleukin-4 (IL-4) and granulocyte/macrophage colony stimulating factor (GM-CSF)[60-64]. Immature DC are specialized in internalizing and processing antigens, but rather ineffective as antigen presenting cells and in stimulating naïve T cells. Even though they do express both HLA class II and HLA class I, their expression of co-stimulatory molecules such as CD86, CD40 and CD80 is low. Surface expression of the mannose receptor (CD206) has been regarded a differentiation hallmark of monocyte-derived immature DC, in contrast to monocytes and mature DC[63, 65]. However, when the immature DC encounter inflammatory or infectious signals such as lipopolysaccharide (LPS) or tumor necrosis factor-α (TNF-α), they are capable of differentiating into a mature state. CD83 is a specific marker of mature DC, and in contrast to the immature DC, these mature cells are professional antigen presenting cells and effectively stimulate T cell responses[64, 66-68].

In **Paper II**, we performed a study to elucidate some of the short-term effects of ECP treatment on monocytes and in vitro generated monocyte-derived immature DC. We also wanted to compare the effect of gamma irradiation as an alternative method to induce apoptosis of monocytes and DC. In addition we were interested in evaluating the post-treatment differentiating capacity of the cells.

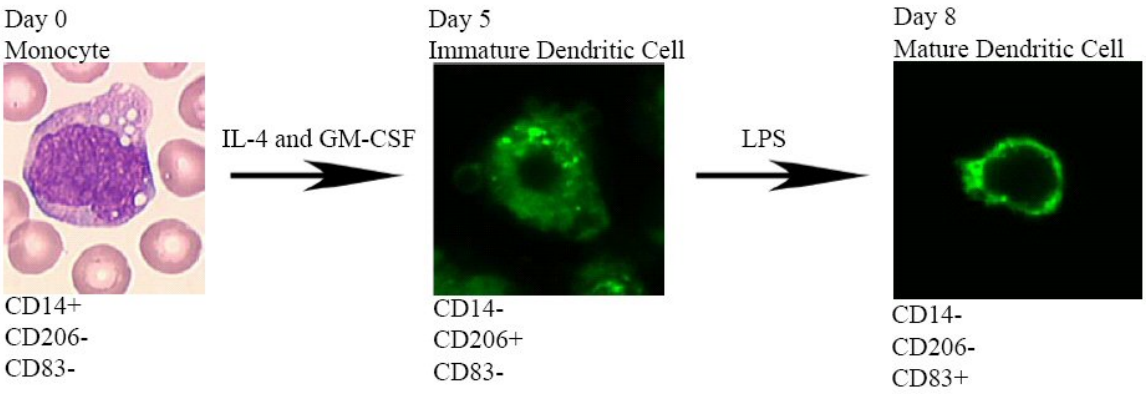

Fig. 4 Generation of dendritic cells in vitro. *Immature dendritic cells can be generated from CD14+ monocytes during 5 days of cultivation with the cytokines IL-4 and GM-CSF. When stimulated with lipopolysaccharide (LPS) they are capable of differentiating into a mature state*

CD4+ helper T cells: at a glance

CD4+ T cells orchestrate the adaptive immune responses and play a central role in the body's immune defence. For instance, they produce different cytokines and chemokines to recruit neutrophils, eosinophils and basophils to the site of inflammation. They activate B cells to produce antibodies. They induce differentiation of monocytes into macrophages, and activate them to enhance their microbicidal activity.

Traditionally, immunologists have divided the CD4+ T cell population into two distinct subsets based on functional criteria and cytokine production. The inflammatory helper T cells 1 (Th1), that mainly produce IFN-γ, and helper T cells 2 (Th2), that mainly produce IL-4 as their hallmark cytokine [69, 70]. Later, it has been discovered that this distinction was overly simplified, and at present there are several different subsets of interest. Some of the CD4+ T cell populations are distinguishable from the others already as they leave the thymus, such as the "natural" regulatory T cells (nTreg) [71] and natural killer T cells (NK T cells)[72]. However, at least 4 other subsets can be differentiated from naïve CD4+ T cells based on the signals they receive: Th1, Th2, Th17 and "induced" regulatory T cells (iTreg).

Identification and function of Th1, Th2 and Th17 cells

Th1 cells have an important role in the immune defence against intracellular pathogens [73, 74] such as viral and mycobacterial infections, by expanding the CD8+ cytolytic T cells. Dysfunction in the Th1 subset is also related to certain autoimmune diseases. IFN-γ, IL-2 and lymphotoxin-α are the main cytokines secreted. Th1 cells express several cytokine and chemokine receptors upon activation, such as IL-12Rβ, CXCR3 and CCR5 [75-77]. T-bet is considered to be the principal Th1 cell transcription factor. It is up-regulated during Th1 differentiation, induce IFN-γ production and inhibit IL-4 secretion[78]. In addition, Stat4, an IL-12 signal transducer, is important in the amplification of Th1 responses[79, 80].

Th2 cells, on the other hand, play a leading role in fighting extracellular parasites, as well as in the induction and persistence of different allergies and asthmatic disease[73, 74]. As mentioned previously, IL-4 is their hallmark cytokine. In addition, they produce IL-5, IL-9, IL-10, IL-13 and IL-25[81, 82]. Without going into detail, these cytokines may mediate the recruiting of eosinophils, expulsion of parasites, or induction of airway hypersensitivity. Th2 cells may be identified, among other surface antigens, by their expression of the chemokine receptor CCR3[83]. When blocking the transcription factor GATA-3, development of and maintenance of Th2 cells is abrogated[84, 85].

When it comes to the induction of many organ-specific autoimmune disorders, in addition to the immune response against extracellular bacteria and fungi, Th17 cells are thought to be responsible[86]. They produce the cytokines IL-17a, IL-17f, IL-21 and IL-22, which recruit and activate neutrophils, T cells, B cells, NK-cells and dendritic cells in the setting of an infection with fungi or extracellular microbes. Th17 cells may be identified by their high expression of IL-23R, as well as CCR6 and CCR4[87]. RORγτ and RORα have been proven to be important transcription factors for Th17 cell differentiation, as a deletion of both completely abolish IL-17 production[88, 89].

Regulatory T cells

The idea of T cells with a suppressive character arose in the early 1970s, as T cells seemed necessary for the induction of B cell tolerance[90, 91]. Suppressor T cells, as these negative regulatory cells became known, were assumed to be a distinct cell population and were intensively investigated. In the 1980's, investigators began to raise questions about the interpretation of the previous studies, and within short time the term "suppressor T cell"

almost disappeared. However, new studies suggest that there is in fact a subset of T cells having a critical function in the prevention of autoimmune disease. These T cells, named "regulatory T cells" (Treg), have now become a major focus of cellular immunological investigation, and constitute a subset of T cells with immunosuppressive properties[92]. As these cells are being further investigated in detail, additional classification has been required based on origin, phenotype and suppressive mechanism, creating a rather complex list of abbreviations and names: "natural" Treg (nTreg), "induced" CD4+ Treg (iTreg) and Tr1-cells, Th-3 cells (involved in mucosal tolerance in the gut) and CD8+ iTreg.

Identifying discriminatory cell-surface markers for the characterization and isolation of Treg cells has been a crucial goal for some time. Treg cells were first identified as helper T cells (CD4+) that strongly expressed CD25 (IL2-Rα), both in mice and humans[93, 94]. In humans, however, it has been shown that activated T cells generally up-regulate CD25 expression[95], thus decreasing the purity of Treg cell isolation by this marker and limiting its utility when studying Treg cells. It is known that Treg cells express several other characteristic molecules in addition to being CD25high, such as glucocorticoid-induced tumour necrosis factor receptor (GITR), cytotoxic T-lymphocyte antigen 4 (CTLA4) and L-selectin (CD62L). The above mentioned cell-surface- or intracellular molecules are, unfortunately, also expressed in activated T cells, thus making an exclusive isolation of viable Treg cells a substantial problem. At present the transcription fator Forkhead box p3 (Foxp3) is considered the most specific marker for Treg cells. This protein is however intracellular and not available for isolation of living cells. The search for Treg specific cell-surface markers continues, and several candidates have recently been assessed[96]. The importance of Foxp3 was demonstrated by showing that Foxp3-mutant mice have a Treg cell-deficiency and develop a severe lymphoproliferative autoimmune syndrome[97]. Similarly, humans lacking Foxp3 suffer from an aggressive autoimmune syndrome, known as immuno dysregulation, polyendocrinopathy and enteropathy, X-linked (IPEX) syndrome, a rare recessive disorder that results in early death[98]. When first discovered in mice, Foxp3 was thought to exclusively identify Treg cells also in humans, but recent evidence support that Foxp3 indeed can be induced upon T cell activation, and that these Foxp3+ effector T cells are without immunosuppressive properties. Hence, low expression of specific cell-surface markers should also be exploited to assess and identify Treg cells, for instance that they have low expression of CD127 (known as IL7-Rα), whereas activated, effector T cells are CD127high[99, 100]. Treg cells are thought to be capable of inhibiting immune responses against a variety of

antigens, including the ones expressed by malignant cells[101]. In preventing autoimmune disease, the presence of Treg cells is naturally thought to be beneficial, whereas in the context of CTCL high numbers of these regulatory cells have mainly been supposed to inhibit the surveillance and clearance of tumour cells. Indeed, investigators have suggested that there is an association between a high tumour burden or tumour progression and an increase in Treg cells[102]. CD103, an αEβ7 integrin that mediates T cell retention in the epithelial compartment, has been shown to be an excellent marker for identifying *in vivo*-activated (induced) Treg cells[103, 104]. These CD103+CD4+Foxp3+ Treg cells have been shown to be more potent suppressors in autoimmune arthritis[103] and in reversing chronic GVHD, than primary CD4+CD25high Treg cells[105]. CD39 is a member of the ectonucleoside triphosphate diphosphohydrolase family, and is expressed on the surface of various cells. Its function is to degrade ATP, which acts as a "danger signal" that activates the immune system, to AMP. In combination with CD73, another ectonucleotidase, it results in the production of adenosine, that exhibit inhibitory and antiproliferative effects. Recently, it has been shown that CD39 is confined to a subset of the Treg cells, thought to represent regulatory cells with effector/memory-like properties. In the same study, the expression of CD39 among the Treg cells was also reflected in the capacity to degrade ATP, as well as in suppressive capacity[106]. CD45RA is a surface marker of primary (naïve) T cells in contrast to secondary (antigen-stimulated) T cells.

There are four basic mechanisms of how the Treg cells are thought to function as suppressors.
The first mechanism involves suppression by secretion of inhibitory cytokines, especially Interleukin-10 (IL-10) and Transforming Growth Factor-beta (TGF-β). The importance of these cytokines are, however, a matter of debate due to the general perception that Treg cells function in a contact-dependent manner[107, 108]. Interleukin-35 (IL-35) has also recently been reported to be a necessity to achieve maximal suppressive activity of Treg cells[109].
A second mechanism reported is suppression by cytolysis, through the secretion of granzymes, which ultimately induce cytolysis of effector T cells. This has long been thought to be the mechanism of action by natural killer cells and CD8+ cytotoxic T cells. However, there are publications that support that granzyme-deficient Treg cells have reduced suppressive activity[110].
A third mechanism of the immunosuppressive action of Treg cells, is a theory about suppression by metabolic disruption. For instance, that CD25+ Treg consume the Interleukin-

2 (IL-2) available, and thus starves the effector T cells by consuming the IL-2 they need to survive[108, 111]. This theory is, however, under debate.

Finally, suppression by targeting dendritic cells has been reported. By modulating the maturation and hence decreasing dendritic cell function, i.e. decreasing effector T cell activation[112]. It has also been shown that Treg cells induce down-regulation of the expression of the co-stimulatory molecules CD80 and CD86 in dendritic cells[113].

Paper III is a study performed to determine the relative numbers of Treg cells, characterized by the phenotype CD3+CD4+Foxp3+CD127-, prior to ECP and after 6 months of ECP treatment in patients with CTCL or GVHD. Further characterization was done to analyze the expression of CTLA4, CD25, CD45RA, CD103 and CD39 among the Foxp3+ cells. In addition we assessed whether there was any change towards a tolerogenic profile, by assessing the serum concentrations of IL-4, IL-6, IL-10, IL-17, IFN-γ and TGF-β, before and after 6 months of ECP treatment.

Aims

Paper I

Since 1992, St. Olavs Hospital has been the only centre in Norway authorized to practice ECP treatment, and an evaluation of the therapy is essential to improve the management of the patients in need of ECP. During these years, lymphocytes and sera have been collected from patients at different intervals during treatment, and stored as frozen samples.

While positive results of ECP have been reported in patients with graft vs. host disease[7] and in preventing graft rejection in heart and renal transplantation[10-12], the effect on CTCL is variable. Only about one half of the treated CTCL patients demonstrate sufficient reduction in disease severity to be classified as "responders" to ECP treatment[36]. Reliable predictive criteria for selecting patients who will respond to ECP are lacking, and such criteria would naturally be of great benefit. As described in the introduction part, the potential predictive criteria investigated in this study include T cell clonality and serum factors as: soluble interleukin-2 receptor (sIL-2R), lactate dehydrogenase (LD), neopterin, beta2-microglobulin (β_2-M) and granzyme B.

The serum values of the above mentioned parameters were measured pre-treatment, after 6 months and 12 months of treatment.

Paper I - Summary of aims:

1. Evaluate the response rate to ECP in CTCL patients treated at St. Olavs Hospital.
2. Retrospectively investigate the potential usefulness of determining: T cell clonality in peripheral blood, and sIL-2R, LD, neopterin, β_2-M and granzyme B in patient sera as predictive markers for response to ECP.

Paper II

This part of the thesis is an attempt to elucidate the effects of ECP treatment on monocytes and dendritic cells. Strong evidence support the fact that extracorporeal photochemotherapy induces complete apoptosis in lymphocytes. However, until recently the opinion has been that monocytes are resistant to the exposure of 8-MOP and UVA-light in combination. Later studies have on the other hand suggested that also the monocytes become apoptotic by the treatment, but somewhat slower than the lymphocytes[50, 53, 58].

Berger et al. [51] reported that repeated adhering to the plastic membranes in the ECP system induces activation and differentiation of the monocytes into immature DC, by stimulating the cells to secrete IL-1, TNF-α and IL-6. Further on, they suggested that an over-night incubation step, before returning the treated cells to the patient will allow the DC to phagocytise apoptotic CTCL cells in high concentration. This would generate the time to process engulfed tumour cells and display tumour antigen on their HLA class I and class II, and finally improve the antigen presentation *in vivo* when reinfused. A prerequisite for this concept must be that the monocytes actually survive the ECP-treatment. If the monocytes survive the treatment they have to be able to differentiate into phagocytising immature DC, as well as stay viable long enough to reach the antigen presenting mature state. If this is the case, an incubation period before reinfusing the cells will probably increase the phagocytosis of apoptotic CTCL-cells and thus improve the following antigen presentation in vivo.

On the other hand, knowing that the ECP treatment ultimately leads to incorporation of 8-MOP in all treated cells and a covalent cross binding between double-stranded DNA, makes it somewhat hard to fully comprehend that the monocytes should remain unaffected by the treatment. Even if the monocytes are viable after treatment, their function and ability to differentiate may be reduced. Therefore, elucidating the effects of ECP treatment on monocytes will give information about the viability and capability to differentiate after treatment, as well as clarifying the importance of such an over-night incubation step.

We also wanted for comparison to investigate the effects of gamma irradiation on monocytes and DC, as an alternate inductor of apoptosis.

Paper II - Summary of aims:

1. Elucidate the effect of ECP on *monocytes*, regarding induction of apoptosis and ability to differentiate into immature dendritic cells when stimulated with IL-4 and GM-CSF.
2. Elucidate the effect of ECP on in vitro differentiated *immature dendritic cells*, regarding induction of apoptosis and ability to progress to mature dendritic cells when stimulated with lipopolysaccaride
3. Use gamma irradiation as an alternative method of apoptosis induction, and follow the cells regarding induction of apoptosis and ability to differentiate.

Paper III

Regulatory T cells (Treg) have been reported to protect against chronic GVHD, and the effect is thought to be mediated through the triggering of certain tolerance mechanisms such as inhibition of pro-inflammatory cytokines, stimulation of Treg cells and production of anti-inflammatory cytokines [23, 27]. A major problem with clinical ECP in CTCL is that we still do not know what effect we aim at obtaining. Due to the findings of monoclonal T cells in peripheral blood, one explanation has been that ECP induces an anti-clonotypic cytolytic immunity directly against the tumour cells[56]. An essential issue in CTCL is whether ECP is inducing specific cytotoxicity against the lymphoma cells, or alternatively inducing specific or non-specific Treg cells controlling the CTCL proliferation. The success obtained with priming dendritic cells in cancer therapy could point to the first possibility [114, 115], whereas the effect of ECP in GVHD, transplant rejection and autoimmunity favours the other view [7, 10-14].

This study was performed to determine the relative numbers of Treg cells, characterized by the phenotype CD3+CD4+Foxp3+CD127-, prior to ECP and after 6 months of ECP treatment in patients with CTCL or GVHD. Further characterization was done to analyze the expression of CTLA4, CD25, CD45RA, CD103 and CD39 among the Foxp3+ cells. In addition, we have assessed whether there is any change in a representative serum cytokine profile of helper T effector cells and Treg cells among these patient groups during ECP, by assessing the serum concentrations of IL-4, IL-6, IL-10, IL-17, IFN-γ and TGF-β, during 6 months of ECP treatment.

Paper III - Summary of aims:

1. Compare the relative fraction of regulatory T-cells among healthy controls, patients with CTCL and patients with GVHD.
2. Investigate whether there is a change in the relative fraction of regulatory T cells in CTCL or GVHD patients during 6 months of ECP treatment.
3. Study if there are any differences in the relative fraction of regulatory T cells between CTCL patients responding to treatment, compared to the non-responders.
4. Investigate whether there is a measurable change in serum concentrations of IL-4, IL-6, IL-10, IL-17, IFN-γ or TGF-β after 6 months of ECP treatment.

General Material and Methods

(See enclosed publications for details)

Study populations

In **Paper I**, Serum and peripheral blood mononuclear cells (PBMC) from 16 CTCL patients, diagnosed with MF or SS and treated with ECP, between 1993 and 2003, were evaluated in an open, retrospective study. The clinical manifestations of the patients were heterogeneous and are summarized in Table 1 (Paper I). The CTCL patients were classified at initiation of ECP according to a skin score test defined as the product of skin disease severity score multiplied by percent affected body surface area[6]. The skin disease severity score is according to the following scale: 0 = normal, 1 = barely detectable erythema and scaling, 2 = readily detectable erythema, edema and scaling, 3 = marked erythema and exfoliation, and 4 = fissuring, maximal erythema, induration and tumors. This makes 400 (4 x 100%) the maximal total skin score. The clinical response of the CTCL patients to ECP was defined as a reduction in skin score by at least 50% as registered after 12 months of ECP treatment. Skin biopsies were taken of all patients, and peripheral blood was examined for atypical cells. Lymph node biopsies were performed on all patients with palpable lymphadenopathy. X-ray or CT-scan of the chest, as well as ultrasound or CT-scan of the abdomen was performed to evaluate clinical stage. For this retrospective study, blood samples were obtained immediately before the first ECP procedure, after a 6 months course of therapy and 12 months after initiating therapy when the clinical effect of ECP was assessed. Sera were stored at -70ºC and PBMC in 10% DMSO at −135°C.

In **Paper II**, PBMC were isolated from healthy blood donor buffycoats using Lymphoprep (Axis-Shield, Oslo, Norway). Monocytes were then positively extracted by immunomagnetic sorting, using magnetic anti-CD14-MicroBeads and MACS separation columns (Miltenyi Biotec, Bergisch Gladbach, Germany), and cultivated after treatment with in vitro ECP or gamma irradiation.

In **Paper III**, stored serum and PBMC from a total 58 individuals were evaluated. This study population included patients with CTCL (n=24) or GVHD (n=14) treated with ECP between 1993 and 2008, as well as controls obtained from healthy blood donors (HC, n=20). The CTCL and GVHD patients were evaluated pre treatment, and after 6 months of ECP treatment. See Table 1 and 2 (Paper III) for individual data on CTCL and GVHD patients, respectively.

Evaluation of T cell clonality in skin biopsies and peripheral blood by TCRγ chain PCR

To get a uniform and qualified re-evaluation of occurrence of monoclonal T cells, DNA was extracted from stored paraffin-embedded skin biopsies and from peripheral blood mononuclear cells stored at –135°C. The DNA was quantified by fluorometry. Specific consensus primers were used for each family of the variable (V) and joining (J) regions of the TCRγ gene. The primers used were based on those described by Vega *et al*[116]. Two parallel PCR reactions were performed using DNA in two different dilution folds. PCR amplification was performed in a thermal cycler (GeneAmp PCR system 2700). Following the PCR reactions, the samples were loaded on a DNA sequence analyser (ABI 3100, Applied Biosystems). Fluorescence data were analysed using GeneScan software. The presence of monoclonality was determined by the presence of 1 or 2 peaks. DNA isolated from T lymphoma cell line Jurkat was used as positive control for both primer sets. The presence of T cell monoclonality in peripheral blood was valid when an identical fluorescence peak emerged for a corresponding skin biopsy sample of the same patient.

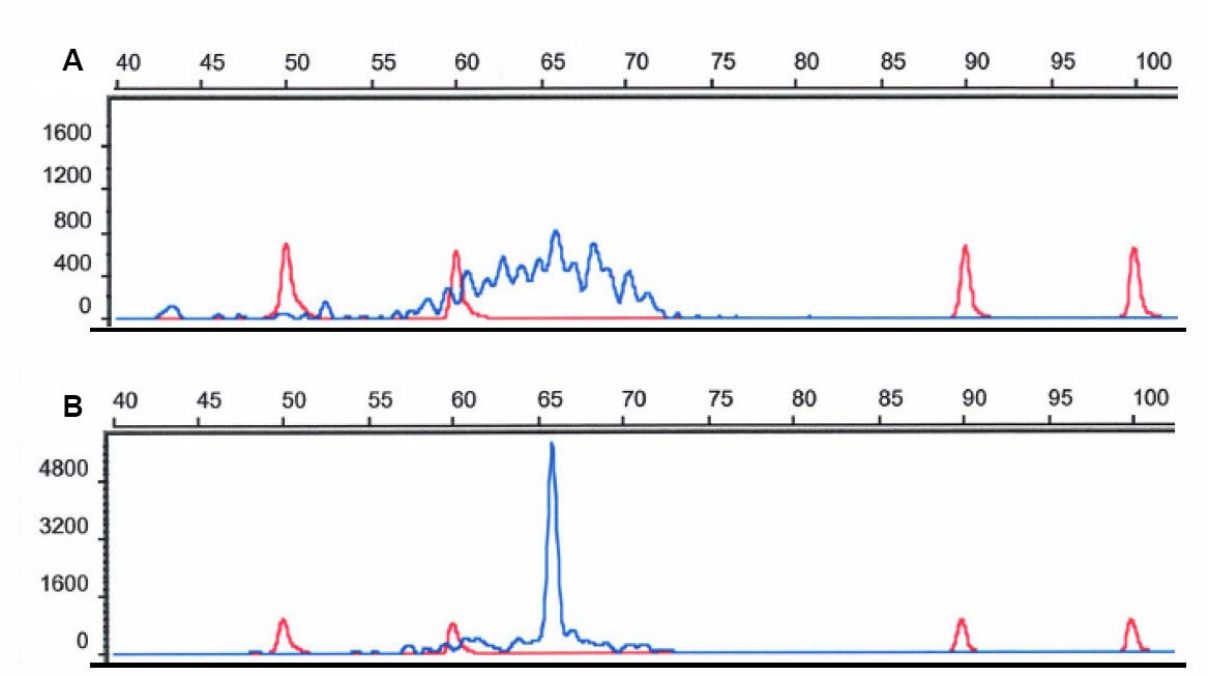

Fig. 5 TCR clonality. *An example of how the presence of T cell reseptor γ chain (TCRγ) clonality in peripheral blood was assessed. After PCR, the samples were loaded on a DNA sequence analyzer, and the fluorescence data from the PCR product were analyzed using GeneScan Software. The blue peaks represent the distribution of the TCRγ chain in a polyclonal (A) and a monoclonal (B) sample. The red peaks represent a known scale or "ruler".*

Flow cytometry

Cells were assessed freshly drawn, directly from cell cultures or after storage at - 135°C, and stained according to manufacturer's instructions. Briefly, the cells were first stained for surface antigens, before permeabilized and stained for intracellular antigens when required. Flow cytometric analysis was performed using a FACScan™ or FACSCanto™ instrument (both obtained from Becton Dickinson Biosciences, San Jose, CA, USA). The FACScan™ cytometer is capable of detecting antibodies in 3-color combinations, whereas samples processed with the FACSCanto™ instrument were stained in 5- or 6-color combinations. See individual papers for further details.

ELISA

As described in Paper I, the soluble IL-2R, Granzyme B and neopterin enzyme-linked immunosorbent assay kits were obtained respectively from Immunotech (Marseille, France), Sanquin Reagents (Amsterdam, The Netherlands) and BRAHMS (Hennigsdorf, Germany). The tests were performed as recommended by the instruction manuals, and the results presented as mean of duplicates.

Serum lactate dehydrogenase (LD) and beta2-microglobulin (β2-M) were analysed at the Hospital Department of Medical Biochemistry. LD was analysed by an enzyme assay (Roche, Basel, Switzerland) and performed according to standard procedures. β2-M was analyzed by radio immunoassay (RIA) techniques (B2-micro RIA 100, Pharmacia Diagnostics AB, Uppsala, Sweden).

Cytokine assays

In Paper III, serum stored at -70ºC, obtained pre-treatment and after 6 months of ECP treatment, was thawed and the concentrations of IL-4, IL-6, IL-10, IL-17 and IFN-γ were measured in duplicates, using a Milliplex Human Cytokine/Chemokine Immunoassay (Millipore Corporation, Billerica, MA, USA). The concentration of TGF-β was measured, also in duplicates, using a LINCOplex TGF-β1Single Plex Kit (LINCO Research, St. Charles, MO, USA). Both kits were applied according to the manufacturer's instructions. Data were then collected on a Luminex 100 IS analyzer using the provided software (Luminex Corp., Austin, TX, USA).

In vitro ECP of monocytes and dendritic cells

As described in detail in Paper II, monocytes were positively extracted by immunomagnetic sorting using anti-CD14-MicroBeads and MACS separation columns (Miltenyi Biotec, Bergisch Gladbach, Germany). Either CD14+ monocytes immediately after immunomagnetic extraction (extracted CD14+ cells were >90% pure as measured with flow cytometry), or monocyte-derived immature DC after 5 days in culture with GM-CSF and IL-4, were incubated with 200 ng/mL 8-methoxypsoralen (8-MOP) for 15 minutes on ice in the dark. Subsequently the cells were exposed to 2 J/cm^2 UVA-light using the Intercept illuminator (Cerus Corporation, Concorde, CA, USA) to simulate extracorporeal photochemotherapy (ECP).

Alternatively, gamma irradiation was provided at doses of 30Gy or 90Gy while keeping cells on ice (^{137}Cs, Sorø, Denmark).

Immediately after treatment, the cells were cultured at 37 °C and 5% CO_2 in 6-well flat-bottom culture plates at a density of 1x10^6 cells/mL in a total of 3mL RPMI 1640 medium w/glutamine, supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 ug/mL of streptomycin, 80 ng/mL each of IL-4 and GM-CSF (R&D Systems Europe, UK). To induce maturation of the immature DC into mature DC, 10 ng/mL lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, MO, USA) was added on day 5 of culture.

Apoptosis was evaluated with flow cytometry, by measurement of staining with annexin V and propidium iodide (PI) with a commercially available kit (IQProducts, Groningen, The Netherlands). Annexin V binds to phosphatidylserine, and externalization of phosphatidylserine is an early sign of apoptosis. Hence, cells stained with annexin V were classified as 'apoptotic'. Annexin V-/PI- cells were classified as 'viable', i.e. non-apoptotic.

Main results and summary of papers

Paper I

Reliable predictive criteria for selecting CTCL patients who will respond to ECP are mainly lacking, and such criteria would naturally be of great benefit. In the first paper, we wanted to evaluate the response rate in 16 patients with CTCL treated with ECP, as well as to assess a selection of parameters retrospectively to investigate their potential usefulness as predictive markers. The occurrence of T cell clonality in skin biopsies and peripheral blood samples obtained before initiating ECP are presented in Table 1 (Paper I). Twelve of the patients (75%) in this study had T cell monoclonality detected in their skin biopsies by using PCR and DNA fragment analysis. Three of the four patients who did not have monoclonal T cells detected in their skin biopsies were patients included in the study as "suspicious of" MF, and diagnosed as Red Man Syndrome (RM). Clinically, the fourth patient was histologically diagnosed as MF, but without any T cell clone being detected in skin or blood. Eleven patients were found to have monoclonal T cells in peripheral blood, and among these the four patients diagnosed with MF in the responding group all had a detectable clone in both skin and blood. There were, however, no significant differences in distribution of these patients into the ECP responding and non-responding groups. The non-responding patient group on average had higher pre-treatment values of serum sIL-2R compared to the responding group, but this was not statistically significant. However, after 6 as well as 12 months of ECP treatment the difference in average serum sIL-2R between the two patient groups was significant (Fig. 1, Paper I). An individual reduction in serum sIL-2R concentration was well correlated to clinical remission measured as a reduction in skin score during 6 months of treatment.

In conclusion, seven out of 16 patients were classified as responders to ECP. Neither T cell clonality nor any of the other serum markers assessed pre-treatment could reliably predict the response to ECP treatment. However, the individual relative changes in sIL-2R, neopterin and β2-M during 6 months of ECP treatment coherently displayed correlation to the clinical response, as assessed after 12 months of treatment.

Paper II

In Paper II, we wanted to elucidate the effects of ECP treatment on monocytes and dendritic cells (DC). Strong evidence support the fact that extracorporeal photochemotherapy induces complete apoptosis in lymphocytes. However, until recently the opinion had mainly been that

monocytes are resistant to the exposure of 8-MOP and UVA-light in combination, and rather differentiate into antigen presenting DC. In this study, we investigated the effect of ECP and gamma irradiation on lymphocytes, monocytes and immature DC in vitro, and followed the ability of the cells to differentiate and survive post treatment. We found that ECP induced apoptosis in all treated cells within 72 hours following treatment. This was in contrast to cells treated with 30Gy gamma irradiation, which seemed mainly to kill the lymphocytes. The small fraction of surviving ECP treated monocytes presented a reduced ability to differentiate into immature DC within this time frame. We also demonstrated that the few surviving immature DC lost their normal ability to mature on stimulation with lipopolysaccharide after ECP treatment. 30Gy gamma irradiation did not seem to hamper the monocytes' ability to differentiate or the immature DC ability to mature. Monocytes and immature DC treated with 2J/cm^2 of UVA without incubation with 8-MOP or 8-MOP without UVA did not differ from the untreated control cells (not shown).

In conclusion, as both monocytes and immature DC seemed to be doomed by the ECP treatment, it was suggested that the therapeutic effect of ECP is caused by in vivo effects of reinfused apoptotic cells, rather than by infusion of monocytes induced to differentiate into functional immature DC.

Paper III

In GVHD, the effect of ECP is reported to be mediated through induction of certain tolerance mechanisms, such as regulatory T cell (Treg) stimulation. In the case of CTCL an essential issue in explaining the mechanism of effect of ECP treatment, is whether ECP is inducing specific cytotoxisity against lymphoma cells, or alternatively, specific or non-specific Treg cells controlling CTCL proliferation. We included 24 patients with CTCL and 14 patients with GVHD to assess the relative fraction of Treg cells during 6 months of ECP treatment, compared to data obtained from 20 healthy controls. Individual data on CTCL and GVHD patients are presented in Table 2 and 3 (Paper III), respectively. In addition we studied whether there was a measurable change in a representative cytokine profile of effector T cells and Treg cells, by assessing the serum concentrations of 6 cytokines, IL-4, IL-6, IL-10, IL-17, IFN-γ and TGF-β, during the same treatment interval. We found that the mean relative amount of Treg cells was twice as high in the CTCL patients, compared to both the patients with GVHD and the healthy controls. Pre-treatment, the anti-inflammatory cytokine TGF-β was on average 3 times higher in GVHD than in CTCL. Both CTCL and GVHD patients had a small, but significant increase of TGF-β after 6 months of ECP treatment. There was also a

significant decrease of the inflammatory cytokine IL-17 in the GVHD patients. No differences were found among the other cytokines assessed.

In conclusion, our results may indicate a strengthened Treg cell function in both patient groups as a result of ECP. Elevated TGF-β may indicate high Treg cell activation in GVHD, whereas an increased number of Treg cells in CTCL could be interpreted as a response that possibly is involved in down-regulating the lymphoma cells.

Discussion

In search of predictive markers

Based on cutaneous symptoms, histological examination of skin biopsies and detection of T cell monoclonality in skin and peripheral blood, patients were selected for ECP therapy and later included in the study presented in **Paper I**. A re-classification was done after a retrospective evaluation of the T cell monoclonality in skin biopsies and peripheral blood. Three patients were then classified as "suspicious of MF", but still chosen to enter the study as Red Man Syndrome (RM), since they had accomplished the ECP therapy. Classification of the patients into responders and non-responders was based on the change in skin score and performed 12 months after initiation of the ECP. Based on the given criteria and including the RM patients, seven patients were classified as responders to ECP. Nine patients were classified as non-responders. This gives a response rate of 44% (7/16 patients) and is somewhat lower than the observations made by other investigators[36]. Further on, if excluding the RM patients, the response rate is even lower in this material (4/13 patients, 31%). This may however be incidental due to the small size of the patient material.

As presented in Table 1, Paper I, T cell clonality was detected in most patients both in the responding as well as the non-responding group. Detection of T cell clonality in skin or peripheral blood is important in assessing a diagnosis of CTCL, but in this evaluation, circulating clonal T cells does not seem to be a decisive factor in trying to predict the effect of ECP therapy. However, the exact physiologic effect of ECP is still not fully understood. Treatment with UVA and psoralen has in some publications been shown to induce apoptosis in the circulating malignant and non-malignant T cells, but not in monocytes[50, 58, 117]. ECP activation of dendritic cells that is leading to increased phagocytosis and antigen presentation of apoptotic cells has been demonstrated. ECP has been shown to increase MHC I surface antigen expression on CD4+ T cells, facilitating a cytolytic T cell response specifically against the pathogenic CD4+ T cells[54]. However, other mechanisms besides direct killing of malignant T cells have also been investigated. The phagocytosis of lymphoma cells has been supposed to result in production of specific suppressor T cells acting against clonal T cells[52, 55-58]. On the other hand, Berger et al. have proposed that CTCL may be a malignant proliferation of regulatory T cells (Treg), i.e. cells expressing a regulatory phenotype (CD25+, CTLA4+, Foxp3+), being stimulated by dendritic cells loaded with apoptotic CTCL-cells. These CTCL-Treg cells also secreted IL-10 and TGF-β, both cytokines

that will maintain dendritic cells immature and ensure continued phagocytosis and antigen presentation, inducing further CTCL-cell proliferation. That CTCL cells adopt Treg cell functions may explain the immunosuppressive effect seen in progressive CTCL disease[118]. However, Gjerdrum et al demonstrated that in skin biopsies the degree of Treg cell infiltration in CTCL was correlated to patient survival, and suggested that Treg cells can directly suppress the function of malignant T cells[119]**.** We think that their findings also contradict that CTCL cells have a Treg phenotype, as infiltrating lymphoma cells should clearly not correlate positively to patient survival. In addition, a report from Tiemessen et al suggested that Treg cells in late stage CTCL are dysfunctional[120], which may explain the lack of tumour control seen in progressive disease.

The presence of a T cell clone in blood anyhow would seem to be essential for obtaining response to ECP. T cell receptor PCR and DNA fragment analysis is probably sufficiently sensitive for detecting clonality. However, in this study two patients classified as RM have responded well to ECP in spite that no clone was detectable in peripheral blood. Other essential prerequisites for activating an effective immune reaction are not so easily detected. The individual HLA constitution might be one factor of importance in relation to presentation of relevant antigens from the monoclonal cells. In any case an apparent therapeutic effect or not will be the result of a balance between an anti CTCL immune reaction and the proliferating capacity of the T cell lymphoma. A high tumor burden is likely to be unfavorable. Other mechanisms that might seem to be less dependent on the occurrence of circulating clonal T cells have been proposed. Some researchers have suggested that CTCL is a malignant proliferation of helper T cells type 2 (Th2). ECP appears to increase the helper T cells type 1 (Th1), and consequently to reverse the abnormally high Th2-cell level[121-123]. ECP has also been shown to induce the release of pro-inflammatory cytokines like TNF-α, IL-1 and IL-6, which implicates monocyte activation[124, 125].

It has been published by other investigators that increased serum levels of sIL-2R, β2-M as well as LD indicate an unfavorable response to ECP[42-45]. Our results indicate that non-responders to ECP on average do have a higher pre-treatment serum level of sIL-2R than responders do. The difference is small and statistically insignificant, however, and thus not useful in predicting whether a CTCL patient will be a responder to ECP or not. Contrary to this we think that individual regress in serum sIL-2R level at 6 months of ECP is characterising responding patients and that this parameter can contribute to identify patients who can possibly benefit from further ECP treatment, either directly or at later relapse. In this material, we observed that the individual relative changes in serum concentrations of

neopterin and β2-M during 6 months of ECP were coherently correlated to the change in clinical disease, i.e. skin score. Both factors are indicators of immune activation. We found in this material only slightly elevated mean values of LD and indeed the responding group was the higher one. The difference is not statistically significant however and may be just incidental or because the age of the responders was on average 10 years higher than the age of the non-responders. Monitoring of granzyme B levels in serum does not seem to be of general interest in following up ECP of CTCL patients. We had hoped it could be used to reveal an ongoing cytolytic reaction against a T cell lymphoma, being released from activated responsive CD8+ cells. However, the test can probably be used to monitor tumor burden in the minority of CTCLs that are of cytolytic T cell (CD8+) origin. According to the one case reported in Paper I, the test does not seem to be very sensitive for this purpose, as granzyme B in serum was not detected before the CD8+ clone had reached 8% of circulating leukocytes, and it was not detected at the points of time when T cell monoclonality in skin and peripheral blood was first detected.

In evaluating this rather small patient group of CTCL, we have not been able to identify any pre-treatment factor that can predict the clinical response of ECP. A reduction in serum sIL-2R, neopterin and β2-M during the first course of ECP can however, add in the prediction of a better prognosis on the long run. Even if being a relative insensitive test granzyme B in serum can probably be used in monitoring the progress of cytolytic CD8+ T cell lymphomas. However, it should be assessed in combination with other markers such as skin score, skin biopsy, CD4/CD8-ratio and Sezary cell-count, when trying to assess disease severity, therapy and prognosis.

Differences in results between the study presented in **Paper I** and studies made by other investigators may be due to the complexity of CTCL and thus of the patients included in different studies. This also means variation in duration of the disease, in treatment given before ECP and in intensity and duration of the ECP course. Most therapeutic centres have limited number of patients referred for ECP. Patient survival would be a stronger parameter to follow up than clinical response measured as skin score improvement. So far, the patients described here are too few for such a follow-up study.

ECP induces apoptosis in monocytes and dendritic cells

As expected, UVA-light ($2J/cm^2$) without incubation with 8-MOP or 8-MOP without illumination with UVA did neither induce apoptosis nor affect the cells ability to differentiate.

However, when using 8-MOP and UVA in combination, we confirmed in our *in vitro* experiments presented in **Paper II** the previous observations that ECP made lymphocytes undergo apoptosis[50, 58, 126], and that this took place at the same kinetic rate as in gamma-irradiated lymphocytes. The opinion on what happens to monocytes and DC after ECP has, however, been a matter of debate. In contrast to some previous reports, we observed that also the majority of monocytes underwent apoptotic cell death during the first 24 hours post-treatment[52, 58, 127]. A limited number of investigators have followed the effect of ECP on monocytes for more than 24 hours post treatment. It has recently been reported, like in our investigation, that also monocytes turn apoptotic after ECP[53, 128-130]. Any discrepancies on this topic may partly be due to the methodology chosen for revealing apoptosis. For detecting early apoptosis we think that the use of annexin V is a favorable method[131, 132]. It has also been reported that DC harvested from peripheral blood of GVHD patients survive ECP[133]. We observed that monocyte-derived immature DC as well as DC harvested from peripheral blood underwent apoptosis as a result of ECP, the kinetics of which was much like that observed for lymphocytes. These observations led us to the conclusion that within 72 hours post-treatment almost all ECP treated cells become apoptotic, the majority even earlier. The few DC surviving ECP showed a reduced ability to mature when stimulated with LPS.

Somewhat surprisingly, monocytes seem to tolerate 30Gy of gamma irradiation without reduced viability and without losing their ability to differentiate into immature DC. When stimulating these cells in a next step with LPS for 72 hours, they gained the phenotype of mature DC. As a follow-up, both monocytes and immature DC were exposed to doses as high as 90Gy, and even though this resulted in a slightly increased fraction of apoptotic cells compared to 30Gy, it was still to a lesser extent than apoptosis induced by ECP (data not presented). In coherence with our findings, Merrick et al reported in 2005 that monocyte-derived immature DC indeed do tolerate doses of gamma irradiation that induce apoptosis in lymphocytes, and they seem to maintain their differentiating, phagocytic and migratory capability to the same extent as untreated cells. However, an impact on their function was shown, as the irradiated DC produced less IL-12, and were less effective in priming naïve T cells compared to untreated control cells[59].

The main objective of the present investigation was to elucidate the primary *ex vivo* effect of ECP on monocytes and DC, in an attempt to partly clarify this basic issue, before trying to disclose the main principal mechanism behind the clinical *in vivo* effect of ECP. Monocytes

are blood cells thought to have few other functions than being precursors to phagocytes, such as macrophages and DC, and it seems to be essential that they go through such a differentiation in order to function as antigen-presenting cells. Immature DC are specialized to phagocytize, and when maturing they get increasing capacity to present antigens on HLA class II and I, and being able to stimulate a primary immune response[64, 66, 67]. We demonstrated in this study that ECP treatment reduced the ability of surviving monocytes to differentiate into immature DC when cultured with IL-4 and GM-CSF, compared to untreated control cells. 24 hours post treatment, the control cells and the gamma-irradiated cells, in contrast to the ECP-treated cells, acquired the CD206 antigen, which is characterizing immature DC. From our series of experiments we observed that the differentiation from CD14+/CD206- monocytes to immature CD14-/CD206+ DC in response to IL-4 and GM-CSF stimulation took 5 days.

How functional are ECP treated monocytes and DC as antigen-presenting cells after reinfusion to the patient? This is a central question to follow a theory that recently has been presented as "transimmunization", suggesting that clinical ECP would be more effective if including an overnight incubation step of the treated cells before returning the cells to the patient[51, 134]. This would, in theory, give the monocytes time to differentiate into immature DC which again can phagocytize the apoptotic lymphocytes at a high concentration, and thus, following reinfusion increase the efficiency of in vivo antigen presentation. However, our series of experiments indicated that when the ECP treated cells are incubated overnight, they should not be expected to differentiate into phagocytes, but rather to undergo apoptotic cell death. Maeda et al[129] very convincingly demonstrated in a mouse *in vivo* experiment by use of a dinitrofluorobenzene hapten antigenic system that ECP treated DC are indeed able to induce CD4+CD25+ antigen-specific regulatory T cells. Their applied DC had however, been exposed to the antigen in vivo for 5 days before submitted to *ex vivo* ECP and subsequently adoptively transfused to a second animal for tolerance induction. In their model the DC might have had a 'flying' start and hence time to exert their tolerizing effect before dying. In traditional clinical ECP, as well as in the proposed transimmunization model, the *ex vivo* treated monocytes/DC will primarily be induced to apoptosis, and they will not start their supposed antigen processing before the lymphocytes are dying. Our findings also indicated that a one-step procedure based on 30Gy gamma-irradiated cells instead of ECP-treated cells ought to be tried when testing out the transimmunization model as proposed by Berger et al[51], based on that monocytes/DC, contrary to lymphocytes, retained their viability and

differentiating potential following 30Gy gamma irradiation. This could give information about the effect of viable monocytes/DC in a procedure similar to ECP. The potential oncogenic risk of reinfusing viable gamma-irradiated cells is probably negligible, but should be evaluated before performing such a study.

With traditional clinical ECP, a major problem is that we still do not really know which kind of an effect we aim at obtaining. One explanation has been that ECP induces an anticlonotypic cytolytic immunity directly against tumor cells, due to the findings of monoclonal T cells in the peripheral blood of patients with CTCL[56]. However, response has also been observed in CTCL patients lacking such a clone[135]. In CTCL, we do not know whether ECP is inducing specific cytotoxicity to the lymphoma cells or alternatively is inducing specific or non-specific regulatory T cells controlling the CTCL proliferation. The success obtained with priming DC in cancer therapy could point to the first possibility[114, 115], whereas the effect of ECP in GvHD, transplant rejection and autoimmunity favor the other view[7-15]. Several potential mechanisms of action in GvHD has recently been reviewed, including production or inhibition of cytokines, inhibition of activated T cells or induction of regulatory T cells[27]. There are many issues that have to be addressed before a fully rational exploitation of ECP can be obtained. Which maturity state of the DC and which potential accessory stimuli should preferably be applied? We do not know the importance of what can be obtained by *ex vivo* phagocytosis compared to what takes place when reinfused apoptotic cells end up in the specialized phagocytic compartments of liver and spleen.

Regulatory T cells and ECP

Even if rarely curative, ECP can give clinical improvement to both CTCL and GVHD, two conditions that are pathogenetically very different, but have in common that they are very resistant to conventional therapeutic regimens. In the case of GVHD it may seem easy to understand the supposed effector-mechanism of ECP. This disease is mainly mediated by allogeneic effector T cells recognizing the transplant recipient as foreign, and with skin, intestine and liver as main target organs for an immunological attack. It is understandable that a therapy that can improve the Treg function can down-regulate the immunological attack and bring clinical alleviation to the patient, and that this may be the probable effect of ECP. When it comes to CTCL this issue is still not solved. What is the biology of Treg cells in this condition, and what is the effective mechanism mediated by ECP? The explanation that could seem to be the most likely possibility is that ECP strengthen a T cell cytolytic effect on the

CTCL analogous to what can be obtained by immunotherapy of solid tumours[115]. It seems paradoxical however, that ECP should improve a T cell regulatory function in one kind of patients and a cytolytic one in another. This is further complicated by the findings of Berger et al that the CTCL cells can by stimulation convert into a Treg phenotype[118], and Tiemessen et al have demonstrated that Treg cells in late stage CTCL are dysfunctional[120]. The potential mechanisms of immune regulation with ECP, which imply infusion of apoptotic dying cells, have recently been reviewed[27]. In addition to stimulation of Treg cells, inhibition of pro-inflammatory cytokines, such as IL-1, IL-6 and TNF-α, as well as production of anti-inflammatory cytokines, such as TGF-β and IL-10, has been reported[136-139]. The appropriate concentrations of these cytokines may regulate the immune response at inflammatory sites.

The study presented in **Paper III** was designed to make a comparison between two patient groups; CTCL and GVHD submitted to the same kind of ECP therapy during a parallel time course of some years. We chose to test freeze-stored serum and PBMC samples collected at start and ending of the standard 6 months course of ECP. The serums were tested for a cytokine profile representing the three kinds of T helper effector cells; Th1, Th2 and Th17, as well as Treg cells. As will be seen from Fig. 2 and Fig. 3 (Paper III) the patients displayed great individual variations in the cytokine levels. For most patients however, their individual levels were surprisingly similar before and after the 6 months ECP-course. The only significant changes in average concentration during the ECP course were a small increase in TGF-β in both patient groups and a small decrease in IL-17 in the GVHD patients, which can be in accordance with a supposed mechanism of Treg cell-mediated down-regulation of an immune reaction. The mean level of TGF-β was on average more than three times higher in the GVHD than the CTCL patients, and the GVHD patients also had the highest increase during the ECP course (Fig. 3B, Paper III). In spite of the great individual variations the cytokine profile was quite similar in the two patient groups for IL-4, IL-6, IL-10, IL-17 and IFN-γ. Th1 and Th17 are supposed to be the main effector T cells mediating of GVHD[140, 141], and one might have expected their marker cytokines IFN-γ and IL-17, respectively, to be elevated especially in the GVHD patients. Nevertheless, these findings may emphasize that it is the local milieu and concentrations of cytokines that ultimately are decisive for immune regulation without necessarily being reflected in the serum concentrations.

We have looked especially for the relative concentration of Treg cells among the helper T cells (CD3+CD4+) in the two patient groups. We chose to define the Treg cells as; CD3+CD4+Foxp3+CD127-[142] and not to use the CD25 marker as it may be problematic to identify precisely the CD25high fraction which represents the original hallmark of Treg cells[94, 143]. In the present study, we found that among the healthy controls (HC) the fraction of Treg cells was on average 4,1%±1,9 (mean±SD), which is in coherence with previously reported percentage of the Treg cell subset[94, 144]. The fact that these cells are also predominantly CTLA4+ in both patient groups and HC seems to support their Treg identity. The most striking finding was that the Treg fraction in the GVHD patients was at a level close to the HC, whereas the CTCL patients had a Treg level more than twice as high (Fig. 4A, Paper III). Our finding of an elevated Treg fraction in the CTCL patients is at variance with the report from Tiemessen et al. who found equal Treg levels in CTCL patients and controls[120]. Klemke et al reported paucity of Foxp3 cells in peripheral blood of Sèzary patients[145]. Our CTCL patients only include one Sèzary case so our finding may not necessarily be contrasting to this. It may seem to be a paradox that the GVHD patients have the highest serum concentration of TGF-β, but close to normal Treg counts in peripheral blood, whereas the CTCL patients have elevated Treg count, but low levels of TGF-β. One explanation could be that the GVHD patients may have activated Treg cells, and that these are mainly not in circulation, but have migrated into the tissues where the pathogenic alloreaction is taking place. We cannot really know how the distribution of circulating cells correlates to the total body distribution. Generally, the reported level of Treg cells in chronic GVHD remains inconclusive, with both increased[146] and decreased[147] levels reported, together with studies that demonstrate a normalization of the Treg cells after ECP[148]. In this study, the patients with GVHD had close to normal levels of Treg cells, although slightly elevated pre-treatment compared to the HC. After 6 months of ECP, this small difference was completely abolished. Miyara et al recently published a convincing study that shows that the CD4+Foxp3+ Treg cell population can be divided into three distinct subsets, based on the degree of Foxp3 and CD45RA expression[149]. These subpopulations are CD45RA-Foxp3^{high} activated Treg cells (aTreg), and CD45RA+Foxp3^{low} resting Treg cells (rTreg), both potently suppressive. They also describe a third population consisting of CD45RA-Foxp3^{low} non-suppressive Treg cells, secreting proinflammatory cytokines, such as IL-17. The latter may correspond to the activation-induced Foxp3+ cells that transiently express Foxp3, but lack suppressive properties. The key-findings of Miyara's group are the demonstration of that CD45RA+ rTreg and CD45RA- aTreg cells represent a differentiation linage of the same T

cells, and that expression of CD45RA can be used to differentiate between the activation state of the subsets. Finally, they demonstrate that aTreg cells can suppress rTreg cells, thus creating a negative feedback loop to prevent further activation of resting Tregs. A reduction of naïve CD45RA+ Treg cells was also observed in our study, possibly reflecting an increase in the proportion of secondary, activated cells. Our findings of increased secondary cells are similar to those reported by Biagi et al[150]. Tiemessen et al found that Treg from CTCL patients who did not respond to ECP treatment were dysfunctional and expressed lower levels of Foxp3 than normal cells[120]. We could not detect by flow cytometry any lowered expression of Foxp3 in the CTCL patients, and we have not tested the Treg cells functionally. However, our findings of high Treg counts and low serum TGF-β in the CTCL patients could possibly be related to the findings of Berger's[118] and Tiemessen's[120] groups that the Treg population, or part of it, in the CTCL patients is pathological and dysfunctional. The slight, but significant increase in serum TGF-β and lowered Treg count following the ECP course could represent an effect of ECP towards normalization also in the CTCL patients. The patient materials that we present here are small, and even if our findings are supported by statistical significance, the conclusions should not be too far-fetched. However, it is reasonable to relate our results to the recent findings of Gjerdrum et al who demonstrated that the degree of Treg infiltration in CTCL was correlated to patient survival, and they suggested that Treg cells can directly suppress the function of malignant T cells[119]**.** We think that their findings also contradict that CTCL cells have a Treg phenotype as infiltrating CTCL cells should clearly not correlate positively to patient survival. We have here demonstrated an increasing serum concentration of TGF-β in both patient groups as a result of the ECP; in the GVHD patients also a reducing level of IL-17. This might point to an amplified Treg function, a finding recently confirmed in GVHD patients[151]. Parallel to this both patient groups exhibited a reduced primary (CD45RA+) fraction among the Treg cells following ECP. This could imply an antigen-driven expansion of secondary Treg cells. Elevated level of CD39 in both patient groups, and even significantly increasing in the GVHD patients following ECP could imply an elevated suppressive quality of the Treg cells. Of the GVHD patients 11 out of 14 had responded well to the ECP therapy (Table 2, Paper III), whereas 13 out of 24 CTCL patients had responded, but to a more varying degree. Except for the 3 patients included in the study as RM, the remaining patients were considered as late-stage CTCL (Table 1, Paper III). When comparing the non-responding and responding patients one would expect to find increased CD39 expression as a sign of improved Treg cell function in the responding group. We were, however, not able to detect any differences in the CD39 expression, but both groups

displayed a small increase after ECP. This may indicate that additional factors mediating treatment success are involved, or perhaps that the patient material is too small to detect any difference. The especially elevated serum TGF-β in the GVHD patients could reflect the very strong immune reaction taking place in these patients. Even if seemingly not increased in number, the activation of the Treg cells may be strong to counterbalance the strong and destructive GVH reaction. Immunologically, CTCL supposedly imply a more indolent condition than GVHD, not activating the Treg cells strongly, but an elevated number of Treg cells might represent a homeostatic proliferative reaction evolving to curb the expansion of malignant T cells in accordance with the mentioned report by Gjerdrum et al.

Conclusions

This thesis approaches different issues concerning the ECP treatment, and the main results and conclusions can be summarized in the following way, together with which aim they are intended cover (see *Aims* p.25-27):

Paper I

Seven patients were classified as responders to ECP. Nine patients were classified as non-responders. This gives a response rate of 44%, and is somewhat lower than the observations made by other investigators. If we exclude the RM patients, the response rate is even lower in this material, 4/13 patients, i.e. 31%. This may be incidental due to the small size of the patient material *(Aim 1)*.

Neither T cell clonality nor any of the serum markers assessed pre-treatment could reliably predict the clinical response to ECP. A reduction in serum sIL-2R, neopterin and β2-M during the first course of ECP can however, add in the prediction of a better prognosis on the long run *(Aim 2)*.

Paper II

Monocytes and immature DC become apoptotic within 72 hours when treated with ECP, the majority even earlier. Since monocytes and immature DC seem to have a reduced ability to differentiate after ECP treatment, it is likely that the therapeutic effect of ECP is caused by *in vivo* effects of reinfused apoptotic cells, rather than by infusion of monocytes induced to differentiate into immature DC *(Aims 1 and 2)*.

Monocytes and immature DC seem to tolerate doses of gamma irradiation that induce apoptosis in lymphocytes, and maintain their differentiating capability to the same extent as untreated cells *(Aim 3)*. These findings validates that a one-step procedure based on 30Gy gamma irradiated cells instead of ECP treated cells ought to be tried when testing out the transimmunization model proposed by other investigators.

Paper III

The relative fraction of Treg cells was twice as high in the patients with CTCL, compared to both GVHD-patients and healthy controls *(Aim 1)*, and could be interpreted as a response

involved in down-regulating the lymphoma cells. A reduction of Treg cells was observed in both patient groups during 6 months of treatment, but without reaching a statistically significant difference *(Aim 2)*. The non-responding CTCL patients had on average a higher relative amount of Treg cells than the responding patients both before and after treatment, but the difference was not significant *(Aim 3)*. Finally, we observed that the anti-inflammatory cytokine TGF-β was on average 3 times higher in GVHD than in CTCL. Both CTCL and GVHD patients had a small, but significant increase of TGF-β after 6 months of ECP treatment, whereas the GVHD patients additionally showed a decrease in IL-17, which is associated with inflammation *(Aim 4)*. In conclusion, we think our data support the notion that the clinical effect of ECP in both GVHD and CTCL most probably is mediated by a strengthening of the Treg cell function.

Future aspects

Patient survival would be a stronger parameter to follow up than clinical response measured as skin score improvement. So far, the patients included in this thesis are too few for such a follow up, but it should be considered when more patients get included. A predictive marker for response would naturally be of great benefit and should be searched for.

Monocytes and dendritic cells were observed to undergo apoptosis within 72 hours after ECP therapy, the majority even earlier. It would, however, be interesting to do functional studies of the cells immediately after treatment. Are the treated cells capable to phagocytize and present engulfed antigen to the immune system before they die?

We observed elevated numbers of Treg cells in the CTCL patients, and further characterization and isolation of these cells would be a natural next step as an attempt to determine the origin and function of the cells (especially cytokine production and suppressive capacity).

As CTCL and GVHD are rather rare conditions, there are limited numbers of patients available. A larger study population seems essential to obtain more conclusive results, and inclusion in international multi-center studies should be an aim in the future.

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Cutaneous T cell lymphoma and graft-versus-host disease: A comparison of *in vivo* effects of extracorporeal photochemotherapy on Foxp3+ regulatory T cells ☆

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Abstract Extracorporeal photochemotherapy (ECP) is a well established treatment for both cutaneous T cell lymphoma (CTCL) and graft-versus-host disease (GVHD). However, the general effector mechanism is not fully settled. Twenty-four patients with CTCL and 14 patients with GVHD were included to assess the relative numbers of regulatory T cells (Treg) and any change in the serum cytokine profile during 6 months of ECP therapy. The relative amount of Treg cells was twice as high in CTCL compared to GVHD and healthy controls. TGF-β was on average three times higher in GVHD than in CTCL. Both patient groups had a small but significant increase in TGF-β after treatment. Our results indicate a strengthened Treg function as a result of ECP. Elevated TGF-β may indicate high Treg activation in GVHD, whereas an increased number of Treg cells in CTCL could be interpreted as a response that is involved in down-regulating the lymphoma cells.

Introduction

Extracorporeal photochemotherapy (ECP), or photopheresis, was introduced by Edelson et al. in 1987 [1] as a treatment for cutaneous T cell lymphomas (CTCL). Encouraged by the promising results in CTCL, ECP has later been applied to a variety of T cell mediated diseases, such as graft-versus-host disease (GVHD) [2], preventing graft rejection in heart and renal transplantation [3–5], as well as in other autoimmune diseases [6,7]. The ECP procedure is based on leucapheresis, followed by extracorporeal treatment of the leucocytes with 8-methoxypsoralen (8-MOP) and illumination with ultraviolet light A (UVA) before being reinfused to the patient. The therapeutic effect of ECP is supposed to be explained by 8-MOP

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binding covalently to DNA in circulating lymphocytes when illuminated with UVA. This ultimately leads to cell proliferation arrest and induction of apoptosis in the treated cells [8,9].

GVHD is a condition that evolves when allogeneic immunocompetent cells are introduced into an immunoincompetent host, and was first described by Billingham in 1959 [10]. The main occurrence is after haematopoietic cell transplantation, but GVHD is also seen after solid organ transplantations and blood transfusions. The pathophysiology of GVHD is T cell recognition of target tissues as being foreign with a subsequent induction of an inflammatory and cytolytic response leading to injury. Regulatory T cells (Treg) have been reported to protect against chronic cutaneous GVHD [11]. Immunomodulation by phototherapeutic agents has been proven beneficial for some patients, especially with chronic GVHD in the skin. PUVA is most widely used, but success has also been reported with ECP [12,13]. The effect is thought to be mediated through triggering of certain tolerance mechanisms such as inhibition of pro-inflammatory cytokines, stimulation of Treg cells and production of anti-inflammatory cytokines [14–16].

CTCL represents a group of low-grade, non-Hodgkins' lymphomas, such as mycosis fungoides (MF) and Sezary syndrome (SS). Approximately one half of the patients with CTCL treated with ECP demonstrates a reduction in skin score by at least 50% within 12 months of treatment, and is categorized as responders [17]. A major problem with clinical ECP in CTCL is that we still do not know which kind of effect we aim at obtaining. Due to the findings of monoclonal T cells in peripheral blood, one explanation has been that ECP induces an anti-clonotypic cytolytic immunity directly against the lymphoma cells [18]. An essential issue in CTCL is whether ECP is inducing specific cytotoxicity against the lymphoma cells, or alternatively inducing specific or non-specific Treg cells controlling the CTCL proliferation. The success obtained with priming dendritic cells in cancer therapy could point to the first possibility [19], whereas the effect of ECP in GVHD, transplant rejection and autoimmunity favours the other view [2–7].

Treg cells constitute a subset of T cells with immunosuppressive properties, and identifying discriminatory cell-surface markers for the characterization and isolation of Treg cells has been a crucial goal for some time. Treg cells were first identified as T helper cells (CD4+) that strongly expressed CD25 (IL2-Rα), both in mice and humans [20,21]. In humans, however, it has been shown that activated T cells generally up-regulate CD25 expression [22], thus decreasing the purity of Treg cell isolation by this marker and limiting its utility when studying Treg cells. It is known that Treg cells express several other characteristic molecules in addition to being CD25high, such as glucocorticoid-induced tumour necrosis factor receptor (GITR), cytotoxic T-lymphocyte antigen 4 (CTLA4) and L-selectin (CD62L). The above mentioned cell-surface or intracellular molecules are, unfortunately, also expressed in activated T cells, thus making an exclusive isolation of viable Treg cells a substantial problem. At present the transcription factor Forkhead box p3 (Foxp3) is considered the most specific marker for Treg cells. This protein is, however, intracellular and not available for isolation of living cells. The search for Treg specific cell-surface markers continues, and several candidates have recently been assessed [23]. The importance of Foxp3 was demonstrated by showing that Foxp3-mutant mice have a Treg cell-deficiency and develop a severe lymphoproliferative autoimmune syndrome [24]. Similarly, humans lacking Foxp3 suffer from an aggressive autoimmune syndrome, known as immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, a rare recessive disorder that results in early death [25]. When first discovered in mice, Foxp3 was thought to exclusively identify Treg cells also in humans, but recent evidence supports that Foxp3 indeed can be induced upon T cell activation, and that these Foxp3+ T effector cells are without immunosuppressive properties. Hence, low expression of specific cell-surface markers should also be exploited to assess and identify Treg cells, for instance, that they have low expression of CD127 (known as IL7-Rα), whereas activated, effector T cells are CD127high [26,27]. Treg cells are thought to be capable of inhibiting immune responses against a variety of antigens, including the ones expressed by malignant cells [28]. In preventing autoimmune disease, the presence of Treg cells is naturally thought to be beneficial, whereas in the context of CTCL high numbers of these regulatory cells have mainly been supposed to inhibit the surveillance and clearance of tumour cells. Indeed, investigators have suggested that there is an association between a high tumour burden or tumour progression and an increase in Treg cells [29]. CD103, an αEβ7 integrin that mediates T cell retention in the epithelial compartment, has been shown to be an excellent marker for identifying *in vivo*-activated (induced) Treg cells [30,31]. These CD103+CD4+Foxp3+ Treg cells have been shown to be more potent suppressors in autoimmune arthritis [30] and in reversing chronic GVHD than primary CD4+CD25high Treg cells [32]. CD39 is a member of the ectonucleoside triphosphate diphosphohydrolase family, and is expressed on the surface of various cells. Its function is to degrade ATP, which acts as a "danger signal" that activates the immune system, to AMP. In combination with CD73, another ectonucleotidase, it results in the production of adenosine, that exhibit inhibitory and antiproliferative effects. Recently, it has been shown that CD39 is confined to a subset of the Treg cells, thought to represent regulatory cells with effector/memory-like properties. In the same study, the expression of CD39 among the Treg cells was also reflected in the capacity to degrade ATP, as well as in suppressive capacity [33]. CD45RA is a surface marker of primary (naïve) T cells in contrast to secondary (antigen-stimulated) T cells.

This study was performed to determine the relative numbers of Treg cells, characterized by the phenotype CD3+CD4+Foxp3+CD127−, prior to ECP and after 6 months of ECP treatment in patients with CTCL or GVHD. Further characterization was done to analyze the expression of CTLA4, CD25, CD45RA, CD103 and CD39 among the Foxp3+ cells. In addition we studied whether there was a measurable change towards a tolerogenic profile, by assessing the serum concentrations of IL-4, IL-6, IL-10, IL-17, IFN-γ and TGF-β, before and after 6 months of ECP treatment.

Materials and methods

Study populations

Freeze-stored serum and peripheral blood mononuclear cells (PBMC) from a total of 58 individuals were evaluated. The

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study population included patients with CTCL ($n=24$) or GVHD ($n=14$) treated with ECP between 1993 and 2008, as well as healthy controls obtained from blood donors (HC, $n=20$). The CTCL and GVHD patients were evaluated before treatment and after 6 months of ECP therapy. The stored material was unfortunately not complete for all patients when it came to the availability of both serum and PBMC before and after 6 months of ECP treatment. Hence, 20/24 CTCL patients and 12/14 GVHD patients were assessed with the cytokine assays, whereas 20/24 CTCL and 11/14 GVHD patients were included in the flow cytometric analysis (Tables 1 and 2).

CTCL patients

The clinical manifestations of the CTCL patients were heterogeneous (Table 1). The included patients were all erythrodermic when initiating ECP or immediately before ECP started, and had skin biopsies "diagnostic of" or "consistent with" mycosis fungoides (MF) (20/24), whereas 3/24 patients were histologically classified as "suspicious" of MF, and included in the study as "Red man syndrome" (RM). One patient had Sezary syndrome (SS), defined as having more than 20% abnormal, cerebriform, circulating lymphocytes (Sezary cells) of the total leukocyte count in peripheral blood, according to a proposal from ISCL [34,35]. During the treatment period, all patients initially received ECP as a monotherapy. However, the patients in the non-responding group, i.e., patients who lacked clinical response or had progressive disease, received in addition to ECP interferon-alfa after 6 months of ECP monotherapy, but without improved effect on their clinical skin disease. The CTCL patients were classified at initiation of ECP according to a skin score test defined as the product of skin disease severity score multiplied by percent affected body surface area [1]. The skin disease severity score is according to the following scale: 0=normal, 1=barely detectable erythema and scaling, 2=readily detectable erythema, edema and scaling, 3=marked erythema and exfoliation, and 4=fissuring, maximal erythema, induration and tumours. This makes 400 (4×100%) the maximal total skin score. The clinical response of the CTCL patients to ECP was defined as a reduction in skin score by at least 50% as registered after 12 months of ECP treatment. Skin biopsies were taken of all patients, and peripheral blood was examined for atypical cells. Lymph node biopsies were performed on all patients with palpable lymphadenopathy. X-ray or CT-scan of the chest, as well as ultrasound or CT-scan of the abdomen, were performed to evaluate clinical stage.

Table 1 CTCL patients treated with extracorporeal photochemotherapy.

Patient	Age[a]/sex	Diagnosis[b]	Stage[c]	TNMB[c]	Skin score[d]	Response[e]	Serum[f]	PBMC[f]
1	82/M	MF	IIB	T3NxM0B0	NA	NR	✓	NA
2	40/M	MF	IIIB	T4N1M0B0	270	PD	✓	✓
3	77/M	MF	IIIA	T4N0M0B0	356	MR	✓	✓
4	63/M	MF	IIIA	T4N0M0B1	270	PR	✓	✓
5	76/F	MF	IIIA	T4N0M0B1	400	NR	✓	✓
6	75/M	MF	IIIA	T4N0M0B0	189	MR/PR	✓	✓
7	66/M	MF	IVA	T4N2M0B0	255	NR	✓	✓
8	56/F	MF	IIIA	T4N0M0B0	144	MR	✓	✓
9	73/M	MF	IIIA	T4N0M0B0	288	MR/PR	✓	✓
10	66/M	SS	IVA	T4N3M0B2	26	PD	✓	✓
11	64/M	MF	IVA	T4N3M0B0	285	NR	✓	✓
12	61/F	MF	IIIA	T4N0M0B1	170	PR	✓	✓
13	59/F	MF	IIIA	T4N0M0B0	285	NR	✓	✓
14	77/F	MF	IIIA	T4N0M0B0	122	NR/PD	✓	✓
15	75/M	MF	IIIA	T4N0M0B1	162	NR	✓	✓
16	70/M	MF	IIIA	T4N0M0B0	249	NR	✓	✓
17	77/F	MF	IIIA	T4N0M0B0	NA	NR	✓	NA
18	36/M	MF	IIIB	T4N1M0B0	380	NR	✓	✓
19	72/F	MF	NA	NA	NA	NR	✓	NA
20	58/M	MF	IIIA	T4N0M0B0	400	NR	✓	NA
21	70/F	RM			300	PR/CR	NA	✓
22	80/M	RM			300	PR	NA	✓
23	76/M	RM			400	PR	NA	✓
24	76/F	MF	IIIA	T4N0M0B0	300	PR	NA	✓

NA, not available.
[a] Age at ECP start.
[b] MF, mycosis fungoides; SS, Sezary syndrome; RM, Red man syndrome.
[c] Staging according to Bunn and Lamberg [54]. Patients with RM have no indication for staging.
[d] Skin score pre-treatment. ND, not done/available.
[e] CR, complete remission; PR, partial remission (>50% reduction in skin score); MR, minor response (25–50% reduction in skin score); NR, no response/stable disease; PD, progressive disease (>25% increase in skin score).
[f] ✓ indicates the availability of stored serum or peripheral blood mononuclear cells (PBMC) for cytokine assays or flow cytometric analysis, respectively.

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Table 2 GVHD patients treated with extracorporeal photochemotherapy.

Patient	Age[a]/ sex	Pre-treatment (mg/week)[b]		6 months (mg/week)[b]		GVHD/organ	Response[c]	Serum[d]	PBMC[d]
		Pred	Cyclo	Pred	Cyclo				
1	52/M	280	1400	52,5	1400	Oral/genital mucosa/eyes	R	✓	✓
2	35/M	35	1400	0	1400	Skin/oral mucosa/eyes	R	✓	✓
3	45/F	70	0	35	0	Skin/eyes	R	✓	NA
4	25/M	0	1400	0	1400	Liver/oral mucosa/eyes	NR	✓	✓
5	21/M	0	0	0	0	Oral/genital mucosa	R	✓	✓
6	43/M	280	0	87,5	0	Skin	NR	✓	✓
7	62/F	0	700	0	700	NA	NR	✓	NA
8	27/F	27,5	0	0	0	Skin/oral mucosa/eyes	R	✓	NA
9	55/M	105	2625	0	2275	Oral mucosa	R	✓	✓
10	32/F	70	1400	35	1400	Oral/genital mucosa/eyes	R	✓	✓
11	48/F	110	0	52,5	0	Skin/oral mucosa/eyes	R	✓	✓
12	43/F	0	1400	0	1400	Oral mucosa	R	✓	✓
13	58/M	0	0	0	0	Skin/oral mucosa/eyes	R	NA	✓
14	21/M	0	0	0	0	Skin/eyes	R	NA	✓

NA, not available.
[a] Age at ECP start.
[b] Pred, prednisolone; Cyclo, cyclosporine.
[c] R, responder; NR, no response.
[d] ✓ indicates the availability of stored serum or peripheral blood mononuclear cells (PBMC) for cytokine assays or flow cytometric analysis, respectively.

GVHD patients

A total of 14 patients with chronic GVHD after allogeneic bone marrow transplantation were included in the study. They had all been treated with bone marrow transplantation for hematological malignancies, and received ECP either as monotherapy or in combination with other immunosuppressive drugs. See Table 2 for individual data of immunosuppressive treatment and clinical response.

Cells and reagents

For this retrospective study, blood and serum samples from CTCL or GVHD patients were obtained immediately before each ECP procedure. Serum was stored at −70 °C. Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on Lymphoprep (Axis-Shield, Oslo, Norway) according to standard procedures, and stored in 10% DMSO in RPMI1640/PHS at −135 °C. As healthy controls (HC), we used frozen samples of PBMC that were isolated and stored as described above, obtained from blood donors.

Cytokine assays

Serum stored at −70 °C, obtained pre-treatment and after 6 months of ECP treatment, was thawed and the concentrations of IL-4, IL-6, IL-10, IL-17 and IFN-γ were simultaneously measured in duplicates, using a Milliplex Human Cytokine/Chemokine Immunoassay (Millipore Corp., Billerica, MA). The concentration of TGF-β was measured, also in duplicates, using a LINCOplex TGF-β1 Single Plex Kit (LINCO Research, St. Charles, MO). Both kits were applied according to the manufacturer's instructions. Data from the cytokine measurements were then retrieved from a Luminex 100 IS analyzer using the instrument software (Luminex Corp., Austin, TX).

Immunophenotyping

Cells stored at −135 °C were thawed for immunophenotyping. Following the manufacturer's instructions, the cells were stained with the following antibodies in five- or six-color combinations: anti-CTLA4 APC, anti-CD3 FITC, anti-CD4 APC-H7, anti-CD25 APC (all four obtained from BD Biosciences, San Jose, CA), anti-CD103 PE-Cy5 (Abcam, Cambridge, UK), anti-CD127 FITC, anti-CD45RA PE-Cy7, anti-CD3 PerCp-Cy5.5 and anti-CD39 PE-Cy7 (all four obtained from eBioscience, San Diego, CA). For intracellular staining, a PE anti-human Foxp3 Staining Set (clone PCH101) was used (also obtained from eBioscience). Briefly, after thawing, the PBMCs were washed with phosphate buffered saline with 0.1% bovine serum albumin (PBS w/0.1% BSA) and stained for surface antigens by incubation for 20 min at room temperature protected from light. After washing with PBS w/0.1% BSA, the cells were permeabilized with the provided Fix/Perm buffer for 45 min at 4 °C, washed twice with Perm buffer and stained for intracellular Foxp3 and CTLA4 at 4 °C for 30 min. After intracellular staining, the cells were washed twice with Perm buffer, and finally resuspended in 500 μl PBS w/0.1% BSA for flow cytometric analysis.

Flow cytometry

After surface and intracellular staining as described above, the cells were processed through a FACSCanto™ flow cytometer (BD Biosciences), using forward vs. side scatter properties to distinguish viable from dead cells. The T lymphocytes were identified using anti-CD3, and a minimum of 30,000 CD3+ events were acquired. With five- or six-color staining the percentage of Treg cells (defined as CD3+CD4+Foxp3+CD127−/CD3+CD4+ lymphocytes) was determined as

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shown in Fig. 1, using gates previously set with isotype background controls. Further characterization was done to analyze the fraction of CTLA4, CD25, CD45RA, CD103 and CD39 among the CD3+CD4+Foxp3+CD127− cells.

Statistical analysis

Patient samples were evaluated before and after 6 months of ECP treatment. With SPSS 15.0 software (SPSS, Inc., Chicago, IL), any differences between the patient groups were evaluated using a two-tailed Mann–Whitney U-test, whereas the Wilcoxon matched pairs test was used to evaluate individual differences during treatment. The significance level of the statistical tests (p-values) was set to <0.05. In Figs. 4 and 5, only significant differences are displayed.

The study was approved by The Regional Medical Research Ethics Committee of Central Norway and conducted according to Declaration of Helsinki principles.

Results

Cytokine assays

Serum concentrations of IL-4, IL-6, IL-10, IL-17, IFN-γ and TGF-β in patients with GVHD ($n=12$) and CTCL ($n=20$) were evaluated before and after 6 months of ECP treatment. In the CTCL patients there was a small but significant increase in mean TGF-β from 3.3 to 3.9 ng/ml during 6 months of ECP treatment ($p<0.01$). No significant difference in the mean serum concentrations of IL-4, IL-6, IL-10, IL-17 or IFN-γ was found during the same period of ECP (Fig. 2). Further on, we found no differences between the responding and non-responding CTCL patients in the cytokine measurements (not shown). As displayed in Fig. 3A, an increase in mean TGF-β from 11.7 to 15.6 ng/ml was also found in the patients with GVHD during 6 months of ECP treatment ($p<0.05$). In the same patients, the mean concentration of IL-17 decreased from an average of 41.7 to 23.5 pg/ml after 6 months of ECP treatment compared to pre-treatment ($p<0.05$). As for the CTCL patients, there was no significant change in the serum concentrations of the remaining cytokines that were evaluated. When comparing the two patients groups, we observed that the GVHD patients had significantly higher mean serum concentrations of TGF-β than the CTCL patients: 11.7 vs. 3.3 ng/ml ($p<0.01$) pre-treatment and 15.6 vs. 3.9 ng/ml ($p<0.01$) after 6 months of ECP (Fig. 3B). No differences were found between the two patient groups, regarding IL-4, IL-6, IL-10, IL-17 or IFN-γ.

Flow cytometric analysis of PBMC

Stored samples of PBMC from healthy controls (HC, $n=20$) and patients with GVHD ($n=11$) or CTCL ($n=20$) were thawed for immunophenotyping and flow cytometric analysis, to

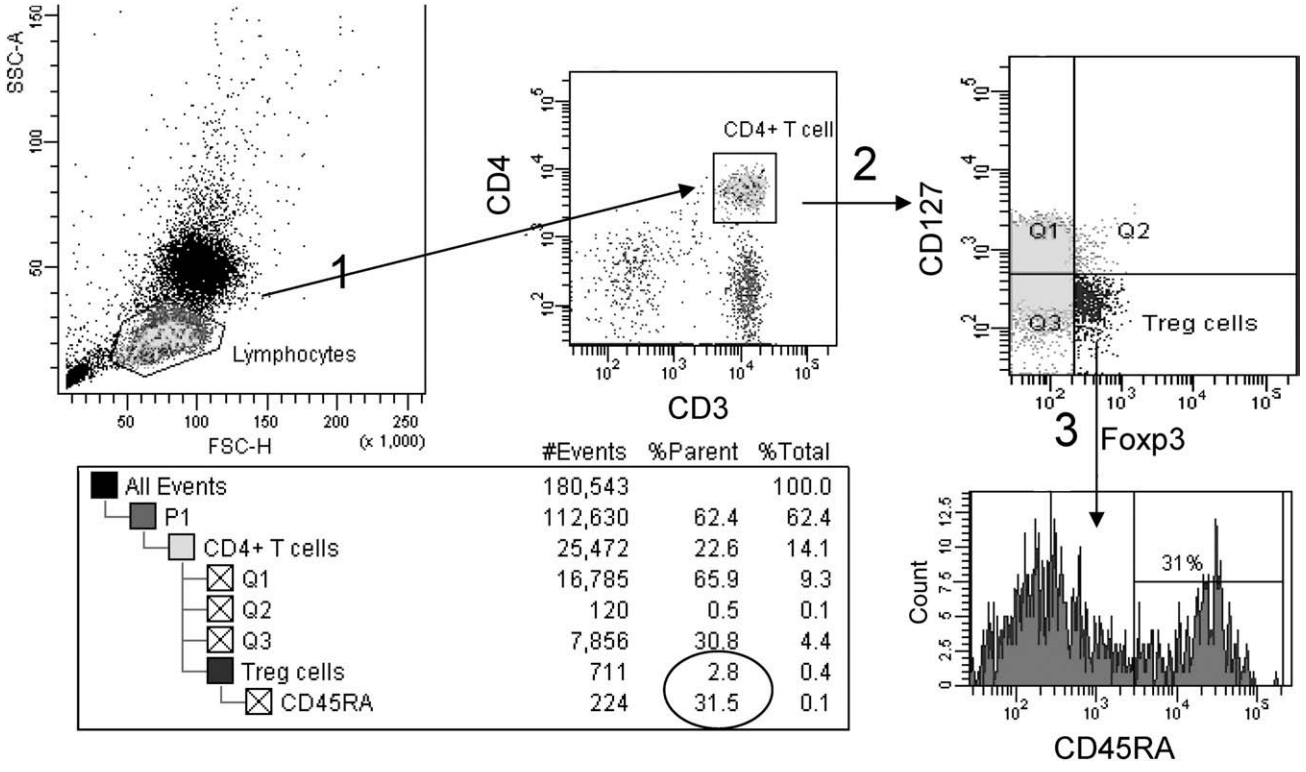

Figure 1 Flow cytometric gating strategy. A region was set around the lymphocytes using the forward vs. side scatter properties (1), before gating on CD3+CD4+ double positive cells (2). Then, the expression of Foxp3+CD127− among the CD3+CD4+ cells was determined by using quadrants previously set with background controls (3). Finally, further characterization was done to analyze the fraction of CD45RA (in this example), CD25, CTLA4, CD103 and CD39 among the Treg cells. Data displayed in this example are obtained from a healthy blood donor considered as representative.

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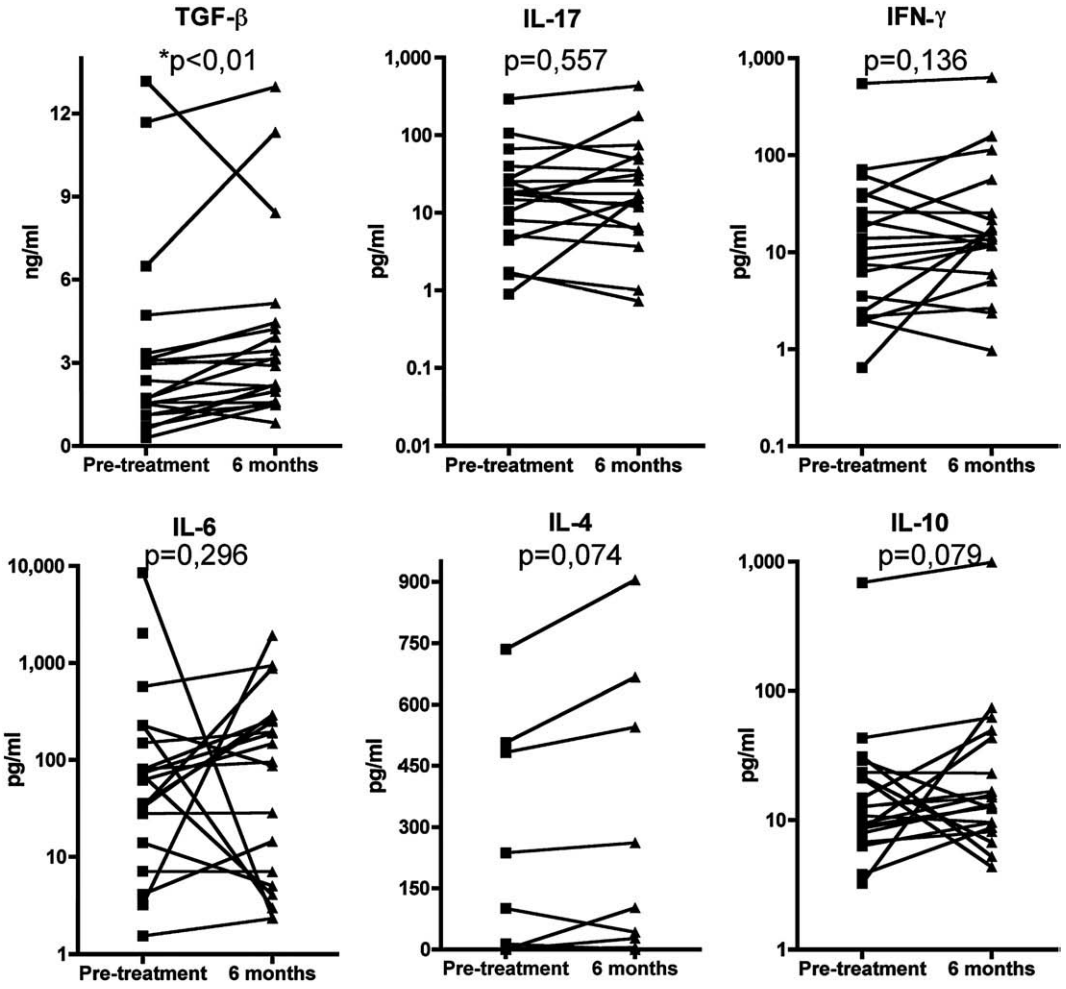

Figure 2 Individual serum cytokine profiles in CTCL. In the CTCL patients the serum concentration of TGF-β increased during 6 months of ECP treatment ($p<0.01$). There were no statistical differences in IL-17, IFN-γ, IL-6, IL-4 or IL-10 during the treatment period.

assess the percentage of CD4+Foxp3+CD127− Treg cells out of the total CD4+ T lymphocyte subset, pre-treatment and after 6 months of ECP treatment. In the HC, the percentage of Treg cells was on average 4.1%. In the peripheral blood of the CTCL patients, the relative amount of Treg cells was higher compared to the HC both before (11.7%, $p<0.001$) and after ECP treatment (10.1%, $p<0.001$), as well as when compared to the patients with GVHD (4.9% pre-treatment and 4.1% after 6 months of ECP treatment, $p<0.01$) (Fig. 4A). A slight but not significant decrease of the Treg cells was observed during the treatment period in both patient groups. The GVHD patients had only slightly elevated levels of Treg cells pre-treatment, but the differences were not statistically significant compared to the HC (4.9% vs. 4.1%, $p=0.44$). After 6 months of ECP treatment, the relative amount of Treg cells seemed to further normalized (Fig. 4A).

The CTCL patients were categorized as 'non-responders' or 'responders' to ECP (see Table 1) in order to see whether there were any differences in Treg cells. Pre-treatment, both groups were significantly higher than the HC (14.8% and 8.5% vs. 4.1%, $p<0.001$ and $p<0.01$, respectively), and the non-responders had on average a higher relative amount of Treg cells than the responders, but the difference was not significant (Fig. 4B). As described when assessing the CTCL patients without regard to their responder status, both the non-responders and the responders had a slight decrease in the percentage of Treg cells after 6 months of ECP treatment (from 14.8% to 12.1% and from 8.5% to 8.0%, respectively).

Expression of CTLA4, CD103, CD45RA and CD39 among the Treg cells

Further characterization was done to analyze the expression of CTLA4, CD103, CD45RA and CD39 among the Treg cells. CTLA4 was near equally expressed in the Treg cells of CTCL (81.6%) and GVHD (75.6%) patients, as well as in the HC (88.0%) (Fig 5A). As shown in Fig. 5B–D, a higher percentage of the Treg cells in GVHD patients expressed CD103 compared to the HC pre-treatment (43.8% vs. 33.6%, $p<0.01$). There was a decrease in the expression of both CD103 (31.1%) and CD45RA (from 64.8% to 52.3%) after 6 months of ECP treatment (both $p<0.05$), whereas the expression of CD39 increased from 66.4% to 73.2% ($p<0.05$). A similar trend, with a decrease in the expression of CD103 and CD45RA and an increase in CD39, was also observed among the Treg cells in the CTCL patients during ECP therapy (Fig. 5B–D). No differences were found between the responding and non-responding CTCL patients

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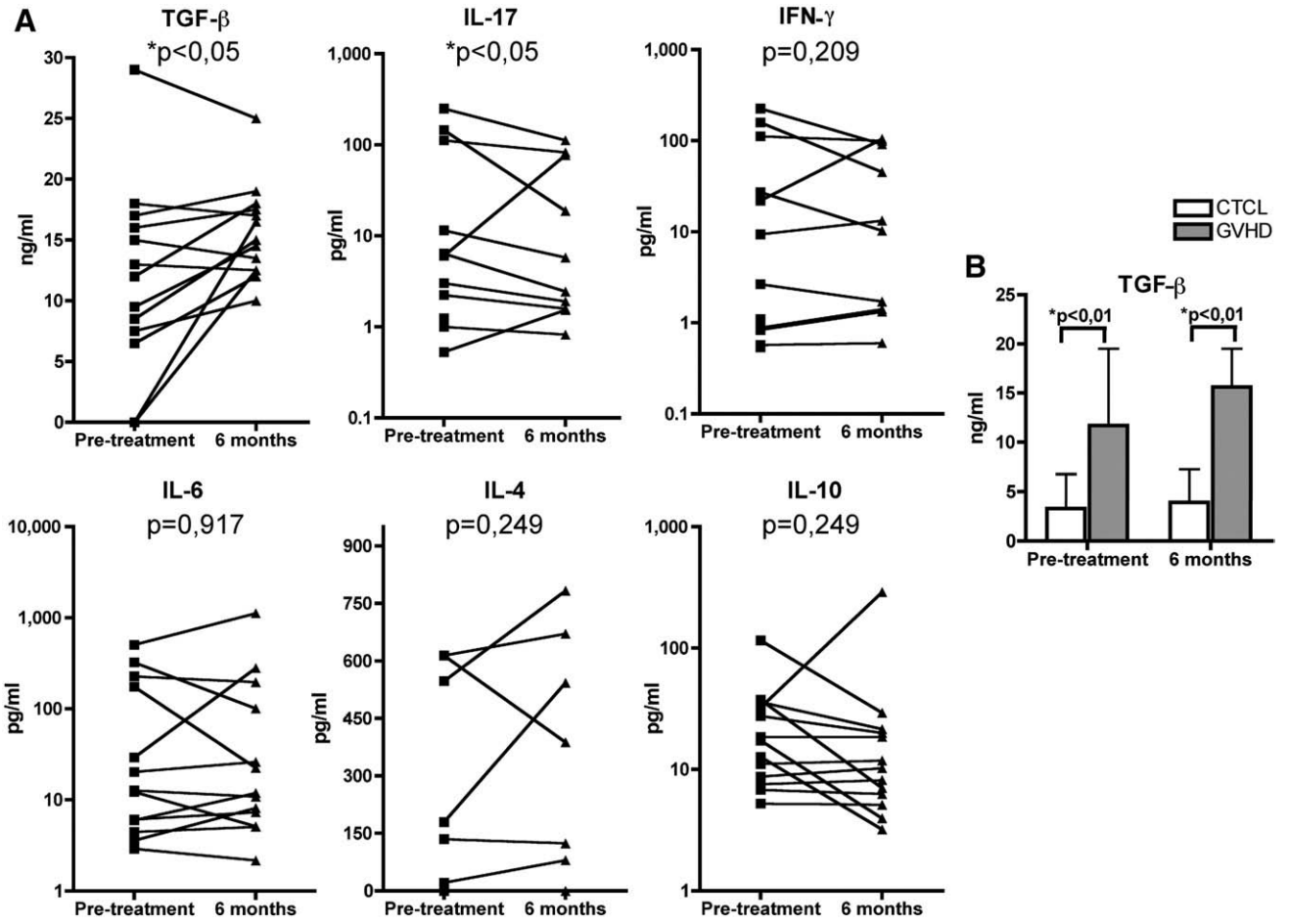

Figure 3 Individual serum cytokine profiles in GVHD. In the GVHD patients the serum concentration of TGF-β increased ($p<0.05$), whereas IL-17 decreased ($p<0.05$) during 6 months of ECP treatment. There were no statistical differences in IFN-γ, IL-6, IL-4 or IL-10 during the treatment period (A). When comparing the two patient groups, the GVHD patients had significantly higher serum concentrations of TGFβ than the patients with CTCL, both before and after 6 months of ECP treatment ($p<0.01$) (B). The columns represent the mean, and the error bars display the standard deviation (SD).

regarding the Treg cell expression of CD39, CD45RA or CD103 (not shown).

Discussion

CTCL and GVHD are the diseases that have been found most widely accepted for therapy with ECP. Even if rarely curative, ECP can give clinical improvement to both of these conditions which are pathogenetically very different, but have in common that they are very resistant to conventional therapeutic regimens. In GVHD it may seem easy to understand the supposed effector mechanism of ECP. This disease is mainly mediated by allogeneic T effector cells recognizing the transplant recipient as foreign, and with skin, intestine and liver as main target organs for an immunological attack. It is understandable that a therapy that can improve the Treg function can down-regulate the immunological attack and bring clinical alleviation to the patient, and that this may be the probable effect of ECP. When it comes to CTCL this issue is still not solved. What is the biology of Treg cells in this condition, and what is the effective mechanism mediated by ECP? The explanation that could seem to be the most likely possibility is that ECP strengthen a T cell cytolytic effect on the CTCL analogous to what can be obtained by immunotherapy of solid tumors [19]. It seems paradoxical, however, that ECP should improve a T cell regulatory function in one kind of patients and a cytolytic one in another. This is further complicated by the findings of Berger et al. that the CTCL cells can by stimulation convert into a Treg phenotype [36], and Tiemessen et al. have demonstrated that Treg cells in late stage CTCL are nonfunctional [37]. The potential mechanisms of immune regulation with ECP, which imply infusion of apoptotic dying cells, have recently been reviewed [15]. In addition to stimulation of Treg cells, inhibition of pro-inflammatory cytokines, such as IL-1, IL-6 and TNFα, as well as production of anti-inflammatory cytokines, such as TGF-β and IL-10, have been reported [38–41]. The appropriate concentrations of these cytokines may regulate and decrease the immune response at inflammatory sites.

The present study was designed to make a comparison between two patient groups, CTCL and GVHD, submitted to the same kind of ECP therapy during a parallel time course of

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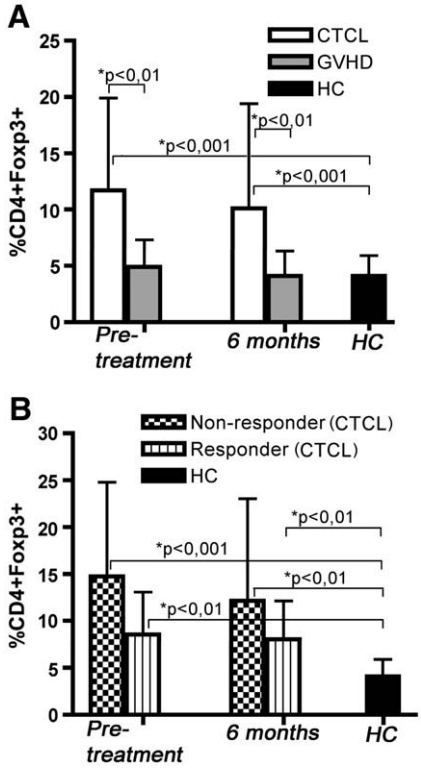

Figure 4 Relative mean percentage of Treg cells in CTCL and GVHD. Treg cells were elevated in the CTCL patients, compared to both the HC and GVHD patients ($p<0.001$ and $p<0.01$, respectively) (A). The CTCL patients were categorized as 'non-responders' or 'responders.' Both groups had elevated Treg cells compared to the HC ($p<0.001$ and $p<0.01$). Non-responders had a higher mean of Treg cells than responders, but not significantly (B). The columns represent the mean, and the error bars display the standard deviation (SD).

some years. Of the GVHD patients 11 out of 14 had responded well to the ECP therapy (Table 2), whereas 13 out of 24 CTCL patients had responded but to a more varying degree. Except for the three patients included in the study as RM, the remaining patients were considered as late-stage CTCL (Table 1). We chose to test freeze-stored serum and PBMC samples collected at start and ending of the standard 6-month course of ECP. The serums were tested for a cytokine profile representing the three kinds of T helper effector cells, Th1, Th2 and Th17, as well as Treg cells. As will be seen from Figs. 2 and 3 the patients displayed great individual variations in the cytokine levels. For most patients, however, their individual levels were surprisingly similar before and after the 6 months of ECP course. The only significant changes in average concentration during the ECP course were a small increase in TGF-β in both patient groups and a small decrease in IL-17 in the GVHD patients, which can be in accordance with a supposed mechanism of Treg cell-mediated down-regulation of an immune reaction. The mean level of TGF-β was on average more than three times higher in the GVHD than the CTCL patients, and the GVHD patients also had the highest increase during the ECP course (Fig. 3B). In spite of the great individual variations the cytokine profile was quite similar in the two patient groups for IL-4, IL-6, IL-10, IL-17 and IFN-γ. Th1 and Th17 are supposed to be the main T effector cells mediating GVHD [42,43], and one might have expected their marker cytokines IFN-γ and IL-17, respectively, to be elevated especially in the GVHD patients. Nevertheless, these findings may emphasize that it is the local milieu and concentrations of cytokines that ultimately are decisive for immune regulation without necessarily being reflected in the serum concentrations.

We have looked especially for the relative concentration of Treg cells among the T helper cells (CD3+CD4+) in the two patient groups. We chose to define the Treg cells as CD3+CD4+Foxp3+CD127− [44] and not to use the CD25 marker as it may be problematic to identify precisely the CD25high fraction which represents the original hallmark of Treg cells [21,45]. Due to the retrospective design, we did not have data on absolute cell counts and are therefore only assessing the relative changes in the cell populations. In the present study, we found that among the healthy controls (HC) the fraction of Treg cells was on average 4.1 ± 1.9% (mean ± SD), which is in coherence with previously reported percentage of the Treg cell subset [21,46]. The fact that these cells are also predominantly CTLA4+ in both patient groups and HC seems to support their Treg identity. The most striking finding was that the Treg fraction in the GVHD patients was at a level close to the HC, whereas the CTCL patients had a Treg level more than twice as high (Fig. 4A). Our finding of an elevated Treg fraction in the CTCL patients is at variance with the report from Tiemessen et al. who found equal Treg levels in CTCL patients and controls [37]. Klemke et al. reported paucity of Foxp3 cells in peripheral blood of Sezary patients [47]. Our CTCL patients only include one Sezary case so our finding may not necessarily be contrasting to this. It may seem to be a paradox that the GVHD patients have the highest serum concentration of TGF-β, but close to normal Treg counts in peripheral blood, whereas the CTCL patients have elevated Treg count, but low levels of TGF-β. One explanation could be that the GVHD patients may have activated Treg cells, and that these are mainly not in circulation, but have migrated into the tissues where the pathogenic alloreaction is taking place. We cannot really know how the distribution of circulating cells correlates to the total body distribution. Generally, the reported level of Treg cells in chronic GVHD remains inconclusive, with both increased [48] and decreased [49] levels reported, together with studies that demonstrate a normalization of the Treg cells after ECP [50]. In this study, the patients with GVHD had close to normal levels of Treg cells, although slightly elevated pre-treatment compared to the HC. After 6 months of ECP, this small difference was completely abolished. A reduction in naïve CD45RA+ Treg cells was also observed, possibly reflecting an increase in the proportion of secondary, memory cells. Our findings of increased secondary cells are similar to those reported by Biagi et al. [51]. Tiemessen et al. found that Treg cells from CTCL patients who did not respond to ECP treatment were dysfunctional and expressed lower levels of Foxp3 than normal cells [37]. We could not detect by flow cytometry any lowered expression of Foxp3 in the CTCL patients, and we have not tested the Treg cells functionally. However, our findings of high Treg

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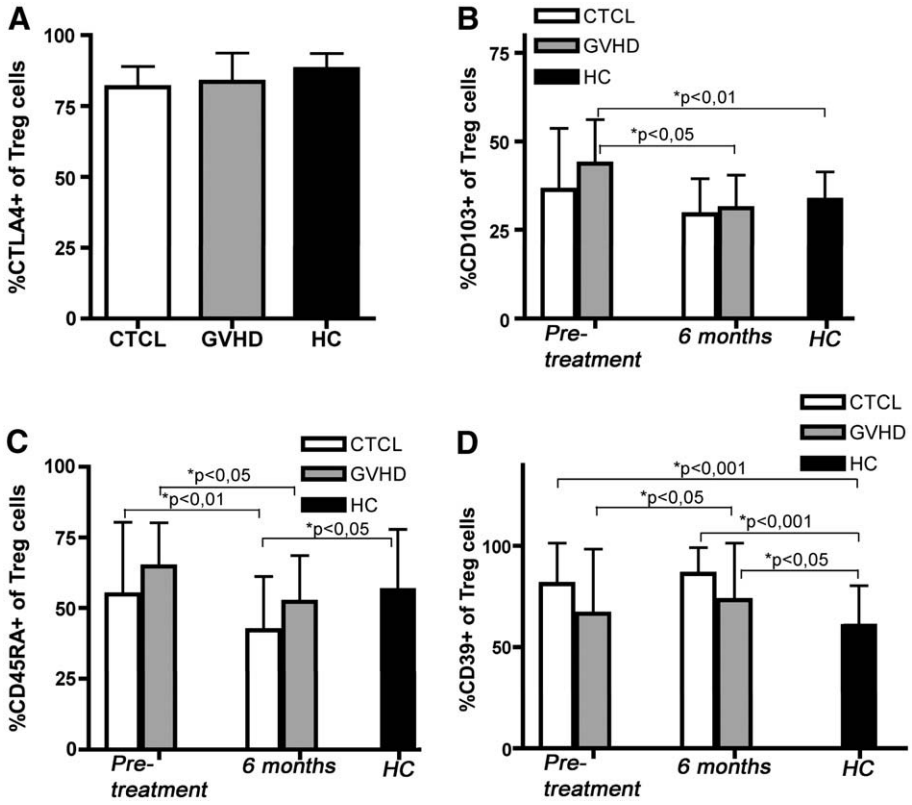

Figure 5 Functional markers on Treg cells. CTLA4 was near equally expressed on Treg cells in patients and HC (A). CD103 (B) and CD45RA (C) displayed a decrease as result of ECP while CD39 showed a slight increase (D). The columns represent the mean, and the error bars display the standard deviation (SD).

counts and low serum TGF-β in the CTCL patients could possibly be related to the findings of Berger's [36] and Tiemessen's [37] groups that the Treg population, or part of it, in the CTCL patients is pathological and dysfunctional explaining the lack of tumor control seen in progressive CTCL disease. The slight but significant increase in serum TGF-β and lowered Treg count following the ECP course could represent an effect of ECP towards normalization also in the CTCL patients. The patient materials that we present here are small, and even if our findings are supported by statistical significance, the conclusions should not be too far-fetched. However, it is reasonable to relate our results to the recent findings of Gjerdrum et al. who demonstrated that the degree of Treg infiltration in CTCL was correlated to patient survival, and they suggested that Treg cells can directly suppress the function of malignant T cells [52]. We think that their findings also contradict that CTCL cells have a Treg phenotype, as infiltrating lymphoma cells should clearly not correlate positively to patient survival. We have here demonstrated an increasing serum concentration of TGF-β in both patient groups as a result of the ECP; in the GVHD patients also a reducing level of IL-17. This might point to an amplified Treg function, a notion recently confirmed in GVHD patients [53]. Parallel to this both patient groups exhibited a reduced primary (CD45RA +) fraction among the Treg cells following ECP. This could imply an antigen-driven expansion of secondary Treg cells. Elevated level of CD39 in both patient groups, and even significantly increasing in the GVHD patients following ECP, could imply an elevated suppressive quality of the Treg cells. When comparing the responding and non-responding patients, however, we were not able to detect any differences in the CD39 expression, indicating that additional factors mediating treatment success are involved and should be searched for. The especially elevated serum TGF-β in the GVHD patients could reflect the very strong immune reaction taking place in these patients. Even if seemingly not increased in number, the activation of the Treg cells may be strong to counterbalance the strong and destructive GVH reaction. Immunologically, CTCL supposedly implies a more indolent condition than GVHD, not activating the Treg cells strongly, but an elevated number of Treg cells might represent a homeostatic proliferative reaction evolving to curb the expansion of malignant T cells in accordance with the mentioned report by Gjerdrum et al. Further investigations including functional suppression assays of isolated patient Treg cells are required to ultimately decide whether ECP strengthens the suppressive capacity.

Conclusion

We think our data suggest that the clinical effect of ECP in both graft-versus-host disease and in cutaneous T cell lymphoma is mediated by a strengthening of the regulatory T cell function.

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Conflict of interest statement
None of the authors has any potential financial conflict of interest related to this manuscript.

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80. Olav Haraldseth: NMR SPECTROSCOPY OF CEREBRAL ISCHEMIA AND REPERFUSION IN RAT.
81. Eiliv Brenna: REGULATION OF FUNCTION AND GROWTH OF THE OXYNTIC MUCOSA.

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82. Gunnar Bovim: CERVICOGENIC HEADACHE.
83. Jarl Arne Kahn: ASSISTED PROCREATION.
84. Bjørn Naume: IMMUNOREGULATORY EFFECTS OF CYTOKINES ON NK CELLS.
85. Rune Wiseth: AORTIC VALVE REPLACEMENT.
86. Jie Ming Shen: BLOOD FLOW VELOCITY AND RESPIRATORY STUDIES.
87. Piotr Kruszewski: SUNCT SYNDROME WITH SPECIAL REFERENCE TO THE AUTONOMIC NERVOUS SYSTEM.
88. Mette Haase Moen: ENDOMETRIOSIS.
89. Anne Vik: VASCULAR GAS EMBOLISM DURING AIR INFUSION AND AFTER DECOMPRESSION IN PIGS.
90. Lars Jacob Stovner: THE CHIARI TYPE I MALFORMATION.
91. Kjell Å. Salvesen: ROUTINE ULTRASONOGRAPHY IN UTERO AND DEVELOPMENT IN CHILDHOOD.

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92. Nina-Beate Liabakk: DEVELOPMENT OF IMMUNOASSAYS FOR TNF AND ITS SOLUBLE RECEPTORS.
93. Sverre Helge Torp: *erb*B ONCOGENES IN HUMAN GLIOMAS AND MENINGIOMAS.
94. Olav M. Linaker: MENTAL RETARDATION AND PSYCHIATRY. Past and present.
95. Per Oscar Feet: INCREASED ANTIDEPRESSANT AND ANTIPANIC EFFECT IN COMBINED TREATMENT WITH DIXYRAZINE AND TRICYCLIC ANTIDEPRESSANTS.
96. Stein Olav Samstad: CROSS SECTIONAL FLOW VELOCITY PROFILES FROM TWO-DIMENSIONAL DOPPLER ULTRASOUND: Studies on early mitral blood flow.
97. Bjørn Backe: STUDIES IN ANTENATAL CARE.
98. Gerd Inger Ringdal: QUALITY OF LIFE IN CANCER PATIENTS.
99. Torvid Kiserud: THE DUCTUS VENOSUS IN THE HUMAN FETUS.
100. Hans E. Fjøsne: HORMONAL REGULATION OF PROSTATIC METABOLISM.
101. Eylert Brodtkorb: CLINICAL ASPECTS OF EPILEPSY IN THE MENTALLY RETARDED.
102. Roar Juul: PEPTIDERGIC MECHANISMS IN HUMAN SUBARACHNOID HEMORRHAGE.
103. Unni Syversen: CHROMOGRANIN A. Phsysiological and Clinical Role.

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104. Odd Gunnar Brakstad: THERMOSTABLE NUCLEASE AND THE *nuc* GENE IN THE DIAGNOSIS OF *Staphylococcus aureus* INFECTIONS.
105. Terje Engan: NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY OF PLASMA IN MALIGNANT DISEASE.
106. Kirsten Rasmussen: VIOLENCE IN THE MENTALLY DISORDERED.
107. Finn Egil Skjeldestad: INDUCED ABORTION: Timetrends and Determinants.
108. Roar Stenseth: THORACIC EPIDURAL ANALGESIA IN AORTOCORONARY BYPASS SURGERY.
109. Arild Faxvaag: STUDIES OF IMMUNE CELL FUNCTION *in mice infected with* MURINE RETROVIRUS.

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110. Svend Aakhus: NONINVASIVE COMPUTERIZED ASSESSMENT OF LEFT VENTRICULAR FUNCTION AND SYSTEMIC ARTERIAL PROPERTIES. Methodology and some clinical applications.
111. Klaus-Dieter Bolz: INTRAVASCULAR ULTRASONOGRAPHY.
112. Petter Aadahl: CARDIOVASCULAR EFFECTS OF THORACIC AORTIC CROSS-CLAMPING.
113. Sigurd Steinshamn: CYTOKINE MEDIATORS DURING GRANULOCYTOPENIC INFECTIONS.
114. Hans Stifoss-Hanssen: SEEKING MEANING OR HAPPINESS?
115. Anne Kvikstad: LIFE CHANGE EVENTS AND MARITAL STATUS IN RELATION TO RISK AND PROGNOSIS OF CANCER.
116. Torbjørn Grøntvedt: TREATMENT OF ACUTE AND CHRONIC ANTERIOR CRUCIATE LIGAMENT INJURIES. A clinical and biomechanical study.
117. Sigrid Hørven Wigers: CLINICAL STUDIES OF FIBROMYALGIA WITH FOCUS ON ETIOLOGY, TREATMENT AND OUTCOME.
118. Jan Schjøtt: MYOCARDIAL PROTECTION: Functional and Metabolic Characteristics of Two Endogenous Protective Principles.
119. Marit Martinussen: STUDIES OF INTESTINAL BLOOD FLOW AND ITS RELATION TO TRANSITIONAL CIRCULATORY ADAPATION IN NEWBORN INFANTS.
120. Tomm B. Müller: MAGNETIC RESONANCE IMAGING IN FOCAL CEREBRAL ISCHEMIA.
121. Rune Haaverstad: OEDEMA FORMATION OF THE LOWER EXTREMITIES.
122. Magne Børset: THE ROLE OF CYTOKINES IN MULTIPLE MYELOMA, WITH SPECIAL REFERENCE TO HEPATOCYTE GROWTH FACTOR.
123. Geir Smedslund: A THEORETICAL AND EMPIRICAL INVESTIGATION OF SMOKING, STRESS AND DISEASE: RESULTS FROM A POPULATION SURVEY.

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124. Torstein Vik: GROWTH, MORBIDITY, AND PSYCHOMOTOR DEVELOPMENT IN INFANTS WHO WERE GROWTH RETARDED *IN UTERO*.
125. Siri Forsmo: ASPECTS AND CONSEQUENCES OF OPPORTUNISTIC SCREENING FOR CERVICAL CANCER. Results based on data from three Norwegian counties.
126. Jon S. Skranes: CEREBRAL MRI AND NEURODEVELOPMENTAL OUTCOME IN VERY LOW BIRTH WEIGHT (VLBW) CHILDREN. A follow-up study of a geographically based year cohort of VLBW children at ages one and six years.
127. Knut Bjørnstad: COMPUTERIZED ECHOCARDIOGRAPHY FOR EVALUTION OF CORONARY ARTERY DISEASE.
128. Grethe Elisabeth Borchgrevink: DIAGNOSIS AND TREATMENT OF WHIPLASH/NECK SPRAIN INJURIES CAUSED BY CAR ACCIDENTS.
129. Tor Elsås: NEUROPEPTIDES AND NITRIC OXIDE SYNTHASE IN OCULAR AUTONOMIC AND SENSORY NERVES.
130. Rolf W. Gråwe: EPIDEMIOLOGICAL AND NEUROPSYCHOLOGICAL PERSPECTIVES ON SCHIZOPHRENIA.
131. Tonje Strømholm: CEREBRAL HAEMODYNAMICS DURING THORACIC AORTIC CROSSCLAMPING. An experimental study in pigs.

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132. Martinus Bråten: STUDIES ON SOME PROBLEMS REALTED TO INTRAMEDULLARY NAILING OF FEMORAL FRACTURES.
133. Ståle Nordgård: PROLIFERATIVE ACTIVITY AND DNA CONTENT AS PROGNOSTIC INDICATORS IN ADENOID CYSTIC CARCINOMA OF THE HEAD AND NECK.

134. Egil Lien: SOLUBLE RECEPTORS FOR **TNF** AND **LPS**: RELEASE PATTERN AND POSSIBLE SIGNIFICANCE IN DISEASE.
135. Marit Bjørgaas: HYPOGLYCAEMIA IN CHILDREN WITH DIABETES MELLITUS
136. Frank Skorpen: GENETIC AND FUNCTIONAL ANALYSES OF DNA REPAIR IN HUMAN CELLS.
137. Juan A. Pareja: SUNCT SYNDROME. ON THE CLINICAL PICTURE. ITS DISTINCTION FROM OTHER, SIMILAR HEADACHES.
138. Anders Angelsen: NEUROENDOCRINE CELLS IN HUMAN PROSTATIC CARCINOMAS AND THE PROSTATIC COMPLEX OF RAT, GUINEA PIG, CAT AND DOG.
139. Fabio Antonaci: CHRONIC PAROXYSMAL HEMICRANIA AND HEMICRANIA CONTINUA: TWO DIFFERENT ENTITIES?
140. Sven M. Carlsen: ENDOCRINE AND METABOLIC EFFECTS OF METFORMIN WITH SPECIAL EMPHASIS ON CARDIOVASCULAR RISK FACTORES.

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141. Terje A. Murberg: DEPRESSIVE SYMPTOMS AND COPING AMONG PATIENTS WITH CONGESTIVE HEART FAILURE.
142. Harm-Gerd Karl Blaas: THE EMBRYONIC EXAMINATION. Ultrasound studies on the development of the human embryo.
143. Noèmi Becser Andersen:THE CEPHALIC SENSORY NERVES IN UNILATERAL HEADACHES. Anatomical background and neurophysiological evaluation.
144. Eli-Janne Fiskerstrand: LASER TREATMENT OF PORT WINE STAINS. A study of the efficacy and limitations of the pulsed dye laser. Clinical and morfological analyses aimed at improving the therapeutic outcome.
145. Bård Kulseng: A STUDY OF ALGINATE CAPSULE PROPERTIES AND CYTOKINES IN RELATION TO INSULIN DEPENDENT DIABETES MELLITUS.
146. Terje Haug: STRUCTURE AND REGULATION OF THE HUMAN UNG GENE ENCODING URACIL-DNA GLYCOSYLASE.
147. Heidi Brurok: MANGANESE AND THE HEART. A Magic Metal with Diagnostic and Therapeutic Possibilites.
148. Agnes Kathrine Lie: DIAGNOSIS AND PREVALENCE OF HUMAN PAPILLOMAVIRUS INFECTION IN CERVICAL INTRAEPITELIAL NEOPLASIA. Relationship to Cell Cycle Regulatory Proteins and HLA DQBI Genes.
149. Ronald Mårvik: PHARMACOLOGICAL, PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL STUDIES ON ISOLATED STOMACS.
150. Ketil Jarl Holen: THE ROLE OF ULTRASONOGRAPHY IN THE DIAGNOSIS AND TREATMENT OF HIP DYSPLASIA IN NEWBORNS.
151. Irene Hetlevik: THE ROLE OF CLINICAL GUIDELINES IN CARDIOVASCULAR RISK INTERVENTION IN GENERAL PRACTICE.
152. Katarina Tunòn: ULTRASOUND AND PREDICTION OF GESTATIONAL AGE.
153. Johannes Soma: INTERACTION BETWEEN THE LEFT VENTRICLE AND THE SYSTEMIC ARTERIES.
154. Arild Aamodt: DEVELOPMENT AND PRE-CLINICAL EVALUATION OF A CUSTOM-MADE FEMORAL STEM.
155. Agnar Tegnander: DIAGNOSIS AND FOLLOW-UP OF CHILDREN WITH SUSPECTED OR KNOWN HIP DYSPLASIA.
156. Bent Indredavik: STROKE UNIT TREATMENT: SHORT AND LONG-TERM EFFECTS
157. Jolanta Vanagaite Vingen: PHOTOPHOBIA AND PHONOPHOBIA IN PRIMARY HEADACHES

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158. Ola Dalsegg Sæther: PATHOPHYSIOLOGY DURING PROXIMAL AORTIC CROSS-CLAMPING CLINICAL AND EXPERIMENTAL STUDIES
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160. Christina Vogt Isaksen: PRENATAL ULTRASOUND AND POSTMORTEM FINDINGS – A TEN YEAR CORRELATIVE STUDY OF FETUSES AND INFANTS WITH DEVELOPMENTAL ANOMALIES.
161. Holger Seidel: HIGH-DOSE METHOTREXATE THERAPY IN CHILDREN WITH ACUTE LYMPHOCYTIC LEUKEMIA: DOSE, CONCENTRATION, AND EFFECT CONSIDERATIONS.
162. Stein Hallan: IMPLEMENTATION OF MODERN MEDICAL DECISION ANALYSIS INTO CLINICAL DIAGNOSIS AND TREATMENT.

163. Malcolm Sue-Chu: INVASIVE AND NON-INVASIVE STUDIES IN CROSS-COUNTRY SKIERS WITH ASTHMA-LIKE SYMPTOMS.
164. Ole-Lars Brekke: EFFECTS OF ANTIOXIDANTS AND FATTY ACIDS ON TUMOR NECROSIS FACTOR-INDUCED CYTOTOXICITY.
165. Jan Lundbom: AORTOCORONARY BYPASS SURGERY: CLINICAL ASPECTS, COST CONSIDERATIONS AND WORKING ABILITY.
166. John-Anker Zwart: LUMBAR NERVE ROOT COMPRESSION, BIOCHEMICAL AND NEUROPHYSIOLOGICAL ASPECTS.
167. Geir Falck: HYPEROSMOLALITY AND THE HEART.
168. Eirik Skogvoll: CARDIAC ARREST Incidence, Intervention and Outcome.
169. Dalius Bansevicius: SHOULDER-NECK REGION IN CERTAIN HEADACHES AND CHRONIC PAIN SYNDROMES.
170. Bettina Kinge: REFRACTIVE ERRORS AND BIOMETRIC CHANGES AMONG UNIVERSITY STUDENTS IN NORWAY.
171. Gunnar Qvigstad: CONSEQUENCES OF HYPERGASTRINEMIA IN MAN
172. Hanne Ellekjær: EPIDEMIOLOGICAL STUDIES OF STROKE IN A NORWEGIAN POPULATION. INCIDENCE, RISK FACTORS AND PROGNOSIS
173. Hilde Grimstad: VIOLENCE AGAINST WOMEN AND PREGNANCY OUTCOME.
174. Astrid Hjelde: SURFACE TENSION AND COMPLEMENT ACTIVATION: Factors influencing bubble formation and bubble effects after decompression.
175. Kjell A. Kvistad: MR IN BREAST CANCER – A CLINICAL STUDY.
176. Ivar Rossvoll: ELECTIVE ORTHOPAEDIC SURGERY IN A DEFINED POPULATION. Studies on demand, waiting time for treatment and incapacity for work.
177. Carina Seidel: PROGNOSTIC VALUE AND BIOLOGICAL EFFECTS OF HEPATOCYTE GROWTH FACTOR AND SYNDECAN-1 IN MULTIPLE MYELOMA.

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178. Alexander Wahba: THE INFLUENCE OF CARDIOPULMONARY BYPASS ON PLATELET FUNCTION AND BLOOD COAGULATION – DETERMINANTS AND CLINICAL CONSEQUENSES
179. Marcus Schmitt-Egenolf: THE RELEVANCE OF THE MAJOR hISTOCOMPATIBILITY COMPLEX FOR THE GENETICS OF PSORIASIS
180. Odrun Arna Gederaas: BIOLOGICAL MECHANISMS INVOLVED IN 5-AMINOLEVULINIC ACID BASED PHOTODYNAMIC THERAPY
181. Pål Richard Romundstad: CANCER INCIDENCE AMONG NORWEGIAN ALUMINIUM WORKERS
182. Henrik Hjorth-Hansen: NOVEL CYTOKINES IN GROWTH CONTROL AND BONE DISEASE OF MULTIPLE MYELOMA
183. Gunnar Morken: SEASONAL VARIATION OF HUMAN MOOD AND BEHAVIOUR
184. Bjørn Olav Haugen: MEASUREMENT OF CARDIAC OUTPUT AND STUDIES OF VELOCITY PROFILES IN AORTIC AND MITRAL FLOW USING TWO- AND THREE-DIMENSIONAL COLOUR FLOW IMAGING
185. Geir Bråthen: THE CLASSIFICATION AND CLINICAL DIAGNOSIS OF ALCOHOL-RELATED SEIZURES
186. Knut Ivar Aasarød: RENAL INVOLVEMENT IN INFLAMMATORY RHEUMATIC DISEASE. A Study of Renal Disease in Wegener's Granulomatosis and in Primary Sjögren's Syndrome
187. Trude Helen Flo: RESEPTORS INVOLVED IN CELL ACTIVATION BY DEFINED URONIC ACID POLYMERS AND BACTERIAL COMPONENTS
188. Bodil Kavli: HUMAN URACIL-DNA GLYCOSYLASES FROM THE UNG GENE: STRUCTRUAL BASIS FOR SUBSTRATE SPECIFICITY AND REPAIR
189. Liv Thommesen: MOLECULAR MECHANISMS INVOLVED IN TNF- AND GASTRIN-MEDIATED GENE REGULATION
190. Turid Lingaas Holmen: SMOKING AND HEALTH IN ADOLESCENCE; THE NORD-TRØNDELAG HEALTH STUDY, 1995-97
191. Øyvind Hjertner: MULTIPLE MYELOMA: INTERACTIONS BETWEEN MALIGNANT PLASMA CELLS AND THE BONE MICROENVIRONMENT
192. Asbjørn Støylen: STRAIN RATE IMAGING OF THE LEFT VENTRICLE BY ULTRASOUND. FEASIBILITY, CLINICAL VALIDATION AND PHYSIOLOGICAL ASPECTS

193. Kristian Midthjell: DIABETES IN ADULTS IN NORD-TRØNDELAG. PUBLIC HEALTH ASPECTS OF DIABETES MELLITUS IN A LARGE, NON-SELECTED NORWEGIAN POPULATION.
194. Guanglin Cui: FUNCTIONAL ASPECTS OF THE ECL CELL IN RODENTS
195. Ulrik Wisløff: CARDIAC EFFECTS OF AEROBIC ENDURANCE TRAINING: HYPERTROPHY, CONTRACTILITY AND CALCUIM HANDLING IN NORMAL AND FAILING HEART
196. Øyvind Halaas: MECHANISMS OF IMMUNOMODULATION AND CELL-MEDIATED CYTOTOXICITY INDUCED BY BACTERIAL PRODUCTS
197. Tore Amundsen: PERFUSION MR IMAGING IN THE DIAGNOSIS OF PULMONARY EMBOLISM
198. Nanna Kurtze: THE SIGNIFICANCE OF ANXIETY AND DEPRESSION IN FATIQUE AND PATTERNS OF PAIN AMONG INDIVIDUALS DIAGNOSED WITH FIBROMYALGIA: RELATIONS WITH QUALITY OF LIFE, FUNCTIONAL DISABILITY, LIFESTYLE, EMPLOYMENT STATUS, CO-MORBIDITY AND GENDER
199. Tom Ivar Lund Nilsen: PROSPECTIVE STUDIES OF CANCER RISK IN NORD-TRØNDELAG: THE HUNT STUDY. Associations with anthropometric, socioeconomic, and lifestyle risk factors
200. Asta Kristine Håberg: A NEW APPROACH TO THE STUDY OF MIDDLE CEREBRAL ARTERY OCCLUSION IN THE RAT USING MAGNETIC RESONANCE TECHNIQUES

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201. Knut Jørgen Arntzen: PREGNANCY AND CYTOKINES
202. Henrik Døllner: INFLAMMATORY MEDIATORS IN PERINATAL INFECTIONS
203. Asta Bye: LOW FAT, LOW LACTOSE DIET USED AS PROPHYLACTIC TREATMENT OF ACUTE INTESTINAL REACTIONS DURING PELVIC RADIOTHERAPY. A PROSPECTIVE RANDOMISED STUDY.
204. Sylvester Moyo: STUDIES ON STREPTOCOCCUS AGALACTIAE (GROUP B STREPTOCOCCUS) SURFACE-ANCHORED MARKERS WITH EMPHASIS ON STRAINS AND HUMAN SERA FROM ZIMBABWE.
205. Knut Hagen: HEAD-HUNT: THE EPIDEMIOLOGY OF HEADACHE IN NORD-TRØNDELAG
206. Li Lixin: ON THE REGULATION AND ROLE OF UNCOUPLING PROTEIN-2 IN INSULIN PRODUCING ß-CELLS
207. Anne Hildur Henriksen: SYMPTOMS OF ALLERGY AND ASTHMA VERSUS MARKERS OF LOWER AIRWAY INFLAMMATION AMONG ADOLESCENTS
208. Egil Andreas Fors: NON-MALIGNANT PAIN IN RELATION TO PSYCHOLOGICAL AND ENVIRONTENTAL FACTORS. EXPERIENTAL AND CLINICAL STUDES OF PAIN WITH FOCUS ON FIBROMYALGIA
209. Pål Klepstad: MORPHINE FOR CANCER PAIN
210. Ingunn Bakke: MECHANISMS AND CONSEQUENCES OF PEROXISOME PROLIFERATOR-INDUCED HYPERFUNCTION OF THE RAT GASTRIN PRODUCING CELL
211. Ingrid Susann Gribbestad: MAGNETIC RESONANCE IMAGING AND SPECTROSCOPY OF BREAST CANCER
212. Rønnaug Astri Ødegård: PREECLAMPSIA – MATERNAL RISK FACTORS AND FETAL GROWTH
213. Johan Haux: STUDIES ON CYTOTOXICITY INDUCED BY HUMAN NATURAL KILLER CELLS AND DIGITOXIN
214. Turid Suzanne Berg-Nielsen: PARENTING PRACTICES AND MENTALLY DISORDERED ADOLESCENTS
215. Astrid Rydning: BLOOD FLOW AS A PROTECTIVE FACTOR FOR THE STOMACH MUCOSA. AN EXPERIMENTAL STUDY ON THE ROLE OF MAST CELLS AND SENSORY AFFERENT NEURONS

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216. Jan Pål Loennechen: HEART FAILURE AFTER MYOCARDIAL INFARCTION. Regional Differences, Myocyte Function, Gene Expression, and Response to Cariporide, Losartan, and Exercise Training.
217. Elisabeth Qvigstad: EFFECTS OF FATTY ACIDS AND OVER-STIMULATION ON INSULIN SECRETION IN MAN

218. Arne Åsberg: EPIDEMIOLOGICAL STUDIES IN HEREDITARY HEMOCHROMATOSIS: PREVALENCE, MORBIDITY AND BENEFIT OF SCREENING.
219. Johan Fredrik Skomsvoll: REPRODUCTIVE OUTCOME IN WOMEN WITH RHEUMATIC DISEASE. A population registry based study of the effects of inflammatory rheumatic disease and connective tissue disease on reproductive outcome in Norwegian women in 1967-1995.
220. Siv Mørkved: URINARY INCONTINENCE DURING PREGNANCY AND AFTER DELIVERY: EFFECT OF PELVIC FLOOR MUSCLE TRAINING IN PREVENTION AND TREATMENT
221. Marit S. Jordhøy: THE IMPACT OF COMPREHENSIVE PALLIATIVE CARE
222. Tom Christian Martinsen: HYPERGASTRINEMIA AND HYPOACIDITY IN RODENTS – CAUSES AND CONSEQUENCES
223. Solveig Tingulstad: CENTRALIZATION OF PRIMARY SURGERY FOR OVARAIN CANCER. FEASIBILITY AND IMPACT ON SURVIVAL
224. Haytham Eloqayli: METABOLIC CHANGES IN THE BRAIN CAUSED BY EPILEPTIC SEIZURES
225. Torunn Bruland: STUDIES OF EARLY RETROVIRUS-HOST INTERACTIONS – VIRAL DETERMINANTS FOR PATHOGENESIS AND THE INFLUENCE OF SEX ON THE SUSCEPTIBILITY TO FRIEND MURINE LEUKAEMIA VIRUS INFECTION
226. Torstein Hole: DOPPLER ECHOCARDIOGRAPHIC EVALUATION OF LEFT VENTRICULAR FUNCTION IN PATIENTS WITH ACUTE MYOCARDIAL INFARCTION
227. Vibeke Nossum: THE EFFECT OF VASCULAR BUBBLES ON ENDOTHELIAL FUNCTION
228. Sigurd Fasting: ROUTINE BASED RECORDING OF ADVERSE EVENTS DURING ANAESTHESIA – APPLICATION IN QUALITY IMPROVEMENT AND SAFETY
229. Solfrid Romundstad: EPIDEMIOLOGICAL STUDIES OF MICROALBUMINURIA. THE NORD-TRØNDELAG HEALTH STUDY 1995-97 (HUNT 2)
230. Geir Torheim: PROCESSING OF DYNAMIC DATA SETS IN MAGNETIC RESONANCE IMAGING
231. Catrine Ahlén: SKIN INFECTIONS IN OCCUPATIONAL SATURATION DIVERS IN THE NORTH SEA AND THE IMPACT OF THE ENVIRONMENT
232. Arnulf Langhammer: RESPIRATORY SYMPTOMS, LUNG FUNCTION AND BONE MINERAL DENSITY IN A COMPREHENSIVE POPULATION SURVEY. THE NORD-TRØNDELAG HEALTH STUDY 1995-97. THE BRONCHIAL OBSTRUCTION IN NORD-TRØNDELAG STUDY
233. Einar Kjelsås: EATING DISORDERS AND PHYSICAL ACTIVITY IN NON-CLINICAL SAMPLES
234. Arne Wibe: RECTAL CANCER TREATMENT IN NORWAY – STANDARDISATION OF SURGERY AND QUALITY ASSURANCE

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235. Eivind Witsø: BONE GRAFT AS AN ANTIBIOTIC CARRIER
236. Anne Mari Sund: DEVELOPMENT OF DEPRESSIVE SYMPTOMS IN EARLY ADOLESCENCE
237. Hallvard Lærum: EVALUATION OF ELECTRONIC MEDICAL RECORDS – A CLINICAL TASK PERSPECTIVE
238. Gustav Mikkelsen: ACCESSIBILITY OF INFORMATION IN ELECTRONIC PATIENT RECORDS; AN EVALUATION OF THE ROLE OF DATA QUALITY
239. Steinar Krokstad: SOCIOECONOMIC INEQUALITIES IN HEALTH AND DISABILITY. SOCIAL EPIDEMIOLOGY IN THE NORD-TRØNDELAG HEALTH STUDY (HUNT), NORWAY
240. Arne Kristian Myhre: NORMAL VARIATION IN ANOGENITAL ANATOMY AND MICROBIOLOGY IN NON-ABUSED PRESCHOOL CHILDREN
241. Ingunn Dybedal: NEGATIVE REGULATORS OF HEMATOPOIETEC STEM AND PROGENITOR CELLS
242. Beate Sitter: TISSUE CHARACTERIZATION BY HIGH RESOLUTION MAGIC ANGLE SPINNING MR SPECTROSCOPY
243. Per Arne Aas: MACROMOLECULAR MAINTENANCE IN HUMAN CELLS – REPAIR OF URACIL IN DNA AND METHYLATIONS IN DNA AND RNA
244. Anna Bofin: FINE NEEDLE ASPIRATION CYTOLOGY IN THE PRIMARY INVESTIGATION OF BREAST TUMOURS AND IN THE DETERMINATION OF TREATMENT STRATEGIES

245. Jim Aage Nøttestad: DEINSTITUTIONALIZATION AND MENTAL HEALTH CHANGES AMONG PEOPLE WITH MENTAL RETARDATION
246. Reidar Fossmark: GASTRIC CANCER IN JAPANESE COTTON RATS
247. Wibeke Nordhøy: MANGANESE AND THE HEART, INTRACELLULAR MR RELAXATION AND WATER EXCHANGE ACROSS THE CARDIAC CELL MEMBRANE

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248. Sturla Molden: QUANTITATIVE ANALYSES OF SINGLE UNITS RECORDED FROM THE HIPPOCAMPUS AND ENTORHINAL CORTEX OF BEHAVING RATS
249. Wenche Brenne Drøyvold: EPIDEMIOLOGICAL STUDIES ON WEIGHT CHANGE AND HEALTH IN A LARGE POPULATION. THE NORD-TRØNDELAG HEALTH STUDY (HUNT)
250. Ragnhild Støen: ENDOTHELIUM-DEPENDENT VASODILATION IN THE FEMORAL ARTERY OF DEVELOPING PIGLETS
251. Aslak Steinsbekk: HOMEOPATHY IN THE PREVENTION OF UPPER RESPIRATORY TRACT INFECTIONS IN CHILDREN
252. Hill-Aina Steffenach: MEMORY IN HIPPOCAMPAL AND CORTICO-HIPPOCAMPAL CIRCUITS
253. Eystein Stordal: ASPECTS OF THE EPIDEMIOLOGY OF DEPRESSIONS BASED ON SELF-RATING IN A LARGE GENERAL HEALTH STUDY (THE HUNT-2 STUDY)
254. Viggo Pettersen: FROM MUSCLES TO SINGING: THE ACTIVITY OF ACCESSORY BREATHING MUSCLES AND THORAX MOVEMENT IN CLASSICAL SINGING
255. Marianne Fyhn: SPATIAL MAPS IN THE HIPPOCAMPUS AND ENTORHINAL CORTEX
256. Robert Valderhaug: OBSESSIVE-COMPULSIVE DISORDER AMONG CHILDREN AND ADOLESCENTS: CHARACTERISTICS AND PSYCHOLOGICAL MANAGEMENT OF PATIENTS IN OUTPATIENT PSYCHIATRIC CLINICS
257. Erik Skaaheim Haug: INFRARENAL ABDOMINAL AORTIC ANEURYSMS – COMORBIDITY AND RESULTS FOLLOWING OPEN SURGERY
258. Daniel Kondziella: GLIAL-NEURONAL INTERACTIONS IN EXPERIMENTAL BRAIN DISORDERS
259. Vegard Heimly Brun: ROUTES TO SPATIAL MEMORY IN HIPPOCAMPAL PLACE CELLS
260. Kenneth McMillan: PHYSIOLOGICAL ASSESSMENT AND TRAINING OF ENDURANCE AND STRENGTH IN PROFESSIONAL YOUTH SOCCER PLAYERS
261. Marit Sæbø Indredavik: MENTAL HEALTH AND CEREBRAL MAGNETIC RESONANCE IMAGING IN ADOLESCENTS WITH LOW BIRTH WEIGHT
262. Ole Johan Kemi: ON THE CELLULAR BASIS OF AEROBIC FITNESS, INTENSITY-DEPENDENCE AND TIME-COURSE OF CARDIOMYOCYTE AND ENDOTHELIAL ADAPTATIONS TO EXERCISE TRAINING
263. Eszter Vanky: POLYCYSTIC OVARY SYNDROME – METFORMIN TREATMENT IN PREGNANCY
264. Hild Fjærtoft: EXTENDED STROKE UNIT SERVICE AND EARLY SUPPORTED DISCHARGE. SHORT AND LONG-TERM EFFECTS
265. Grete Dyb: POSTTRAUMATIC STRESS REACTIONS IN CHILDREN AND ADOLESCENTS
266. Vidar Fykse: SOMATOSTATIN AND THE STOMACH
267. Kirsti Berg: OXIDATIVE STRESS AND THE ISCHEMIC HEART: A STUDY IN PATIENTS UNDERGOING CORONARY REVASCULARIZATION
268. Björn Inge Gustafsson: THE SEROTONIN PRODUCING ENTEROCHROMAFFIN CELL, AND EFFECTS OF HYPERSEROTONINEMIA ON HEART AND BONE

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269. Torstein Baade Rø: EFFECTS OF BONE MORPHOGENETIC PROTEINS, HEPATOCYTE GROWTH FACTOR AND INTERLEUKIN-21 IN MULTIPLE MYELOMA
270. May-Britt Tessem: METABOLIC EFFECTS OF ULTRAVIOLET RADIATION ON THE ANTERIOR PART OF THE EYE
271. Anne-Sofie Helvik: COPING AND EVERYDAY LIFE IN A POPULATION OF ADULTS WITH HEARING IMPAIRMENT
272. Therese Standal: MULTIPLE MYELOMA: THE INTERPLAY BETWEEN MALIGNANT PLASMA CELLS AND THE BONE MARROW MICROENVIRONMENT

273. Ingvild Saltvedt: TREATMENT OF ACUTELY SICK, FRAIL ELDERLY PATIENTS IN A GERIATRIC EVALUATION AND MANAGEMENT UNIT – RESULTS FROM A PROSPECTIVE RANDOMISED TRIAL
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