



PÄIVI HANNULA

Immune Deficiency in  
Chronic Kidney Disease



ACADEMIC DISSERTATION

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for public discussion in the Auditorium of Finn-Medi 1,  
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UNIVERSITY OF TAMPERE

ACADEMIC DISSERTATION

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# 1. LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following publications, which are referred to in the text by their Roman numerals:

**I** Antonen J, Hannula P, Pyhälä R, Saha H, Ala-Houhala I, Pasternack A: Adequate seroresponse to influenza vaccination in dialysis patients. *Nephron* 86(1): 56-61, 2000

**II** Antonen J, Pyhälä R, Hannula P, Ala-Houhala I, Santanen R, Ikonen N, Saha H: Influenza vaccination in dialysis patients: cross-reactivity of induced hemagglutination-inhibiting antibodies to H3N2 subtype antigenic variants is comparable with the response of naturally infected young healthy adults. *Nephrology Dialysis Transplantation* 18: 777-781, 2003

**III** Hannula P, Antonen J, Saha H, Koivisto A-M, Krohn K, Pasternack A: Constant, but not pulsed calcitriol suppresses hemodialysis patients' antigen-induced lymphocyte proliferation. *Nephron* 86(2): 139-144, 2000

**IV** Hannula P, Pörsti I, Saha H, Kalliovalkama J, Jolma P, Kööbi P, Ölander R-M, Antonen J. Treatment of hyperphosphatemia, secondary hyperparathyroidism and hypocalcemia with calcium carbonate favourably

modulates vaccination response in uremic rats. *Clinical nephrology* 61(6): 406-412, 2004

**V** Hannula P, Ala-Houhala I, Saha H, Lagerstedt A, Parviainen T, Pasternack A, Antonen J. Erythropoietin treatment alters immune functions in predialysis patients. *Scandinavian Journal of Urology and Nephrology* 40(3): 241-246, 2006

## 2. ABBREVIATIONS

BSA	Bovine serum albumin
CKD	Chronic kidney disease
CMV	Cytomegalovirus
EBV	Ebstein-Barr virus
Epo	Erythropoietin
GFR	Glomerular filtration rate, expresses the degree of kidney function
HD	Hemodialysis
IgG, -A or -M	Immunoglobulin G, A or M: proteins capable of binding to antigens
IL	Interleukin
I.v.	Intravenous
NTX	Nephrectomy
PBMC	Peripheral blood mononuclear cells
PD	Peritoneal dialysis
PHA	Phytohemagglutinin, a mitogen stimulation agent
PMA	Phorbol myristate acetate, a mitogen stimulation agent
PMNL	Polymorphonuclear leukocytes
PPD	Purified protein derivative of tuberculin
PR	Protection rate, proportion of subjects reaching protective titre level following vaccination
Pre-D	Pre-dialysis: patients with CKD who do not yet need dialysis therapy
PTH	Parathyroid hormone



PTX	Parathyroidectomy
PWM	Pokeweed mitogen, a mitogen stimulation agent
RR	Response rate, proportion of subjects with sufficient mean-fold titre rise from prevaccination titre following vaccination
SHPT	Secondary hyperparathyroidism
SRBC	Sheep red blood cells
T <sub>h</sub>	Helper T cell
T <sub>h1</sub>	Helper T cell producing cytokines to support cellular immunity
T <sub>h2</sub>	Helper T cell producing cytokines to support B cells
TNF- $\alpha$	Tumor necrosis factor $\alpha$ (alpha)
T <sub>s</sub>	Suppressor T cell
TSAT	Percent transferrin saturation
TT	Tetanus toxoid
VDR	Vitamin D receptor

### 3. ABSTRACT

**Background.** Chronic kidney disease (CKD) is a globally increasing condition that almost always finally leads to renal replacement therapy (dialysis treatment). CKD is also a state of immunodeficiency with increased susceptibility to infections that are the second most common cause of death after vascular diseases among dialysis patients (Rocco et al. 2002; Collins et al. 2006; Inaguma et al. 2008). In an earlier Finnish doctoral study in 1985, lymphocyte antigen responses of hemodialysis (HD) patients were only 60% of the controls (Huttunen 1985). The vaccination response is also impaired e.g. against influenza (Beyer et al. 1987; Cavdar et al. 2003).

Uremic toxins, lack of supportive cytokines, constructive cellular factors, dialysis modality and the membranes used in HD are all involved in the immune suppression. An important cause of dysfunction in the cells of the immune system is intracellular hypercalcemia related to secondary hyperparathyroidism (SHPT) caused by retention of phosphate in CKD. Patients with advanced CKD also by nature develop hypovitaminosis D, because the kidneys have lost their ability to synthesize calcitriol, which is known to have immunoregulatory actions. This contributes to SHPT and the numerous immune abnormalities. Initiation of erythropoietin (Epo) treatment has also been shown to affect the immune functions. By and large, the effects of the treatments of CKD on immune functions remain to be clarified.

**Aims.** In this series of studies we aimed to study the influenza vaccination response against vaccine antigens in CKD of various degrees (I) and HD-patients' cross-reacting antibody responses to wild influenza virus antigens (II). We also aimed to study whether phosphate binding with calcium carbonate could increase the reduced tetanus vaccination response in uremic rats (IV). We aimed to study the immune effects of ancillary treatments in CKD, calcitriol (III) and Epo (V), on lymphocyte functions.

**Subjects and methods.** The groups in the clinical studies consisted of patients with all stages of CKD and controls from Tampere University Hospital. Pre-dialysis (Pre-D), HD, peritoneal dialysis (PD) and cardiac control patients were vaccinated against influenza and their antibody response against the influenza vaccine antigens A/H3N2, A/H1N1 and B was measured. HD patients' sera were additionally studied for cross-reactivity against subsequent years' virus isolates of A/H3N2 subtype. *In vitro* calcitriol was added in lymphocyte cultures of HD patients in a lymphocyte antigen response study using tuberculin (PPD) and tetanus toxoid (TT) as antigens. In the experimental study rats underwent a 5/6-nephrectomy or a sham operation, and thereafter they were given a high or control calcium carbonate diet, and their tetanus vaccination response was evaluated. Pre-D patients were tested with 1) antibody tests to Epstein-Barr virus (EBV) and cytomegalovirus (CMV), 2) lymphocyte subclass analyses and 3) lymphocyte proliferation tests using phytohemagglutinin (PHA),

pokeweed mitogen (PWM), PPD and TT as stimulants, before and three months after they begun with Epo treatment.

**Results.** Influenza vaccination of CKD patients resulted in post-vaccination titres that were almost comparable to those of the controls. Against A/H3N2 antigen they were 84%, 84% and 96% of the controls' titres (pre-D, HD and PD, respectively). Sixty-one percent of controls and 67% of PD patients reached a protective titre against A/H3N2 but no more than 35% of pre-D and 36% of HD patients. However, the proportion of CKD and control patients that reached protective titres was clearly higher for the two other antigens A/H1N1 and B. Among HD patients, those on intravenous (i.v.) calcitriol seemed to have a better protection than those without i.v. calcitriol ( $p=0.06$ , borderline significant). The antibodies efficiently cross-reacted against wild influenza virus A/H3N2 antigens, similarly in HD patients and controls and even in healthy military conscripts who had suffered from an influenza A infection previously.

In the lymphocyte proliferation studies, the effect of *in vitro* pulse (mimicking i.v.) calcitriol therapy among HD patients had a statistically non-significant enhancing effect on lymphocyte antigen stimulation cultures, whereas having calcitriol continuously in the culture medium was even immunosuppressive to TT ( $p=0.001$ ).

High calcium diet was beneficial to the tetanus vaccination response of rats with 5/6-nephrectomy: the response of the Ca-NTX rats with high calcium

diet was almost as high as (75% of) that of sham-operated (Sham) rats ( $p=NS$ ), while the NTX rats with control calcium diet had a reduced response (42%) compared to the Sham rats,  $p<0.008$ . However, the difference between Ca-NTX and NTX rats was non-significant. Creatinine, phosphate and intact PTH correlated inversely to the tetanus antibody response ( $p=0.002$ ,  $0.03$  and  $0.02$ , respectively).

The initiation of Epo treatment in pre-D patients caused lymphopenia and a decrease in lymphocyte proliferation, but no changes in the general antibody production against EBV and CMV. The changes in iron status, reticulocytes, hemoglobin or glomerular filtration rate (GFR) did not explain the decline.

**Conclusions.** The influenza vaccination responses of regularly monitored patients with CKD were comparable to controls. Especially HD patients' cross-reactivity against several wild viruses was not inferior to that of the controls. In 5/6-NTX animals, the impaired tetanus vaccination response correlated both to GFR and to the control of hyperphosphatemia and PTH level. Calcitriol *in vivo* borderline significantly ( $p=0.06$ ) enhanced the influenza vaccine response and calcitriol *in vitro* pulse treatment slightly (non-significantly) enhanced lymphocyte antigen proliferation of HD patients. Incubation with calcitriol *in vitro* continuously was immunosuppressive to TT response ( $p=0.001$ ). Epo had initial immune depressing actions to lymphocyte number and function. There seemed to be clinical benefits of calcitriol and phosphate binding therapies in vaccination response in CKD.

## 4. TIIVISTELMÄ

**Tutkimuksen tausta.** Munuaisten vajaatoiminta (MV) on diabeteksen ja sydän- ja verisuonisairauksien yleistymisen vuoksi maailmassa lisääntymässä oleva sairaus, jossa joudutaan usein munuaisen glomerulusfiltraation (GFR) heikentyessä keinomunuaishoitoon eli dialyysiin. MV:ssa immuunipuolustus on heikentynyt ja riski infektioille lisääntynyt. Infektiot ovat toiseksi yleisin kuolinsyy dialyysipotilailla sydän- ja verisuonisairauksien jälkeen. Rokotevaste on MV:ssa myös heikentynyt.

Vajaatoiminnassa kertyvät toksiset aineenvaihduntatuotteet, puolustuskykyä tukevien välittäjäaineiden puute, puolustussoluverkoston heikkous, hemodialyysihoidomuoto ja huonosti bioyhteensopivat dialyysikalvot heikentävät immunitettä. Tärkeä toksinen tekijä on solunsisäisen kalsiumpitoisuuden nousu eli intrasellulaarinen hyperkalsemia, joka liittyy fosfaatin kertymiseen ja siitä johtuvaan sekundaariseen lisäkilpirauhasen liikatoimintaan (hyperparatyreoosiin, SHPT). Kalsitriolin eli aktiivisen D-vitamiinin puute voimistaa SHPT:a ja heikentää immunitettä. Vaurioitunut munuainen ei kykene muuntamaan kalsitriolin esiastetta kalsitrioliksi, eikä myöskään tuottamaan riittävästi erytropoietiinia (Epoa). Epohoidon aloittaminen korjaa munuaispotilaalla anemiam, ja sen on myös osoitettu vaikuttavan immuunitoimintoihin. Immuunijärjestelmän toimivuudesta MV:ssa nykyisten hoitomuotojen yhteydessä on niukalti tietoa.

**Tavoitteet.** Tutkimuksen tavoitteena oli selvittää, millainen on influenssarokotuksen aikaansaama vaste rokoteantigeenejä kohtaan munuaisten vajaatoiminnassa (I) ja eristettyjä epideemisiä virusantigeenejä kohtaan dialyysipotilailla (II). Halusimme tutkia vaikuttaako fosfaatin sijaan käytetty kalsiumkarbonaatti tetanusrokotevasteeseen vajaatoimintaisilla rotilla (IV). Tavoitteena oli myös selvittää kalsitriolin (III) ja erytropoietiniin (V) vaikutuksia lymfosyyttien toimintaan.

**Aineistot ja menetelmät.** Kliinisiin tutkimuksiin osallistui eri asteista munuaisten vajaatoimintaa sairastavia sekä kontrollipotilaita. Kokeellisessa tutkimuksessa rotille tehtiin munuaisten 5/6-osapoistoleikkaus (nefrektomia) tai lumeleikkaus, ja tutkittiin korkeakalsiumisen ja kontrolliruokavalion vaikutusta tetanusrokotevasteeseen. Pre-dialyysi- (Pre-D), hemodialyysi- (HD), peritoneaalidialyysi- (PD) ja kontrollipotilaat rokotettiin kolmikantaisella influenssarokotteella, ja vaste rokoteantigeeneille tutkittiin. Lisäksi tutkittiin HD-potilaiden vasta-aineiden kykyä ristireagoida seuraavien vuosien epideemisten virusten A/H3N2-antigeenejä kohtaan. Kalsitriolia lisättiin lymfosyyttiviljelmiin HD-potilaiden lymfosyyttistimulaatioissa *in vitro* jäljitellen suonensisäistä hoitoa, käyttäen tuberkuliinia (PPD) ja tetanustoksoidia (TT) stimulantteina. Pre-D-potilaille tehtiin 1) Epstein-Barr virus- (EBV) ja sytomegalovirus- (CMV) vasta-ainemääritys, 2) lymfosyyttien alaluokkianalyysi ja 3) lymfosyyttistimulaatio fytohemagglutiniinilla (PHA), pokeweed-mitogeenilla (PWM), PPD:lla ja TT:lla, ennen Epo-hoidon aloittamista ja kolme kuukautta sen jälkeen.

**Tulokset.** Influenssarokotevaste MV-potilailla oli melkein kontrollipotilaiden luokkaa. A/H3N2-vasta-ainetitterit olivat pre-D-, HD- ja PD-potilailla 84%, 84% ja 96% (vastaavasti) kontrollien tittereistä. Kontrolleista 61% ja PD-potilaista 67% saavutti suojaavan vasta-ainetason A/H3N2-antigeenia kohtaan, mutta vain 35% pre-D- ja 36% HD-potilaista. Suojaavan tason saavuttaneiden osuus oli kuitenkin korkeampi kaikilla ryhmillä kahta muuta antigeenia, A/H1N1 ja B, kohtaan. HD-potilaista niillä, jotka saivat suonensisäistä kalsitriolia, vaikutti useammalla olevan suojaava titteritaso ( $p=0.06$ , raja-arvoinen). Rokotuksella saavutettiin hyvä vaste epidemioista eristettyjen virusten antigeeneja kohtaan. Vaste oli samankaltainen HD-potilailla, kontrolleilla, ja jopa terveillä varusmiehillä, jotka olivat toipilasvaiheessa sairastettuaan A-influenssan.

Lymfositistimulaatioissa HD-potilaiden *in vitro* kalsitriolipulssihoito kohensi (tilastollisesti merkityksettömästi) antigeenistimulaatiovastetta, kun taas jatkuva kalsitriolin läsnäolo viljelmässä heikensi immuniteettia TT-antigeenistimulaatioissa ( $p=0.001$ ). Soluvälitteistä immuniteettia vaimentava vaikutus on kalsitriolilla tunnettu aiemmin terveillä henkilöillä.

Korkeakalsiuminen ruokavalio edisti tetanusrokotevastetta MV:a sairastavilla rotilla, joille oli tehty 5/6-nefrektomia: korkeakalsiumisella ruokavaliolla olevien Ca-NTX-rottien vaste oli lumeleikattujen rottien vasteen luokkaa ollen 75% siitä ( $p=NS$ ), kun taas kontrolliruokavaliolla olevien NTX-rottien vaste oli alempi kuin (42%) lumeleikattujen rottien,  $p<0.008$ . Ca-NTX- ja NTX-rottien vasteissa ei ollut merkitsevää eroa. Kreatiniinin ( $p=0.002$ ),



fosfaatin ( $p=0.003$ ) ja lisäkilpirauhashormonin ( $p=0.02$ ) pitoisuudet korreloivat käänteisesti tetanusvasteeseen. Fosfaatinsitojan käytön ei aiemmin ole osoitettu vaikuttavan vasta-ainetuotantoon, mutta lisäkilpirauhasen poistoleikkauksesta on samansuuntaista tutkimustietoa (Gaciong et al. 1991).

Epo-hoidon aloitus pre-D-potilailla laski lymfosyyttien lukumäärää ja heikensi lymfosyyttistimulaatiovastetta, mutta ei vaikuttanut yleiseen vasta-aineenmuodostuskykyyn, jota mitattiin IgG-luokan EBV- ja CMV-vasta-aineilla. Muutokset rauta-arvoissa, retikulosyyttimäärässä, hemoglobiinitasossa tai GFR:ssä eivät selittäneet heikentävää vaikutusta.

**Johtopäätökset.** Säännöllisesti seurattujen MV:a sairastavien potilaiden influenssarokotevaste oli lähes kontrollien luokkaa, eikä HD-potilaiden vasta-aineresponssi useita epidemiaviruksia kohtaan ollut kontrolleja huonompi. Osanefrektomoiduilla rotilla heikentynyt tetanusrokotevaste korreloi GFR:een, sekä fosfaatti- ja lisäkilpirauhashormonitasoon. Alentuneella GFR:lla oli itsenäinen rokotevastetta heikentävä vaikutus. Kalsitrioli *in vivo* kohensi influenssarokotevastetta ( $p=0.06$ , raja-arvoinen) ja *in vitro* pulssihoito kalsitriolilla näytti kohentavan lymfosyyttistimulaatiovastetta ( $p=NS$ ) HD-potilailla. Jatkuva tasainen kalsitriolipitoisuus *in vitro* heikensi lymfosyyttien TT-antigeenistimulaatiovastetta ( $p=0.001$ ). Epo heikensi lymfosyyttiproliferaatiota ja vähensi lymfosyyttien ja niiden alaluokkien määrää. MV:ssa kalsitriolilla ja fosfaatinsitojana käytetyllä kalsiumkarbonaatilla saattaisi olla suotuisa vaikutus rokotevasteisiin.

## 5. INTRODUCTION

Prevalence of chronic kidney disease (CKD) is continuously increasing along with hypertension and diabetes (Coresh et al. 2007; Zhang et al. 2008) and is 30% in elderly persons (Zhang et al. 2008). CKD is classified according to the level of kidney function (table 1) (KDOQI 2002). CKD also causes immunodeficiency, and infections are the second most common cause of death after vascular diseases among dialysis patients (Rocco et al. 2002; Collins et al. 2006; Inaguma et al. 2008). Sepsis / bacteremia is the commonest and pulmonary infections are the second most frequent causes of infectious mortality (Powe et al. 1999; Collins et al. 2006). The solutes retained in uremia cause an essential part of the immune disorder. Hemodialysis (HD) treatment induces complement activation and release of proinflammatory cytokines (Horl 2002). In HD patients especially, poor vaccination responses have been observed against tetanus (Girndt et al. 1995; Kruger et al. 2001), influenza (Beyer et al. 1987; Cavdar et al. 2003), and hepatitis B (Fernandez et al. 1996; Peces et al. 1997).

*Table 1.* Classification of the stages of chronic kidney disease according to guidelines by the kidney disease outcomes quality initiative of the United States National Kidney Foundation (KDOQI 2002).

<b>Stage</b>	<b>Description of kidney damage</b>	<b>GFR (ml/min/1.73m<sup>2</sup>)</b>
1	Normal or elevated GFR	≥ 90
2	Mildly decreased GFR	60 – 89
3	Moderately decreased GFR	30 – 59
4	Severely decreased GFR	15 – 29
5	Kidney failure	< 15 (or dialysis treatment)

Calcitriol, i.e. 1,25-(OH)<sub>2</sub>D<sub>3</sub>, is synthesized from its precursor 25-(OH)D<sub>3</sub> in the cortical kidneys. It is the most active metabolite of vitamin D, which beyond multiple other actions modulates the immune responses. In healthy subjects with normal kidney function, supraphysiological doses of calcitriol can cause immunosuppression (Rigby et al. 1984; Tsoukas et al. 1984; Lemire et al. 1985). This finding has led to the development of calcitriol analog therapies such as topical products in dermatology. Vitamin D deficiency is, on the contrary, a worldwide general health risk factor with rickets and osteomalacia being only the tip of the iceberg of its harmful consequences (Holick 2007). A growing number of large studies show an increased risk of cancer, autoimmune disorders and inflammatory and infectious diseases in vitamin D deficient subjects, underlining the effects of vitamin D on the immune system. Many immune modulating effects could be

lost in calcitriol deficiency in patients with CKD because of lack of its synthesis from the cortical kidneys. Both healthy subjects and patients with CKD would benefit from vitamin D and calcitriol supplementation immunologically. Yet, only a few clinical studies have focused on these problems among the patients with CKD (Wolf et al. 2007).

Oversecretion of parathyroid hormone (PTH) due to secondary hyperparathyroidism (SHPT) is an early phenomenon in CKD. Phosphate retention and hypocalcemia trigger the oversecretion in parathyroid glands. Calcitriol deficiency adds to the hypocalcemia so that inadequately suppressed PTH and excess uremic solutes, via intracellular hypercalcemia in lymphocytes and other cells, lead to diminished immune functions (Haag-Weber et al. 1993; Alexiewicz et al. 1996). An essential mechanism in lymphocyte function (e.g. proliferation) is calcium influx into the cell, as a reaction to immune stimulus and activation. Chronic intracellular hypercalcemia leads to lymphocyte preactivation in SHPT, and the calcium influx reaction becomes blunted in lymphocyte proliferation (Ori et al. 1999). Therapies such as calcium channel blockers and calcitriol as well as parathyroidectomy (PTX) ameliorate intracellular hypercalcemia and relieve the immune suppression (Gaciong et al. 1991; Haag-Weber et al. 1993; Alexiewicz et al. 1996; Alexiewicz et al. 1997). However the immune effects of phosphate binding have not been studied, even though hyperphosphatemia is the leading cause of SHPT. The efficacy of calcium carbonate to function as a phosphate binder depends on the quantity of dietary phosphate ingestion and the timing of calcium carbonate intake and

meals. Therefore, a stable environment is essential when the immune effects of calcium carbonate are being studied. Such conditions are almost impossible to accomplish in clinical studies, whereas animal studies can provide with a better control of dietary and other environmental factors.

Anemia is a natural consequence of CKD and develops secondary to deficient erythropoietin (Epo) secretion from the kidney cortex. Previously iron overload, caused by frequent blood transfusions, used to cause immunosuppression in patients with CKD. The development of recombinant Epo has reduced the need for transfusions. Positive immune enhancing effects on vaccine responsiveness of HD patients were reported (Birmingham et al. 1996), when the need for blood transfusions began to decline. However, also negative immune effects of Epo among HD patients have been reported (Barany et al. 1992; Steffensen et al. 1996). Epo therapy is common on pre-dialysis (pre-D) patients, but the studies on immune effects of Epo on pre-D patients are limited.

Calcitriol and Epo therapies together with phosphate binding with calcium carbonate are ancillary treatments of CKD, all of which have direct or indirect immune actions. There is a need to evaluate the contribution of these ancillary treatments and the diminished kidney function itself to the immune deficiency in dialysis patients as well as pre-D patients. This thesis and the literature reviewed in it focuses on current clinical immunity among the patients with CKD.

## 6. REVIEW OF THE LITERATURE

### 6.1 Immune dysfunction in chronic kidney disease

#### 6.1.1 Historical perspectives

Kidney diseases and the uremic state was recognized early: Hippocrates wrote about the prognosis of the patient in relation to urine: “But the most deadly of all kinds of urine are the fetid, watery, black, and thick; in adult men and women the black is of all kinds of urine the worst, but in children, the watery” (Hippocrates -430). Morgagni (1682-1771) as a clinical pathologist found the anatomic findings of atrophied kidneys at autopsy, in association with the signs and symptoms of uremia (Antonello et al. 1999). The history of immunology begins in 1798, when Edward Jenner (1749-1823) carried out smallpox vaccination. The histories of nephrology and immunology converged in the 1930's: the histocompatibility antigens were found and renal transplantation ( $T_x$ ) was pursued. A couple of decades later, an immune dysfunction associated with chronic kidney disease (CKD) was reported, and a large field of studies on the immunological changes in uremia took place. Even today the interrelated causes and consequences remain partly unsolved.

Half a century ago it was discovered that kidney and skin transplants in uremic patients survived longer than expected (Hume et al. 1955; Dammin et al. 1957), and that PHA-induced mixed leukocyte reaction was suppressed in renal failure (Elves et al. 1966). Concordantly, delayed hypersensitivity reaction and the response to lymphocyte mitogen (Huber et al. 1969) and antigen stimulation (Selroos et al. 1973) were found to decrease. However, the responses of uremic lymphocytes were normal when the cells were cultured in serum from a healthy individual, whereas sera from uremic patients had an inhibitory effect on the stimulation of normal lymphocytes (Touraine et al. 1975).

### 6.1.2 The clinical manifestations of the immunodeficiency

The susceptibility to infections in CKD is increased (Montgomerie et al. 1968; Goldblum et al. 1980); and until the 1990's, sepsis and other infections have been the leading cause of morbidity and mortality among HD patients (Lowrie et al. 1973; Mailloux et al. 1991). Today infections are the second most common cause of death after cardiovascular disease (Rocco et al. 2002; Collins et al. 2006; Inaguma et al. 2008), causing up to 23% of mortality in a recent study among 226 pre-D patients with CKD stages 3-4 (Inaguma et al. 2008). Sepsis and infections are equal causes of mortality with cardiovascular diseases in diabetic patients with dialysis therapy (Brunner et al. 1988). Of immunological predictors, high levels of proinflammatory cytokines in dialysis patients are associated with mortality, while improved T-cell number and T-cell antigen proliferation response are

associated with survival (Kimmel et al. 1998). In a follow-up study among around 5000 dialysis patients to detect sepsis, as many as 10% of the patients had at least one episode of sepsis during a seven-year follow-up, the difference between PD and HD being statistically insignificant (Powe et al. 1999). Older age and diabetes were independent risk factors of sepsis in both patient groups. With influenza vaccination, the risk for hospitalization and death among 125000 HD and PD patients decreased, even though only 49% of HD and 39% of PD patients were vaccinated (Gilbertson et al. 2003).

Foot ulcers and lower extremity amputations cause severe health problems and disability. Amputations in patients with CKD are most often performed not only due to diabetes or vascular disease, but also due to gangrene, osteomyelitis and sepsis (Eggers et al. 1999). In diabetes mellitus with CKD foot ulcers and amputations are overexpressed at very early stages of CKD, having a non-vascular etiopathogenesis (Margolis et al. 2008).

A continuously high susceptibility to tuberculosis is a clinical evidence of impaired cellular immunity among patients with CKD. Tuberculosis is especially frequent among those with CKD stage 5, among men more than among women, and extrapulmonary tuberculosis is more common than the pulmonary form (Hussein et al. 2003; Venkata et al. 2007). The tuberculin skin test remains negative in the majority (64%) of CKD patients with tuberculosis (Venkata et al. 2007).



The risk of hepatitis B infection has been decreasing due to uniform attempts to control its spreading. Dialysis patients with hepatitis B infection are often free of symptoms of hepatitis and the disease seldom leads to high aminotransferase activity (Fabrizi et al. 2002). The prevalence of hepatitis C infection is increased among patients who are starting dialysis in the U.S.A. (Bergman et al. 2005).

In dialysis patients there is overexpression of cancer of the kidney, bladder, thyroid and other endocrine organs (organs for which viruses have been suspected as causative agents), more in younger than older patients and more in women than in men (Maisonneuve et al. 1999; Stewart et al. 2003). The cancer risk factors acting specifically in CKD are probably less dependent on age and are more potent than the risk factors that account for most cancers in the general population (Stewart et al. 2003). The risk of cancer is not related to the type of dialysis: it is likely that the uremic state rather than any treatment-related phenomenon contributes to the cause of the increased risk (Maisonneuve et al. 1999).

Current recommendations in preventive health care in management of CKD are aimed at controlling the risk of bacterial, fungal and viral infections by prophylaxis, screening and immunization, and at cancer screening along with monitoring the lipid and blood glucose levels (Choudhury et al. 2008).

### 6.1.3 Findings on lymphocytes and antigen-presenting cells

In primary response to infection, antigen-presenting cells capture microbial structures and process them to provoke the cellular and humoral immune response. Pre-immune naïve T cells recognize the microbial structures, leading to activation and fast expansion. These naïve cells differentiate into either antigen-specific short-lived effector cells or to long-lived memory cells. The memory cells are able to bind to lymphoid organs. The memory cells are maintained and they are able to elicit the full immunologic response rapidly and thus are important in vaccination response. In the immune response, the clonally expanding T cells are of CD4+ T helper (T<sub>h</sub>) and CD8+ T suppressor (T<sub>s</sub>) cell types.

The most dramatic changes in cellular immunity in CKD patients are observed in the T lymphocytes and in the antigen presenting cells. The numbers of circulating T lymphocytes are reduced in HD patients, and the reduction of T<sub>h</sub> cells is more prominent than that of T<sub>s</sub> cells (Raska et al. 1983; Moser et al. 2003; Litjens et al. 2006; Yoon et al. 2006). Especially, the number of naïve and central memory cells is suppressed (Yoon et al. 2006) also among pre-D patients (Litjens et al. 2006).

In uremia, T lymphocytes show an increased amount of activation markers on their cell surfaces and they are in a pre-activated state (Chatenoud et al. 1986; Descamps-Latscha et al. 1995). The amount of pre-activated (CD69+, CD25+) T<sub>h</sub> cells is increased in HD patients despite generally decreased

amounts of T<sub>h</sub> cells among these patients (Meier et al. 2005). As a result of pre-activation, there is an increased apoptotic turnover which has been suggested to explain the lymphopenia (Carracedo et al. 1995; Meier et al. 2002; Moser et al. 2003; Alvarez-Lara et al. 2004; Litjens et al. 2006). The lymphopenia affects also the B cells: the B cells of HD patients are thought to have insufficient support from the T<sub>h</sub> cells, which cannot produce enough of the cytokines that would promote B cell function (see next chapter). B cells as well are subjects to apoptosis (Fernandez-Fresnedo et al. 2000). The co-operation between the T cells and the antigen presenting cells (especially monocytes) is defective leading to subnormal proliferation, and interferon- $\gamma$  and interleukin (IL)-2 production (Meuer et al. 1987; Gerez et al. 1991; Girndt et al. 1993; Brinkkoetter et al. 2005; Meier et al. 2005). The co-operation defect exists because of alterations in receptor-ligand signalling (Girndt et al. 1993; Brinkkoetter et al. 2005).

A Finnish doctoral monography has assessed the cell-mediated immunity in patients with CKD in 1985 (Huttunen 1985). The study group consisted of 48 CKD stage 3 patients with mean creatinine 152 (range 62-299)  $\mu\text{mol/l}$ , 49 uremic CKD stage 5 patients on HD, PD or conservative treatment (i.e. pre-D patients), and 35 transplanted patients. Additionally, 54 controls with normal kidney function were included. The pre-D patients (n=11) had the lowest mean lymphocyte mitogen stimulation responses being 72% of the controls (n=54). The PD patients (n=17) had the best (87% of the controls) mean mitogen responses of the uremic patients while HD patients' response was 76% of the controls. Similarly, the antigen stimulation responses to PPD

were 51% (pre-D), 59% (HD), and 88% (PD) of the controls. The lymphocyte proliferation in the CKD stage 5 pre-D patients was significantly lower than in controls for all mitogens and antigens tested. [The percentages presented here are deduced from Figure 4 p.57 and Figure 5 p.58 (Huttunen 1985)].

#### 6.1.4 Findings on cytokines and immunoglobulin production

The immune deficiency of uremia is coincident with alterations in humoral immunity. The plasma levels of proinflammatory cytokines, such as IL-1b, IL-6 and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), are elevated in CKD (especially HD) due to general overproduction and inadequate clearance of these substances (Pereira et al. 1994; Descamps-Latscha et al. 1995). A continuous complement activation causes a pre-activation state of cytokine-producing monocytes (antigen-presenting cells) and leads to the release of the pro-inflammatory cytokines, instead of IL-10 and other counter-regulatory cytokines (Brunet et al. 1998).

The  $T_h$  cells can differentiate into the  $T_{h1}$  or  $T_{h2}$  subset with different cytokine secretion:  $T_{h1}$  cells produce interferon- $\gamma$  and are effectors of cellular immunity whereas  $T_{h2}$  cells secrete IL-4, which supports humoral immunity. Pre-activation and decreased co-stimulatory activity of antigen-presenting cells drive differentiation of T helper cells towards the  $T_{h1}$  type (Descamps-Latscha et al. 1996; Sester et al. 2000), while the synthesis of  $T_{h2}$  type regulatory cytokines (IL-4, IL-10) is defective (Perianayagam et al. 2002). The pro-inflammatory state in CKD, particularly in HD, also inhibits B

cells by inhibiting the T<sub>h</sub>2 type humoral response (Girndt et al. 2001; Libetta et al. 2001; Nitta et al. 2002). In PD patients, the T<sub>h</sub> cells exhibit an unaltered T<sub>h</sub>1/T<sub>h</sub>2 balance (Ando et al. 2005).

The defects in the immunoglobulin production of B cells are mostly thought to reflect the dysfunction of T cells. Basal secretion of IgG, IgA and IgM can be defective or normal (Degiannis et al. 1987; Beaman et al. 1989). Antigen-specific immunoglobulin production is defective, presumably due to a reduction in memory type B cells (Bouts et al. 2004a). The antibody production of lymphocytes is regulated by expression of lymphocyte cell surface IgG- and complement receptors, which are reduced at least in in uremic dialyzed and pre-D children (Bouts et al. 2004b).

### 6.1.5 Findings on phagocytosis

HD patients dialyzed with non-biocompatible dialysis membranes can have neutropenia due to complement activation and migration of activated neutrophils to the lungs (Kaplow et al. 1968; Craddock et al. 1977). However an increase in the number of polymorphonuclear leukocytes (PMNL) predicts mortality in HD patients (Reddan et al. 2003). The increase in PMNL counts in CKD has been suggested to be a sign of pre-activation (Sela et al. 2005). The number of PMNL increases when the glomerular filtration rate (GFR) decreases (Sela et al. 2005).

The *unstimulated* release of reactive oxygen species (ROS) and superoxide (SO) is increased in dialysis patients and this is thought to represent PMNL pre-activation (Sela et al. 2005; Sardenberg et al. 2006). The *antigen-stimulated* release of ROS or SO is on the contrary decreased in all CKD patients (Cendoroglo et al. 1999; Rao et al. 2004; Sela et al. 2005; Sardenberg et al. 2006). The *mitogen-stimulated* release of ROS with phorbol myristate acetate (PMA) is also decreased in HD patients. The PMNL's ability to phagocytose is related to kidney function, being only 50% of that of healthy controls in CKD patients (Vanholder et al. 1991).

PD treatment corrects PMNL dysfunction linked to increased apoptosis, which is especially marked among pre-D patients and less marked in HD (Sardenberg et al. 2006). The PMNL apoptosis correlates negatively with antigen and PMA-stimulated ROS production and phagocytosis (Sardenberg et al. 2006). Apoptosis is thus considered a parallel marker of phagocyte functional change but probably not a causal factor (Sardenberg et al. 2006).

#### 6.1.6 Findings on vaccination response

Vaccination mimics the challenge of microbial invasion, and it reflects the total function of the immune system. In patients with CKD, decreased antigen-associated co-stimulatory signalling impairs T<sub>h</sub> cell function, leading to less differentiation of B cells into specific antibody producing B cells (Meuer et al. 1987; Girndt et al. 1993).

Vaccines can be divided into T cell independent and T cell dependent ones. There are two kinds of T-independent antigenic stimulants, the stronger ones (type 1) eliciting a mitogenic polyclonal B cell activation (e.g. LPS, the lipopolysaccharide of *Escherichia coli*) and the weaker ones (type 2) activating mature B cells that produce mainly IgM-antibodies (e.g. pneumococcal, meningococcal, or *Haemophilus influenzae* capsular polysaccharide vaccines). The virus vaccines (influenza, hepatitis, polio, or varicella) and the bacterial toxoid vaccines (pertussis, tetanus, and diphtheria) are T-dependent. By conjugating with polypeptide carriers or adjuvants the type 2 T-independent vaccines can also be made T-dependent.

#### *6.1.6.1 Vaccination in experimental kidney disease*

In studies with uremic NTX rats, suppressed antigen responses have been reported (Raskova et al. 1973; Gaciong et al. 1991). Using strong antigenic stimuli such as sheep red blood cells (SRBC), an intact immune response has also been found (Nelson et al. 1980). However, bovine serum albumin (BSA), a T-dependent antigen, elicited a reduced response (Mezzano et al. 1982).

#### *6.1.6.2 Vaccination efficacy*

In study populations the vaccination responses can be evaluated by calculating the proportion of subjects that have protective antibody levels,

the protection rate (PR). Another means of describing the efficacy is calculating the response rate (RR), i.e. the proportion of subjects that exhibit a sufficient mean fold titre increase (the difference between the logarithmated geometric mean titres of post- and pre-vaccination sera).

#### 6.1.6.3 *Tetanus and diphtheria*

The diphtheria immunization response has been among the earliest targets for studies on immune suppression in CKD: Stoloff et al. found that uremic patients were able to produce diphtheria antitoxin only after a booster dose of diphtheria toxoid (Stoloff et al. 1958). The tetanus vaccination (against the bacteria *Clostridium tetani*) of dialysis patients has been shown to produce a good response at first, but the PR rapidly declines from 97% to 62% at six months (Guerin et al. 1992). Also a lower immediate PR of 69% has been presented in HD and pre-D patients (Girndt et al. 1995). At 12 months, PR has been shown to be roughly 50% (Kruger et al. 1999; Kruger et al. 2001). The responses to tetanus and diphtheria vaccinations are comparable (Kruger et al. 1999), but diphtheria immunization elicits lower PR than tetanus (Kruger et al. 2001).

#### 6.1.6.4 *Influenza*

Influenza vaccinations became available in the late 1970's. Studies in dialysis patients from that era showed a similar or weaker seroconversion with a RR of 43-93% compared to controls (Osanloo et al. 1978; Nikoskelainen et al. 1982; Cappel et al. 1983; Beyer et al. 1987). Later



studies showed an even lower RR (7-44%) (Cavdar et al. 2003; Vogtlander et al. 2004). Post-vaccination responses are inversely correlated to pre-vaccination antibody levels (Osanloo et al. 1978), explaining the lowering of the RR in the 1990's and after, with many patients having high prevaccination levels due to earlier vaccinations. On the contrary, the PR of HD patients has clearly increased during the last three decades (from 25-66% to 46-87%, depending on the antigen) (Beyer et al. 1987; Cavdar et al. 2003; Vogtlander et al. 2004). Patients on PD have a higher RR and PR against influenza than HD patients (Beyer et al. 1987; Cavdar et al. 2003).

There is only limited data on influenza vaccination response in adult *pre-D* patients (Osanloo et al. 1978; Nikoskelainen et al. 1982). The mean increase of post-vaccination antibodies has been shown to be similar to that of controls (Osanloo et al. 1978; Nikoskelainen et al. 1982). The influenza vaccination response of HD treated children, 20% of whom had been vaccinated previously, was non-significantly lower than that of controls, being 60/73/93% (RR) and 66/73/80% (PR) against vaccine antigens of subtype H3N2/H1N1/B, respectively (Furth et al. 1995).

#### 6.1.6.5 *Hepatitis B*

Hepatitis B infections were found to spread among HD patients in the United States within a decade from the implementation of HD treatment (Snydman et al. 1976). Shortly afterwards recommendations were published to vaccinate all HD patients and staff members. Yet the vaccination frequency

has remained low even though it has been shown that vaccination diminishes hepatitis B infections of dialysis patients although they respond to hepatitis B vaccination less than healthy individuals (Crosnier et al. 1981; Miller et al. 1999). RRs of only 73-78% (Buti et al. 1992; Kramer et al. 1997; Peces et al. 1997) and a PR of 61% (Fernandez et al. 1996) have been reported. The response is relatively rapidly lost especially in older individuals (Buti et al. 1992). Among pre-D patients (n=165), the RR has been reported to be 82% (DaRoza et al. 2003). Commercial recombinant vaccines have largely superseded plasma vaccines, although in CKD patients no statistically significant difference between these vaccine types has been found according to a Cochrane database review (Schroth et al. 2004).

A reinforced vaccination series brings no additional effect to the routine three-dose recombinant vaccination scheme (Schroth et al. 2004).

In one of the earliest placebo-controlled vaccination studies it was shown that of the 138 vaccinated HD patients, as many as 40% did not reach protective levels. Out of these non-responders, 21% became infected with hepatitis B, but only two infections were detected after completing the whole three-stage injection series. Among the placebo arm of the study, 45% became infected, and as many as 12 infections were detected after the third (placebo) injection (Crosnier et al. 1981). Almost two decades later, the risk of HBV infection was 70% lower in vaccinated patients (Miller et al. 1999). Non-response to hepatitis B vaccination has been related to greater mortality (28% in non-responders versus 8% in responders) and more hospital days in HD patients (Fernandez et al. 1996).

#### 6.1.6.6 *Pneumococcal polysaccharide*

Findings on pneumococcal vaccination outcome among patients with CKD are scarce. A study on vaccination against *Streptococcus pneumoniae* of 57 patients including pre-D, HD and kidney transplant patients, and 33 controls, was conducted in the early 1980's in Finland using a 14-valent pneumococcal capsular polysaccharide vaccine. Antibodies against six pneumococcal antigens were measured before and one and 12 months after the vaccination. HD patients had the weakest antibody responses both in terms of post-vaccination levels and the durability of antibody levels. The IgG-type antibodies were comparable in pre-D patients, kidney transplant patients and controls (Nikoskelainen et al. 1985).

Since the 1980's, pneumococcal vaccines have first evolved into 23-valent ones, and thereafter into conjugated vaccines. In a study among 41 pediatric patients with CKD (including PD, kidney transplant and pre-D patients) using a 23-valent capsular polysaccharide vaccine, the IgG-type antibody response was considered adequate against the two test antigens (serotypes 3 and 14) that were used. Yet the post-vaccination PR at twelve months against serotype 14 was only 59%. Thus, as many as 40% might not reach a protective level to all serotypes (Furth et al. 1996).

#### 6.1.6.7 *Recommendations about vaccinations in CKD*

The National Institute of Health and Welfare recommends free influenza vaccination to all above 65 years or between 6 and 35 months and to risk

groups such as dialysis patients. Those over 65 years and those over five years with CKD should also be vaccinated against *Streptococcus pneumoniae*. Patients with nephrotic syndrome should be revaccinated against *Str. Pneumoniae* every five years. Children younger than five years with CKD should receive the 7-valent conjugated vaccine. They should also later be revaccinated with the 23-valent capsular polysaccharide vaccine. Dialysis patients are recommended to receive four doubled doses of Hepatitis B vaccination. The National Institute for Health and Welfare yearly updates these Finnish recommendations.

## 6.2 Factors that have an impact on the altered immune response

The following section reviews literature that evaluates which factors affect lymphocyte function and vaccination response in uremia. The key functional disturbances lie in the uremic milieu, leading to a disturbed communication between cells involved in antigen presentation. The B cell function seems to be better preserved than T cell and monocyte/ macrophage function.

### 6.2.1 Age, reduced kidney function and related factors

Increased PMNL count and lymphopenia are independent predictors of increased mortality risk in HD patients. In a large study reported by Reddan et al. including 25000 HD patients, an increased neutrophil count was

associated with higher age and higher serum ferritin as well as lower serum albumin, creatinine and percent transferrin saturation (TSAT). The increased risk of death associated with increased neutrophil count could reflect an ongoing inflammatory state (Reddan et al. 2003). A lower lymphocyte count, however, correlated with higher age but also with lower body mass index (BMI), lower hematocrit and lower serum creatinine. The increased mortality risk associated with lymphopenia might reflect increased death risk of protein-calorie malnutrition. Age also correlated inversely with the lymphocyte proliferation response to mitogens in 49 Finnish uremic CKD patients with serum creatinine  $\geq 500 \mu\text{mol/l}$  (mean  $1053 \mu\text{mol/l}$ ) on conservative, HD or PD treatment (Huttunen 1985).

The number of PMNL increases in relation to the GFR decrease ( $p < 0.0001$ ) (Sela et al. 2005). Increased PMNL count and rate of SO release, increased serum C-reactive protein and IL-6 and decreased albumin are all associated with declining GFR (Sela et al. 2005). Also PMNL function (glucose utilization in latex and zymosan phagocytosis) decreases with increasing serum creatinine level and lower creatinine clearance (Vanholder et al. 1991). In the T cell lymphopenia of CKD especially the decreasing naïve (CD45RO+) and central memory T cell numbers correlate with the decreasing GFR (Litjens et al. 2006; Yoon et al. 2006). In pre-D patients, lymphopenia with peripheral blood mononuclear cell (PBMC) apoptosis associates with decreasing creatinine clearance (Martin-Malo et al. 2000). In a study of 230 HD patients, T cell antigen proliferation response inversely

correlated with protein catabolic rate, but not with the dialysis adequacy-measure Kt/V (Kimmel et al. 1998).

Studies on vaccination response on CKD patients have revealed a relationship to GFR. In an early study with 36 CKD patients, tetanus antitoxin titres increased with GFR: the mean  $\log_{10}$  titres were 1.72, 1.32, 2.56 and 2.99 for groups with very low (2.6 ml/min), low (7.3 ml/min), moderate (18.6 ml/min) and normal mean creatinine clearance, respectively. The two groups with lowest GFR had significantly lower titres than either the group with moderate or normal GFR ( $p < 0.05$ ) (Byron et al. 1976). In 56 CKD patients, pneumococcal vaccination response correlated inversely with serum creatinine value and age (Nikoskelainen et al. 1985). The level of GFR independently predicted also hepatitis B vaccination-induced seroconversion rate in multivariate analysis, and  $\text{GFR} < 10 \text{ ml/min}$  associated with high odds of non-response ( $p = 0.001$ ) (DaRoza et al. 2003).

Age and nutritional status correlate with non-responsiveness to hepatitis B vaccination on HD patients (Fraser et al. 1994; Fernandez et al. 1996; Peces et al. 1997; DaRoza et al. 2003). Even in recent studies odds of non-response is high (OR 3.35) for increasing age among dialysis (mainly PD) patients (Chow et al. 2006). In pre-D patients, the correlation of age and hepatitis B vaccination response has been less clear (Fraser et al. 1994; DaRoza et al. 2003; McNulty et al. 2005). Markers of nutritional status (serum albumin and urea concentration) significantly correlate with hepatitis B vaccination response in HD patients, and the percentage of non-responders has been remarkably higher among those with malnutrition

(Fernandez et al. 1996). However, this has not been confirmed by others (Peces et al. 1997).

### 6.2.2 Uremic toxins

Uremic toxins are active solutes that are responsible of the uremic syndrome (Vanholder et al. 2003). They are potential non-classical risk factors of inflammatory and atherosclerotic vascular disease, which occurs earlier and more frequently in uremic patients than in the general population (Vanholder et al. 2003).

Uremic toxins inhibit T cell proliferation of HD patients and healthy controls (Touraine et al. 1975; Dumann et al. 1990; Meier et al. 2005). In HD patients, the IL-2 production and PHA-stimulated proliferation capacity of pre-activated T<sub>h</sub> cells (CD69+/CD4+ cells) is decreased compared with pre-D patients and controls, but with addition of normal serum, the proliferation capacity is restored to normal. B cells of HD patients are intact when cultured in a non-uremic environment (Krishnamurthy et al. 2002). Furthermore, the proliferation of normal pre-activated T<sub>h</sub> cells is decreased by addition of sera from HD patients (Meier et al. 2005).

The early toxin investigations revealed the presence of “middle size” molecules in uremic serum, which are immunosuppressive (Traeger et al. 1980). Albumin fragment molecules, much more clearly than whole BSA molecules, suppressed the vaccination response of both normal and uremic

rats given BSA prior to vaccination (Mezzano et al. 1982). BSA fragment molecules were markedly retained in blood of uremic animals compared to sham-operated controls. On the contrary, the whole BSA molecules were eliminated as efficiently in uremic as in sham-operated rats. BSA fragments and their accumulation were thus causing the antigen specific suppression of immunization in uremic rats given BSA (Mezzano et al. 1982).

Uremic sera from pre-D or PD patients induce many changes in the cells of the immune system but are not as suppressive as the sera of HD patients: both uremia and the HD process cause immunosuppression in HD patients. Various compounds have been isolated from the serum of uremic patients that have been shown to inhibit the function of PMNL (Haag-Weber et al. 1996). Besides heterologous uremic serum, urea accelerates the apoptosis of normal monocyte-derived dendritic cells ( Lim et al. 2007).

### 6.2.3 Dialysis

The effects of the dialysis treatment affecting the immune system can be analysed by comparing the immune functions of pre-D and HD patients, by prospective analyses of subjects initiating HD treatment, and by comparing HD and PD treatments.



### 6.2.3.1 *Pre-dialysis versus hemodialysis stages, and prospective studies*

When cellophane membranes were used, granulocytes and monocytes could be entrapped into lung vessels upon re-infusion to venous blood, as a result of the activation of the complement in contact with the dialyser (Craddock et al. 1977). Since then, more biocompatible membranes have been synthesized (Horl 2002). In a prospective study by Vanholder et al. (Vanholder et al. 1991), the start of HD therapy initially decreased PMNL phagocytosis which reached a nadir at 2-3 weeks. In cuprophan users phagocytosis remained only 67% of the pre-D level at 12 weeks, while in users of biocompatible membranes it was corrected to pre-D levels (Vanholder et al. 1991). Also the phagocytosis of *Candida albicans* of HD patients treated with biocompatible membranes has been shown to be similar to that of controls (Anding et al. 2003). However, compared to the pre-D patients, the PMNL of HD patients were significantly less able to phagocytose *Staphylococcus aureus* or to produce ROS (Sardenberg et al. 2006).

Among HD patients, there is a substantial loss of naïve T cells compared to pre-D patients (Litjens et al. 2006), and the cytokine pattern is changed towards Th1 dominance (Descamps-Latscha et al. 1995; Litjens et al. 2006). HD patients' T cell proliferation is lower than that of pre-D patients with moderately decreased creatinine clearance (Ankersmit et al. 2001). In a prospective analysis, in maximally uremic pre-D patients the T cell proliferation response is diminished but it is clearly repaired when HD treatment is initiated (Kaul et al. 2000). There is a defect in T cells of HD

patients in co-operation with the accessory cells in signalling via the receptors CD28 and CD46 (Girndt et al. 1993; Brinkkoetter et al. 2005). The proportion of T cells committed to activation-induced cell death is substantially higher in HD vs pre-D (Ankersmit et al. 2001). All these changes occur presumably due to pre-activation of the cells in HD patients (Girndt et al. 1993; Ankersmit et al. 2001; Brinkkoetter et al. 2005).

In a prospective analysis, the lymphocyte proliferation response increases regardless of the membrane used when HD therapy is initiated in a uremic state (Kaul et al. 2000). However, PBMC proliferation response is better with the use of polysulphone as compared to cuprophan membranes (Degiannis et al. 1990).

#### *6.2.3.2 Hemodialysis versus peritoneal dialysis*

The PD treatment is considered to be a more physiological means of removal of uremic retention molecules than the intermittent HD, because the patient's own peritoneum functions continuously as the dialyser membrane. The production of ROS and phagocytosis of PMNL are decreased in HD patients, being only 27% of the antigen stimulated ROS-production, 41% of the mitogen (PMA) stimulated ROS-production and 70% of the phagocytosis of the PD patients (Sardenberg et al. 2006). The apoptosis of PD patients' PMNL is similar to that of controls, while in HD patients it is increased (Sela et al. 2005; Sardenberg et al. 2006).

With PD therapy, complement activation is weaker (Brinkkoetter et al. 2005), and Th1 type cytokine secreting cells are less overexpressed (Zamauskaite et al. 1999; Nitta et al. 2002; Ando et al. 2005) than in HD therapy. The plasma levels of soluble TNF- $\alpha$  receptors are significantly lower in PD (Pereira et al. 1994; Moser et al. 2003), even though plasma levels of TNF- $\alpha$  are similar in PD and HD patients (Pereira et al. 1994; Descamps-Latscha et al. 1995). The numbers of T<sub>h</sub> and T<sub>s</sub> cells are higher in PD patients (Moser et al. 2003). In a study of cytokines and PBMC activation markers (soluble CD25 for T cell activation, sCD23 for B cell activation and neopterin for monocytes), the combined results suggested more severe dysfunction in the immune system among HD patients, compared to PD or pre-D patients (Descamps-Latscha et al. 1995). There is less apoptosis of PBMC in PD than in HD or pre-D therapy (Martin-Malo et al. 2000; Moser et al. 2003).

Beyer et al. found that influenza vaccination of PD patients resulted in better RR, PR and mean fold titre increase than in HD patients, so that A/H3N2 and A/H1N1 PR were significantly better in PD. Also mean fold titre increase values for the test antigens were significantly higher in PD (A/H3N2 almost significantly) (Beyer et al. 1987) These results have been confirmed in a later study (Cavdar et al. 2003).

#### 6.2.4 Metabolism of parathyroid hormone

A profound defect in immune function is caused by an impaired metabolism of calcium and phosphate and subsequent SHPT. The effects of PTH have

been studied *in vitro* directly on lymphocytes in healthy subjects and in CKD, and also by observing the effects of PTX in SHPT or primary hyperparathyroidism. PTH enhances PHA-induced T cell response in healthy subjects, but not in HD patients (Alexiewicz et al. 1990a). PTH inhibits the B-cell proliferation response of both healthy subjects and HD patients (Alexiewicz et al. 1990b). As the result of excess amounts and action of PTH in patients with CKD, an elevated level of cytosolic calcium  $[Ca^{2+}]_i$  is seen, which is associated with impaired cellular immune responses (Alexiewicz et al. 1996; Ori et al. 1999). The effect of experimental NTX and PTX has been studied in rats with vaccination with SRBC, BSA and influenza vaccine (Gaciong et al. 1991). Expectedly, rats with CKD had reduced vaccination responses, but those that also underwent PTX, had responses equal or almost equal to the normal control rats (Gaciong et al. 1991). A defect in PMNL phagocytosis of CKD rats can likewise be corrected with PTX (Chervu et al. 1992).

Immune responses have also been studied before and after PTX in prospective studies of patients with *primary hyperparathyroidism* without CKD. Before PTX, T cell proliferation with PHA and Con-A was inhibited compared to that of healthy subjects, and these changes were normalized one month after PTX (Shasha et al. 1989). Similarly, lymphocyte response to PHA was reduced before PTX, but was restored to the level of control subjects six months after PTX (Kotzmann et al. 1998). However, unlike in SHPT, the high PTH (and calcium) levels of patients with primary hyperparathyroidism did not affect immunoglobulin production or lymphocyte

numbers before or after PTX (Kotzmann et al. 1998). When the lymphoproliferative response to PHA was studied *in HD patients with SHPT* who underwent PTX, a significant increase in proliferation was found four months after PTX, compared to the pre-PTX proliferation response (Tzanno-Martins et al. 2000).

A two-month oral treatment with calcium channel blocker nifedipine in HD patients showed that their B-cell proliferation significantly increased as  $[Ca^{2+}]_i$  levels decreased, and the effects were abolished two months since cessation of nifedipine medication (Alexiewicz et al. 1996). The PMNL glucose uptake is also improved with another calcium channel blocker nitrendipine or calcitriol treatments *in vitro* (Haag-Weber et al. 1993).

### 6.2.5 Vitamin D

Vitamin D is an essential fat-soluble nutrient and sunlight-derived hormone influencing bone and mineral metabolism, cell differentiation, inhibition of cell growth, immunomodulation, and control of other hormonal systems. Rickets is a bone and neural disorder caused by severe vitamin D deficiency, which has been recognised in the 17th century (Smerdon 1950; O'Riordan 2006), and associated with lack of sunlight and cured with cod liver oil in the 19th century (Hess 1922; McCollum et al. 2002). Vitamin D deficiency is common worldwide and especially among people living in higher latitudes where sunshine has a longer tangential path (Webb et al. 1988), as well as among elderly housebound medical inpatients (Thomas et al. 1998). The consequences of clinical hypovitaminosis D in adults include

osteopenia, osteomalacia and muscle weakness. Even subclinical vitamin D deficiency also increases the risk of getting fractures and developing cancer (Ahonen et al. 2000; Welsh 2007) or diseases such as type 1 and 2 diabetes or multiple sclerosis (Hypponen et al. 2001; Munger et al. 2004; Mattila et al. 2007; de Boer et al. 2008). Low serum levels of 1,25-(OH)<sub>2</sub>D<sub>3</sub> have been associated with increased mortality in pre-D patients (Inaguma et al. 2008) and also within 90 days of starting HD treatment (Wolf et al. 2007).

Calcitriol, the active vitamin D metabolite (Holick et al. 1971) is produced by hydroxylation in kidneys and liver from calcidiol (25-hydroxycholecalciferol or 25-(OH)-D). Patients with CKD stage 4-5 have a pronouncedly diminished serum calcitriol level. After the finding of receptors for calcitriol on monocytes and activated lymphocytes, it was shown that it had immunoregulatory actions (Provvedini et al. 1983; Tsoukas et al. 1984; Lemire 1992). In healthy individuals, calcitriol promotes maturation of monocytes/macrophages (Manolagas et al. 1989), and it rather enhances than suppresses their antimicrobial functions. However, it inhibits normal T-lymphocyte proliferation and IL-2 production starting from quite low concentrations 10<sup>-12</sup> M (0,42 ng/l) (Rigby et al. 1984) or 10<sup>-11</sup> M (4,2 ng/l) (Tsoukas et al. 1984; Lemire et al. 1985), and it inhibits the production of other lymphokines as well.

Calcitriol is locally produced in cells such as macrophages and in several tissues such as brain, colon, prostate and breast. This production controls cell growth, cellular differentiation, apoptosis and angiogenesis, being important in inhibiting hyperplasia and malignant transformation (Dusso et

al. 2005; Deeb et al. 2007; Welsh 2007). Calcitriol regulates these functions by modulating genomic events via its nuclear receptor, the vitamin D receptor (VDR). In addition to the genomic actions, calcitriol is also able to generate rapid (seconds-minutes) biological responses, which do not require any protein synthesis as slower (minutes-hours-days) genomic actions do (Baran et al. 1994; Norman et al. 1999). These rapid responses seem not to be needed for inhibition of cell proliferation, rather they can modulate the genomic VDR actions (Norman et al. 1999; Dusso et al. 2005). Natural polymorphisms in VDR gene do not contribute to calcitriol-induced inhibition of PHA-stimulated PBMC proliferation in healthy subjects (Colin et al. 2000). Because of the antiproliferative and immunosuppressive effects, vitamin D and its active metabolites and analogues have been studied and used for cell-regulation in autoimmunity, transplantation, dermatology and oncology (Rigby et al. 1984; Manolagas et al. 1989; Casteels et al. 1995; Lemire 2000; Dusso et al. 2005; Deeb et al. 2007).

Vitamin D analogues (such as oral  $1\text{-}\alpha\text{-(OH)D}_3$ ) have been used to correct vitamin D deficiency and to control for excess PTH secretion and renal osteodystrophy. In dialysis patients, oral  $1\text{-}\alpha\text{-(OH)D}_3$  has been shown to enhance depressed PBMC proliferation responses and to restore them nearly to the normal level (Tabata et al. 1986). Orally administered vitamin D derivatives have been shown to enhance IL-2 secretion (Tabata et al. 1988), PMA-induced IL-1 and IL-6 secretion (Riancho et al. 1993) and LPS-induced TNF- $\alpha$  production (Haran et al. 1994) of PBMC. Treatment of HD patients for four weeks with oral calcitriol improved their monocyte superoxide

production and bactericidal capacity (but not PMNL function) (Hubel et al. 1991). Any clear suppression of immune functions of HD patients resulting from *oral* vitamin D therapy has not been shown.

In cell cultures, the addition of calcitriol concentrations up to  $10^{-11}$ M (4,2 ng/l) augmented PHA-induced proliferation response of HD patients, but supraphysiological concentrations ( $10^{-9}$ M- $10^{-7}$ M; 420 ng/l - 42 $\mu$ g/l) inhibited the mitogen response both in HD patients' and normal persons' cells (Zarrabeitia et al. 1990). With intravenous (i.v.) *in vivo* pulse calcitriol therapy supraphysiological concentrations of up to  $10^{-9}$ M (420 ng/l) could be momentarily reached (Slatopolsky et al. 1984; Salusky et al. 1990). In contrast to *in vitro* studies, the effect on HD patients' lymphocyte responses *in vivo* has been shown to be neutral or immune enhancing (Antonen et al. 1996).

#### 6.2.6 Renal anemia and erythropoietin treatment

Erythropoietin (Epo) is a hormone produced by the kidneys. It stimulates red blood cell production in bone marrow. Patients with impaired kidney function have diminished Epo production, which leads to anemia. Recombinant human Epo and its functional analogues are routinely used to correct renal anemia instead of former blood transfusions (Winearls et al. 1986; Eschbach et al. 1987). Epo therapy of pre-D and dialysis patients has cardiovascular and life quality advantages (Jungers et al. 2001; Silverberg et al. 2001). Epo maintains organ function by increasing the oxygen supply through its



erythropoiesis-stimulating action, but Epo has recently been shown to have pleiotropic actions of its own in cerebral stroke, congestive heart failure and chronic kidney disease (Nangaku et al. 2007). It has been shown to improve wound-healing (Haroon et al. 2003), at least partially mediated by Epo-stimulated angiogenesis (Ribatti et al. 1999).

Epo treatment might affect the immune system since it binds to receptors in human bone marrow mononuclear cells (Hoshino et al. 1989). The hypoxia-inducible factor-1 (HIF-1) is a nuclear factor that activates the erythropoietin gene, quickly disintegrates in the presence of oxygen, and relates to tumor hypoxia, angiogenesis and proliferation (Semenza et al. 1991; Jaakkola et al. 2001; Semenza 2007). HIF-1 has also been shown to regulate (B) lymphocyte development and to control autoimmunity (Kojima et al. 2002). Effective erythropoiesis needs iron, and Epo therapy can result in functional or true iron deficiency. Activated and proliferating cells need iron, and the function of PMNL and natural killer cells is defective in iron deficiency, as well as T cell numbers and IL-2 production (Oppenheimer 2001). The effects of Epo therapy on immune functions or nutrient and energy metabolism in CKD are not very well known.

With Epo treatment of dialysis patients, controversial cellular immune responses have been shown. The phagocyte activity is improved with Epo therapy (Veys et al. 1992; Huraib et al. 1997). In lymphocyte stimulation tests, most studies indicate an initial suppression of stimulation responses coinciding with rapid correction of anemia (1-3 months), and a later

improvement (6-18 months) (Pfaffl et al. 1988; Grimm et al. 1990; Barany et al. 1992; Steffensen et al. 1996). In a few studies there has been an increase of cell-mediated response only (Singh et al. 1992; Shurtz-Swirski et al. 1996). The effects on humoral immunity have been shown to be favourable: Epo treatment increases the responses to hepatitis B (Sennesael et al. 1991) and tetanus (Birmingham et al. 1996) vaccinations. It increases the general immunoglobulin production of B-cells (Schaefer et al. 1992). Soluble CD23, a marker of B cell activation and functional status, is initially elevated although later reduced (Altun et al. 1999).

## 7. AIMS OF THE STUDY

The aim of the study was to evaluate the current immunological status of patients with chronic kidney disease (CKD). The underlying hypothesis was that carefully adjusted treatment of coexisting conditions, anemia and secondary hyperparathyroidism, may have a positive influence on the immune function. The specific aims were:

1. To evaluate the protection rate and response rate to influenza vaccination and the repertoire of antibodies obtained by single vaccination against wild influenza viruses in different stages of CKD **(I-II)**.
2. To examine calcitriol *in vitro* on antigen proliferation response of HD patients' lymphocytes **(III)**.
3. To examine the effects of high calcium diet on tetanus vaccination response in rats with hyperphosphatemia and secondary hyperparathyroidism caused by experimental kidney failure **(IV)**.
4. To evaluate effects of Epo *in vivo* on lymphocyte proliferation and basic antibody levels in pre-D patients **(V)**

## 8. SUBJECTS AND METHODS

### 8.1 Human subjects

The human studies (**I-III** and **V**) involved a total of 136 subjects, out of whom 77 were HD patients, 15 were on PD treatment, and 40 were pre-D patients (Table 2).

Influenza vaccination response was assessed in studies **I-II**. In them, altogether 77 study subjects were involved: a subgroup of 23 HD patients from **I** took part in **II**. There were 31 cardiac control patients in **I**, out of whom 26 were analysed in **II**. Additionally, 26 military conscripts who had suffered from a confirmed influenza A infection, were included in **II** (Table 2). Of the dialysis patients most (86%) had been vaccinated against influenza earlier. The median serum creatinine of the pre-D patients was 221  $\mu\text{mol/l}$  (range 137-540  $\mu\text{mol/l}$ ). The clinical characteristics are shown in Table 3.

In **III** the immunological effects of calcitriol *in vitro* were investigated. Twelve HD patients were involved, out of whom 4 were females and 8 were males. Half of the patients (n=7) were the same as the HD patients in **I**. Their mean age was 58.5 (range 22-77) years. Two test control subjects were involved.

The effects of Epo *in vivo* were investigated in **V** in 24 pre-D patients (one of whom took part in I), of whom 13 were females and 11 were males. Their mean age was 59 (range 26-88) years and they had moderate-to-severe CKD with mean serum creatinine 393 (range 125-847)  $\mu\text{mol/l}$ . They had anemia with a mean hemoglobin of 102 g/l.

*Table 2.* The patients and controls in studies **I-III** and **V**

		<b>I</b>	<b>II</b>	<b>III</b>	<b>V</b>
	HD	42	23	12	
Patients	PD	15			
	Pre-dialysis	20			24
	Cardiac patients	31	26		
Controls	Military conscripts		26		
	Healthy subjects			2	

*Table 3.* The age and sex distribution of patients in study **I**

	<b>HD</b>	<b>PD</b>	<b>Pre-D</b>	<b>Cardiac</b>
Mean age, years	56	55	48	62
Age range, years	20-79	34-79	24-69	33-79
Sex (F/M)	12/30	7/8	6/14	12/19

## 8.2 Animals

Sprague-Dawley rats were involved in the animal study. Out of the total of 55 young male rats, 29 were made uremic by a partial NTX and 26 were non-uremic sham-operated controls. Forty-nine rats were included in the analyses in the study.

*Table 4.* Rat subgroups in study **IV** according to diet

	<b>High calcium (3%) diet</b>	<b>Control calcium (0.3%) diet</b>
5/6-nephrectomy	11	16
Sham operation	8	14

## 8.3 Study protocols

### 8.3.1 Studies I-II

In study I, all subjects were vaccinated against influenza, and antibody titres were measured from serum samples obtained before vaccination and at five weeks (Figure 1). Also 10-week samples were taken, but the 5-week samples were chosen for the analyses, because an outbreak of influenza made the 10-week samples unreliable for the analyses, and because a 5-week period is known to be long enough to evoke a maximal antibody

response (Pyhälä et al. 1994). Creatinine was measured from the initial serum samples of all the study subjects. The blood count and levels of serum albumin, ionized calcium, phosphate, calcitriol and intact PTH were also measured from the dialysis patients. In study II, the antibody responses of HD patients were tested against drift variant virus antigens from five consecutive seasons (1995-2000), and compared to those of control groups (figure 2).

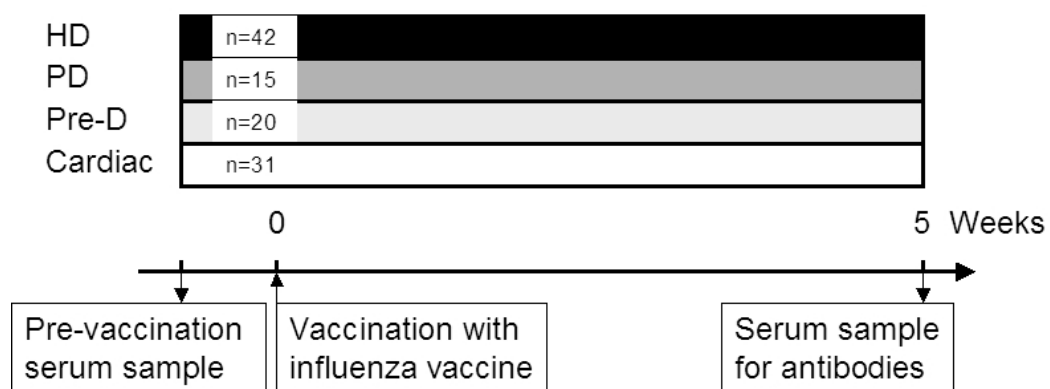


Figure 1. Protocol of study I.

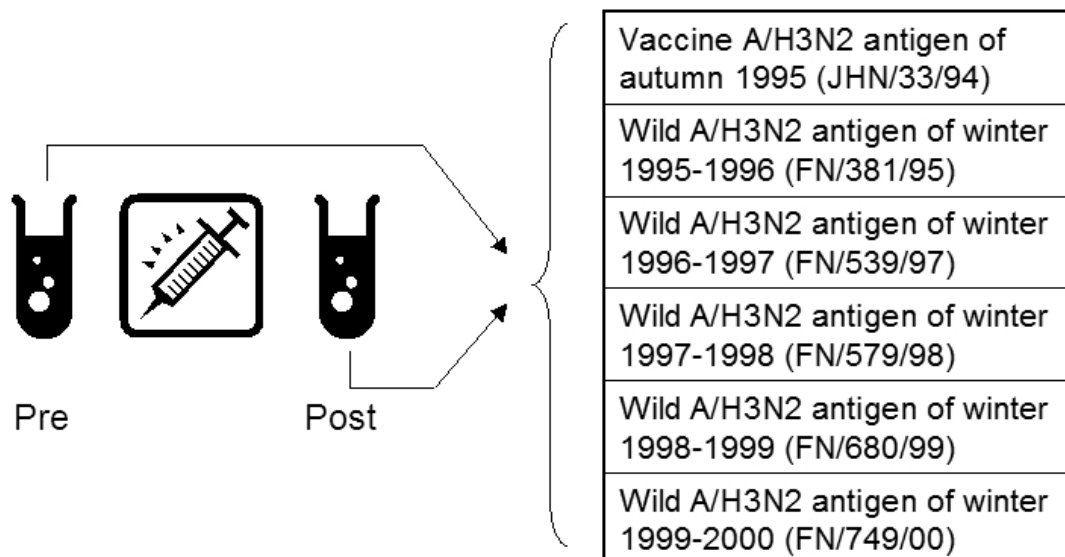


Figure 2. Protocol of study II. The pre- and post-vaccination sera obtained in autumn 1995 were tested against influenza antigens for cross-reactivity.

### 8.3.2 Study III

In study III, peripheral blood mononuclear cells (PBMC) of dialysis patients were tested for lymphocyte antigen proliferation, and the effect of *in vitro* constant and intermittent (pulsed) calcitriol (1,25-(OH)<sub>2</sub>D<sub>3</sub>) on the lymphocyte antigen response was studied. On day 1, PBMC cultures were generated with or without antigens using constant calcitriol concentration (of 0, 42 and 105 ng/l). On day five the culture medium was displaced and replaced with a pulsing medium, i.e. a medium with a high concentration (315 ng/l) of calcitriol for two hours. After re-centrifugation, the pulsing media were replaced with the original culture media and the antigen proliferation tests were continued until labeling and harvesting.



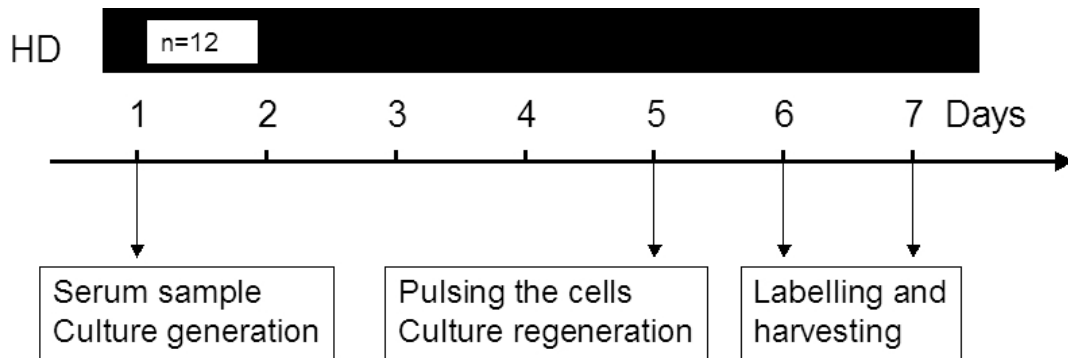


Figure 3. Protocol of study III

### 8.3.3 Study IV

In study IV the tetanus response was evaluated in NTX rats, which were fed either a control (0.3%, n=30) or a high (3%, n=19) calcium diet. The rats underwent 5/6-NTX, and 6 weeks thereafter vaccination against tetanus. Their responses were compared with control rats with the same diets, but normal renal function. Creatinine, hemoglobin, PTH, ionized calcium, phosphate, 1,25-(OH)<sub>2</sub>D<sub>3</sub> and weight were measured, and the effect on the vaccination response was calculated.

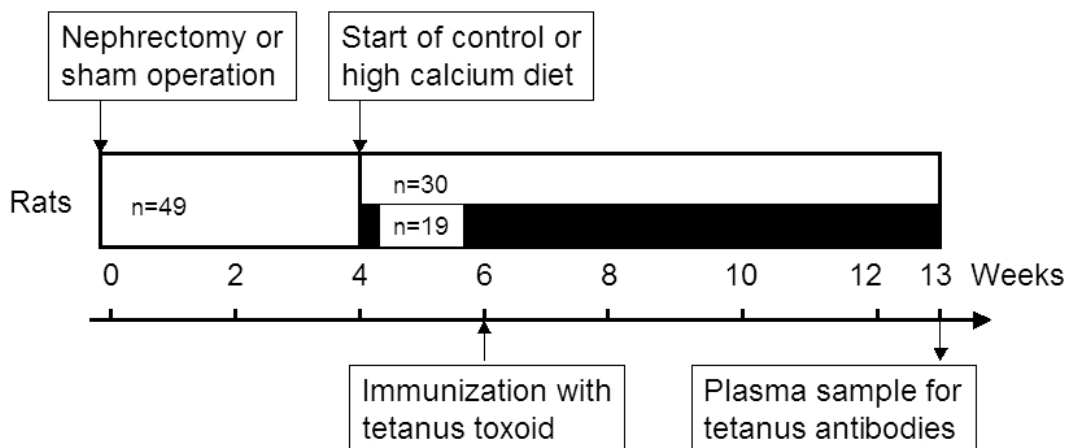


Figure 4. Protocol of study IV

### 8.3.4 Study V

In study **V**, pre-D patients with a moderate renal insufficiency and anemia who were to be treated with Epo were investigated before and three months after the start of the Epo treatment. Those with a ferritin level of  $<200 \mu\text{g/l}$  and/or transferrin saturation  $<20\%$  were given i.v. bi-weekly injections of polynuclear iron (III) hydroxide saccharate starting at one month after the initiation of Epo. The immunological tests (PBMC proliferation tests, subclass distribution analyses and measurements for EBV and CMV antibody titres) were performed. Blood samples which reflected the reticulocyte expansion, GFR change and iron status were collected at initiation, at two weeks, one month, two months and at three months.

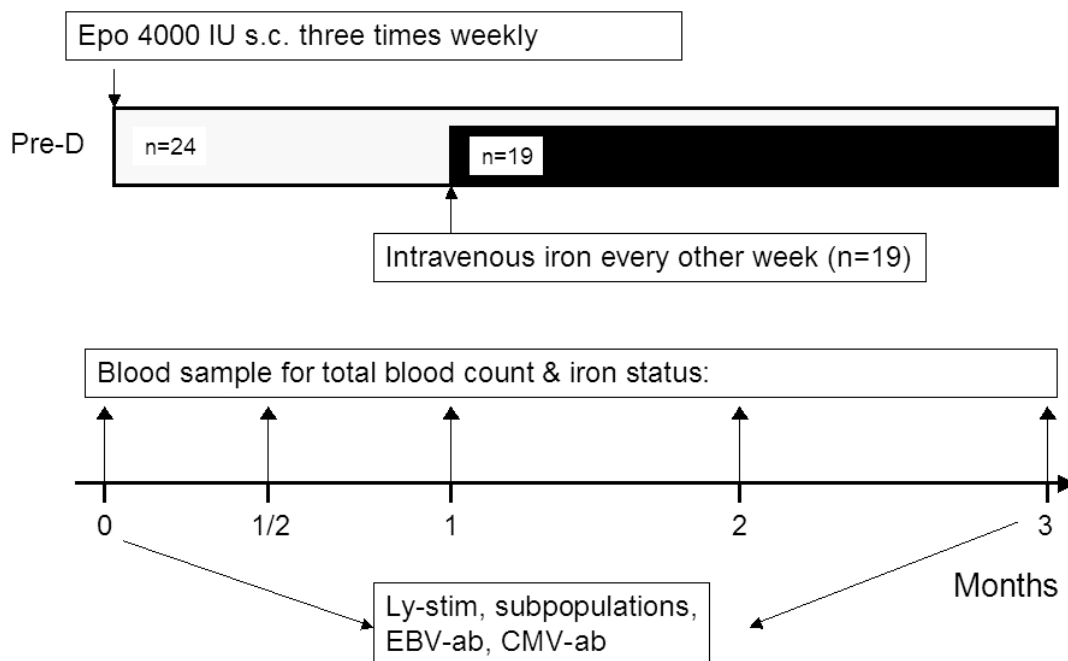


Figure 5. Protocol of study **V**. Ly-stim = lymphocyte proliferation test, Subpopulations = lymphocyte subclass distribution, ab = antibodies, s.c. = subcutaneously.

## 8.4 Vaccinations and antibody measurements

All the subjects in study **I** were vaccinated with a commercially available inactivated trivalent influenza vaccine (containing antigens of the subtypes A/H1N1, A/H3N2 and B) in the autumn of 1995. For antibody measurements in study **I**, the sera were treated with *Vibrio Cholerae* filtrate and studied for hemagglutination-inhibiting (HI) antibodies at dilutions from 1:10 onwards (Pyhälä et al. 1994), using goose erythrocytes and antigens of the vaccine virus subtypes A/H1N1, A/H3N2 and B. Stored aliquots of the sera of HD patients were used in study **II**, in which also similarly treated sera of military conscripts were used.

In the animal study (**IV**), the rats were immunized with TT emulsified with Complete Freund's Adjuvant (3:7), so that each rat received a 100ul (1.5Lf) vaccine intramuscularly. The tetanus antitoxin concentrations were determined in the National Control Laboratory of Vaccines and Sera. The antitoxin concentrations were measured from plasma samples with a double layer antigen ELISA test, which detects protecting, mainly type IgG tetanus antibodies (Kristiansen et al. 1997).

In study **V**, antibodies to EBV and CMV were measured with commercial kits. These are two common viruses that most people obtain antibodies against after encountering these viruses usually in youth. A low-normal basic level of antibodies was assumed, because no antigenic stimulation

(infection) was expected. The IgG-class antibodies before and after a three-month treatment with Epo were evaluated.

## 8.5 Antigen preparation and sequence studies

The influenza antigen preparations and also the nucleotide sequence studies for **I-II** were done in the National Public Health Institute of Finland. For study **I**, two vaccine virus strains and one other strain, antigenically related to the third vaccine virus component, were cultivated in embryonated eggs and they served as antigens in the HI tests. For study **II**, A/H3N2 antigens were prepared, representing the vaccine strain and live influenza virus strains from five consecutive seasons. The vaccine virus strains were cultivated in embryonated eggs. The live influenza virus drift variants had been isolated and cultivated in epithelial-like canine cell line MDCK cultures. Antigenic relationships of the vaccine virus and epidemic viruses were analysed in the HI tests using rat antiserum (Pyhälä et al. 2001b). RNA from the vaccine virus and the five drift variants was extracted and used for cDNA preparation, to be amplified and sequenced for the variable HA1 domain of the virus hemagglutinin (Pyhälä et al. 2001a).

## 8.6 Lymphocyte stimulations and the *in vitro* addition of calcitriol

In study III, blood samples from HD patients were drawn preceding a dialysis session. Peripheral blood mononuclear cells were separated using the Ficoll-Hypaque layer centrifugation method (Boyum 1968). After washing, cell cultures were generated on U-bottom microtitre plates using culture medium (RPMI) and 10% autologous (own) plasma with 100000 cells in a volume of 100  $\mu$ l per well. The cultures were stimulated with the antigens PPD (0, 12.5, 25 or 50 mg/l) or TT (0, 1000, 5000 or 10000 limits of flocculation per liter, LF/l).

Calcitriol was applied during the culture generation phase on day one at a physiological (42ng/l) or supraphysiological (105 ng/l) level. These calcitriol concentrations mimick the *in vivo* normal to high concentrations reached with oral vitamin D analogue treatment of SHPT (Zarrabeitia et al. 1990). Control cultures were stimulated but not supplied with calcitriol. At culture generation, the plates were generated as two identical duplicates. Each cell culture was generated in triplicate wells per plate. The identical plates were treated on the fifth culture day with either the culture medium only, or with calcitriol in a high concentration (final concentration being 315 ng/l), corresponding the mean serum level reached during first two hours post i.v. calcitriol treatment (Salusky et al. 1990). Before pulse treatment, the original culture medium (supernatant) was removed temporarily by centrifugation. The pulse-medium was added for a two-hour incubation time

and washed off by another centrifugation. The culture was then regenerated and grown for another two days using the original culture media. The cells were labelled with tritiated thymidine on the sixth culture day and the stimulation response was measured on the seventh culture day as the intake of the radiolabel.

In study **V**, peripheral mononuclear cells, after separating from blood using the Ficoll-Hypaque layer centrifugation, were cultured 10000 cells in a volume of 100  $\mu$ l per well and stimulated with mitogens PHA & PWM and antigens PPD & TT. Cell cultures were harvested and the stimulation responses measured on the third (PHA) or on the seventh (PWM, PPD, TT) culture day. In this study, the lymphocyte subclass analyses were made for B and NK cells, and the T cells were analysed for the surface antigens CD3, CD4, CD8, HLA-DR, CD45RO and CD38.

## 8.7 Nephrectomy

The surgical procedures of the animals were performed by the co-authors from the Department of Pharmacological Sciences in University of Tampere. At the age of eight weeks, male Sprague-Dawley rats were subjected to either uremia-inducing 5/6-NTX or to sham operation, in which both kidneys were decapsulated. In 5/6- NTX, the upper and lower poles of the left kidney were cut off, and the right kidney was removed (Ylitalo et al. 1976). The surgery was performed under anesthesia and antibiotic and analgesic

treatments were given after the procedure. For blood sampling, the carotid artery was cannulated under anesthesia.

## 8.8 Statistics

In all the studies, the significance level was set at  $p < 0.05$ . All  $p$  values are two-tailed. In study **I**, differences in the antibody concentrations between HD, PD, pre-D and control patients were analysed with the non-parametric Mann-Whitney U-test (between two groups), while in study **II**, differences between the HD, cardiac (control) and military conscript groups in the antibody titres were analysed with Kruskal-Wallis test (between several groups). The differences in the PR were tested with the Chi-square test, and the pre- and post-vaccination titres were evaluated for difference with the Signed-Rank test. The factors explaining the post-vaccination titres were analysed with multiple regression.

In study **III**, analysis of variance was used, in which calcitriol pulse treatment and antigen concentration were used as fixed factors (classified variables) and patient as a block factor that takes into account that samples were dependent within patients. The statistics were analysed in Tampere School of Public Health.

In study **IV**, 10-based logarithmic conversions of tetanus antitoxin concentrations were analysed with the Mann-Whitney U-test for uremic versus control rats, or oneway analysis of variance with LSD test as a post-

hoc method for comparisons between the four groups (uremic and control rats with high calcium or control calcium diets). A simple factorial analysis of variance was performed to study the NTX and the diet as fixed factors to explain the tetanus vaccination response. Bivariate correlation analysis was used to study factors affecting the tetanus response of the uremic rats.

In study **V**, the differences in values obtained at the start and at three months were analysed with the Signed-Rank test. Leukocyte numbers at five points during the study were analysed with a general linear model. The difference (change) variables (calculated by subtracting the start value from the three-month-value) were analysed with Mann-Whitney U-test. Bivariate correlation was used to study the factors explaining the difference variables. The statistics were analysed in Tampere School of Public Health.

## 8.9 Permissions and ethical aspects

All the subjects in the human studies gave their written informed consent to participate in the studies, which were all approved by the local ethical committee. The studies were performed in compliance with the Helsinki Declaration. The experimental design of the animal study was approved by the Animal Experimentation Committee of the University of Tampere and the Department of Social Affairs and Health in the Provincial Government of Western Finland.



## 9. RESULTS

### 9.1 Influenza vaccination response in CKD

The protection obtained with the influenza vaccination was measured as antibody (HI) titre levels five weeks after vaccination. The RR of HD and PD patients were modest, as their pre-vaccination antibody levels were high. This is because as many as 86% of dialysis patients while only 10% of pre-D and cardiac patients had been vaccinated earlier. Yet the post-vaccination titres did not differ between any of the groups: against A/H3N2 they were 84 (HD), 84 (pre-D) and 96% (PD) of the controls' titres. Only the HD patients' post-vaccination titres against influenza B antigen were lower than those of the others' (being 78% of the controls',  $p=0.002$ ).

Among the patients, the PD patients most abundantly (in 67%) attained a protective level of 1.6 (log) against A/H3N2-antigen. Thirty-six percent of HD, 35% of pre-D and 61% of control patients reached the protective level against A/H3N2. The protective level was reached even more frequently against influenza A/H1N1: 80%, 60%, 70% and 71% and influenza B: 80%, 76%, 90% and 97% (PD, HD, pre-D and controls, respectively).

Age, sex, dialysis duration or albumin level did not explain the antibody response. Only calcitriol treatment almost significantly ( $p=0.06$ ) explained the response. In fact, every HD patient (100%) treated with i.v. calcitriol reached an antibody titre considered protective against influenza B, 50% against A/H1N1 and 83% against A/H3N2. The corresponding percentages were only 68, 27 and 50% for those HD patients without vitamin D treatment. Patients with a protective level for at least two out of three antigens were considered responders.

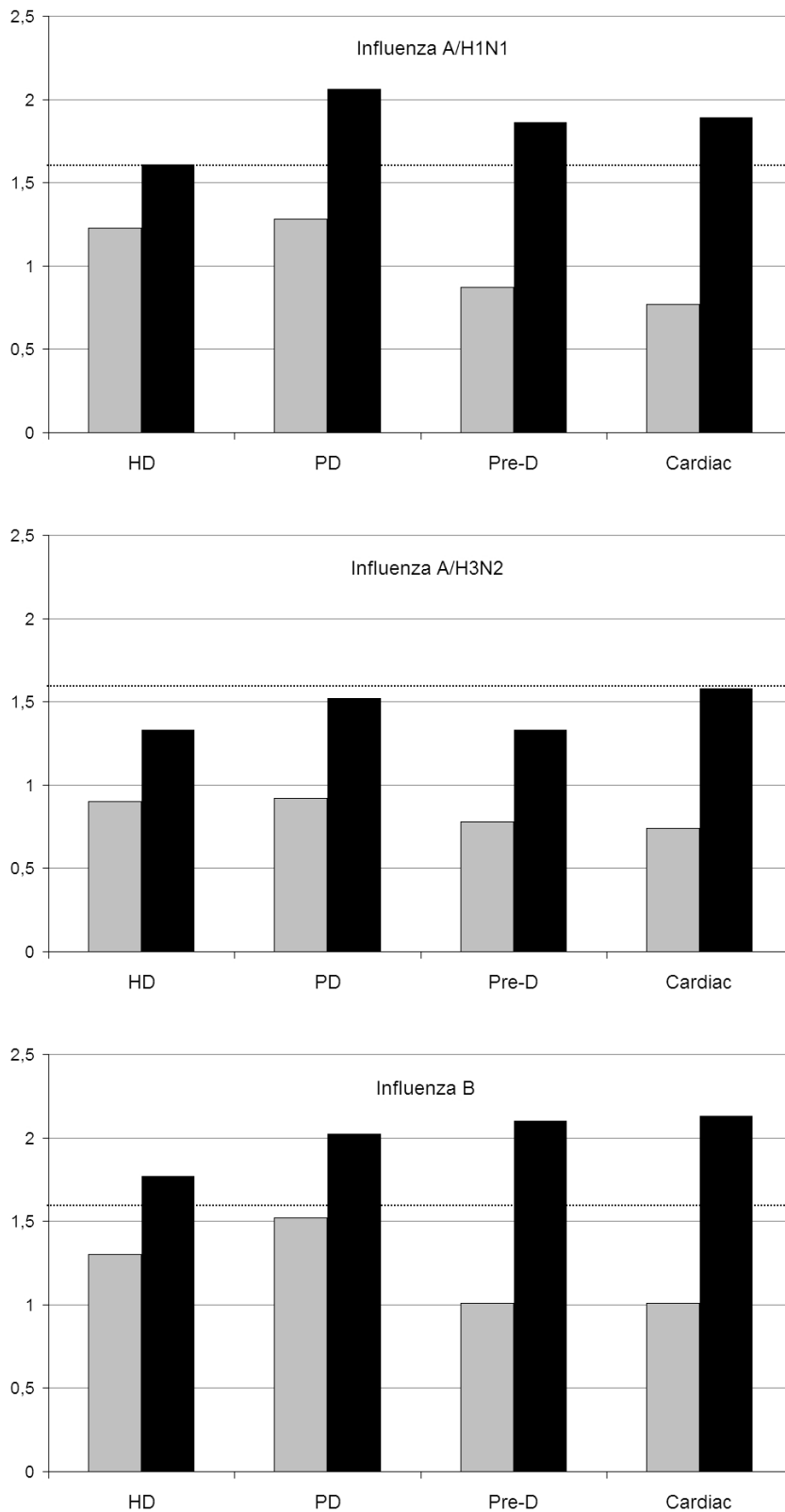


Figure 6. The mean pre- (gray) and postvaccination (black) HI titres against the influenza A/H1N1, A/H3N2 and influenza B in HD, PD, pre-D and cardiac patients (controls). The protective level (dotted line) was 1.6 (log).

## 9.2 Continuity of the influenza vaccination response

The anti-influenza antibodies in the post-vaccination samples of the HD patients were also able to react with future years' antigens. In fact, the cross-reactive antibody levels of HD patients were as high as those of the vaccinated cardiac patients or the convalescent phase military conscripts, who had a confirmed influenza A infection during the same year the patients were vaccinated. There was a yearly decline in reactivity among all three groups until the last season's virus isolate (FN/749/00). According to nucleotide sequences, there were a growing number of amino acid differences in the antigen (in the HA1 domain of the virus hemagglutinin) between the vaccine virus of autumn 1995 (JHN/33/99) and the drift variants. The last years' wild type virus antigen was more similar to the vaccine virus, which explains why the reactivity of the antibodies in the 1995 sera was stronger in all study groups against the latest drift variant antigen (FN/749/00).

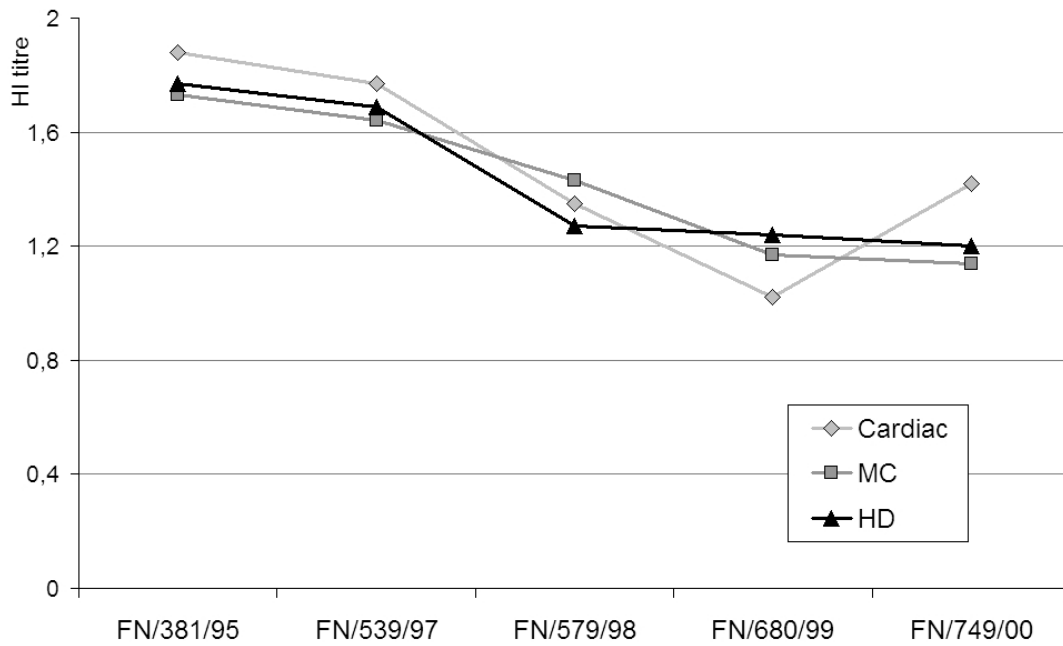


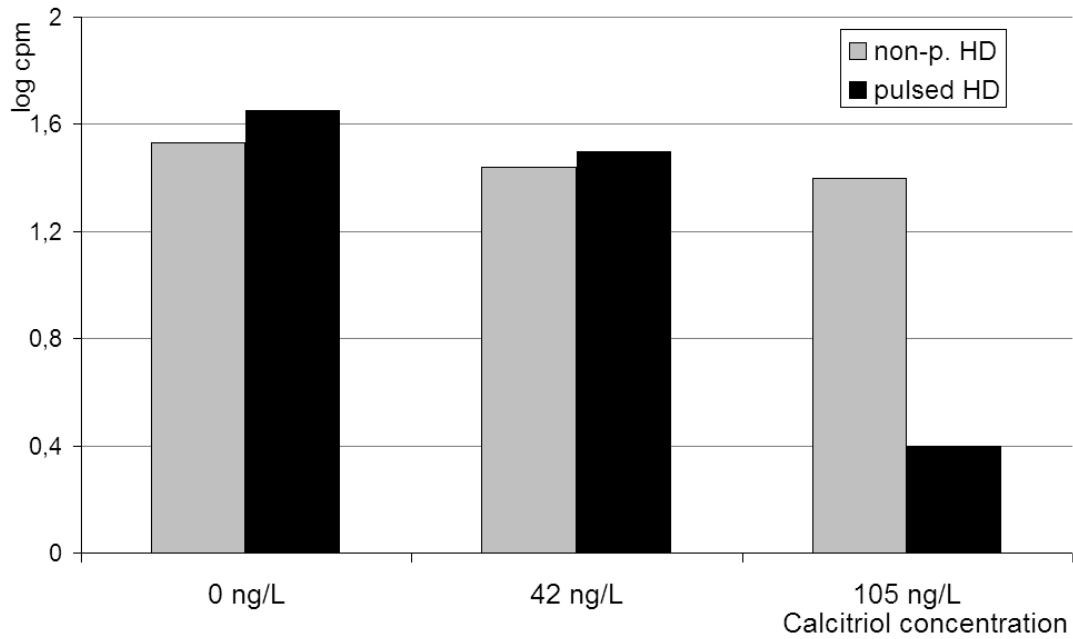
Figure 7. The cross-reacting antibodies in each individuals' post-vaccination serum samples to subsequent years' influenza A/H3N2 antigens, obtained with a single influenza vaccination the first year. MC = military conscripts, HD = hemodialysis patients.

Table 5. The influenza response (HI titres in post-vaccination sera) in percent of the controls (the cardiac patients).

Influenza A/H3N2 specific antigen	Pre-D	HD	PD
Vaccine virus 1995 (JHN/33/94)	84%	84%	96%
Drift variant of season 1995-6 (FN/381/95)		94%	
Drift variant of season 1996-7 (FN/539/97)		95%	
Drift variant of season 1997-8 (FN/579/98)		94%	
Drift variant of season 1998-9 (FN/680/99)		103%	
Drift variant of season 1999-2000 (FN/749/00)		85%	

### 9.3 Calcitriol and the lymphocyte stimulation response of dialysis patients

The lymphocytes of HD patients were pulse treated with high dose calcitriol, and/or incubated with constant calcitriol in physiological or supraphysiological concentrations. The HD patients' lymphocyte proliferation responses to PPD or TT did not increase with the addition of constant calcitriol in the culture medium. In fact the tetanus antigen-induced proliferation response of the constant calcitriol treated cells of HD patients was inversely related to the calcitriol concentration ( $p=0.001$ ); the higher the calcitriol concentration, the lower the response. The cells that were pulse-treated had a (non-significantly) higher response than the constant calcitriol treated cells: the response was 1.89 (non-pulsed) and 1.94 (pulse-treated) log cpm for PPD (mean of the three PPD concentrations), and 1.53 (non-pulsed) and 1.65 (pulse-treated) log cpm for TT (mean of the three TT concentrations). Intermittent but not constant calcitriol thus showed a beneficial trend and was not immunosuppressive on the antigen proliferation *in vitro*. Only the pulsing of the HD patients' lymphocytes that were incubated with the supraphysiological concentration of constant calcitriol clearly lowered the lymphocyte stimulation response (Figure 8).



*Figure 8.* The TT-induced lymphocyte stimulation responses (mean log cpm) of hemodialysis (HD) patients (n=12) in cultures containing 0 pmol/l, 42 ng/l or 105 ng/l of calcitriol. Duplicate cultures of each were additionally pulse-treated with calcitriol (pulsed) and half of the cultures were left non-pulsed (non-p).

#### 9.4 Calcium and the tetanus response of nephrectomized rats

The results of the tetanus vaccination of the NTX and sham-operated rats are shown in Table 6. The NTX rats that were fed with a high calcium diet (Ca-NTX) obtained a tetanus antibody response that was not statistically different from that of the healthy non-uremic (Ca-Sham) rats. The high calcium diet in Ca-NTX rats thus ameliorated the suppression of the tetanus vaccination response seen in the NTX rats with a control calcium diet.

The Ca-NTX and NTX groups' antitoxin levels did not differ. The uremic rats' mean (regardless of diet; both groups combined) antitoxin concentration was 55% in proportion of that of non-uremic control rats. The mean antitoxin level of Ca-NTX rats was 75% in proportion of the mean of Sham groups while the mean concentration of NTX rats was only 42 percent of that of the non-uremic Sham rats. There was an inverse correlation between the tetanus vaccination response and creatinine ( $p=0.002$ ), phosphate ( $p=0.02$ ) and PTH ( $p=0.03$ ). There was also a correlation between creatinine and 1,25-(OH)<sub>2</sub>D<sub>3</sub> (inversely,  $p=0.003$ ), PTH (directly,  $p=0.007$ ) and phosphate (directly,  $p=0.01$ ).

*Table 6.* Mean rat tetanus antitoxin concentrations (log of mean concentration).

NTX= 5/6-NTX rats. Sham= placebo-operated rats, including both high and control calcium diet groups.

	<b>N</b>	<b>Log of mean</b>	<b>SD</b>	<b>p vs Sham</b>
NTX rats	16	0.25	0.32	0.005
Ca-NTX rats	11	0.45	0.44	0.122
Sham rats	22	0.60	0.24	



## 9.5 Erythropoietin treatment in predialysis patients

Epo treatment of initially anemic pre-D patients was associated with a decrease in lymphocyte number ( $p=0.006$ ) and all main lymphocyte subpopulations. The proliferation responses all decreased:  $-0.37$  log cpm (PHA,  $p=0.001$ ),  $-0.19$  log cpm (PWM,  $p=0.073$ ),  $-0.29$  log cpm (PPD,  $p=0.046$ ) and  $-0.42$  log cpm (TT,  $p=0.039$ ). Only the absolute number of memory cells and the cells with activation markers remained equal and their proportion therefore increased. Of the 24 pre-D patients, five did not need i.v. iron supplementation at one month's control due to sufficient ferritin and TSAT levels. The iron-supplemented patients and those not treated did not differ in any parameter that was followed up (blood count or markers of iron status) in statistical analyses.

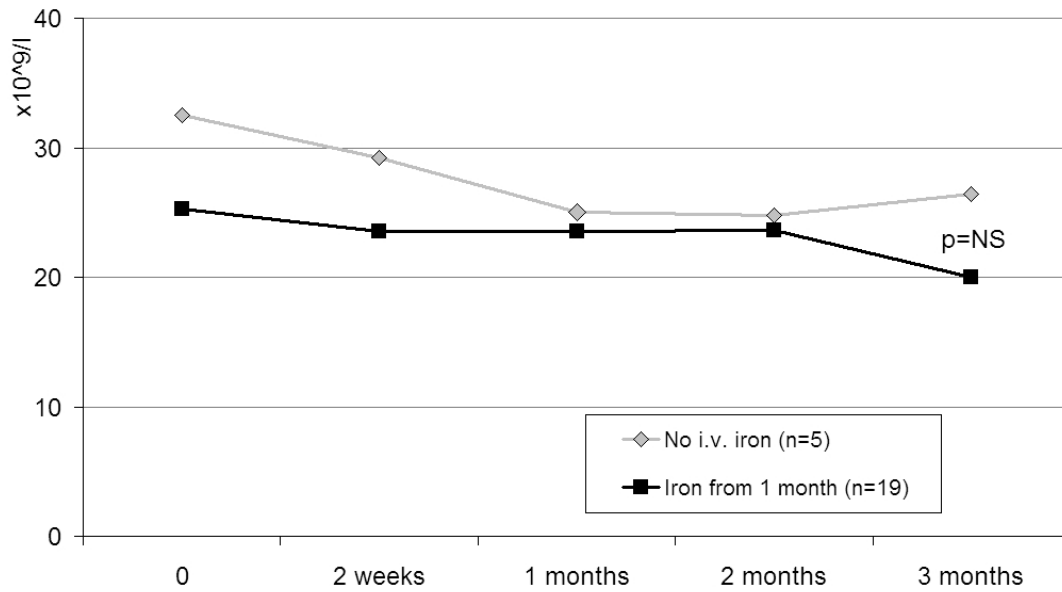


Figure 9. The mean lymphocyte count ( $\times 10^9 / l$ ) of pre-D patients who started Epo treatment. There was no difference between the iron-treated and non-treated individuals (Mann-Whitney U-test). The decline in lymphocyte counts (start value vs 3 months value) was significant ( $p=0.006$ , Wilcoxon's signed ranks test).

The level of IgG class antibodies against CMV and EBV in sera, reflecting non-stimulated basal secretory capacity of B cells, did not change during the three months of treatment with Epo.

## 10. DISCUSSION

Chronic kidney disease (CKD) is becoming increasingly common as the incidence of type 2 diabetes is advancing. There is an immune deficiency related to CKD, which becomes prevalent already in the early stages of CKD. Even the main causes of death in patients with CKD are cardiovascular and infectious diseases, both of which are processes linked with immune function. There are several factors, which contribute to the immune deficiency, many of them being organic compounds and peptides that are retained due to impaired renal function as uremic toxins. Secondary hyperparathyroidism (SHPT) and decreased calcitriol and erythropoietin (Epo) production probably have a prominent role in the immune deficiency. Vaccination of patients with CKD is widely recommended, but the prevention of infections by vaccination is challenging, as the clinical responses to vaccinations have been shown to be sub-optimal among patients with CKD.

### 10.1 Influenza vaccination

We studied the vaccination response to a trivalent (A/H3N2, A/H1N1, B) influenza vaccine in dialysis and pre-D patients. Most dialysis patients (86%)

had been vaccinated earlier. This is much more than in an American study of vaccination frequency, in which up to 49% of dialysis patients had received influenza vaccination (Gilbertson et al. 2003). The RR are often low among groups that have received earlier vaccinations (Cavdar et al. 2003; Vogtlander et al. 2004), like in our study. While the RR was modest, however the proportion of patients that reached a protective antibody level (titre of 1.6 log), i.e. PR, can be considered good. Among all study groups, the lowest PR's were achieved with the A/H3N2 vaccine antigen, and the highest with the influenza B vaccine antigen. In HD patients, the PR's were 36-76% against the different antigens. This is comparable to HD patients' PR (46-87%) in earlier studies (Beyer et al. 1987; Cavdar et al. 2003; Vogtlander et al. 2004).

It has previously been shown that patients on PD have better RR and PR than HD patients (Beyer et al. 1987; Cavdar et al. 2003). Similarly in our study the PD patients reached the highest post-vaccination titres, despite their high pre-vaccination titres due to earlier vaccinations. It is typical that HD patients have clearly weaker vaccination responses than PD and pre-D patients (Beyer et al. 1987; Cavdar et al. 2003). However, even the HD patients' post-vaccination titres did not differ from those of cardiac patients or the other groups (except for B antigen). Regular re-vaccinations could thus contribute to an even better postvaccination protection. In a study of two sequential seasonal influenza vaccinations of children and teenagers with CKD it was also shown that pre-vaccination antibody levels did not become chronically elevated after the first vaccination, and yet the PR even

increased after the second vaccination (Brydak et al. 2000). The efficiency of the repeated influenza vaccinations is not weakened among elderly people either (Gross et al. 1995). Our results are thus in line with these findings, and speak for an increased vaccination-induced protection among patients with CKD. The protective response obtained with the single vaccination was also maintained against several subsequent years' epidemic wild viruses, i.e. drift variants, that had been isolated from patients during the subsequent years, and which were thus capable of causing an epidemic. The post-vaccination titres of HD patients were only slightly less than the titres of cardiac patients, and even comparable to those of the military conscripts having suffered from influenza A. We have thus shown that influenza vaccination of CKD patients leads to an improvement in immune protection against wild influenza viruses.

When we evaluated factors affecting the antibody response of the HD patients, none of the parameters including age, sex, dialysis duration or albumin level explained the antibody production. In fact, these parameters have not uniformly been shown to be explanatory factors either in large studies on hepatitis B vaccination of pre-D (DaRoza et al. 2003; Chow et al. 2006) or dialysis (mainly PD) patients (Chow et al. 2006). Calcitriol treatment in HD patients nearly significantly explained the antibody response of the HD patients ( $p=0.06$ ). Every HD patient who had i.v. calcitriol therapy reached a protective antibody titre against influenza B, and 50% against A/H1N1 and 83% against A/H3N2. The PD or pre-D patients did not receive i.v. calcitriol. A beneficial effect of HD patients' i.v. calcitriol treatment has earlier been

shown in lymphocyte antigen proliferation (Antonen et al. 1996) but such findings have not previously been made concerning vaccination results.

## 10.2 Calcitriol and the lymphocyte antigen response

Vitamin D derivatives can be used since the early stages as treatment of SHPT. There are limitations for an extensive use because of a risk of hyperphosphatemia and hypercalcemia. HD patients have suppressed immunity on the other hand, and reduced serum active vitamin D metabolite levels on the other, which is thought to add to their immune deficiency. Only clearly supraphysiological concentrations of calcitriol have been shown to be immunosuppressive in HD patients *in vitro* (Zarrabeitia et al. 1990). There are therefore arguments for vitamin D or calcitriol therapy in patients with CKD: it can be used to correct the hormonal deficiency (to increase muscular strength and bone quality), and also to control the overproduction of PTH caused by hyperphosphatemia and even to directly regulate several immune functions.

Because of the beneficial *in vivo* effect of i.v. calcitriol treatment on the antigen response of HD patients particularly devoid of 1,25-(OH)<sub>2</sub>D<sub>3</sub> shown by our group before (Antonen et al. 1996), we studied the lymphoproliferative response of HD patients to TT and PPD with an *in vitro* method. The concurrent effects of constantly physiological or supraphysiological calcitriol level in culture conditions with intermittently high calcitriol pulse-treatment were studied. Intermittent administration of calcitriol

as a two-hour pulse did not have any significant effect on the responses. Calcitriol affects activated lymphocytes: it would be of interest to find out whether an earlier pulsing time point would have affected the response, but the study settings allowed us to use day five only as the pulsing time point. Nevertheless, all pulse-treated cultures (those that had been grown with continuous calcitriol in culture medium and those grown without it), showed a (non-significant) trend towards an increase in the response.

The antigen responses of dialysis patients have earlier been evaluated in an academic dissertation (Huttunen 1985). In that study the PPD-induced response was only 59% of that of the controls, while in our study, the lymphoproliferative response against PPD or TT seemed not to be much reduced at all, compared to the response of two healthy test control individuals. The HD patients' initially well-preserved responses resembled those of healthy individuals in earlier studies in that their TT-induced proliferation responses decreased in constantly calcitriol-enriched medium.

### 10.3 Phosphate binding and tetanus response

The treatment of SHPT and subsequent intracellular hypercalcemia with PTX or calcitriol normalize or improve the immune functions in CKD (Gaciong et al. 1991; Chervu et al. 1992; Haag-Weber et al. 1993; Alexiewicz et al. 1996). Intracellular hypercalcemia can lead to chronic inflammation-linked calcification even in subjects with normal kidney function, which can paradoxically be relieved with sufficient calcium intake:

extracellular *hypocalcemia* can associate with intracellular *hypercalcemia* (Arvola et al. 1993; Massry et al. 1993; Lijnen et al. 1995; Ori et al. 1999; Fujita et al. 2000). Phosphate-binding calcium salts are used to prevent uremia-associated hyperphosphatemia and SHPT (Teruel et al. 1999), and because a high calcium diet beneficially decreases lymphocyte intracellular free calcium concentration in rats (Wuorela et al. 1992), we investigated the effect of increased calcium carbonate intake on tetanus vaccination response in subtotally NTX rats.

The “pre-dialysis phase” uremic rats in our study had a moderate but significant renal failure. The severity of CKD was correlated to the impairment of tetanus vaccination response, and kidney function significantly anticipated the tetanus response in analysis of variance, as well. In earlier studies with uremic rats, suppressed antigen responses in NTX rats have been reported (Raskova et al. 1973; Gaciong et al. 1991), but a correlation has not been earlier demonstrated in well-controlled experimental studies between declining GFR and antibody production. In an early human study, an association between declining GFR and tetanus response has been observed (Byron et al. 1976).

Factors other than GFR that explained the uremia-related depression in the tetanus antibody response were plasma phosphate and intact PTH levels. These factors have not before been demonstrated either to lie behind or to inversely correlate with immune suppression of CKD. Beneficial immunological actions of treatment of SHPT are known (Gaciong et al.



1991), but there are no studies other than ours on the effects of phosphate binding with calcium carbonate on immune functions in CKD. Our rodent model enabled to control the phosphate ingestion, a situation that is very difficult to obtain in human studies. The phosphate level was in fact the lowest of all groups in uremic animals on high calcium diet, which fact speaks for a good phosphate binding effect of calcium carbonate.

The main result was that the tetanus antitoxin response of uremic rats on high calcium diet (Ca-NTX) was as high as 75% of that of sham-controls ( $p=0.005$ ). These findings are equal with those obtained by PTX of uremic (NTX) rats (Gaciong et al. 1991). In that study the anti-SRBC response (AUC of 20 days of HA titres) of PTX-NTX rats was 89% of that of normal rats, and the difference was statistically non-significant except for only the latest study day (day 20) when the anti-SRBC response became significantly lower ( $p<0.05$ ) (Gaciong et al. 1991). The immunization results (ELISA titres) of the PTX-NTX rats (on day 20) with anti-BSA-IgG (92%), BSA-IgM (58%), influenza-A-IgG (82%) and influenza-A-IgM (99.6%) were not different from those of the normal rats, except for anti-IgM-BSA ( $p<0.01$ ). Just with increased calcium carbonate intake in our study it was possible to obtain immunization results almost comparable to PTX.

The NTX rats on control calcium diet had the lowest response, which was only 42% of that of the sham-operated control animals. The responses of the non-parathyroidectomized NTX rats in the study of Gaciong et al. were similarly low: anti-SRBC (58%), anti-BSA-IgG (28%), anti-BSA-IgM (16%),

anti-influenza A-IgG (46%) and anti-influenza A-IgM (40%) of the response of the controls (Gaciong et al. 1991). In their study, these were always statistically significantly lower than the PTX-NTX rats' responses, whereas in our study the difference between NTX and Ca-NTX rats was not statistically significant. In our study, when immunized, the NTX rats had a more pronounced kidney failure (creatinine 93-97 mmol/l) compared to those of the study in comparison (creatinine 61-92 mmol/l). Also the time span from immunization to blood testing was longer in our study (7 versus 3 weeks). In fact, even in that study, the difference between NTX and PTX-NTX rats became non-significant towards the end of the study (anti-SRBC on day 20). The creatinine level in our study was found to be the essential explaining factor of antibody response to tetanus antitoxin in analysis of variance ( $p=0.019$ ), although both kidney function and the diet together anticipated the response in analysis of variance ( $p=0.011$ ). Both methods, i.e. PTX and high calcium diet are very effective in lowering PTH levels in a setting in which phosphate intake can be regulated.

#### 10.4 Erythropoietin and immunity

Correction of anemia with Epo treatment caused a significant increase in hemoglobin and the reticulocyte count in pre-D patients, but a decrease in lymphocytes and no change in neutrophils. There were no differences between iron-supplemented and non-supplemented patients in any of the parameters that were studied (except for ferritin and TSAT at one month, which was the time point for those with low ferritin or TSAT to start receiving

iron). Anemia causes a state of tissue hypoxia, and the correction of anemia with the initiation of Epo therapy causes changes in the nutritional environment and the iron and energy metabolism. The changes in the proportions of the blood cells probably reflect the coincident metabolic changes. We also saw a clear decrease in lymphocyte proliferation responses induced by mitogens and antigens – only the response to the B-cell mitogen PWM less clearly decreased ( $p=0.073$ ). Because hypoxia-inducible factor 1 (HIF-1 $\alpha$ ) deficient B cells have a proliferation defect and abnormal development (Kojima et al. 2002), it is possible that hypoxia-inducible factors, increased erythropoiesis, and the decrease in cell proliferation we observed, are interrelated.

The serum IgG class antibodies against EBV and CMV were low, thus antigenic stimulation or CMV- or EBV-infection was ruled out. Epo did not cause any change in basal serum antibody levels in three months. This could reflect the fact that immunoglobulin production is relatively well conserved in CKD (Degiannis et al. 1987; Beaman et al. 1989). These results are in line with previous studies in HD patients regarding B cell function (Grimm et al. 1990; Barany et al. 1992; Steffensen et al. 1996).

When evaluating factors affecting the change of lymphocyte count or proliferation responses, only transferrin level positively correlated with the decline of the mitogens. The reason for this is unknown to us, but perhaps it relates to a shift in energy metabolism towards anabolism caused by stimulation of erythropoiesis. There was a slight decrease in GFR

( $p=0.055$ ) during the three months, which did not correlate with the change in lymphocyte stimulation either. The possible role of the iron status can only be guessed by our results: as none of our patients had received blood transfusions in the preceding months of the study, it is likely that no iron overload could blunt the proliferation responses. There are no previous studies in which immune function and iron and Epo treatment are evaluated together in pre-D patients. T cells expressing activation markers increased, which could reflect the rise in transferrin receptor (=CD71, an activation marker of lymphocytes as well), which also acts as a marker of functional iron deficiency and erythropoiesis. The HIF-1 function in similar situations is also unknown.

# 11. SUMMARY AND CONCLUSIONS

We investigated the degree of immune deficiency in chronic kidney disease and showed that the influenza vaccination response of patients with CKD was similar to that of the controls (cardiac patients). Depending on the influenza vaccine subtype antigen used, a high percentage (from 37 to 76%) of HD patients became protected, which was not much less than with pre-D, PD or the control patients. Despite a trend of being a little lower, the HD patients' postvaccination titres did not significantly differ from titres of the other groups. Vaccination response was especially well preserved in PD patients. These results are similar or even slightly better than those shown in earlier studies (Beyer et al. 1987; Cavdar et al. 2003). The vaccination-induced antibodies were able to react against five subsequent years' epidemic A-virus antigens: the cross-reactivity was of the same magnitude in HD patients as in military conscripts in the convalescent phase of influenza A infection. The HD patients' cross-reactivity was also similar to the cardiac patients' cross-reactivity, being 85-103% (mean 94%) of the titres. Thus, vaccination of HD patients leads to an efficient immune protection against wild influenza viruses.

We observed that every HD patient, who had i.v. calcitriol therapy, reached a protective antibody titre against influenza B, 50% against influenza

A/H1N1 and 83% against A/H3N2. In multiple regression analysis, calcitriol therapy was the only factor borderline significantly explaining the antibody response ( $p=0.06$ ) among the HD patients.

In a previous work by our group calcitriol *in vivo* enhanced lymphocyte function of HD patients with low serum  $1,25\text{-(OH)}_2\text{D}_3$ . We now studied calcitriol *in vitro* treatment in lymphocyte PPD- and TT-antigen proliferation of HD patients, and observed that incubation of the lymphocytes continuously with calcitriol during the antigen-stimulation was immunosuppressive to TT response. Nevertheless, *in vitro* intermittently administered pulse treatment with calcitriol during the antigen-stimulation slightly (but non-significantly) enhanced the antigen response. Thus, the effect of calcitriol was inhibitory to hemodialysis patients' cells in the same way as to normal lymphocytes (Colin et al. 2000), but when given as a two-hour pulse this immunosuppressive effect could not be seen.

Prevention of the immunologically detrimental actions of secondary hyperparathyroidism (SHPT) could be possible by an early control of hyperphosphatemia. We showed that phosphate binding with calcium carbonate could ameliorate the reduced tetanus vaccination response in uremic rats. A high calcium diet efficiently inhibited PTH levels from increasing after a 5/6-nephrectomy (NTX) of rats, and the tetanus vaccination response of these rats was comparable to that of sham-operated rats with a normal kidney function. The 5/6-NTX uremic rats with a control diet had significantly lower responses than the sham-operated rats.

The severity of CKD correlated with the impairment of tetanus vaccination response, and the combination of nephrectomy and diet also statistically explained the tetanus response. Our findings that the prevention of SHPT in uremic rats has positive effects to the antibody response are in line with previous findings obtained with parathyroidectomy of rats (Gaciong et al. 1991).

Anemia due to a lack of erythropoietin (Epo) production from the kidneys leads to a state of tissue hypoxia and energy depletion, but the correction of anemia with Epo has not uniformly been shown to have beneficial immunological effects. When studying the immune effects of Epo in pre-D patients we observed changes in cellular immunity simultaneously with the correction of anemia during the three-month study: lymphopenia, and as a separate phenomenon, a decrease in lymphocyte mitogen and antigen proliferation responses. A minute decrease in GFR during the study did not explain the change in lymphocyte stimulations caused by Epo: only transferrin level positively correlated with the decline in the mitogen responses. Coincident changes in nutritional environment and energy metabolism possibly contribute to the changes that we observed and probably not Epo alone. The erythropoietin treatment -related suppression in lymphocyte stimulation responses and the decrease in lymphocyte number are in line with previous studies (Barany et al. 1992; Steffensen et al. 1996).

*We conclude* that the immune functions of regularly monitored patients with all stages of CKD are relatively well preserved. Their influenza vaccination responses to vaccine antigens are almost comparable to controls. The HD patients' cross-reacting antibody responses against isolated wild virus antigens are high and actually their influenza vaccination response seemed to improve with *in vivo* calcitriol. *In vitro* intermittent calcitriol has a trend-like ameliorating effect on HD patients' lymphocyte proliferation, and constant calcitriol has a suppressive effect on TT-induced responses. Phosphate binding with calcium carbonate, to treat and prevent secondary hyperparathyroidism, ameliorates the immune suppressing effect that associates with low GFR in partially nephrectomized uremic rats. Erythropoietin (Epo), and possibly metabolic changes related to the initiation of Epo treatment, induce lymphopenia and decrease the lymphocyte proliferation responses in anemic pre-D patients.

The clinical immune responses are thus not disturbed by calcitriol therapy that can safely be used in patients with CKD. Despite the decrease in lymphocyte proliferation, we still believe that Epo treatment is probably clinically immunologically safe. In chronic kidney diseases, the defects in cellular immunity probably contribute to the clearly less pronounced defects seen in humoral immunity and the vaccination response.



*Table 7.* Summary of the immune deficiency in chronic kidney disease in the studies I-V (column on the left). HD = hemodialysis patients, Subgroup = HD patients with different vitamin D treatments, PD = peritoneal dialysis patients, Pre-D = predialysis patients. Cell-mediated immunity: lymphocyte proliferation test. Humoral immunity: vaccination response to influenza or tetanus. Results of the influenza vaccination: both the relation of the postvaccination titre to the controls, and the proportion (%) of the patients who reached protective titres ( $\geq 1.6$ ) against two of the vaccine antigens A/H1N1, A/H3N2 or B are shown. Results of the tetanus vaccination: the relation (of the postvaccination titre) of 5/6-nephrectomised to the sham-operated rats is shown.

	<b>Patients</b>	<b>n</b>	<b>Treatment</b>	<b>Cell-mediated immunity</b>	<b>Humoral immunity</b>
	HD	42			Influenza: similar to controls, 55% protected
	<i>Subgroup</i>	6	<i>Intravenous 1,25(OH)<sub>2</sub>D<sub>3</sub></i>		<i>83% protected</i>
	<i>Subgroup</i>	14	<i>Oral 1<math>\alpha</math>-OH-D<sub>3</sub></i>		<i>57% protected</i>
I-II	<i>Subgroup</i>	22	<i>No vitamin D substitution</i>		<i>45% protected</i>
	PD	15			Influenza: similar to controls, 87% protected
	Pre-D	20			Influenza: similar to controls, 70% protected
		12	Constant 1,25(OH) <sub>2</sub> D <sub>3</sub>	Decreased	
III	HD	12	Pulsed 1,25(OH) <sub>2</sub> D <sub>3</sub>	Not changed (trend: higher)	
		11	High calcium carbonate diet		Tetanus: similar to controls
IV	Uremic rats	16	Control calcium diet		Tetanus: Lower (p=0.005)
V	Pre-D	24	Subcutaneous Epo	Decreased	

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Tampere, July 2009

A handwritten signature in cursive script, appearing to read "Panu".

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# Adequate Seroreponse to Influenza Vaccination in Dialysis Patients

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## Key Words

Vaccination · Influenza · Dialysis · Calcitriol

## Abstract

**Background:** Hemodialysis (HD) patients are immunocompromised, and they have been shown to react suboptimally to recommended vaccinations. Advances in dialysis therapy and other supportive measures may theoretically result in better immune system functions. Clinical evidence supporting this theory has, however, not been presented. With influenza vaccination response, we tried to address this question. **Methods:** 42 HD and 15 continuous ambulatory peritoneal dialysis (CAPD) patients were vaccinated with a trivalent influenza vaccine, and the seroresponses at 5 weeks were measured. The results were compared with those of similarly vaccinated 20 nephrology outpatient clinic patients with varying degrees of renal insufficiency and those of 31 cardiac patients with normal renal function. **Results:** The dialysis patients had higher prevaccination titers of hemagglutination-inhibiting (HI) antibodies to all three vaccine virus antigens than the other groups due to more frequent previous vaccinations. The dialysis patients exhibited lower antibody increases, but an almost comparable proportion of them reached a protective antibody level (HI titers

≥ 40) 5 weeks after vaccination [A/H3N2: 61% (cardiac patients), 35% (nephrology outpatient clinic patients), 67% (CAPD), and 36% (HD); A/H1N1: 71, 70, 80 and 60; B: 97, 90, 80, and 76%, respectively]. Among the HD group, all patients receiving parenteral calcitriol except 1 (83%), but only 50% of the other HD patients produced protective antibody titers at least to two out of three vaccine virus antigens. No other patient- or HD treatment-associated parameter was significantly related to the vaccination-induced antibody response. **Conclusions:** We conclude that influenza vaccination of dialysis patients according to current recommendations may be effective. Additionally, our results suggest that parenteral calcitriol treatment may augment the immune response of HD patients even in a clinically relevant way, an effect so far shown only in in vitro studies.

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## Introduction

Influenza vaccination is generally recommended to dialysis patients and even to patients with milder forms of renal impairment [1], although the vaccination responses have been shown to be suboptimal to hepatitis B, influenza, and pneumococcus [2–4]. In adult dialysis patients the

**Table 1.** Clinical characteristics of the 42 HD and the 15 CAPD patients

	HD		CAPD	
	mean $\pm$ SD <sup>a</sup>	range	mean $\pm$ SD <sup>a</sup>	range
Age, years	56 $\pm$ 15	20–79	55 $\pm$ 14	34–79
Female/male ratio	12/30		7/8	
Dialysis duration, months	29 $\pm$ 27	1–136	24 $\pm$ 17	3–59
Kt/V	1.30 $\pm$ 0.19	0.84–1.98	2.13 $\pm$ 0.60	1.50–3.59
Plasma intact parathyroid hormone, pmol/l	20.3 $\pm$ 26.5	1.1–116.1	19.5 $\pm$ 22.7	1.0–65.9
Serum ionized calcium, mmol/l	1.25 $\pm$ 0.08	1.09–1.45	1.26 $\pm$ 0.07	1.11–1.39
Serum phosphate, mmol/l	1.65 $\pm$ 0.41	0.87–2.55	1.50 $\pm$ 0.29	0.99–1.94
Serum 1,25-(OH) <sub>2</sub> D <sub>3</sub> , pmol/l	36 (median)	<20–75	34 (median)	<20–49
Serum albumin, g/l	35 $\pm$ 3	28–43	34 $\pm$ 7	20–43

The nephrological diagnosis of the HD patients: chronic glomerulonephritis 13, diabetic nephropathy 12, hypertensive nephropathy 4, chronic interstitial nephritis 4, polycystic kidney disease 4, miscellaneous 5; those of CAPD patients: chronic glomerulonephritis 5, diabetic nephropathy 7, hypertensive nephropathy 2, miscellaneous 1.

<sup>a</sup> Values are expressed as mean  $\pm$  SD if not otherwise stated.

effect of influenza vaccination has, however, not been evaluated during the advanced dialysis therapy of today.

Patients with impaired renal function are prone to infections which, again, are the main reason for hospitalization and the second most common cause of death [5, 6]. The host defense mechanisms are widely affected in uremia both by factors related to end-stage renal disease and by dialysis therapy [5]. Advances in hemodialysis (HD) treatment, such as the use of more biocompatible dialysate membranes and erythropoietin treatment, have been shown to positively influence in vitro immune functions [7, 8], but no clinical data supporting these findings have been presented. Previous findings suggest that, in addition to positive effects on calcium-phosphorus metabolism and the prevention of the secondary hyperparathyroidism, intravenous calcitriol treatment may be immunologically advantageous to HD patients [9, 10].

The purpose of this study was to evaluate the present efficacy of influenza vaccination in dialysis patients and to find out factors contributing to vaccination-induced antibody responses.

## Patients and Methods

### Patients and Controls

Forty-two HD patients and 15 patients on continuous ambulatory peritoneal dialysis (CAPD) were, in autumn of 1995, vaccinated against influenza. Commercially available inactivated trivalent vaccine (Vaxigrip®; Pasteur Merieux Serums et Vaccins) containing 15  $\mu$ g of antigen from the component strains A/Johannesburg/33/94

(H3N2), A/Texas/36/91 (H1N1), and B/Harbin/7/94 was administered into the deltoid muscle according to standard recommendations. Twenty nephrology outpatient clinic patients (NOP; mean age 48, range 24–69 years; 6 women, 14 men) with varying degrees of renal function impairment [serum creatinine median 221  $\mu$ mol/l (2.50 mg/dl) and range 137–540  $\mu$ mol/l (1.55–6.10 mg/dl)] and 31 cardiac patients (mean age 62, range 33–79 years; 12 women, 19 men) with normal serum creatinine were similarly vaccinated. Patients receiving immunosuppressive therapy were excluded. The clinical characteristics of the dialysis patients are shown in table 1. All the HD patients were dialyzed with synthetic biocompatible (polysulfone, cellulose acetate, cuprammonium) dialysis membranes. Some HD patients were, because of secondary hyperparathyroidism, treated with calcitriol (6 with intravenous calcitriol, Calcijex®, Abbott 2–4  $\mu$ g/week, and 14 with oral calcitriol, Etalpha®, Lövens, 0.25–0.50  $\mu$ g/day). The doses were unchanged 1 month before vaccination and during the study period. Serum 1,25-(OH)<sub>2</sub>D<sub>3</sub> (pmol/l) and plasma intact parathyroid hormone (pmol/l) were 27.7  $\pm$  14.1 and 14.2  $\pm$  15.5 (no calcitriol), 37.9  $\pm$  14.6 and 11.5  $\pm$  10.6 (peroral calcitriol), and 35.2  $\pm$  9.3 and 63.2  $\pm$  43.2 (intravenous calcitriol), respectively. 34 HD and 8 CAPD patients were treated with erythropoietin (Eprex®, Janssen-Cilag). No dialysis patient had hepatitis B or C infection. HD patients' major infections (those requiring hospitalization) were recorded for 18 months after the study. The data concerning previous influenza vaccinations were based on information given by patients. All patients gave their informed consent, and the study was approved by the local ethical committee.

### Influenza Antibody Titers

Blood samples for influenza antibody determinations were collected at the time of vaccination and 5 and 10 weeks thereafter. The sera were treated with *Vibrio cholerae* filtrate and studied for hemagglutination-inhibiting (HI) antibodies at dilutions from 1:10 onwards as presented previously [11]. Two vaccine virus strains (A/Johannesburg/33/94 and B/Harbin/7/94) cultivated in embryonated eggs served as antigens in the HI tests. The third strain in the tests was

**Table 2.** Prevacination and 5-week postvaccination influenza antibody titers (log, mean  $\pm$  SD) in cardiac patients (n = 31), in NOP (n = 20), in CAPD patients (n = 15), and in HD patients (n = 42)

Vaccine virus antigen	Cardiac		NOP		CAPD		HD
<i>Prevaccination titers</i>							
A/H3N2	0.74 $\pm$ 0.13	0.01*	0.78 $\pm$ 0.24	0.06	0.92 $\pm$ 0.35	NS	0.90 $\pm$ 0.34
A/H1N1	0.77 $\pm$ 0.20	<0.0001	0.87 $\pm$ 0.27	0.001	1.28 $\pm$ 0.50	NS	1.23 $\pm$ 0.43
B	1.01 $\pm$ 0.34	0.006	1.01 $\pm$ 0.37	0.02	1.52 $\pm$ 0.62	NS	1.30 $\pm$ 0.46
<i>Postvaccination titers (and x-fold antibody rises)</i>							
A/H3N2	1.58 $\pm$ 0.64 (2.1 $\times$ )	NS*	1.33 $\pm$ 0.50 (1.7 $\times$ )	NS	1.52 $\pm$ 0.54 (1.7 $\times$ )	NS	1.33 $\pm$ 0.44 (1.5 $\times$ )
A/H1N1	1.89 $\pm$ 0.77 (2.5 $\times$ )	NS	1.86 $\pm$ 0.63 (2.1 $\times$ )	NS	2.06 $\pm$ 0.54 (1.6 $\times$ )	0.02	1.61 $\pm$ 0.50 (1.3 $\times$ )
B	2.13 $\pm$ 0.58 (2.1 $\times$ )	0.002	2.10 $\pm$ 0.56 (2.1 $\times$ )	0.01	2.02 $\pm$ 0.61 (1.3 $\times$ )	0.06	1.77 $\pm$ 0.50 (1.4 $\times$ )

\* Statistical significance when compared to HD patients (Mann-Whitney U test); NS = not significant ( $p \geq 0.10$ ).

Comparison of HD patients' pre- and postvaccination titers (signed-rank test): A/H3N2:  $p = 0.000003$ ; A/H1N1:  $p = 0.00005$ ; B:  $p = 0.0000008$ .

egg-grown A/Taiwan/1/86, which was antigenically related but not identical with the vaccine component A/Texas/36/91. Ether treatment was used to enhance the sensitivity of influenza B virus antigen to HI antibodies. In tables and for calculations titers are expressed as log values, and a titer of  $<10$  is arbitrarily set at 5. The 5-week postvaccination titers were used for statistical analysis, for this period is known to be long enough for evoking a maximum level of vaccination-induced antibodies [11]. Besides, the A/H3N2 subtype influenza outbreak in winter 1995/96 made the 10-week samples unreliable for the analysis.

#### Statistical Analysis

Differences in postvaccination influenza antibody titers were analyzed with the nonparametric Mann-Whitney U test, the differences in the proportions of patient groups, reaching protective antibody titers were tested with the chi-square contingency tables. The differences in the pre- and postvaccination titers of the HD patients were calculated with the signed-rank test. The evaluation of the contributing factors influencing the vaccination response of the HD patients was performed with multiple regression analysis.  $p < 0.05$  was considered significant. All the statistics were calculated using Statgraphics® Plus 2.0 statistical software.

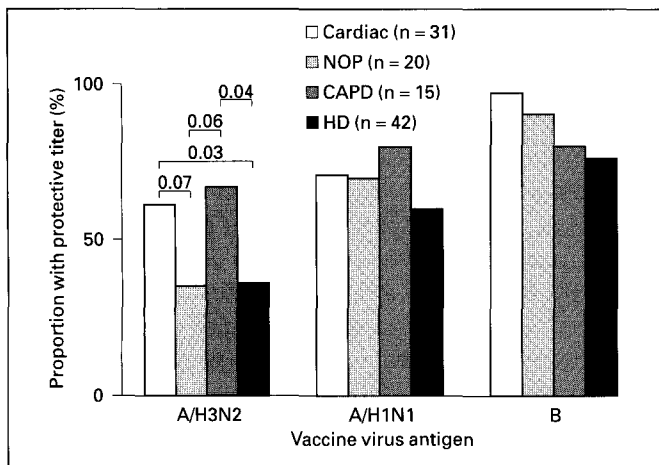
## Results

### Vaccination Response

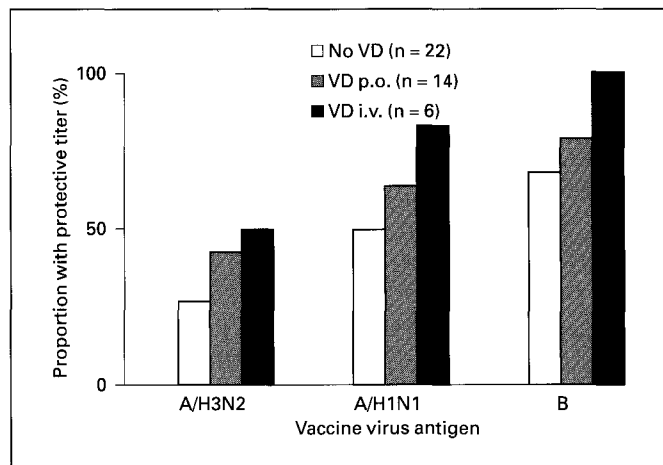
Almost all of the dialysis patients (86%), but only 10% of the NOP and cardiac patients had previously been vaccinated. This was reflected in higher prevaccination titers to all measured vaccine virus antigens in the dialysis group. These and the 5-week postvaccination titers are shown in table 2. The HD patients' postvaccination titers to all measured vaccine virus antigens were significantly higher than their prevaccination titers, but the other

groups had even higher postvaccination titers. The only significant differences were, however, seen in the postvaccination titers to influenza B antigen both in comparison with NOP and with cardiac patients. The patients on CAPD exhibited comparable postvaccination titers both to cardiac patients and NOP to all three antigens, and their titers to A/H1N1 were significantly higher than the titers of HD patients. A titer of 1.6 (log), which is considered protective [11], was reached almost as often in HD patients as in NOP and cardiac patients to A/H1N1 and to influenza B antigens. HD patients and NOP produced less effectively protective antibody levels to A/H3N2 than cardiac patients (fig. 1). The CAPD patients' postvaccination antibody status was, even in this analysis, comparable to the status of cardiac patients and better than that of HD patients, and possibly even better than the postvaccination antibody status of NOP. Among the HD patients the proportion of those who had a protective titer to vaccine antigens increased from prevaccination levels: for A/H3N2 from 7 to 36%, for A/H1N1 from 31 to 60%, and for influenza B from 38 to 76%.

Among the NOP, there was a weak correlation between degree of renal impairment and postvaccination antibody status: the 6 patients who reached a protective titer to all three antigens had a serum creatinine level of  $226 \pm 69$   $\mu\text{mol/l}$  ( $2.56 \pm 0.78$  mg/dl; mean  $\pm$  SD); in contrast, the serum creatinine level of the 6 patients with protective titer to none or only to one vaccine virus antigen was  $344 \pm 144$   $\mu\text{mol/l}$  ( $3.89 \pm 1.63$  mg/dl). This difference was, however, not statistically significant ( $p = 0.10$ , Mann-Whitney U test).



**Fig. 1.** Proportion of patients with a postvaccination protective antibody titer ( $\log \geq 1.6$ ) to the vaccine virus antigens. The statistical significances have been calculated with chi-square test.



**Fig. 2.** Effect of vitamin D supplementation (no VD = no calcitriol therapy,  $n = 22$ ; VD p.o. = oral vitamin D supplementation,  $n = 14$ ; VD i.v. = intravenous calcitriol supplementation,  $n = 6$ ) upon the proportion of HD reaching a protective postvaccination antibody titer ( $\log \geq 1.6$ ) to the vaccine virus antigens.

### Factors Influencing the Antibody Production of HD Patients

A postvaccination protective titer ( $\log \geq 1.6$ ) for two out of the three antigens was considered indicative of an effective response to the present and previous vaccinations as well as, perhaps to some extent, to natural infections. In the analysis of factors affecting this response, a stepwise multiple regression analysis, including the parameters age, sex, duration of dialysis treatment, dialysis efficacy (Kt/V), serum albumin, plasma intact parathyroid hormone, blood leukocyte levels, erythropoietin treatment, and calcitriol treatment (either oral or intravenous), was done. The only almost statistically significant parameter explaining the response was calcitriol treatment ( $p = 0.06$ ). In figure 2 the HD patients are classified according to their vitamin D treatment: 5 out of the 6 (83%) HD patients who had intravenous calcitriol therapy exhibited a protective antibody titer to at least two out of the three antigens, whereas only 45% (10/22) of the non-vitamin-D-treated patients and 57% (8/14) of the patients with oral vitamin D exhibited comparable antibody levels. This was reflected in somewhat higher postvaccination titers among the intravenous calcitriol treated patients than among the patients without vitamin D supplementation: A/H3N2:  $1.45 \pm 0.53$  (intravenous calcitriol treated patients,  $n = 6$ ) vs.  $1.29 \pm 0.41$  (patients without vitamin D therapy,  $n = 22$ ); A/H1N1:  $1.80 \pm 0.24$  vs.  $1.57 \pm 0.60$ ; influenza B:  $2.00 \pm 0.65$  vs.  $1.76 \pm 0.50$ .

### Vaccination Response and Major Infections

Eight hemodialysis patients had major infections requiring hospitalization during the 18-month follow-up period. Only 1 patient out of the 13 (8%) who exhibited a protective postvaccination titer to all three vaccine antigens had a major infection during the follow-up period. The other 7 patients (7/29, 24%) with major infections were among those who exhibited lower antibody levels.

### Discussion

The etiology of dialysis patients' immunosuppression is multifactorial and affects many aspects of host defense, including cell-mediated immunity and antibody production [12]. The vaccination response is the combination of these two [13]. Vaccination is an effective way to enhance immunity to many viral and even bacterial diseases. Unfortunately, individuals who, due to their ineffective immune defense, are at a higher risk to get serious complications even with common viral infections, including influenza, respond suboptimally to vaccinations. Previous influenza vaccination studies with dialysis patients have shown that their responses are weaker than those of subjects with a normal kidney function [2, 14, 15]. With enhanced vaccination protocols, it has, however, been possible to induce better responses than with conventional vaccination schedules [16], indicating quantitative rather than qualitative immune abnormalities.

Our results showed that CAPD patients' postvaccination antibody status was comparable to that of cardiac patients and NOP, which is in accordance with previous findings on vaccination-induced antibody responses [17]. HD patients' vaccination responses were weaker, owing to higher prevaccination antibody status, perhaps mainly due to previous vaccinations. In spite of the lower postvaccination antibody status, almost a similar proportion of HD patients reached protective antibody levels to A/H1N1 and influenza B antigens, thus showing the efficacy of vaccination. To A/H3N2, however, fewer NOP and HD patients exhibited protective titers than cardiac patients and CAPD patients. Recently published results of influenza vaccination in pediatric dialysis patients (including both HD and CAPD patients) also showed responses comparable to those of controls [18]. Corresponding to previous findings, there were differences in the responses to A/H3N2 and to A/H1N1, but in contrast to the results of Beyer et al. [14], we found a lower rate of protective titers to A/H3N2 than to A/H1N1. This is presumably due to antigenic diversity within the influenza A virus subtypes and different antigenic experiences of the vaccinees. In this study, we were not able to analyze the clinical protective effect of vaccination which may be suboptimal in spite of adequate antibody responses to the vaccine virus strains. Although the antibody production among the HD patient group was, as indicated by postvaccination antibody status, almost comparable to that of the other groups, there were still individual variations.

In multivariate analysis, calcitriol therapy was the only parameter somewhat explaining the overall response. HD patients are typically highly deficient of the active vitamin D metabolite, 1,25-(OH)<sub>2</sub>D<sub>3</sub> or calcitriol, and oral vitamin D and intravenous calcitriol therapies are nowadays widely used to prevent and treat secondary hyperparathyroidism [19, 20]. Calcitriol has well-known in

vitro immunological effects which for the most have been immunosuppressive at least at supraphysiological concentrations higher than 10<sup>-10</sup> mol/l [21, 22]. With oral vitamin D administration, effective serum levels within 10<sup>-12</sup> to 10<sup>-11</sup> mol/l can be reached, but with intravenous calcitriol therapy, it is possible to get effective serum concentrations of even 10<sup>-9</sup> mol/l [23]. The influenza vaccination response associated parameters of dialysis patients have previously been analyzed by Beyer et al. [14]. In their study only a dialysis duration over 8 years had a positive effect on vaccination response. The response to hepatitis B vaccination has, in contrast, been shown to be positively related to younger age, but neither to dialysis duration nor to calcitriol therapy [24]. In that study the overall response rate was good, 82% of the vitamin D treated patients seroconverted in comparison to 72% of the vitamin D naive patients. The vitamin D administration route was not given, which makes the results difficult to interpret. According to our results neither oral vitamin D nor intravenous calcitriol is immunosuppressive, but especially intravenous calcitriol administration may be immunostimulatory in HD patients. The present study is the first evidence showing that high-dose calcitriol therapy may, in addition to positive effects on calcium metabolism, have relevant advantageous effects on the immune response, thus being in accordance with the previous findings showing positive effects on HD patients' lymphocyte functions in vitro [9, 10].

In conclusion, we have shown (1) that influenza vaccination of HD patients according to current recommendations results in an effective humoral immune response; (2) that the influenza vaccination response of CAPD patients is comparable to that of patients with a normal kidney function, and (3) our results suggest that the correction of vitamin D deficiency of HD patients may improve their immune response even in a clinically relevant way.

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# Influenza vaccination of dialysis patients: cross-reactivity of induced haemagglutination-inhibiting antibodies to H3N2 subtype antigenic variants is comparable with the response of naturally infected young healthy adults

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## Abstract

**Background.** Annual influenza vaccination is recommended for patients with chronic renal failure, although vaccination responses in haemodialysis (HD) patients may be suboptimal. Typically, the seroreactivity has been analysed against the vaccine virus or the corresponding year's epidemic virus. No studies analysing cross-reactivity against subsequent years' viruses have been presented.

**Methods.** Twenty-three chronic HD patients and 26 cardiac patients were, in autumn 1995, vaccinated with a trivalent influenza vaccine. The cross-reacting haemagglutination-inhibiting antibodies to five consecutive years' (the last season 1999–2000) drift variants of H3N2 subtype influenza A virus were measured and compared with those of vaccinated cardiac patients and with those of 26 healthy military conscripts who suffered a serologically confirmed influenza A infection in the season 1995–1996.

**Results.** The influenza vaccination in HD patients resulted in comparable cross-reacting antibodies to the antibodies induced both by vaccination in cardiac patients and by natural infection in military conscripts. After a steady decline, the cross-reactivity to the latest epidemic virus improved in all the groups. This may be due to two reverted amino acid changes in the HA1 domain of the virus haemagglutinin.

**Conclusions.** Influenza vaccination in HD patients is as effective as the vaccination of cardiac patients with normal kidney function. The cross-reactivity of vaccination-induced antibodies is even as good as that of antibodies induced by natural infection of young healthy males. Additionally, vaccination seems

to prime the individual beneficially against subsequent years' influenza viruses.

**Keywords:** cross-reactivity; haemodialysis; influenza; vaccination; vitamin D

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## Introduction

Influenza vaccination is a safe and relatively effective way to prevent morbidity and mortality [1]. Uraemic patients are especially vulnerable to infections [2] and it is generally recommended to vaccinate patients with chronic renal insufficiency yearly against influenza [3]. In patients on haemodialysis (HD) the influenza vaccination response has been considered suboptimal [4], but recent studies, including our own, have shown almost comparable responses with healthy controls [5,6].

Influenza vaccination may be less prone in stimulating serum antibodies of high avidity than natural infection [7]. Additionally, the antigenic match between the vaccine viruses and the actual epidemic viruses is frequently incomplete. The protective efficacy of the vaccine is, however, dependent on its ability to provoke cross-reacting antibodies to the actual epidemic viruses.

Homologous serum antibody responses to the H3N2 vaccine virus and the cross-reactivity of the induced antibodies to the epidemic virus were recently shown to decline with the increasing age of the people being vaccinated [8], and T-cell dependent B-cell functions, for example influenza vaccine response, are compromised in uraemia [9]. These facts raise a concern about the cross-reactivity of the antibodies induced by the vaccination of HD patients. In the present study we

measured the cross-reactivity of antibodies induced by influenza vaccination in HD patients. This was, in addition to the corresponding year's epidemic virus, also done for the five following seasons' drift variants of H3N2 subtype epidemic influenza A virus. This is the first time the effect of influenza vaccination on the formation of cross-reacting antibodies to subsequent years' drift virus variants has been studied in HD patients.

## Subjects and methods

The HD and cardiac patients were part of a larger study group, of which the basic vaccination results have been reported previously [5]. Twenty-three HD patients (mean age 55 years, six women, 17 men) dialysed (mean time in HD treatment 24 months, range 1–55 months) with synthetic biocompatible dialysis membranes, and 26 patients (mean age 63 years, 10 women, 16 men) from a cardiac ward who had a normal kidney function and received no immunosuppressive therapies, were, in the autumn of 1995, vaccinated with a commercially available inactivated trivalent vaccine (Vaxigrip<sup>®</sup>; Pasteur Merieux Serums et vaccins). The vaccine contained 15 µg of antigen from the component strains A/Johannesburg/33/94 (H3N2), A/Texas/36/91 (H1N1) and B/Harbin/7/94. Almost all HD patients (20/23, 87%) had previously received influenza vaccinations: four patients before HD treatment and 16 while on HD. Only two (8%) cardiac patients had been vaccinated previously.

The blood specimens were collected at the time of vaccination (pre-vaccination sample) and 5 weeks thereafter (post-vaccination sample). Paired sera collected during the 1995–1996 epidemic season from 26 healthy male military conscripts (age range 18–24 years) in the acute and convalescent phases of serologically confirmed (a 4-fold or greater antibody rise in a standard complement-fixation antibody test) influenza A infections were at our disposal. The military conscripts had not been vaccinated during that season. All the sera were stored at –20°C until studied for haemagglutination-inhibiting (HI) antibodies in autumn 2000.

HI assay was performed as outlined previously [10], using goose erythrocytes instead those of hens. The H3N2 subtype vaccine virus A/Johannesburg/33/94 (JHN/33/94) and five virus strains isolated during the five epidemic seasons since 1995–1996 in Finland served as antigens in the HI tests. The drift variants were isolated and cultivated exclusively in

MDCK cell cultures and the vaccine strain in embryonated eggs. Antigenic relationships of the vaccine virus (JHN/33/94) and epidemic viruses were analysed in the HI tests using rat antisera as described previously [11].

The vaccine virus and the five drift variants were studied for their nucleotide sequences coding for the variable HA1 domain of the virus haemagglutinin. Detailed techniques (RNA extraction, cDNA synthesis, amplification procedures with PCR and sequencing) have been described previously [12]. Four virus strains were sequenced for the present study: JHN/33/94, Finland/381/95, Finland/680/99 and Finland/749/00. Two strains were sequenced previously: Finland/539/97 and Finland/579/98.

The statistical significances of the differences in influenza A antibody levels among and between the three groups were calculated with the Kruskal–Wallis test. A  $P < 0.05$  was considered significant. The statistics were calculated using the SPSS statistical software (SPSS Inc., USA).

All the patients gave their informed consents and the study was approved by the local ethical committee.

## Results

Table 1 illustrates the antigenic relationship of the vaccine virus strain JHN/33/94 and the drift variants isolated in Finland during the five consecutive seasons from 1995–1996 to 1999–2000. The antisera produced against the vaccine strain (JHN/33/94) reacted to epidemic virus strains isolated in years 1995–1998, but not to those viruses isolated in 1999 and 2000 (FN680/99, FN/749/00). The antisera to the epidemic strains of 1995–1996 (FN/381/95) and 1997–1998 (FN/539/97) reacted at low titres both with the vaccine strain of 1995 (JHN/33/94) and with all the drift variants, even with the most recently isolated one (FN/749/00). The antisera to the epidemic strains after the season 1997–1998 (FN/579/98, FN/680/99, FN/749/00) did not react with the vaccine virus of the year 1995 (JHN/33/94).

Amino acid changes in the variable HA1 domain of virus haemagglutinin are listed in Table 2. Twelve amino acid substitutions in HA1 differentiated the vaccine virus (JHN/33/94) from the corresponding year's epidemic virus FN/381/95. The number of amino acid substitutions of the following successive epidemic virus variants increased gradually to 19, 22, 24 and 30.

**Table 1.** Cross-reactivities of the vaccine virus of autumn 1995 (JHN/33/94) and representative H3N2 subtype influenza A virus variants isolated in Finland (FN) during the epidemic seasons 1995–1996 to 1999–2000

Virus strains	HI titers of rat antisera against					
	JHN/33/94 vaccine virus	FN/381/95 1995–1996	FN/539/97 1997–1998	FN/579/98 1997–1998	FN/680/99 1998–1999	FN/749/00 1999–2000
JHN/33/94	<b>320</b>	80	20	< 10	< 10	< 10
FN/381/95	640	<b>640</b>	160	40	10	< 10
FN/539/97	320	320	<b>320</b>	80	20	10
FN/579/98	20	80	40	<b>160</b>	80	40
FN/680/99	< 10	40	20	40	<b>160</b>	160
FN/749/00	< 10	40	40	80	320	<b>640</b>

Homologous titres are shown in bold.



**Table 2.** Amino acid differences in the HA1 domain of the virus haemagglutinin between the vaccine virus of autumn 1995 (JHN/33/94) and five successive drift variants from the epidemic seasons 1995–1996 to 1999–2000

	0000000011111111111111111111111122222222222222
	034445699222333444556799911223677779
	5356772261240357245680226769691215681
JHN/33/94	GQSSPRKKNINNVDKYGVNKEKGTVRDYQRGNDGNSD
FN/381/95	...S...T.G.....K...D..QNSI.S..D.N.
FN/539/97	..G.SQ.TKTKG..T...K...D..QNSVXSS.D.N.
FN/579/98	...S.E..N.S.NT.RIKQ..D.AQNSVISS..KNE
FN/680/99	...SQE..N.S.NTSRIKQKRD.AQNSV.SS..KN.
FN/749/00	VH.FSQET.N.SINTSR.KQK.EIAQNSVISSN.KN.

The most recent virus variant exhibited two reverted amino acid changes at residues 144 and 160.

Although the most recent variant (FN/749/00) had more substitutions than the preceding years' variants, it exhibited two reverted amino acid changes (at residues 144 and 160 amino acids changed to those seen in the vaccine virus).

Table 3 shows the cross-reacting HI antibodies induced by the vaccinations of the HD and the cardiac patients in autumn 1995 and induced by influenza A infections of the conscripts in winter 1995–1996. The pre-vaccination antibody levels of the HD patients to each of the five epidemic virus strains were higher than those of the cardiac patients and the military conscripts. The proportion of the HD patients who exhibited protective antibody titres to the epidemic virus FN/381/95 rose from 35 to 78% (18/23) by vaccination, which is comparable (73 and 77%, respectively) with the response induced by vaccination in the cardiac patients and after natural infection in the military conscripts. The mean fold increase of HI antibody titres of the HD patients amounted to 3.3 (from 1.27 to 1.77 in log values). The corresponding mean fold increase of the cardiac patients and the conscripts were 11 and 8, respectively.

Cross-reactivity of the antibodies induced by vaccinations and natural infections decreased gradually

when epidemic virus variants from the seasons 1995–1996 to 1998–1999 were used as antigens in HI tests (Table 3). This decrease is consistent with the antigenic relationship of the drift variants demonstrated using rat antisera in HI tests (Table 1). After the decrease, the cross-reactivity enhanced in all study groups, which is seen as the increased rate of protective titres in the post-vaccination and convalescent phases. The vaccination induced a substantial increase in HD patients' antibody levels, as the post-vaccination antibody levels were significantly higher than the pre-vaccination ones against all studied virus variants except that of year 1998–1999 (FN/680/99). The significance levels were in chronological order:  $P=0.002$ ,  $P=0.003$ ,  $P=0.014$ ,  $P=0.11$  and  $P=0.03$ .

## Discussion

Vaccination-induced antibody response is a clinically relevant way to study immune functions, as it reflects both antigen recognition and processing and humoral response in a real life situation. The vaccination of HD patients in 1995 proved to be effective in provoking HI antibodies to the actual epidemic H3N2 subtype influenza virus of the following season (MDCK-grown FN/381/95). The post-vaccination protection rate (78%) reached even a higher value than previously detected for the vaccine virus strain, the egg-grown JHN/33/94 (36%) [5]. This is not surprising, for MDCK-grown virus variants correspond better than the egg-grown variants to the virus excreted by human host [13,14]. In the HD patients the protection rate and the mean fold increase of HI antibody titres were above the requirements of the Committee for Proprietary Medicinal Products (CPMP 1997) [15].

In HD patients the mean fold increase of HI antibody titres was smaller than in cardiac patients and in naturally infected conscripts. This reflects the

**Table 3.** HI antibodies to the five drift variants of Table 1 in pre-vaccination and 5-week post-vaccination sera of 23 HD and 26 cardiac patients and in acute and convalescent phase sera of 26 young military conscripts (MC) with a serologically confirmed influenza A infection

	FN/381/95 1995–1996	FN/539/97 1997–1998	FN/579/98 1997–1998	FN/680/99 1998–1999	FN/749/00 1999–2000
HD pre-vaccination	1.27 (0.55)	1.21 (0.52)	1.08 (0.24)	1.14 (0.15)	0.90 (0.30)
Protection rate (%)	35	30	4	0	4
HD post-vaccination	1.77 (0.45)	1.69 (0.54)	1.27 (0.25)	1.24 (0.20)	1.20 (0.53)
Protection rate (%)	78	65	26	4	26
Cardiac pre-vaccination	0.85 (0.32)	0.79 (0.24)	0.80 (0.19)	0.90 (0.15)	0.75 (0.16)
Cardiac post-vaccination	1.88 (0.77)	1.77 (0.75)	1.35 (0.37)	1.02 (0.22) <sup>a</sup>	1.42 (0.55)
Protection rate (%)	73	62	35	4	38
MC acute	0.86 (0.34)	0.82 (0.30)	0.84 (0.27)	0.93 (0.15)	0.72 (0.08)
MC convalescent	1.73 (0.65)	1.64 (0.66)	1.43 (0.43)	1.17 (0.26)	1.14 (0.49)
Protection rate (%)	77	65	46	12	27

Mean logarithmic titres (SD) and the protection rates (percentages of subjects exhibiting post-vaccination and convalescent phase protective antibody titres,  $\geq 1.60$ ) are shown. Additionally, the pre-vaccination protection rates of HD patients are shown. The statistical significances have been calculated comparing HD patients' post-vaccination titres to those of cardiac patients and to the convalescent titres of military conscripts.

<sup>a</sup> $P=0.003$ .

higher pre-vaccination titres in the HD patients than in the other groups. Almost 90% of the HD patients had been vaccinated previously against influenza, but the cardiac patients and the conscripts were practically influenza vaccine naïve, explaining the difference in pre-vaccination titres. However, HD patients did not differ from the other two groups in the post-vaccination antibody levels and correspondingly in the proportion of the persons mounting a protective post-vaccination titre to the five different epidemic virus variants. As the vaccination of HD patients induced a significant increase in antibody levels (when compared with pre-vaccination antibody levels) against the studied virus variants except that of year 1998–1999 (FN680/99), it seems justified to propose that this particular vaccination was effective. This suggests that in the case of influenza vaccination B cells get appropriate co-stimulatory signals from antigen presenting cells and T cells, although there is a shift towards Th-1 type cell functions not favouring antibody formation in uraemia [9].

The cross-reactivity declined among all the groups until the last studied epidemic variant, FN/749/00 isolated in 2000. Surprisingly, the protection rate among all the groups increased from 4–12 (as determined against FN/680/99 isolated in 1999) to 26–38% (against FN/749/00 isolated in 2000). Our sequence analysis of viral haemagglutinin suggests that two reverted amino acid changes in the antigenic sites A (residue 144) and B (residue 160) may be involved in the enhanced cross-reactivity. Both are located in the protruding loops close to the receptor-binding site and are thus suitable for avid antibody binding and may participate in antibody-mediated neutralization and inhibition of haemagglutination [16]. Our results on the enhanced cross-reactivity suggest that the advantage of vaccination-induced immunity is not necessarily restricted only to the season following vaccination. At least occasionally, the antigenic evolution of influenza viruses may result in a situation in which previous vaccinations and natural infections have favourably primed a part of the host population. Previous studies have shown that the influence of anamnestic antigenic experiences for the vaccination-induced immunity may greatly vary from year to year depending, for example on the antigenic evolution of the influenza virus [17]. Our results are valid for H3N2 type influenza viruses, and the situation might be different with H1N1 type or with influenza B virus [5,18].

In conclusion, we found first that influenza vaccination of HD patients provokes corresponding levels of cross-reacting H3N2 subtype influenza A virus HI antibodies to cardiac patients with normal kidney function. The induced antibodies are even comparable with those detected after natural influenza A infection in young healthy males. Secondly the antigenic drift of influenza A virus may after a decline in cross-reactivity result in enhanced cross-reactivity again. This emphasizes the importance of yearly influenza vaccination, which may be advantageous not only for the

corresponding season, but even for the subsequent years. Additionally, the decline of antibody levels among HD patients seen after hepatitis B vaccination [19] further underscores the need for yearly influenza vaccination, while no evidence of decreasing protection with repeated influenza vaccination has been shown [20].

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*Conflict of interest statement.* None declared.

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# Constant, but Not Pulsed Calcitriol Suppresses Hemodialysis Patients' Antigen-Induced Lymphocyte Proliferation

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## Key Words

Calcitriol · Hemodialysis · Antigen response · Lymphocyte proliferation

## Abstract

**Background/Aims:** In vitro constant calcitriol [1,25-(OH)<sub>2</sub>D<sub>3</sub>] inhibits healthy individuals' T lymphocyte proliferation at supraphysiological concentrations. In contrast, among hemodialysis patients, intravenous 1,25-(OH)<sub>2</sub>D<sub>3</sub> pulse therapy of secondary hyperparathyroidism has been shown to be even immunostimulatory. We studied the effect of in vitro constant and intermittent 1,25-(OH)<sub>2</sub>D<sub>3</sub> on lymphocyte antigen response of hemodialysis patients. **Methods:** Twelve hemodialysis patients' peripheral blood mononuclear cells were stimulated with purified protein derivative of tuberculin (12.5, 25 and 50 mg/l) or tetanus toxoid (TT; 1,000, 5,000 and 10,000 Lf/l, limit of flocculation) for 7 days. Constant 1,25-(OH)<sub>2</sub>D<sub>3</sub> was added to all cultures at concentrations of 0, 10<sup>-10</sup> or 0.25 × 10<sup>-9</sup> mol/l (0, 42 and 105 ng/l) and to half of the cultures additionally as a 0.75 × 10<sup>-9</sup> mmol/l (315 ng/l) pulse on the 5th culture day. **Results:** TT-induced lymphocyte proliferation was statistically related to a constant 1,25-(OH)<sub>2</sub>D<sub>3</sub> concentration (p = 0.001, analysis of variance). With constant 1,25-(OH)<sub>2</sub>D<sub>3</sub> concentrations of 0, 42 and 105 ng/l, the TT-induced responses were

1.53, 1.44 and 1.40 log cpm, respectively (mean of TT concentrations). The responses of the (additionally) pulse-treated cells [1.65, 1.50 and 1.40 log cpm; concentrations of constant 1,25-(OH)<sub>2</sub>D<sub>3</sub> as above] were similar to those of the nonpulsed cells. Thus constant, but not pulsed 1,25-(OH)<sub>2</sub>D<sub>3</sub> decreased the TT responses. On the purified protein derivative of tuberculin response, neither constant nor pulsed 1,25-(OH)<sub>2</sub>D<sub>3</sub> had any significant effect. **Conclusions:** The decline of TT response with constant 1,25-(OH)<sub>2</sub>D<sub>3</sub> corresponds with findings on immunosuppressive action of 1,25-(OH)<sub>2</sub>D<sub>3</sub> in previous studies done on normal subjects' cells. This was not seen with intermittently applied 1,25-(OH)<sub>2</sub>D<sub>3</sub>. These results support the previous concept that intermittent 1,25(OH)<sub>2</sub>D<sub>3</sub> therapy is not immunosuppressive in hemodialysis patients.

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## Introduction

Calcitriol [1,25-(OH)<sub>2</sub>D<sub>3</sub>], the active metabolite of vitamin D, is produced by hydroxylation in kidneys and liver. Patients with end-stage renal disease have a pronouncedly diminished serum 1,25-(OH)<sub>2</sub>D<sub>3</sub> level. In healthy individuals, calcitriol promotes maturation of monocytes/macrophages [1], and it rather enhances than

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**Table 1.** Patient characteristics

Patient No.	Sex	Age years	Time on hemodialysis months	Dialyzer membrane <sup>a</sup>	Duration of erythropoietin treatment months	Dosage of erythropoietin IU/week	Serum 1,25-(OH) <sub>2</sub> D <sub>3</sub>		Serum intact parathyroid hormone		Diagnosis
							pmol/l	ng/l	pmol/l	µg/l	
1	female	49	5	CA	9	4,000	not done		6.0	57.0	chronic glomerulonephritis
2	male	30	60	PS	24	15,000	33.0	13.9	11.0	104.5	chronic glomerulonephritis
3	male	22	20	PS	14	5,000	36.0	15.1	13.0	123.5	chronic glomerulonephritis
4	male	73	6	CA	18	8,000	42.0	17.6	12.0	114.0	chronic glomerulonephritis
5	male	77	35	PS	33	7,000	36.0	15.1	10.0	95.0	chronic glomerulonephritis
6	male	62	17	CA	17	4,000	<20.0	<8.4	10.0	95.0	diabetic nephropathy
7	female	72	5	CA	5	15,000	44.0	18.5	50.0	475.0	diabetic nephropathy
8	female	77	50	CA	49	6,000	23.0	9.7	12.0	114.0	diabetic nephropathy
9	female	47	66	PS	34	6,000	36.0	15.1	40.0	380.0	polycystic renal disease
10	male	67	7	CA	2	8,000	39.0	16.4	15.0	142.5	polycystic renal disease
11	male	51	3	CA	–	–	<20.0	<8.4	15.0	142.5	glomerulosclerosis
12	male	75	15	CA	15	4,000	21.0	8.8	12.0	114.0	hypertensive renal disease

<sup>a</sup> CA = Cellulose acetate; PS = polysulfone.

suppresses their antimicrobial functions [2, 3]. However, it inhibits T lymphocyte proliferation and interleukin (IL) 2 production at concentrations as low as 10<sup>-11</sup> mol/l (4.2 ng/l) [4, 5] or 10<sup>-12</sup> mol/l (0.42 ng/l) [6] as well as the production of other lymphokines. Because of potential immunosuppressive effects, vitamin D and its active metabolites and analogues have been reviewed and studied for cell-regulating use in oncology, autoimmunity, transplantation and dermatology [1, 3, 7–12].

1,25-(OH)<sub>2</sub>D<sub>3</sub> is widely used in the treatment of secondary hyperparathyroidism in end-stage renal disease. In dialysis patients, orally administered vitamin D derivatives have been shown to enhance T cell lymphoproliferative responses [13], IL-2 secretion [14], phorbol myristate acetate-induced IL-1 and IL-6 secretion [15], lipopolysaccharide-induced tumor necrosis factor alpha production [16] of peripheral blood mononuclear cells and superoxide generation and bactericidal capacity of monocytes [17]. Any clear suppression of immune functions of hemodialysis patients resulting from oral vitamin D therapy has not been shown. In cell cultures, the addition of 1,25-(OH)<sub>2</sub>D<sub>3</sub> at concentrations of up to 10<sup>-11</sup> mol/l (4.2 ng/l) augmented phytohemagglutinin-induced lymphocyte response of hemodialysis patients, but supraphysiological concentrations (10<sup>-9</sup> to 10<sup>-7</sup> mol/l; 420 ng/l to 42 µg/l) inhibited mitogenesis [18] – (the mean physiological serum 1,25-(OH)<sub>2</sub>D<sub>3</sub> concentration in the Finnish adult normal population is roughly 100 × 10<sup>-12</sup> mol/l (42 ng/l) [19]). With intravenous pulse 1,25-(OH)<sub>2</sub>D<sub>3</sub> therapy supraphysiological concentrations of up to 10<sup>-9</sup> mol/l (420 ng/l) can be reached [20, 21]; with that therapy

the effect upon hemodialysis patients' T cell responses has been shown to be beneficial [22].

It is controversial that 1,25-(OH)<sub>2</sub>D<sub>3</sub>, given to healthy subjects acts immunosuppressively, but given to hemodialysis patients, it augments immune functions. It is important to determine which doses of 1,25-(OH)<sub>2</sub>D<sub>3</sub> have an immunosuppressive effect in hemodialysis patients, considering the varying pharmacokinetics of the hormone according to its administration route. In *in vivo* studies, it is difficult to obtain a controlled environment essential for the analysis of the extent of potential toxicity. The aim of this study was to analyse *in vivo*, using a lymphocyte stimulation assay, the actions of physiological and supra-physiological constant and intermittent 1,25-(OH)<sub>2</sub>D<sub>3</sub> on antigen-induced T cell proliferation responses of hemodialysis patients.

## Patients and Methods

Twelve hemodialysis patients with no systemic autoimmune disease and no immunomodulating therapy were studied. Their hemodialysis treatment had lasted for at least 3 months. They had previously not been on any vitamin D therapy. Also 2 healthy individuals 30 (female) and 33 (male) years of age were included as test controls. All subjects gave an informed consent, and the local ethical committee approved the study. Patient characteristics are shown in table 1.

Heparinized blood samples were drawn preceding a dialysis session, and peripheral blood mononuclear cells were separated using the Ficoll-Hypaque layer centrifugation method [23]. After careful washing, cell cultures were grown on two identical U-bottom microtiter plates (Nunc, Roskilde, Denmark) in triplicate, using RPMI culture medium and 10% autologous plasma. The cultures were stimu-

lated either with purified protein derivative of tuberculin (PPD) at concentrations of 50, 25, 12.5 or 0 mg/l (Staatens Serum Institut, Copenhagen, Denmark) or tetanus toxoid (TT) at concentrations of 1,000, 5,000, 10,000 or 0 Lf/l (limit of flocculation; Immuno, Vienna, Austria). These antigens are useful in the Finnish population, as over 99% of the Finnish children follow the Finnish State Program on Immunization and are vaccinated against tuberculosis with BCG (bacilles Calmette Guérin) vaccine and against tetanus with PDT (pertussis-diphtheria-tetanus) vaccine. According to Finnish experience, the reactivity, when reached, is often long lasting [24].

1,25-(OH)<sub>2</sub>D<sub>3</sub> was applied during the culture generation phase at  $1 \times 10^{-10}$  mol/l (42 ng/l),  $2.5 \times 10^{-10}$  mol/l (105 ng/l) or none. The lower concentration represented a physiological level. The higher concentration, a potentially immunosuppressive level, reached the peak phase of oral 1,25-(OH)<sub>2</sub>D<sub>3</sub> treatment with a 143-fmol/g (60-pg/g) [21]. The identical plates were pulse treated on the 5th culture day either with 1,25-(OH)<sub>2</sub>D<sub>3</sub> or with RPMI. In the 1,25-(OH)<sub>2</sub>D<sub>3</sub> pulse treated plate, the final 1,25-(OH)<sub>2</sub>D<sub>3</sub> concentration was  $7.5 \times 10^{-10}$  mol/l (315 ng/l) for 2 h. This concentration corresponds to the mean serum concentration levels reached during the first 2 h of intravenous 1,25-(OH)<sub>2</sub>D<sub>3</sub> pulse treatment with a 143-fmol/g (60-pg/g) dose [21]. The 1,25-(OH)<sub>2</sub>D<sub>3</sub> pulse treatment required temporary removal of the original culture medium by centrifugation (3–4 min, 660 rpm) and displacement of the supernatant, a 2-hour incubation period with a pulse concentration of 1,25-(OH)<sub>2</sub>D<sub>3</sub> or RPMI, a wash-off of the pulse medium by centrifugation, and a regeneration period of the culture using the original culture medium. One of the two plates was hence pulse treated with 1,25-(OH)<sub>2</sub>D<sub>3</sub> and the other (control plate) with RPMI only. The stimulation response was measured as the intake (cpm) of tritiated thymidine (methyl-<sup>3</sup>H-thymidine, 5 Ci/mmol, 1 μCi/ well; Amersham, Little Chalfont, UK) during the last (7th) culture day (24 h). The specific response was calculated by subtracting the triplicate mean counts per minute of the unstimulated control culture – with the corresponding 1,25-(OH)<sub>2</sub>D<sub>3</sub> concentration) – from the triplicate mean counts per minute of the stimulated cultures. The data were transformed into ten-based logarithmic values [25]. The data of PPD and TT -stimulations were analyzed separately. The (numeric) results are given as the mean specific response of the 12 patients (i.e., the triplicate mean values of the 12 patients have been pooled together), if not otherwise stated (i.e., as mean of the three antigen concentrations).

#### Statistics

The data were analyzed using analysis of variance in which 1,25-(OH)<sub>2</sub>D<sub>3</sub> concentration, pulse treatment and antigen concentration were used as fixed factors, i.e., classified variables, and patient as a block factor which takes into account that samples were dependent within patients. Analyses were done with Statistica/Win (edition 98) software.  $p < 0.05$  was considered significant.

## Results

#### Serum Levels of 1,25-(OH)<sub>2</sub>D<sub>3</sub>

All patients had abnormally low serum 1,25-(OH)<sub>2</sub>D<sub>3</sub> levels, ranging from <20 pmol/l (8.4 ng/l) to 44 pmol/l (18.5 ng/l), while the normal values in our laboratory are 50–215 pmol/l (21.0–90.3 ng/l).

**Table 2.** Hemodialysis patients' proliferative responses (log cpm; mean ± SD)<sup>a</sup>

Antigen		Patients (n = 12)	Controls (n = 2) <sup>b</sup>
PPD	12.5 mg/l	1.91 ± 0.77	1.50
	25 mg/l	1.89 ± 0.73	1.53
	50 mg/l	1.88 ± 0.66	1.62
TT	1,000 Lf/l	1.48 ± 0.70	1.35
	5,000 Lf/l	1.41 ± 0.85	0.95
	10,000 Lf/l	1.70 ± 0.74	1.60

<sup>a</sup> Antigen responses without additional 1,25-(OH)<sub>2</sub>D<sub>3</sub> in the culture medium.

<sup>b</sup> Mean values of 2 healthy individuals.

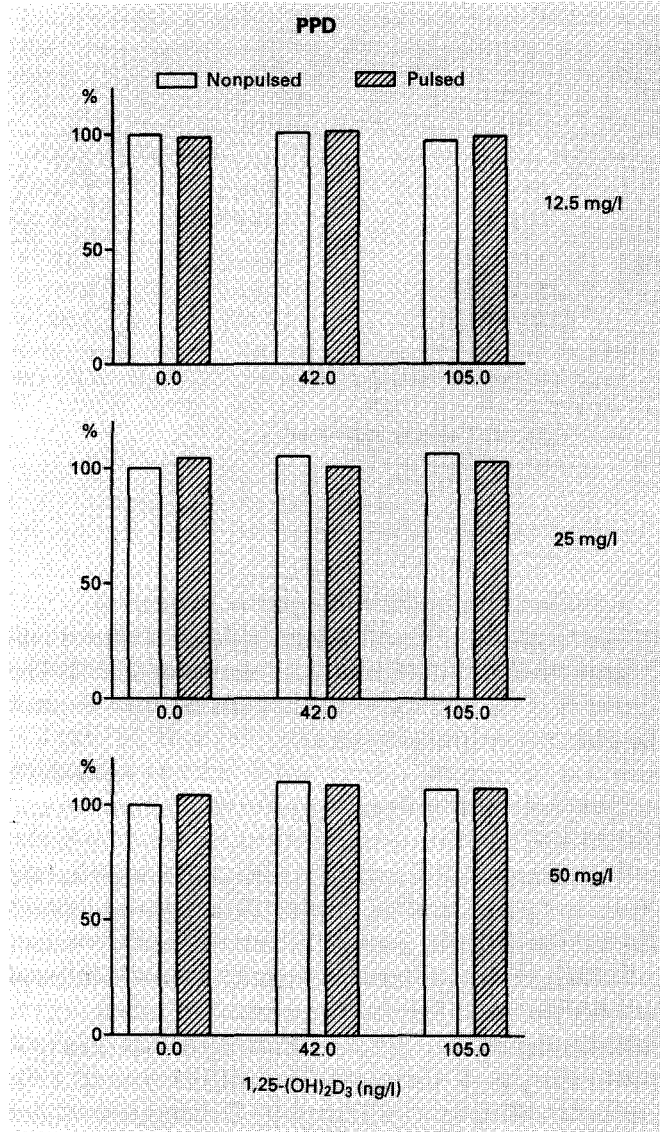
#### Lymphocyte Stimulation Responses in General

The patients' overall lymphocyte stimulation responses without 1,25-(OH)<sub>2</sub>D<sub>3</sub> are shown in table 2. The responses of the patients were not different from those of the 2 healthy control individuals.

#### Lymphocyte Stimulation Response in the Presence of 1,25-(OH)<sub>2</sub>D<sub>3</sub>

The stimulation responses with PPD antigen are shown in figure 1 and those with TT antigen in figure 2 and in table 3. Using analysis of variance, only constant 1,25-(OH)<sub>2</sub>D<sub>3</sub> concentrations explained the TT-induced lymphocyte proliferation response: constant 1,25-(OH)<sub>2</sub>D<sub>3</sub> slightly lowered the TT-induced responses ( $p = 0.001$ , analysis of variance). The responses were 1.53, 1.44 and 1.40 log cpm for 1,25-(OH)<sub>2</sub>D<sub>3</sub> concentrations of 0, 42 and 105 ng/l, respectively (mean of the three TT concentrations). The responses of the additionally pulse-treated cells – 1.65, 1.50 and 1.40 log cpm; with constant 1,25-(OH)<sub>2</sub>D<sub>3</sub> concentrations as above – did not differ from those of the respective non-pulsed cells ( $p = \text{NS}$ , analysis of variance).

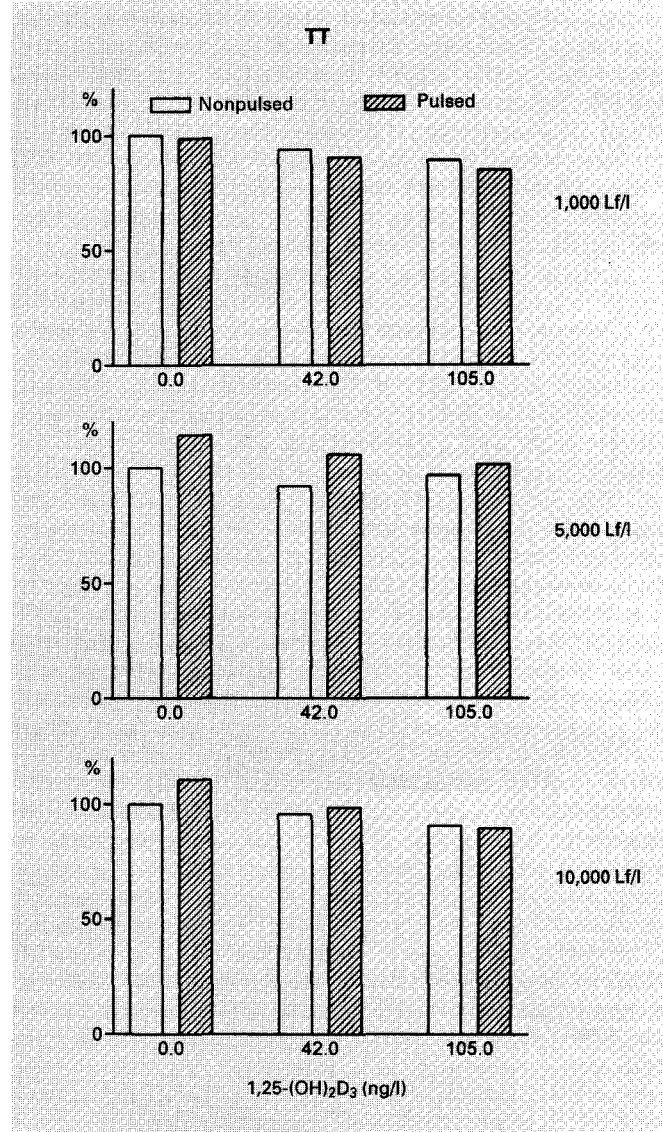
Neither constant nor pulsed 1,25-(OH)<sub>2</sub>D<sub>3</sub> had any significant effect on PPD responses: the nonpulsed PPD responses were 1.89, 1.99 and 1.95 log cpm (mean of the three PPD concentrations), respectively, for 1,25-(OH)<sub>2</sub>D<sub>3</sub> concentrations of 0, 42 and 105 ng/l, and the respective (mean) pulsed PPD responses were 1.94, 1.96 and 1.95 log cpm.



**Fig. 1.** Mean relative lymphocyte stimulation response (expressed in percentages) with PPD as the antigen. PPD was used at concentrations of 12.5, 25 and 50 mg/l. The category axis shows the 1,25-(OH)<sub>2</sub>D<sub>3</sub> concentration in the culture medium. In the value axis, the mean lymphocyte response of the nonpulsed, no 1,25-(OH)<sub>2</sub>D<sub>3</sub>-containing cultures of each PPD concentration is given the value 100%. Using analysis of variance, neither 1,25-(OH)<sub>2</sub>D<sub>3</sub> concentration nor pulsing explained log cpm values ( $p = \text{NS}$ ).

## Discussion

Both intravenous and oral calcitriol are considered important in the treatment of secondary hyperparathyroidism of end-stage renal disease [26–28]. As hemodialy-



**Fig. 2.** Mean relative lymphocyte stimulation response (expressed in percentages) with TT as the antigen. The antigen concentrations were 1,000, 5,000 and 10,000 Lf/l. The category axis shows the 1,25-(OH)<sub>2</sub>D<sub>3</sub> concentration in the culture medium. In the value axis, the mean lymphocyte response of the nonpulsed, no 1,25-(OH)<sub>2</sub>D<sub>3</sub>-containing cultures of each TT concentration, is given the value 100%. Using analysis of variance, the slight downward trend for log cpm with increasing 1,25-(OH)<sub>2</sub>D<sub>3</sub> concentration was statistically significant ( $p = 0.001$ ), but pulsing had no significant effect.

sis patients have a state of immunodeficiency on the one hand and reduced serum 1,25-(OH)<sub>2</sub>D<sub>3</sub> levels on the other, it is of interest to know whether a low serum 1,25-(OH)<sub>2</sub>D<sub>3</sub> concentration could contribute to their immune deficiency state and whether treatment of hyperparathy-

**Table 3.** Ten-based logarithmic values (log cpm) of 12 hemodialysis patients' TT antigen stimulation responses, (mean  $\pm$  SD)

TT antigen Lf/l	1,25-(OH) <sub>2</sub> D <sub>3</sub>		
	0 ng/l (0 mol/l)	42 ng/l (10 <sup>-10</sup> mol/l)	105 ng/l (0.25 $\times$ 10 <sup>-9</sup> mol/l)
<i>Nonpulsed</i>			
1,000	1.48 $\pm$ 0.70	1.39 $\pm$ 0.80	1.31 $\pm$ 0.72
5,000	1.41 $\pm$ 0.85	1.30 $\pm$ 0.76	1.36 $\pm$ 0.78
10,000	1.70 $\pm$ 0.74	1.62 $\pm$ 0.67	1.54 $\pm$ 0.91
Mean	1.53	1.44	1.40
<i>Pulsed</i>			
1,000	1.46 $\pm$ 0.77	1.33 $\pm$ 0.84	1.25 $\pm$ 0.82
5,000	1.61 $\pm$ 0.75	1.49 $\pm$ 0.66	1.43 $\pm$ 0.77
10,000	1.88 $\pm$ 0.75	1.67 $\pm$ 0.80	1.52 $\pm$ 0.92
Mean	1.65	1.50	1.40

roidism with 1,25-(OH)<sub>2</sub>D<sub>3</sub> affects this state. Regarding normal subjects, it has been proposed that an optimal 1,25-(OH)<sub>2</sub>D<sub>3</sub> concentration exists for optimum immune system function [29]. For hemodialysis patients, such conclusions have not been made, rather an immune system enhancing effect of 1,25-(OH)<sub>2</sub>D<sub>3</sub> treatment has been proposed [13–17, 22]. However, supraphysiological concentrations have been shown to be immunosuppressive even in hemodialysis patients in vitro [18].

Zarrabietia et al. [18] showed that in vitro 1,25-(OH)<sub>2</sub>D<sub>3</sub> administered during the culture generation phase at physiological concentrations (up to 10<sup>-11</sup> mol/l; 4.2 ng/l) increased mitogen-induced proliferation, but that supraphysiological concentrations (10<sup>-9</sup> to 0<sup>-7</sup> mol/l; 420 ng/l to 42  $\mu$ g/l) incited an inhibition. In our study, the 1,25-(OH)<sub>2</sub>D<sub>3</sub> concentrations represent those reached in every-day oral and intravenous treatment of secondary hyperparathyroidism in hemodialysis patients [21]. The concentrations we used for constant 1,25-(OH)<sub>2</sub>D<sub>3</sub> (10<sup>-10</sup> to 2.5  $\times$  10<sup>-10</sup> mol/l) were in the range where 1,25-(OH)<sub>2</sub>D<sub>3</sub> treatment turned from enhancing to suppressing in the study of Zarrabietia et al. [18]. Similarly, for one of the antigens that we used (TT), these high constant concentrations turned out to be immunosuppressive. We administered the highest concentration (7.5  $\times$  10<sup>-10</sup> mol/l; 315 ng/l) only as a 2-hour pulse to simulate intravenous 1,25-(OH)<sub>2</sub>D<sub>3</sub> treatment. This did not act as immune system suppressing on our patients' cells.

We used a novel method for studying 1,25-(OH)<sub>2</sub>D<sub>3</sub> treatment. Normally, in a lymphocyte stimulation test, peripheral blood mononuclear cells are applied onto

cell culture plates together with antigens or mitogens and a culture medium, after which the plates are incubated in a cell culture incubator for a required time period (days). In addition to this, we added 1,25-(OH)<sub>2</sub>D<sub>3</sub> together with the culture medium during the culture generation phase. We replaced the culture medium once during the cell culture period, adding pulsed 1,25-(OH)<sub>2</sub>D<sub>3</sub> or control (RPMI) culture medium for 2 h. It is noteworthy that using this method, cells undergo manipulation. According to our results, this procedure had no harmful effect on cell proliferation.

When hemodialysis patients' lymphocyte proliferation responses were studied by Antonen et al. [22], a clear enhancement was seen by intravenous 1,25-(OH)<sub>2</sub>D<sub>3</sub> treatment in patients with initially low serum 1,25-(OH)<sub>2</sub>D<sub>3</sub> levels who also had initially decreased antigen stimulation responses. In our study, no such effect could be confirmed (not shown). The lymphocyte stimulation responses of hemodialysis patients – in the absence of 1,25-(OH)<sub>2</sub>D<sub>3</sub> – in our study were similar to those of 2 healthy test control individuals, and, accordingly, we considered the responses to be good. In lymphocyte stimulation tests, the measures (cpm) reached depend on the isotope used, its specific activity and the test settings in many ways. It is not possible to directly compare our results to other's, but in a previous study, the PPD-induced median response of uremic patients (n = 49, of whom 38 were on dialysis treatment) was only 63% of that of controls (n = 54; p < 0.02) [30]. Also in the study by Tabata et al. [13], the mean mitogen-induced response of 7 hemodialysis patients was significantly lower than that of 6 controls. In our study, with good lymphocyte stimulation responses, no significant enhancement by any enriching factor in the culture medium can be expected. Our finding that a high pulsed concentration of 1,25-(OH)<sub>2</sub>D<sub>3</sub> is not toxic thus becomes all the more important.

Better nutrition and more efficient dialysis with the use of high-flux membranes [31] and erythropoietin [32, 33] might be factors contributing to a better immune response in today's dialysis patients. Even humoral vaccination responses of hemodialysis patients might not be defective any more [34–36]. Yet any therapies with a potential risk of an immune system suppressing action should be avoided in the treatment of hemodialysis patients. Constantly administered high concentration calcitriol might act as such, as it was slightly immune response suppressing for TT antigen in our study. However, in vitro pulsed 1,25-(OH)<sub>2</sub>D<sub>3</sub> even at supraphysiological concentrations does not significantly suppress cellular immune responses of dialysis patients.



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# Treatment of hyperphosphatemia, secondary hyperparathyroidism and hypocalcemia with calcium carbonate favorably modulates vaccination response in uremic rats

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## Key words

calcium carbonate – immune function – hyperparathyroidism – phosphate binding – hyperphosphatemia

**Abstract.** **Aims:** Immune dysfunction is characteristic of renal failure, leading to suboptimal antibody generation and increased susceptibility to infections. We tested whether the treatment of uremic phosphate retention by increased calcium carbonate intake will beneficially influence vaccination response in 5/6-nephrectomized rats. **Methods:** The nephrectomized (uremic) and sham-operated (control) rats were either fed 0.3% calcium diet (NTX and Sham groups, respectively) or 3% high-calcium diet (Ca-NTX and Ca-Sham groups). All rats were immunized with tetanus toxoid 6 weeks after the operations, and antitoxin levels were measured 7 weeks later. **Results:** Plasma creatinine was significantly elevated after the nephrectomy: the values (mean  $\pm$  SD) in the NTX (n = 16), Ca-NTX (n = 11), Sham (n = 14) and Ca-Sham (n = 8) groups were  $97 \pm 14$ ,  $93 \pm 17$ ,  $66 \pm 7$ , and  $69 \pm 8$   $\mu$ mol/l, respectively. The NTX group developed phosphate retention and secondary hyperparathyroidism, which were completely prevented by the high calcium diet. The mean tetanus antitoxin concentrations of the groups were: NTX  $0.25 \pm 0.32$ ; Ca-NTX  $0.45 \pm 0.44$ ; Sham  $0.58 \pm 0.24$  and Ca-Sham  $0.64 \pm 0.25$  IU/ml (log of geometric mean concentration). The antibody response in the NTX group was significantly lower, i.e. 43% of that in the Sham group (p = 0.003), while the response in the Ca-NTX group was not different from that in the Sham group. The tetanus response of all the uremic rats inversely correlated with the plasma levels of phosphate (r = -0.447, p = 0.02), parathormone (r = -0.409, p = 0.03) and creatinine (r = -0.578, p = 0.002). **Discussion:** We conclude that renal failure impairs

vaccination response in rats, the impairment of which can be favorably modulated by phosphate-binding and PTH-suppressing high-calcium diet.

## Introduction

Immunological dysfunction is common in uremia. Patients with renal failure exhibit deficient phagocytosis, defects in the cooperation and function of antigen-presenting cells and T cells, and lowered specific antibody responses and vaccination seroconversion rates [Cohen et al. 1997, Descamps-Latscha and Chatenoud 1996, Girndt et al. 1999]. The underlying mechanisms of the immune deficiency in uremia are incompletely understood. With the present therapies of renal patients, more adequate immunological responses have been reported, as vitamin D therapy and other treatments of secondary hyperparathyroidism and also the correction of renal anemia with erythropoietin, have been thought to contribute to this progress [Antonen et al. 2000, Sennesael et al. 1991, Tabata et al. 1986].

In experimental uremia, the development of secondary hyperparathyroidism has been suggested to impair antibody production, the impairment of which can be reversed by parathyroidectomy [Gaciong et al. 1991]. High intracellular calcium concentration in end-stage renal disease and secondary hyper-

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parathyroidism is harmful for lymphocyte proliferation and activation [Ori et al. 1999]. A high-calcium diet per se decreases lymphocyte intracellular free calcium concentration in rats [Wuorela et al. 1992]. In clinical practice, calcium salts are widely used in the prevention and treatment of uremia-associated hyperphosphatemia and secondary hyperparathyroidism [Teruel et al. 1999, Yudd and Llach 2000]. The 5/6-nephrectomized rat offers a well-controlled model to study chronic renal failure [Gaciong et al. 1991, Miller and Stewart 1980, Raskova and Morrison 1973]. We applied this rat model to study the effects of renal failure on antibody response to tetanus vaccination, and to examine the effect of high calcium carbonate diet upon this antibody response. In particular, we tested the hypothesis whether treatment of hypocalcemia, hyperphosphatemia and secondary hyperparathyroidism by oral calcium carbonate administration is beneficial to antibody generation in uremia.

## Material and methods

Fifty-five young male Sprague-Dawley rats were housed 2 to a cage with free access to drinking fluid and food pellets (Lactamin AB, Stockholm, Sweden). At the age of 8 weeks, the rats were subjected either to uremia-inducing 5/6 surgical nephrectomy, in which the upper and lower poles of the left kidney were cut off and the right kidney was subsequently removed (uremic rats,  $n = 29$ ) [Ylitalo et al. 1976], or to sham operation, in which both kidneys were decapsulated (control rats,  $n = 26$ ). The surgical procedures were performed under ketamine/diazepam anesthesia (75 mg/kg and 2.5 mg/kg intraperitoneally, respectively). Intraperitoneal antibiotics (metronidazole 60 mg/kg and cefuroxim 225 mg/kg) were given after the surgery, and pain was relieved with buprenorphine (0.2 mg/kg subcutaneously) 3 times a day during the first 3 postoperative days.

### *Treatment of hyperphosphatemia and high PTH in uremic rats*

Four weeks after the operation (at study week 4), when the rats were 12 weeks old,

sham-operated and nephrectomized rats were divided to control calcium (Sham  $n = 17$ , and NTX  $n = 18$ ) and high calcium groups (Ca-Sham  $n = 9$ , and Ca-NTX  $n = 11$ ) of equal mean systolic blood pressures and body weights. Commercial diets were used, which were ordered directly from the manufacturer and contained either control 0.3% or high 3.0% calcium (Lactamin R34 rat chow, AnalyCen, Lindköping, Sweden). Otherwise, the 2 diets were identical and contained 0.80% phosphate, 1,500 IU/kg vitamin D and 16.5% protein. At study week 6, all rats were immunized with tetanus toxoid (50 Lf/ml in saline; Lf = limes flocculationis; an Immunoprotect antigen supplied by Immuno AG, Vienna, Austria) emulsified with complete Freund's adjuvant (3 : 7), so that each rat received a 100  $\mu$ l (1.5 Lf, approximately 4.5  $\mu$ g) vaccine in the buttock musculature. At study week 13, the rats were weighed and anesthetized by the intraperitoneal administration of urethane (1.3 g/kg). The carotid artery was cannulated and blood samples for plasma creatinine, hemoglobin, intact parathyroid hormone, ionized calcium, phosphate, 1,25-(OH)<sub>2</sub>D<sub>3</sub> and tetanus antibodies were drawn. The experimental design of the study was approved by the Animal Experimentation Committee of the University of Tampere, Finland, and the Provincial Government of Western Finland Department of Social Affairs and Health, Finland.

### *Clinical variables and tetanus antitoxin concentrations*

The blood samples were collected into chilled tubes (except for ionized calcium, which was drawn into glass capillaries) with heparin as anticoagulant. Creatinine in plasma was determined by kinetic colorimetric assay (Cobas Integra analyzer, F. Hoffman-La Roche Ltd., Basel, Switzerland). Plasma phosphate was determined by colorimetric end-point dry chemistry (Vitros 950 analyzer, Johnson and Johnson Clinical Diagnostics, Rochester, NY, USA). Ionized calcium was measured by an ion-selective electrode (Ciba Corning 634 Ca<sup>2+</sup>/pH analyzer, Ciba Corning Diagnostics, Sudbury, UK), and hemoglobin by photometric analysis (Technicon H\*2, Technicon Instruments Corporation, Tarry-

Table 1. Study protocol.

Study week	0	4	6	13
Age of rats	8 weeks	12 weeks	14 weeks	21 weeks
Procedure	5/6-nephrectomy/ sham operation	Start of diets	Vaccination	Collection of samples

town, NY, USA). Plasma intact parathormone was determined with N-tact PTH IRMA reagent kits from Incstar Corporation (Stilwater, Minnesota, USA). Tetanus antitoxin concentrations were measured from plasma samples with a double antigen ELISA test, which detects protecting, mainly type IgG tetanus antibodies [Kristiansen et al. 1997]. The antitoxin concentrations are expressed in international units per ml (IU/ml). Forty-nine rats (16 NTX, 11 Ca-NTX, 14 Sham and 8 Ca-Sham) were included in these analyses in this study. Additionally, the active vitamin D  $1,25(\text{OH})_2\text{D}_3$  concentration was measured together from 28 rats (15 NTX and 13 Sham rats), methods as previously described [Saha et al. 1993].

### Statistics

For the calculation of geometric mean concentrations, the tetanus antitoxin concentrations were converted into 10-based logarithmic values. SPSS 7.5 for Windows was used for the statistical analyses. The results were analyzed using the Mann-Whitney U-test (U-test; for uremic versus control rats) or 1-way analysis of variance (ANOVA) with LSD test as a post-hoc multiple comparison method (for comparisons between Sham, Ca-Sham, NTX and Ca-NTX groups). In the whole study population, a simple factorial analysis of variance (experimental method) was yet carried out to find out whether kidney status (nephrectomy or not) and/or diet (high calcium or not), as fixed factors, would have an influence on tetanus response (as the dependent variable). When factors explaining the antibody response among the NTX rats were analyzed, bivariate correlation test was applied. Values were generally expressed as mean  $\pm$  SD unless otherwise indicated. A  $p < 0.05$  was considered significant.

## Results

### Clinical variables

The clinical chemistry and weights in the 4 rat groups are shown in Table. In the 2 main groups (uremic, i.e. NTX + Ca-NTX, versus control, i.e. Sham + Ca-Sham), the nephrectomy induced a significant renal failure and anemia: in uremic rats, creatinine was significantly higher and hemoglobin lower than that of control rats (creatinine  $95.4 \pm 15.0$  vs  $67.2 \pm 7.4$   $\mu\text{mol/l}$ ,  $p = 0.001$ , and hemoglobin  $167.8 \pm 15.6$  vs  $180.3 \pm 7.9$   $\text{g/l}$ ,  $p < 0.001$ , U-test). The uremic rats had lower  $1,25(\text{OH})_2\text{D}_3$  than the control rats ( $84 \pm 16$  vs  $97 \pm 18$   $\text{pmol/l}$ ,  $p = 0.034$ , U-test), although no significant differences were found in the plasma levels of active vitamin D between the 4 study groups.

### Effects of high calcium diet on plasma phosphate, PTH and ionized calcium

NTX rats on control 0.3% calcium diet developed phosphate retention and secondary hyperparathyroidism, which, on the other hand, were clearly prevented by 3.0% high calcium diet in the Ca-NTX group. Additionally, high calcium diet induced an increase in serum ionized calcium both in the Ca-NTX and Ca-Sham groups (Table 2).

### Tetanus antibodies

The geometric means  $\pm$  SD of tetanus antitoxin concentrations of the 4 groups are shown in Figure 1. Tetanus vaccination-induced antibody response of uremic rats ( $0.33 \pm 0.38$  IU/ml) was significantly lower than that of the control rats ( $0.60 \pm 0.23$  IU/ml,  $p = 0.006$ , U-test). The mean response of NTX rats was significantly lower, i.e. only 43% ( $0.25/0.58$ ) of that of the (corresponding) sham rats ( $p = 0.003$ , U-test; Figure 1). The mean tetanus response of Ca-NTX rats was 70% ( $0.45/0.64$ ) of Ca-sham rats' response, and this difference was not statistically significant ( $p = \text{NS}$ , U-test, Figure 1). The difference in the tetanus response between NTX

Table 2. Laboratory variables and weights (mean  $\pm$  SD) of 5/6-nephrectomized (NTX) and sham-operated (Sham) rats according to the diets (control or high calcium (Ca) diet). Diet-related differences among the subgroups (Ca-Sham, NTX or Ca-NTX versus Sham, and Ca-NTX versus NTX) were tested with 1-way Anova + LSD.

	Sham n = 14	Ca-Sham n = 8	NTX n = 16	Ca-NTX n = 11
Creatinine (mmol/l)	66 $\pm$ 7	69 $\pm$ 8	97 $\pm$ 14*	93 $\pm$ 17*
Hemoglobin (g/l)	181 $\pm$ 7	180 $\pm$ 10	164 $\pm$ 19*	173 $\pm$ 6
PTH(intact) (pg/ml)	201 $\pm$ 82	40 $\pm$ 6	514 $\pm$ 293*	52 $\pm$ 21**
Ca(ion) (mmol/l)	1.36 $\pm$ 0.07	1.48 $\pm$ 0.04*	1.29 $\pm$ 0.05*	1.47 $\pm$ 0.04*,**
Phosphate (mmol/l)	1.51 $\pm$ 0.20	1.12 $\pm$ 0.25*	1.75 $\pm$ 0.40	1.06 $\pm$ 0.33*,**
1,25(OH) <sub>2</sub> D <sub>3</sub> (pmol/l) <sup>a</sup>	91 $\pm$ 12	101 $\pm$ 22	83 $\pm$ 8	84 $\pm$ 21
Weight (g)	470 $\pm$ 21	469 $\pm$ 43	465 $\pm$ 21	460 $\pm$ 21

\* =  $p < 0.01$  versus the sham group, \*\* =  $p < 0.001$  versus the NTX group, <sup>a</sup>n = 6 (Sham), n = 7 (Ca-Sham), n = 7 (NTX), n = 8 (Ca-NTX).

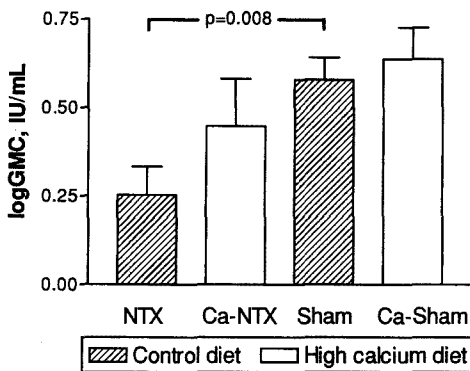


Figure 1. Mean (SD) tetanus response (logGMC, IU/ml) of 5/6-nephrectomized (NTX) and sham-operated (Sham) rats according to diet received (control or high-calcium (Ca) diet). Statistics: Mann-Whitney U-test. Mean response in the high-calcium diet uremic rats (Ca-NTX) did not differ from that in the sham groups. Between NTX and Sham,  $p = 0.008$ , and NTX and Ca-Sham,  $p = 0.005$  (Mann-Whitney U-test).

and Ca-NTX groups was not statistically significant.

In analysis of variance, kidney status and diet together significantly explained vaccination-induced antibody response to tetanus antitoxin ( $p = 0.011$ ), as kidney status by itself also significantly anticipated the tetanus response ( $p = 0.019$ ).

## Factors explaining the antibody response in nephrectomized rats

The relationship of tetanus antibody response of the uremic rats (all nephrectomized

rats) to hemoglobin, creatinine, ionized calcium, PTH, phosphate, 1,25(OH)<sub>2</sub>D<sub>3</sub>, and weight was examined. The tetanus response inversely correlated with phosphate ( $r = -0.447$ ,  $p = 0.02$ ), PTH ( $r = -0.409$ ,  $p = 0.03$ , Figure 2b) and creatinine ( $r = -0.578$ ,  $p = 0.002$ , Figure 2a). Creatinine correlated with 1,25(OH)<sub>2</sub>D<sub>3</sub> ( $r = -0.719$ ,  $p = 0.003$ ), PTH ( $r = 0.508$ ,  $p = 0.007$ ) and phosphate ( $r = 0.471$ ,  $p = 0.01$ ) but not with the other factors.

## Discussion

Chronic renal failure suppresses immune functions. Uremic toxins cause metabolic and functional changes in polymorphonuclear leukocytes, and impair the function and cytokine production of antigen presenting cells and T cells, leading to defects in B cell function [Cohen et al. 1997, Girndt et al. 1999]. Elevated cytosolic free calcium, a probable consequence of high circulating parathyroid hormone levels and uremia [Alexiewicz et al. 1990, Massry and Fadda 1993, Ori et al. 1999], is toxic to numerous cell functions, including immune responses [Fujita and Palmieri 2000, Massry and Fadda 1993]. The treatment of hyperparathyroidism with parathyroidectomy or calcitriol administration, and also the administration of calcium channel blockers, normalize the elevated cytosolic free calcium, and simultaneously normalize or improve the function of neutrophils and B cells, in uremic patients and in experimental uremia [Alexiewicz et al. 1995, 1996, Chervu et al. 1992, Gaciong et al. 1991, Haag-Weber et al. 1993, Horl et al. 1995].

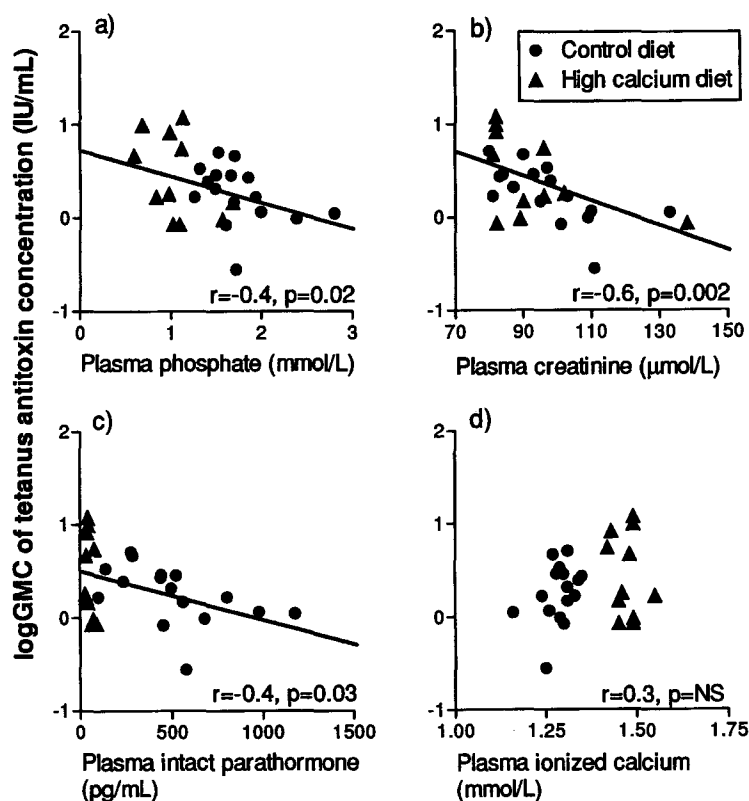


Figure 2. The correlation of tetanus response and (a) plasma phosphate, (b) plasma creatinine, (c) plasma intact parathormone and (d) plasma ionized calcium concentrations of 5/6-nephrectomized rats, according to diet received.

We induced renal failure with a subtotal nephrectomy, and investigated the effect of phosphate binding with increased calcium carbonate intake on tetanus vaccination response in rats. The 5/6-nephrectomy caused a moderate but significant renal failure with a relatively small variation in plasma creatinine, which suggests that the study groups were homogenous, and reliable conclusions about immune responses could be made. Vaccination is a useful method to examine immune functions, because both cellular and humoral systems are involved in the subsequent immune response. Accordingly, the antibody response reflects the summary function of both of these systems [Liu 1998].

In the present study, chronic renal failure was associated with depressed tetanus response, and the impairment of immune function was correlated with the severity of renal insufficiency. Similar findings of suppressed antibody response to antigens in nephrectomized rats have been previously reported [Gaciong et al. 1991, Raskova and Morrison

1973]. On the contrary, in 1 report [Nelson et al. 1980], there was no difference in the antibody response between nephrectomized and control rats, but in this study the antigens were injected on the day of induction of renal failure before the development of full-blown uremia. In our study, the antigen was inoculated later at a phase when the rats already had renal insufficiency, which protocol better simulates the situation in chronic uremia. In analysis of variance, renal failure (kidney status) powerfully explained antibody response obtained by tetanus vaccination. Thus, our results confirm the harmful effects of renal failure on vaccination response [Gaciong et al. 1991, Raskova and Morrison 1973], and show that this impairment significantly correlates with the level of remaining renal function.

The vaccination response of the uremic rats on the high calcium diet did not differ from sham-operated controls. Uremic rats on the control diet, however, had a lower tetanus response than the sham-operated rats. Metabolic changes, such as treatment of hyperphosphatemia and secondary hyperparathyroidism probably have contributed to the improved tetanus response among the rats on high-calcium diet. In earlier studies, parathyroidectomy has normalized vaccination-induced antibody production and phagocytosis of polymorphonuclear leukocytes (PMNL) in rats with chronic renal failure [Chervu et al. 1992, Gaciong et al. 1991]. Vitamin D therapy (to control hyperparathyroidism) has also positive effects on immune functions, such as lymphocyte function and vaccination response, in chronic renal failure [Antonen et al. 1996, 2000, Tabata et al. 1986].

Despite all reports on the beneficial immunological actions of therapies of secondary hyperparathyroidism, there are no earlier studies on the actions of phosphate-binding with calcium carbonate ingestion on immune functions in renal failure. In addition, the possible effects of hyperphosphatemia per se on immune functions are currently unknown. We found a negative relationship between plasma phosphate level and tetanus vaccination response. We propose that by preventing hyperphosphatemia and subsequently the development of hyperparathyroidism, calcium carbonate seems to have some positive immunological effects, mimicking those of

parathyroidectomy or vitamin D therapy. Regardless of exact mechanism (reversal of hypocalcemia, hyperphosphatemia or secondary hyperparathyroidism), the impact of the therapeutic intervention, calcium carbonate treatment, was beneficial: the tetanus response of Ca-NTX rats was statistically indifferent from that of control (sham-operated) rats, and there were inverse correlations between tetanus response and phosphate and parathormone levels among all uremic rats. Future experiments should be designed to differentiate, whether phosphate binding by other means in addition to oral calcium carbonate administration (e.g. aluminum-containing salts, sevelamer) can beneficially influence vaccination response and other immunological actions in renal failure. Therefore, the present results must be transferred to therapy of human renal failure with caution.

In summary, experimental uremia leads to a significant impairment of tetanus vaccination-induced antibody response, which is related to the severity of renal failure. Increased dietary calcium intake, by treating and preventing the development of hyperphosphatemia, hypocalcemia and secondary hyperparathyroidism in 5/6-nephrectomized rats, helped to maintain a fair tetanus vaccination response in 5/6-nephrectomized rats, although renal function was not affected.

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ORIGINAL ARTICLE

## Effects of erythropoietin treatment on cell-mediated immune responses in predialysis patients

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### Abstract

**Objective.** The effects of erythropoietin (EPO) treatment on the immune functions of dialysis patients have been shown to be controversial and there are only limited data concerning predialysis patients. **Material and methods.** Twenty-four predialysis patients with renal anemia were assigned to subcutaneous EPO treatment, and those in need ( $n = 19$ ) were additionally treated with i.v. iron every other week. We analyzed the effect of the start of EPO treatment on (i) lymphocyte and lymphocyte subclass counts, (ii) lymphocyte stimulation functions and (iii) persisting IgG-class antibody levels to the viral antigens of Epstein–Barr virus and cytomegalovirus. **Results.** Our main findings were a decrease in the absolute lymphocyte count, combined with decreases in all the main lymphocyte subclass counts. The absolute number of cells with activation and memory markers remained constant, and therefore their proportion slightly increased. The proliferation responses to phytohemagglutinin, tuberculin and tetanus declined significantly, while the amount of IgG-class viral antibodies remained unchanged, meaning that the humoral side of immunity was not affected by the start of the EPO treatment. Similarly, the proliferation response to pokeweed mitogen, a B-cell mitogen, was unchanged. **Conclusions.** EPO treatment has a suppressive effect on cellular immune functions of predialysis patients. This suppression does not correlate with erythropoiesis, kidney function or iron status.

**Key Words:** Immunodeficiency—secondary, proliferation, renal immunology, T cells

### Introduction

Erythropoietin (EPO), a hormone produced by the kidneys, stimulates red blood cell production in bone marrow. Patients with impaired renal function have diminished EPO production, and subsequently develop renal anemia. Recombinant human EPO and its functional analogs are widely used in the correction of renal anemia [1–3]. EPO may affect the immune system, as it binds to specific receptors in human bone marrow mononuclear cells [4].

In dialysis patients, coinciding with the rapid correction of anemia, EPO treatment causes an initial suppression of lymphocyte stimulation responses followed by a subsequent improvement (6–18 months) [5–8], although solely positive effects have been reported as well [9,10]. EPO increases the responses to some vaccinations [11,12] and increases

immunoglobulin production [13]. The amounts of total T cells, helper and suppressor T-cell subsets and B cells may remain unchanged [5,8,14–16] or decrease [5,14], but the helpers and suppressors have been shown to increase as well [16]. In contrast to dialysis patients, there are so far only limited data on the immunological effects of EPO treatment in predialysis patients: Steffensen et al. [8] studied cell-mediated immunity as a result of EPO treatment in predialysis, hemodialysis and peritoneal dialysis patients (five subjects in each group), and found no differences between the groups.

We analyzed the numbers of lymphocytes and lymphocyte subsets, assessed lymphocyte function and measured the antibody levels to two common viruses in predialysis patients who started EPO treatment. We hypothesized that, even in predialysis

patients, EPO treatment affects immune cells and their function.

## Material and methods

### Patients and clinical parameters

Twenty-four predialysis patients with renal anemia were included in the study. Exclusion criteria were as follows: untreated anemia due to folic acid or vitamin B12 deficiency; iron supplementation treatment or blood transfusion within 1 month prior to enrollment; previous EPO treatment; corticosteroid treatment; and rheumatoid arthritis and other diseases and conditions (including acute infectious diseases) clearly affecting immunological status. The patients were enrolled at the Tampere University Hospital Nephrological Outpatient Clinic. All subjects gave their informed consent to participate in the study, which was approved by the local ethical committee.

The predialysis patients (mean age 59 years; range 26–88 years) had moderate-to-severe chronic kidney disease (serum creatinine 125–847  $\mu\text{mol/l}$ ) together with renal anemia (Table I). They had a mild to moderate degree of secondary hyperparathyroidism (mean parathyroid hormone level  $19.4 \pm 11.5$  pmol/l). Ten of the 24 patients had diabetic nephropathy, five had polycystic kidney disease, two had glomerulonephritis, two had chronic interstitial nephritis, two had obstructive uropathy, one had focal segmental glomerulosclerosis and in two the diagnosis was unknown. The decision to start EPO treatment was based on the clinical findings of renal anemia.

The starting dose of EPO (all patients received  $\alpha$ -EPO, with the exception of two who received  $\beta$ -EPO) was 150 U/kg/week subcutaneously. Patients received i.v. iron supplementation (Venofer<sup>®</sup>; complex of polynuclear iron (III) hydroxide in sucrose) after 1 month if they had a ferritin level of  $<200$   $\mu\text{g/l}$  and/or transferrin saturation  $<20\%$ . After an initial dose of 50 mg, the subjects received a 200-mg dose every other week throughout the study. The subjects were typically given 650–850 mg of iron during the study.

Serum creatinine and urea were measured at the start of the study and at 3 months. The blood count (together with leukocyte differential distribution analysis), hemoglobin (Hb), reticulocytes (retic), ferritin, percentage saturation of transferrin (TSAT) and transferrin receptor content (TFR) were measured from blood samples obtained at the start of EPO treatment and after 2 weeks and 1, 2 and 3 months. The glomerular filtration rate (GFR) was calculated according to the formula of Cockcroft and Gault [17].

### Immunological tests

The immunological tests were performed immediately before the onset of EPO treatment and at 3 months.

*Leukocyte and lymphocyte subset analyses.* Lymphocyte subclass analyses were measured from EDTA blood samples for B and natural killer (NK) cells, as well as for the surface markers CD3, CD4, CD8, HLA-DR, CD45RO and CD38.

*Lymphocyte stimulation tests.* Heparinized blood samples were drawn, and peripheral blood mononuclear cells were separated using the Ficoll–Hypaque layer centrifugation method [18]. After careful washing, cell cultures were generated in flat-bottomed microtiter plates (Nunc, Roskilde, Denmark) in triplicate, using Roswell Park Memorial Institute medium and 10% autologous plasma. The antigen response cultures were stimulated with purified protein derivative (PPD) of tuberculin (Staatens Serum Institut, Copenhagen, Denmark), at concentrations of 10, 1 or 0 mg/l, or tetanus toxoid (TT; Immuno AG, Vienna Aizzo, Austria), at concentrations of 1000, 10 000 or 0 limits of flocculation (LF)/l. These antigens are useful in the Finnish population as  $>99\%$  of Finnish children are included in the Finnish State Programme on Immunization and are vaccinated against tuberculosis with bacillus Calmette–Guérin (BCG) vaccine, and against tetanus with pertussis–diphtheria–tetanus (PDT) vaccine. Ac-

Table I. Clinical parameters (mean  $\pm$  SD) at the start of the study and at 1 and 3 months.

Parameter	At start	At 1 month	$p^a$	At 3 months	$p^a$
Hb (g/l)	101.8 $\pm$ 7.0	114.6 $\pm$ 8.2	$<0.001$	126.9 $\pm$ 8.3	$<0.001$
Creatinine ( $\mu\text{mol/l}$ )	393.0 $\pm$ 191.7			453.7 $\pm$ 220.6	0.036
GFR (ml/min)	23.5 $\pm$ 14.9			20.2 $\pm$ 12.3	0.055
Ferritin ( $\mu\text{g/l}$ )	173.5 $\pm$ 169.7	82.5 $\pm$ 95.1	$<0.001$	191.5 $\pm$ 124.0	0.330
TSAT (%)	21.9 $\pm$ 6.4	13.8 $\pm$ 6.9	$<0.001$	17.3 $\pm$ 7.6	0.004
TFR (mg/l)	1.49 $\pm$ 0.35	2.88 $\pm$ 0.67	$<0.001$	2.29 $\pm$ 0.57	$<0.001$

<sup>a</sup>Versus start.

cording to Finnish experience, reactivity, when achieved, is often long-lasting [19]. The mitogen response cultures were stimulated with phytohemagglutinin (PHA) at concentrations of 1, 0.1 or 0 mg/l (HA16; Wellcome, Dartford, UK) or pokeweed mitogen (PWM) at dilutions of 1:1000, 1:5000 or 0. Cell cultures were harvested on the third (PHA) and seventh (PWM, PPD, TT) days. The stimulation response was measured as the intake [counts per minute (cpm)] of tritiated thymidine (methyl-<sup>3</sup>H-thymidine; 5 Ci/mmol, 1 µCi/well; Amersham, Little Chalfont, UK) during the last 6 h on the last (third or seventh) culture day. The specific response was calculated by subtracting the mean intake of tritiated thymidine (in triplicate) of the unstimulated control culture from that of the stimulated cultures. The data were transformed into logarithmic values [20]. The data for different antigens and mitogens were analyzed separately.

*CMV and EBV antibodies.* To evaluate competence for general antibody formation, IgG-class antibodies against two common infectious agents, cytomegalovirus (CMV) and Epstein-Barr virus (EBV), were measured.

#### Statistics

A non-parametric paired samples test (Wilcoxon's signed ranks test) was performed for the analysis of EPO treatment to evaluate differences in values obtained at the start of the study and at 3 months. A general linear model for repeated measures was used for analyses of leukocyte numbers at five time points of EPO treatment. To describe changes in variables between the start and end of the study, difference variables (change) for the immune parameters were calculated by subtracting the start value from the 3-month value. These were analyzed with the Mann-Whitney U-test. Spearman's bivariate correlation was used to evaluate the impact of the most important clinical and iron status parameters on difference variables from the lymphocyte stimulation tests. All statistical analysis was performed with SPSS version 11.5.  $p < 0.05$  was considered significant.

## Results

### Effects of EPO treatment

The general effects of EPO treatment on Hb, renal function and common iron status parameters are shown in Table I. At 1 month, most patients (19/24) had developed iron deficiency and received iron supplementation during the remainder of study.

The rise in Hb (from  $103 \pm 6$  to  $128 \pm 6$  g/l in the iron-treated group, and from  $96 \pm 7$  to  $120 \pm 10$  g/l in the non-iron-treated group) and the fall in GFR (from  $14.6 \pm 4.3$  to  $13.9 \pm 1.6$  ml/min, and from  $25.1 \pm 16$  to  $22.6 \pm 14$  ml/min, respectively) did not differ between the iron-treated and non-iron-treated groups (Mann-Whitney U-test).

### Results of the immunological tests

The leukocyte count (mean  $\pm$  SD) remained unchanged ( $6.81 \pm 2.36 \times 10^9/l$  at the start and  $6.82 \pm 2.51 \times 10^9/l$  at 3 months), and so did the absolute neutrophil count ( $4.45 \pm 1.96 \times 10^9/l$  and  $4.69 \pm 2.01 \times 10^9/l$ , respectively). Lymphocytes decreased significantly during EPO treatment, from  $1.64 \pm 0.55 \times 10^9/l$  to  $1.35 \pm 0.48 \times 10^9/l$  ( $p = 0.006$ ). The absolute and subclass lymphocyte counts are shown in Table II. B cells, CD3-, CD4- and CD8-positive cells all decreased. The CD4:CD8 ratio did not change ( $1.9 \pm 1.0$  vs  $2.1 \pm 2.2$ ;  $p = 0.097$ ). Only the absolute number of memory cells (CD4+CD45RO+ and CD8+CD45RO+) and the absolute number of cells showing activation markers (CD4+HLADR+, CD8+HLADR+, CD4+CD38+ and CD8+CD38+) remained constant, and accordingly their proportion increased (Table II).

The individual patients' responses to EPO treatment are shown in Figure 1. The lymphocyte stimulation responses clearly and consistently declined under EPO treatment: PHA 1.0 mg/l from  $3.46 \pm 0.32$  to  $3.09 \pm 0.50$  cpm ( $p = 0.001$ ); PWM 1:1000 from  $2.50 \pm 0.63$  to  $2.31 \pm 0.65$  cpm ( $p = 0.073$ ); PPD 10 mg/l from  $2.59 \pm 0.58$  to  $2.30 \pm 0.66$  cpm ( $p = 0.046$ ); and TT 10 LF/l from  $2.54 \pm 0.65$  to  $2.12 \pm 0.74$  cpm ( $p = 0.039$ ). The decline in stimulation responses at the lower concentrations of the mitogens and antigens was non-significant.

There was no change in the concentrations of IgG-class viral antibodies to CMV ( $5.0 \pm 4.6$  vs  $5.3 \pm 5.0$  U/ml) or EBV ( $168.1 \pm 120.5$  vs  $160.6 \pm 108.4$  U/ml).

### Effect of clinical factors on the absolute lymphocyte count and the results of the stimulation tests

The ferritin level (mean  $\pm$  SD) of the iron-treated patients changed from  $44.6 \pm 44.9$  (at 1 month; immediately before i.v. iron was given) to  $175.2 \pm 110.8$  µg/l (at 3 months) and TSAT changed from  $12.2\% \pm 4.6\%$  to  $17.8\% \pm 7.7\%$  (respectively). The ferritin level of the non-iron-treated patients changed from  $211.6 \pm 111.8$  (at 1 month) to  $188.3 \pm 69.6$  µg/l (at 3 months), and TSAT changed from

Table II. Number of lymphocytes and lymphocyte subpopulations ( $\times 10^9$  cells/l; mean  $\pm$  SD) of 24 predialysis patients before and after 3 months of EPO treatment.

	At start	At 3 months	<i>p</i>
Lymphocytes	1.64 $\pm$ 0.55	1.35 $\pm$ 0.48	0.006
CD3+ cells	1.28 $\pm$ 0.51	1.11 $\pm$ 0.40	0.027
CD4+ cells	0.84 $\pm$ 0.35	0.72 $\pm$ 0.29	0.044
CD8+ cells	0.50 $\pm$ 0.22	0.44 $\pm$ 0.18	0.044
B cells	0.12 $\pm$ 0.06	0.10 $\pm$ 0.06	0.034
NK cells	0.22 $\pm$ 0.12	0.20 $\pm$ 0.15	0.258
CD4+CD45RO+	0.65 $\pm$ 0.28	0.59 $\pm$ 0.25	0.106
CD8+CD45RO+	0.25 $\pm$ 0.12	0.23 $\pm$ 0.12	0.448
CD4+CD38+	0.51 $\pm$ 0.27	0.44 $\pm$ 0.20	0.050
CD8+CD38+	0.23 $\pm$ 0.09	0.22 $\pm$ 0.11	0.728
CD4+HLADR+	0.20 $\pm$ 0.32	0.19 $\pm$ 0.22	0.689
CD8+HLADR+	0.17 $\pm$ 0.13	0.18 $\pm$ 0.13	0.884

19.6%  $\pm$  10.7% to 18.3%  $\pm$  2.3% (respectively). Markers of erythropoiesis, kidney function and iron status (mean retic, mean GFR, difference variable of GFR, and mean ferritin, transferrin, TSAT and TFR) were tested for possible correlations with the changes in lymphocyte number and the results of the stimulation tests. None of the markers of erythropoiesis or kidney function correlated with the change in lymphocyte stimulations, and neither did the iron status markers ferritin, TSAT or TFR. Only mean transferrin correlated significantly with the decline of the mitogens PHA ( $r = -0.621$ ;  $p = 0.003$ ) and PWM ( $r = -0.521$ ;  $p = 0.015$ ).

## Discussion

Present-day treatment of renal failure aims at preserving kidney function and preventing secondary hyperparathyroidism, both of which are essential

elements contributing to immune functions in kidney failure [21–23]. EPO and its analogs are widely used in the treatment of predialysis patients. In addition to positive effects in anemia, EPO treatment has been thought to positively affect humoral immunity of dialysis patients by increasing immunoglobulin production [13] and vaccination response [11], but its actions on cellular immunity have been shown to be suppressive during the initial months of treatment [8].

Our main findings from EPO treatment were a clear decrease in the lymphocyte count, which occurred equally in all the main lymphocyte subclasses, and a decline in lymphocyte proliferation. The decline was similar to that shown in earlier studies done on hemodialysis patients [6–8]. The absolute numbers of cells with activation and memory-related markers remained constant, and subsequently their proportion slightly increased. The IgG-class viral antibodies (to CMV and EBV)

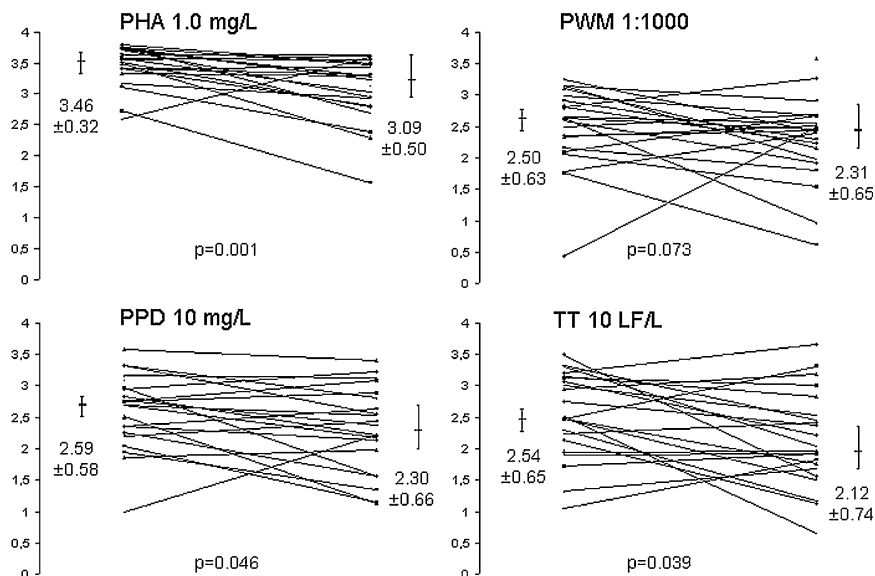


Figure 1. Lymphocyte stimulation responses of 24 predialysis patients at the start of EPO treatment and after 3 months. The results are shown as logarithmically transformed cpm values. The vertical lines with horizontal bars represent medians and 25% and 75% quartiles.

remained unchanged, meaning that the humoral side of immunity was not affected by the start of EPO treatment.

In line with our results, in the study of Steffensen et al. [8] a similar (but non-significant) trend towards an EPO-related reduction in lymphocyte count was seen, and likewise the proportions of CD4+ and CD8+ cells and the CD4:CD8 ratio remained unchanged. Collart et al. [14] also reported an unchanged proportion of T cells, only a minimal decrease in the proportion of B cells and unchanged proportions of CD4+ and CD8+ cells. In our study the subpopulation proportions remained constant, except for those of the activation and memory markers (which increased). Our finding of a proportional increase in T cells expressing activation markers could reflect the rise in TFR during the study. This is because TFR (=CD 71), in addition to being a marker of functional iron deficiency and erythropoiesis, is also an activation marker of T lymphocytes [24].

The reduction in lymphocyte function is not directly linked to the reduction in lymphocyte number. In lymphocyte proliferation tests, a constant amount of cells are used in the microtiter plate. The declines in number and function are thus different phenomena that occur simultaneously, probably for the same reason(s).

The EPO treatment-associated decreases in lymphocyte number and proliferation ability could be due to direct effects of EPO on the immune system or to simultaneous changes in other clinical factors in the patients. The receptors for EPO have been found on human bone marrow cells [4], but not on lymphocytes [25]. For lymphocytes, direct (positive) effects have been suggested only on B cells [13,26,27]. It is not possible to conclude whether the changes seen in our study are more likely to relate to direct effects of EPO or to simultaneous changes in other parameters of the patients. We found no correlation between the mean number of reticulocytes or the GFR and the changes in lymphocyte number and proliferation. The change in GFR was most likely too small to induce any alterations in immune functions.

The changes in iron status could potentially explain some changes in lymphocyte number and function. Since the era of repeated blood transfusions and subsequent iron overload, iron deficiency has become much more common in renal patients undergoing EPO treatment [28,29]. Iron deficiency in particular leads to a decrease in T-cell numbers and to decreased thymocyte and lymphocyte proliferation, but transfusional iron overload has also been associated with a blunted response to mitogens [30,31]. In our study, of the markers of iron status

(ferritin, transferrin, TSAT and TFR), only transferrin correlated with lymphocyte number or proliferation. The patient-to-patient variations in ferritin and TSAT were relatively high (Table I). Based on the results of our study, we cannot exclude the possible effect of iron status or other clinical parameters underpinning the effect of EPO treatment on the decline in lymphocyte number and function.

## Conclusions

The start of EPO treatment in predialysis patients caused a decline in the lymphocyte count, no changes in activation or memory-cell markers and a decline in a wide range of lymphocyte proliferation markers. We conclude that EPO administration has an initial inhibitory action on lymphocyte number and function in predialysis patients. On the basis of current results it is not possible to determine either the clinical significance or permanence of this inhibition.

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