Glucocorticoid Treatment in Childhood Nephrotic Syndrome

Weighing the Cornerstone

Nynke Teeninga

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Glucocorticoid Treatment in Childhood Nephrotic Syndrome

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De behandeling van nefrotisch syndroom bij kinderen met glucocorticoïden

het wegen van de hoeksteen

Proefschrift

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INTRODUCTION

CHAPTER 1

Childhood nephrotic syndrome: one picture, many faces

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Nephrotic syndrome is the most common manifestation of glomerular disease in childhood. The clinical presentation typically includes a relatively sudden onset of either localised or general edema, often accompanied by oliguria, abdominal pain and general discomfort. These symptoms result from impaired function of the glomerular filtration barrier, leading to heavy proteinuria, hypoproteinemia, decreased plasma oncotic pressure, and salt and water retention. Underlying causes are usually unknown and are likely to be multifactorial. Unless treated, this condition can lead to serious morbidity and mortality.

1.1 Clinical presentation and epidemiology

Nephrotic syndrome (NS) is defined by massive proteinuria (≥200 mg protein/mmol creatinin) and hypoalbuminemia (≤25 g/L).[1] Edema, due renal retention of salt and water and decreased plasma oncotic pressure, ranges from puffy eyes to ascites and pleural effusion. The nephrotic state induces hyperlipidemia, hypercoagulability and increased risk of infection.[2]

Hypertension and microscopic hematuria are both seen in around 30% of children in their acute (nephrotic) phase of NS and usually subside after remission is induced.[3] Macroscopic or persisting microscopic hematuria is uncommon. It is associated with therapy resistance,[4] and should prompt clinicians to consider certain underlying glomerular diseases.[5,6] Remission is defined as disappearance of proteinuria (<20 mg protein/mmol creatinin or <1+ of protein on urine dipstick for at least 3 consecutive days).

The worldwide incidence of NS in children is estimated to be 1-7 per 100.000.[2,7,8,9] The incidence is highest in children from Asian descent. Nephrotic syndrome is seen more often in African and African-American children than in Caucasians.[8,6] A recently published Dutch survey reported a yearly incidence of 1.5 per 100.000 children in the Netherlands.[9]

An unexplained male preponderance is observed, with male to female ratio's ranging from 1.5-3.1.[8,10-14] Childhood nephrotic syndrome typically presents in the first decade of life; the average reported age at onset is 3.5-5.5 years.[15,16,12,8] Congenital (before 3 months age) and infantile (3-12 months) NS mostly result from genetic mutations or congenital infections and are therefore clearly distinguished from the far more common 'idiopathic' nephrotic syndrome in childhood (> 12 months age).[2]

This thesis focuses on a clinically classified group of children with nephrotic syndrome without characteristics of glomerulonephritis or congenital/infantile NS (Box 1). The patients reported in this thesis have Steroid Sensitive Nephrotic Syndrome (SSNS), unless otherwise indicated. The practical reason for the chosen classification is that neither renal histology, nor pathogenetic investigations are part of clinical practice in the vast majority of children presenting with nephrotic syndrome. The use of the term Idiopathic Nephrotic Syndrome (INS) for patients presenting with a first episode of NS can be confusing. It is basically a pathogenetic category while thorough investigations such as a renal biopsy or serology are usually not performed at this stage.

Box 1. Classifications of nephrotic syndrome in childhood

Classifications based on clinical presentation

- Age at presentation:
  - <3 months: congenital NS
  - 3-12 months: infantile NS
  - >12 months: childhood NS
- The absence or presence of symptoms indicating glomerulonephritis: persisting hematuria/hypertension and renal failure
- Initial response to predniso(lo)ne:
  - Steroid Sensitive Nephrotic Syndrome (SSNS)
  - Steroid Resistant Nephrotic Syndrome (SRNS)

Histopathological classification

- Minimal Change Nephrotic Syndrome (MCNS)
- Focal Segmental Glomerulosclerosis (FSGS)
- Membranous Nephropathy
- Membranoproliferative Glomerulonephritis
- Other

Pathogenetic classification

- Primary NS
  - 'Idiopathic' NS: cause unidentified
  - Genetic
- Secondary NS:
  - (Post)infectious (e.g. group A beta-haemolytic streptococci)
  - Systemic diseases (e.g. Systemic Lupus Erythomatosus)
  - Syndrome-associated (e.g. Nail-Patella Syndrome)
  - Toxic (e.g. drug reaction)
  - Other

1.2 Pathophysiology

Massive proteinuria as seen in nephrotic syndrome results from increased permeability of the glomerular filtration barrier for proteins. This barrier is comprised of the fenestrated capillary endothelium, the glomerular basement membrane and intercalated foot processes of the visceral cells of the glomerulus, termed podocytes (Figure 1). Increased permeability for proteins can result from both structural changes within the glomerular filtration barrier (GFB) and loss of negatively charged molecules.[17] Recent developments have demonstrated the podocyte and the slit diaphragm in-between foot processes to be of key importance in the pathophysiology of NS.[18,19]

Nephrotic range proteinuria is associated with morphologic changes of podocytes, including retraction and flattening of foot processes (Figure 2). During remission, podocytes have resumed their original shapes. The events triggering podocyte effacement are complex and not fully understood. Potential mechanisms leading to podocyte effacement include impaired signal transduction within the slit diaphragm, increased production of lysosomal proteases, loss of cell polarity and loss of negatively charged proteins from the glomerular filtration barrier.[20,19,21,18] Changes in the cytoskeletal structure of the podocyte may result from both frailty of the podocyte and slit diaphragm architecture and from circulating plasma factors.[18,22,23,24,21] Steroid sensitive nephrotic syndrome is currently thought to entail reversible conformational changes of podocytes, whereas irreversible conformational changes are associated with steroid resistance and disease progression.[18]

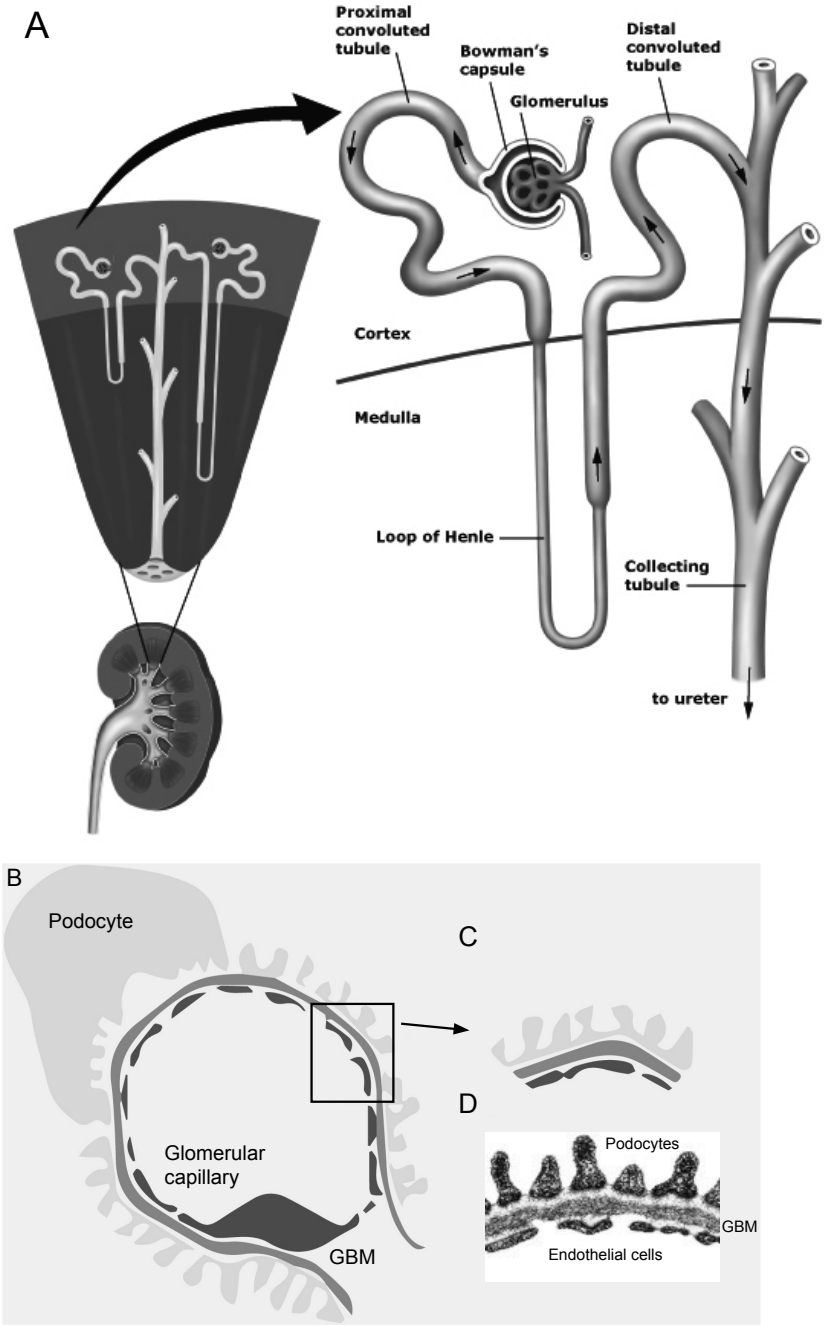

Figure 1. A: The glomerulus as part of a nephron (functional unit) in the kidney. B: The glomerular filtration barrier (GFB) consists of fenestrated endothelial cells, the glomerular basement membrane (GBM) and podocytes, which have intercalated foot processes. C: schematic representation of the GFB. D: transmission electron micrograph of the GFB. Adapted from: Lennon *et al.*[229]

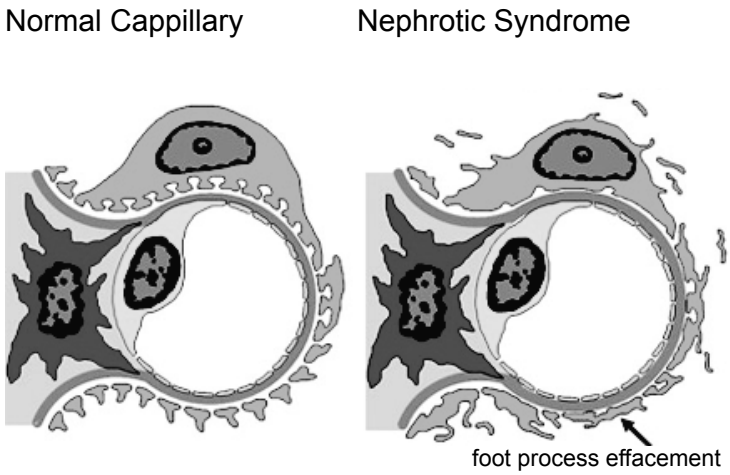

Figure 2. Schematic view of normal podocyte foot processes (left) and fused podocyte foot processes in nephrotic syndrome (right).[230]

Genetic mutations resulting in vulnerability of the podocyte cytoskeleton and slit diaphragm
Pathogenic mutations are rare in childhood NS and have predominantly been found in congenital NS of the Finnish-type and familial focal segmental glomerulosclerosis (FSGS), both characterized by severe and progressive disease.[19] Primary disruption of the podocyte architecture can result from mutations in genes encoding essential components of the slit diaphragm, such as podocin and nephrin. Hereditary types of childhood nephrotic syndrome are beyond the scope of this thesis.

Circulating plasma factors
A body of evidence suggests that a circulating 'permeability factor' induces proteinuria in non-hereditary childhood NS.[25] This could explain the observation that proteinuria can reoccur shortly after kidney transplantation in NS patients,[26] while proteinuria as well as podocyte effacement can resolve when kidneys from patients with NS are donated to recipients without NS.[27] Further support is found in the observations that proteinuria can be temporarily reduced by plasma exchange[28] and that plasma from nephrotic patients induces podocyte effacement and proteinuria in rats.[29] The plasma (permeability) factor hypothesis is however still surrounded by mystery, since neither the plasma factor itself nor the cells producing it have been revealed as yet.

Plasma factors are likely produced by lymphoid cells
Research supporting the plasma factor theory mostly points towards factors produced by immune cells. Relapses are often seen after events triggering the immune system,

such as infections,[30,31,8,32] allergies,[33] and stressful events.[30] These events may contribute to seasonal variation in the incidence of relapses.[34]

Evidence for activated T-cells and B-cells

Particularly abnormal activity of T lymphocytes has been put forward within this context.[35,29] Suppression of T-cells seems to facilitate remission, as was learned from rapid remission seen after measles infection,[36,37] and treatment with (cell-mediated) immunosuppressive therapy.[38] The association between certain HLA class II antigens and susceptibility to NS contributes to the theory of enhanced T-cell activity in NS.[39,40,41]

A role for B lymphocytes in the pathophysiology of NS is proposed from two indirect observations. First, a beneficial effect is seen after B-cell mediated immune suppression (cyclophosphamide, rituximab).[38] Second, several studies reported relatively high incidences of atopy (15-45%) and elevated IgE levels (32-60%) in children with NS.[33]

Though a causal relationship between allergy and NS has not been established, a common ground has been suggested, with interleukin-13 (IL-13) as the potential connector. IL-13 is produced by activated T-cells and is an important stimulator of IgE production by B-cells. IL-13 is also thought to be involved in processes affecting the glomerular protein barrier.[33] Lai *et al*. found podocyte effacement and proteinuria in rats that overexpressed IL-13.[33] In children with NS, both elevated levels of IL-13[42] and increased upregulation of IL-13 in T-cells[43] have been found during the nephrotic phase.

Several studies that analyzed cytokine profiles in patients with NS revealed a predominance of type 2 T-helper (Th2) cytokines over type 1 T-helper (Th1) cytokines during the nephrotic phase.[44] Th2 cytokines, including IL-13, are engaged in allergic responses and responses against parasites. This last finding might explain the therapeutic effect of levamisole, which is thought to restore the balance between Th1 and Th2 immune responses in NS.[45] The associations between IL-13 and NS described above support a Th2 dominance during the nephrotic phase.[33]

Whether these immunologic phenomena are specific for the pathophysiology of NS is still subject of debate.[24,22,18,44] From the hypothesis that NS and allergy may share a common immunological origin, one may speculate that the majority of children outgrow the disease before adulthood is reached following maturation of the immune system.[46]

(Plasma) factors from non-lymphoid cells

In the quest for (plasma) factors causing proteinuria in NS, several non-inflammatory candidates have been put forward as well, including vascular endothelial growth factor (VEGF), heparanase,[47] hemopexin[48] and angiopoeietin-like-4.[49] As some of these factors are in fact upregulated by podocytes in NS, it is possible that the disease in part originates within podocytes themselves.[49]

Until now, none of the proposed (plasma) factors, either inflammatory or non-inflammatory, was claimed to be the holy grail in the pathophysiology of NS. Since many different pathways may lead to impaired GFB function and subsequent nephrotic proteinuria, it is possible that NS reflects a heterogenous group of glomerulopathies (or podocytopathies). Interindividual differences in etiological pathways may well contribute to the variability in clinical course in childhood NS.

1.3 Renal Histology

Today, therapeutic response to glucocorticoids at diagnosis rather than histology is regarded the major prognostic indicator of renal function in childhood NS.[50,51] As a result, renal biopsy is now performed only when children are therapy resistant (5-10%) or when clinical and laboratory evaluations are atypical, e.g. persistent hematuria and/or hypertension, impaired renal function, or a positive family history.

The most common histological type of NS in childhood is minimal change NS (MCNS), in which minor abnormalities of glomeruli are seen at electron microscopic level, while no abnormalities are seen on light microscopy. These abnormalities are found in many diseases accompanied by proteinuria and are therefore not unique to MCNS. MCNS is seen in 80-90% of patients aged one to six years and in 20-30% of adolescent patients with NS.[52,53] Less common histological patterns are focal segmental glomerular sclerosis (FSGS), diffuse mesangial proliferation, membranous nephropathy and (rarily) others. These histological types are characterised by marked structural abnormalities seen on light microscopy[22] and are associated with an increased risk of renal failure.

1.4 Treatment and clinical outcome

Since the 1950's, glucocorticoids represent the cornerstone of the treatment for nephrotic syndrome, as these induce remission of proteinuria in 90-95% of patients.[54,44,55,3] In conjunction with the introduction of antibiotic therapy, glucocorticoids have caused a remarkable reduction of mortality from 35-50% to less than 3%.[3]

The initial treatment generally consists of highly dosed oral prednisone or prednisolone. Prednisone is inactive and is converted into its active metabolite prednisolone by hepatic enzymes. Systemic bioavailability of prednisolone is generally equal when oral administration of prednisone is compared to prednisolone.[56] Supportive care during the nephrotic phase consists of fluid and salt restriction, diuretics, and occasionally albumin infusion.[54,55,3]

Steroid responsiveness at diagnosis is of major prognostic importance in NS with regard to kidney function, which is generally preserved well in steroid sensitive nephrotic syndrome (SSNS).[57,58] This is in contrast with primary and secondary steroid resistant nephrotic syndrome (SRNS), seen in 5-10% and 1-3% of children with NS respectively.[2,59] Steroid resistant patients are prone to progressive disease and renal failure.[2]

Despite the high initial response rate, relapses occur in 60-90% of the initial responders.[3,7] Relapse frequency is highly variable among patients. Around 20-60% of patients develop frequent relapses (generally ≥2 relapses within six months after initial treatment, or ≥4 relapses per year). Children with NS form a heterogeneous and therapeutically challenging group as they suffer from relapses and glucocorticoid toxicity to a varying degree. Those in need of numerous courses of glucocorticoid therapy are at risk of serious infectious,[60] as well as adverse effects on growth and bone mineral density, obesity, hypertension, changes in behaviour and cataract. These patients often need other immunomodulatory agents (cyclophosphamide, ciclosporin, mycophenolate mofetil, levamisol, rituximab) in order to reduce adverse effects of glucococorticoid therapy.

Interestingly, NS usually subsides before or during adolescence,[3] yet can extend into adulthood. Few reports describe relapse patterns beyond adolescence. Those that do offer a wide spread in the incidence of relapses continued in adulthood (6-44%). The retrospective design of these studies may have resulted in selection bias.[57,61,62,63] In general, it is believed that children with NS who develop glucocorticoid dependence or secondary resistance to therapy are likely to face a protracted disease course.[2,64]

1.5 Lack of clinical and biochemical predictors

Many efforts were made to predict relapse patterns in children with NS, yet it has been impossible to provide clinicians with a clear-cut set of risk indicators. Studies focusing on the prognostic value of demographic and clinical features have yielded conflicting results. The low incidence of NS complicates studying these parameters in a prospective setting.

Male gender has only occasionally been correlated to frequent relapses[12]. Most reports have not found a significant effect of gender on clinical course in terms of (frequent) relapses or other morbidity.[10,13-15,65]

Age at onset has been proposed as an indicator of clinical outcome in NS. In adolescents presenting with NS, atypical features and steroid resistance are seen more often,[4,66,67,6] whereas young age at diagnosis (1-6 years of age) has been associated with frequent relapses, steroid dependency and/or a longer duration of disease.[15,12,54] A possible explanation for the higher incidence as well as the increased number of relapses at a young age could be a higher frequency of potentially triggering events, such as viral infections.[31,8] Some reports however did not find any effect of age on clinical course.[16,31,68,13]

Low birth weight has been associated with unfavourable clinical outcome. In children with low birth weight, steroid dependence[69,70] and hypertension[69,70,71] were observed more often compared to children with normal birth weight. A lower nephron number in patients with low birth weight has been suggested as part of the underlying mechanism for this relationship, though this requires further clarification. All studies were retrospective and had small sample sizes.

Rapid remission within 7-9 days of glucocorticoid treatment has been correlated with fewer relapses and/or less steroid dependence.[16,31,68,14,72] Again, this relationship was not confirmed in several other studies.[73,12]
In steroid sensitive NS, hypertension at diagnosis does not have a significant impact on the risk of relapse.[14]

HLA-typing may aid in predicting steroid resistance, and certain HLA-subtypes may be an indicator of (frequent) relapses. These associations are restricted to certain regions.[39,40,74-77] Biopsy is no longer considered a standard procedure in childhood SSNS and histology therefore is not a suitable indicator for relapses in SSNS.

A wide variety of genetic and biochemical factors have been put forward as biomarkers for the clinical course of NS in children. The focus of these studies has mainly been on differentiating SSNS from SRNS in the early phase of the disease. Few studies have concentrated on biomarkers for relapses within SSNS patients.

Plasma levels of electrolytes, proteins, lipids and creatinin have not been correlated with response to steroids, relapses or histopathological findings.[3,14] In steroid sensitive NS, microscopic hematuria is not associated with the risk of relapse.[16,31,12,14,3]

The protein selectivity index, defined as the clearance ratio of IgG and albumin or transferrin and IgG, is based on the degree of loss of selectivity of the glomerular basement membrane. Studies correlating protein selectivity with histological findings in NS are contradictory.[78,79,51] The selectivity index was not studied in relation to relapses. The urinary protein/creatinin ratio at diagnosis might discriminate between SSNS and SRNS,[80] yet does not predict relapses.[14] Recent research in the field of proteomics has revealed associations between urinary protein compounds and steroid resistance. Relapse patterns have not been analyzed in these studies. Since patient numbers were small and the techniques used posed several difficulties, we should interpret these studies with caution.[81]

Hypocortisolism resulting from adrenal suppression after glucocorticoid therapy has been put forward as a possible risk indicator of (frequent) relapses in NS around 30 years ago.[82,83,84] In 2007, Abeyagunawardena *et al*. reported suboptimal cortisol levels after stimulation with synthetic adrenocorticotropine hormone in 62.5% of children while receiving long-term maintenance prednisolone therapy.[85] They correlated suboptimal adrenal response to an increased risk of relapse. Since cortisol levels were assessed during long-term glucocorticoid therapy, the question remained if impaired adrenal function was either cause or effect in this association. Though the exact role of adrenal suppression in the clinical course of NS in children and the need for testing this in clinical practice needs more clarification, it is interesting that patients with NS vary in their susceptibility to adrenal suppression.

CHAPTER 2

The role of glucocorticoids in childhood nephrotic syndrome

2.1 History of glucocorticoid treatment for nephrotic syndrome
2.2 Understanding the effects of glucocorticoids in nephrotic syndrome
2.3 Glucocorticoid metabolism
2.4 Glucocorticoid sensitivity: the glucocorticoid receptor

Glucocorticoids mediate many essential physiological processes, including stress response, glucose metabolism and anti-inflammatory actions.[86,87] The complex mechanisms of glucocorticoid action give rise to heterogeneity of glucocorticoid sensitivity, which is known to exist in the general population.[88,89,90] It is well known that patients differ in their clinical response to empirically derived doses of prednisolone. Many factors, both intracellular and extracellular, can influence the path from the ingestion to treatment response and side-effects.

Though glucocorticoids have been first choice treatment for nephrotic syndrome for decades, surprisingly little is known about how they work, how much children with NS actually need, and how much this differs between individual patients. Here we will discuss the history of glucocorticoid treatment for childhood nephrotic syndrome, and the currently proposed therapeutic mechanisms. In addition, several determinants of glucocorticoid metabolism and sensitivity are reviewed, with special attention to their potential role in patients with NS.

2.1 History of glucocorticoid treatment for nephrotic syndrome

Before the availability of pharmaceutical glucocorticoids and antibiotics, 35-50% children with nephrotic syndrome died, mainly of infection (80%). Treatment consisted of high protein and low salt diets, diuretic agents, blood-letting, human serum transfusions to restore protein concentrations, removal of focal infectious disease by drainage or resection, or even induction of measles or malaria in experimental settings (Figure 3).[91,92,37]

The history of glucocorticoid treatment for NS is characterized by a substantial amount of 'trial and error'. Glucocorticoids were first applied in the early 1950's in a rather improvised setting, as underlying mechanisms were unclear. Before 1954, glucocorticoids were considered to induce diuresis, thereby resolving edema in NS.[11] Treatment consisted of 5 to 16[93,94] days of adrenocorticotropin (ACTH) or cortisone. Later on, the aim to use glucocorticoids shifted towards inducing remission of proteinuria rather than diuresis.

Duration of treatment schedules was prolonged to 28 days and sometimes entailed higher dosages to achieve a longer lasting state of remission.[93,95] Daily treatment was followed by intermittent treatment that lasted for several weeks up to several months or even longer than one year.[94] Patients treated intensively with glucocorticoids seemed better off in terms of maintaining remission than children who had received smaller amounts. However, this effect was seen only up to five to eight years after onset, at

which point intensive therapy yielded no better results than the previously used less intensive course which was used to induce diuresis. This elicited discussion on whether glucocorticoids were a mere temporary panacea for NS.[94,11]

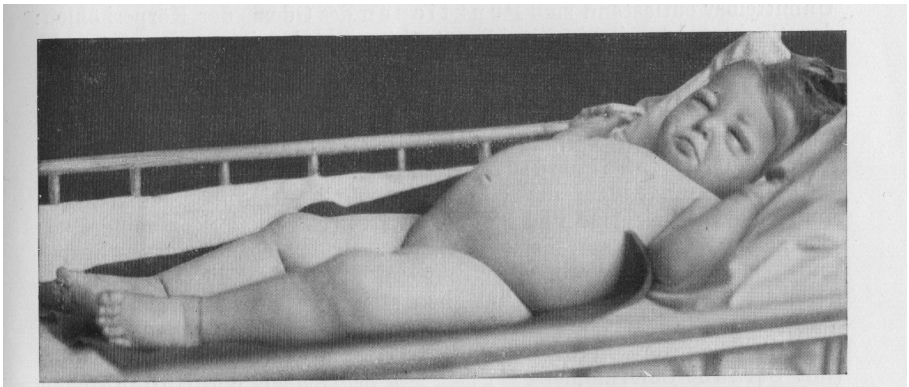

Fig. 119. Genuine Nephrose bei 3 Jahre altem Kind (Sammlung H. Finkelstein).

Die **Behandlung** der Nephrose ist zunächst gegen die Ödeme gerichtet, bringt aber während ihres Anstiegs wenig Erfolge. Auch auf ihrer Höhe vermögen Medikamente (Herzmittel, Diuretika) die Gewebeflüssigkeit nicht ins Blut und an die Niere zu locken; nur Harnstoff (5 bis 10 g pro die in Flüssigkeit gelöst) ist manchmal wirksam, ebenso wie ein kräftiger Aderlaß (100—150—200—300 ccm). Volhards Erfahrung entsprechend sah ich einige Male 1 l Wasser bei knapper Kost die Harnsperre sprengen. Sehr empfehlenswert ist die etwas modifizierte v. Noorden-Volhardsche Hunger-Durstkur: nicht zuviel Fett (salzfreie Butter, Sahne) und reichlich Zucker (dicker Fruchtsirup) neben wenig salzfreiem Brot (bei jedem Bäcker zu bestellen) mit nur soviel Flüssigkeit (Tee, Fruchtsäfte, Wasser, Kakao, Schokolade), als der Gesamtharn des Vortages betrug. Mit Einsetzen der Harnflut gebe man die dem Alter entsprechenden Trinkmengen (s. S. 386). Frauenmilch kann auch beim älteren Kinde ganz oder teilweise die Heilnahrung bilden. Unmittelbar entlastend sind Punktionen des Ödems der Körperhöhlen; das Punktat ist milchig (lipoid) getrübt. Die Ableitung der Anasarka durch Skarifikationen oder durch Drainage mit Curschmannschen Kanülen kann, der notwendigen allerstrengsten Asepsis wegen, allerdings nur im späteren Kindesalter vorgenommen werden. Auch trockene oder feuchte Schwitzpackungen (elektrische Wärmekissen) sind zu versuchen. Urämiebehandlung s. S. 388.

Dem nach Ablauf des Wassers gewöhnlich einsetzenden Hunger muß eine kochsalzarme und nicht zu wasserreiche kalorisch mindestens genügende Kost entsprechen. Sie*) soll der guten Toleranz gegen Eiweiß und seiner starken Verluste gerecht werden, gegen die Ödemtendenz gerichtet sein und sich der allmählich steigenden Kochsalztoleranz und Wasserdurchlässigkeit anpassen. Letztere erkennt man schon aus dem

Figure 3. Page from a German handbook describing treatment of childhood nephrotic syndrome in 1926 - before the availability of glucocorticoids.[231]

When doubt on the long-lasting effects of glucocorticoids came forward, their adverse effects gained more attention. These effects included Cushingoid appearance, impaired growth, myopathy, osteoporosis, pseudotumors, increased risk of serious infections, hypertension, cataract and steroid induced diabetes. Nevertheless, the beneficial effect of glucocorticoids in terms of reducing proteinuira and edema remained crucial in reducing morbidity and mortality.[11] Together with the use of antibiotics, glucocorticoids had led to 65% of patients being symptom-free after five years of follow up, and a decrease in mortality to 16-22% in the 1960's.[11] Continuing discussion existed concerning the optimal duration and dosage of glucocorticoids treatment.

In 1966, The International Society of Kidney Disease in Children (ISKDC) instituted a standard two-month prednisone treatment schedule for the initial episode of NS and relapses.[96] Today, many modified versions concerning dose and duration of the primary initial treatment schedule exist.

The variability as well as the unpredictability of the clinical course of childhood nephrotic syndrome called for better understanding of underlying mechanisms as well as improvement of current treatment protocols. Several attempts have been made to enhance existing glucocorticoid treatment regimes in order to improve clinical outcome. In 2000, Hodson and colleagues performed a meta-analysis of five clinical trials comparing standard two-month predniso(lo)ne treatment with longer regimes. Longer, more intensive glucocorticoid treatments were associated with a reduced relapse risk.[97] The mechanisms explaining this favourable effect remain unknown. Even after several updates of this meta-analysis,[7] it remains unclear if the beneficial effect results from prolonged duration or higher cumulative dose. Today, no worldwide consensus exists on treatment duration and tapering of prednisone.[98,38]

Current Dutch guidelines for glucocorticoid therapy for the initial episode of childhood NS were adopted from German studies performed by the Arbeitsgemeinschaft fur Pädiatrische Nephrologie.[99] This schedule consists of 6 weeks 60mg/m^2 prednis(ol)one daily, followed by 6 weeks 40mg/m^2 predniso(lo)ne on alternate days. When a first relapse occurs, a shorter schedule with similar dosages is used.

2.2 Understanding the effects of glucocorticoids in nephrotic syndrome

Therapeutic effects of glucocorticoids (GC) in NS may be based on the following two perspectives: an immunomodulatory effect may remove the impact from circulating plasma factors, while a direct effect on podocytes and/or their slit diaphragms may promote recovery of the glomerular filtration barrier (Figure 4).[44]

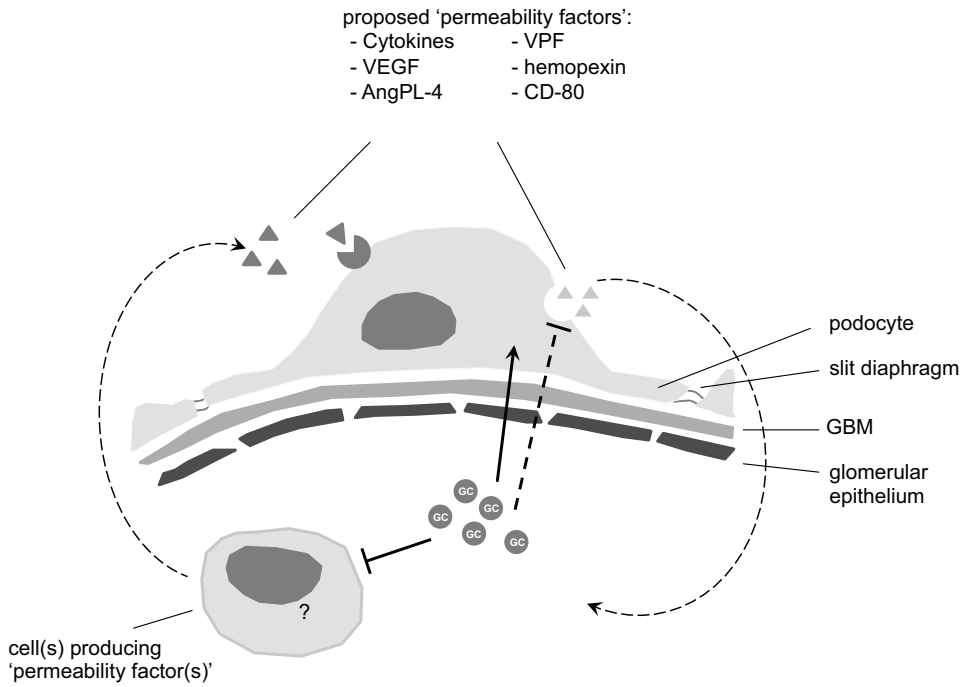

Figure 4. Currently proposed mechanisms of therapeutic glucocorticoid effects in nephrotic syndrome. An immunomodulatory effect may remove the impact from circulating permeability factors, while a direct effect on podocytes and/or their slit diaphragms may promote recovery of the glomerular filtration barrier. VEGF, vascular endothelial growth factor; AngPL-4, angiopoeitin-like 4; VPF, vascular permeability factor.

The glomerular filtration barrier (GFB) owes its highly selective permeability to proteins to both size-selective and charge-selective properties. Increased permeability to protein of the GFB may result from loss of either of these properties.[17] This is believed to result from circulating factors targeting key components within the GFB. Whether NS results from abnormal high levels of these factors or from intrinsic susceptibility of the GFB to these factors remains to be determined .

Immunosuppression

Glucocorticoids such as prednisolone exert their anti-inflammatory effects by inducing apoptosis of lymphoid cells,[86] promoting expression of anti-inflammatory proteins and interrupting cytokine-mediated pro-inflammatory pathways.[100] Studies that focused on immunomodulatory effects of GC in nephrotic syndrome have mainly addressed cytokine-mediated processes.

Several studies have reported either increased plasma cytokine levels or increased cytokine levels in supernatant from cultured cells during the active phase of NS. In most reports, these levels normalized during remission.[101] Cytokines that have predominantly been associated with the nephrotic phase are IL-4, IL-10, IL-13, IL-18 and TNF-α. However, no general agreement exists on this topic, as results from these studies are contradictory and considerable heterogeneity exists among the methods used and the populations studied.[101,44]

A therapeutic effect from GC within this context cannot be explained from direct inhibition of cytokine production at a genetic level, as most genes encoding cytokines that are associated with NS do not contain GC responsive elements. They do however exhibit binding sites for nuclear transcription factors. Nuclear transcription factors mediate sequence-specific DNA binding, enabling them to control the transfer of genetic information from DNA to mRNA. They contain one or more DNA-binding domains, which attach to specific sequences of DNA adjacent to the genes that they regulate.[102] The transcription factors nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) play a pivotal role in activating many proinflammatory genes, resulting in the production of numerous cytokines.[103]

Inhibitory effects on cytokine production by GC are thought to occur indirectly through interaction with nuclear transcription factors, in particular nuclear factor kappa-B (NF-kB).[104,105] NF-kB facilitates pro-inflammatory processes by promoting cytokine expression.[103] Increased activity of NF-kB is associated with auto-immune disease.[106,107] NF-kB activity is regulated within a negative feedback loop enclosing another transcription factor: IkBα. Glucocorticoids exert their influence on this feedback route in two ways: on the one hand by inhibiting NF-kB activity through blocking its binding to DNA, on the other hand by stimulating IkBα gene transcription resulting in decreased NF-kB activity. This way, glucocorticoids effectively impede cytokine production. The inhibitory effect of GC on transcription factors was studied in patients with asthma[108] and rheumatoid disease[107] in humans, and in experimental glomerulonephritis in rats.[109]

A few studies have evaluated the inhibitory effect of GC on transcription factors in patients with NS. Decreased expression in NF-κB subunits in patients with steroid resistant NS (SRNS) was described by Aviles *et al*.[110] NF-κB consists of two subunits, p50 and p65. Upon encountering antigenic stimulation, a p50/p65 heterodimer translocates to the nucleus, where it regulates genes involved in inflammatory reactions.[111] Since the glucocorticoid receptor requires the p65 subunit to interact with NF-κB,[112] decreased

expression of this subunit may result in insensitivity to GC. Aviles *et al*. found reduced expression of the p65 subunit in T-cells from patients with SRNS compared to SSNS patients and healthy controls. Subsequently, they found translocation of predominantly p50/p50 homodimers in patients with SRNS, which they posed as a possible mechanism underlying steroid resistance.[110]

Sahali *et al*. found highly increased DNA-binding activity of NF-kB in peripheral blood mononuclear cells (PBMC's) from patients with SSNS during the active phase, which normalized during remission. This effect was present with and without the use of GC. In addition, they found diminished expression of IkBα during the active phase of NS.[113] This suggests that the NF-kB - IkBα feedback route fails during the active phase of NS, potentiating a pro-inflammatory status. In a small in vitro study with PBMC's from nephrotic children not yet on steroid therapy, Cao *et al*. found increased DNA-binding of NF-kB compared to healthy controls. This was reduced to normal levels in the presence of dexamethasone. These results support the hypothesis that the nephrotic phase is accompanied by an inflammatory state, which responds to glucocorticoids.[114]

An interesting finding in the study by Sahali *et al*. is that serum from nephrotic patients did not increase activity of NF-kB in PBMC's in vitro. This leaves the possibility that in fact mechanisms upstream from the abnormal cytokine production underlie NS and that increased cytokine production may be a consequence rather than a causative phenomenon in NS.[113]

Despite the fact that literature and clinical experience strongly implicate the immune system in the pathophysiology of NS, there is no clear-cut evidence for either the immunologic target cell or the immune-mediated mechanism of action of glucocorticoids in NS. In addition to potential immunomodulatory effects, recent research has introduced other mechanisms of GC action in NS.[115]

Restoration of the glomerular filtration barrier

Recent reports have studied direct therapeutic effects of glucocorticoids on podocytes, which are key cells within the glomerular filtration barrier. Podocytes express glucocorticoid receptors, which translocate to the cellular nucleus in the presence of dexamethasone.[116,117,118] Nuclear transcription factors NF-kB and AP-1 are expressed in podocytes as well, although their specific role in GC effect on podocytes is unclear. Xing *et al*. found no influence of dexamethasone on expression of NF-kB and AP-1 in cultured human podocytes.[117]

GC induce apoptosis in lymphocytes and other cells of hematopoetic origin, whereas other cell types are in fact protected against apoptosis by GC. This protective effect has been established in cells of mammary epithelium, fibroblasts and hepatocytes,[119] as well as in cells of the glomerular basement membrane and podocytes.[120,121,117] GC were found to have a pro-survival effect on podocytes in vitro by inhibiting expression of pro-apoptotic factors p53 and Bax while promoting expression of the antiapoptotic factor Bcl-xL.[121]

Several studies have investigated the role of vascular endothelial growth factor (VEGF) in nephrotic syndrome, as it promotes vascular leakage. Wasilewska *et al*. found increased levels of vascular endothelial growth factor (VEGF) in patients with steroid sensitive nephrotic syndrome,[122] which were reduced after GC treatment. In addition, several studies reported increased VEGF expression by podocytes from patients with NS,[123] and subsequent downregulation of VEGF in podocytes by GC.[117] It should be noted that increased VEGF expression is seen in several proteinuric diseases and is thus not specific for (steroid sensitive) NS.[124] Whether VEGF is implicated in the therapeutic effect of GC in NS is unclear.

Podocytes express proteins that can potentially induce proteinuria, as was recently demonstrated for angiopoeietin-like-4. In both rat models of NS and in humans with (minimal change) NS, Clement and colleagues revealed upregulation of the glycoprotein angioproietin-like-4 by podocytes. Overexpression of angioprotein-like-4 by podocytes in transgenic rats resulted in massive albuminuria, loss of glomerular basement membrane charge and podocyte effacement. In the presence of glucocorticoids, expression of angiopoeitin-like-4 was reduced and proteinuria was reduced subsequently. The mechanism by which this GC effect occurred still needs clarification.[49]

In vitro studies with human podocytes demonstrated that dexamethasone promotes acceleration of maturation and stabilization of the cytoskeleton.[117,125,126] GC stimulate expression and activity of factors involved in actin polymerization such as heat shock protein 27 (hsp-27) and GTP-ase RhoA.[126,126] An amount of dexamethasone equivalent to the in vivo therapeutic potency of prednisolone resulted in upregulation of both the slit diaprahm protein nephrin and the microtubule protein tubulin α in cultured podocytes.[117] These components are important for upholding slit diaphragm function and podocyte morphology. Recent reports associated nephrotic syndrome with decreased phosphorylation of nephrin in podocytes. Phosphorylation of nephrin was increased after treatment with GC via serum/glucocorticoid-induced kinase 1 (SGK1).

Additional experiments indicated that this effect takes place through genomic actions of GC.[127]

These observations strongly suggest that beneficial effects of glucocorticoids in NS involve assisting podocytes in resuming their original shape and rebuilding the functionality of the slit diaphragm, this way re-establishing the glomerular filtration barrier.

2.3 Glucocorticoid metabolism

Availability of glucocorticoid drugs to their site of action depends on patient compliance, drug formulation, pharmacokinetic (PK) properties, individual metabolic clearance, and interactions with other drugs. Here we will focus on predniso(lo)ne, as this is the standard glucocorticoid drug for childhood nephrotic syndrome. For convenience, we will use the term for the active metabolite, prednisolone, unless otherwise specified.

Pharmacokinetic properties of prednisolone

Prednisolone is rapidly absorbed from the gastrointestinal tract after oral ingestion. Maximum plasma levels are achieved within one to two hours after oral administration.[128] The bioavailability after oral administration of prednisone is generally equal to its active metabolite prednisolone.[56] Systemic availability ranges from 75%-98%[128] and rather depends on interindividual differences than on the choice for either the active or the inactive form of the drug. Enteric coated prednisolone is absorbed more slowly than plain tablets and its bioavailability is characterised by a wide between patient variability.[56,129] Elimination of prednisolone mainly occurs through metabolic processing in the liver and through renal excretion. Elimination half life varies from 2-4 hours. Co administration of certain drugs can cause either inhibition or induction of microsomal liver enzymes, affecting metabolic clearance of prednisolone.[130,131,56] Metabolites as well as unchanged prednisolone and prednisone are excreted in urine.[128,132,130] A circadian variation of prednisolone pharmacokinetics and pharmacodynamics has been reported.[133]

Protein binding results in dose-dependent pharmacokinetics

In blood, both endogenous and exogenous GC are mainly bound to glucocorticoid binding globulin (CBG) and albumin.[128] Only the unbound fraction is biologically or pharmacologically active.[56] When saturation of these proteins occurs, the unbound, biologically active fraction of GC will rapidly and non-linearly increase with increasing dose. As a result, the pharmacokinetics of prednisolone behave in a dose-dependent manner.[56,134,135,136]

Hypoalbuminemia affects prednisolone pharmacokinetics

Hypoalbuminemia in NS naturally includes lack of transporting proteins, causing their saturation point in blood to be reached much sooner than in individuals with normal serum protein levels. Therefore, the unbound fraction of prednisolone is higher in patients with NS. Unbound prednisolone molecules readily diffuse through capillary walls to reach tissues and target organs, including those involved in elimination. As a consequence, the volume of distribution is larger, while total prednisolone clearance is increased. Hypoalbuminemia thus results in a smaller total area under the (time-concentration) curve (AUC) for prednisolone.[129,137-141]

While the unbound *fraction* is indeed higher during hypoalbuminemia, the absolute unbound prednisolone plasma *concentrations* are not increased. The unbound fraction drives drug clearance, resulting in lower total prednisolone concentrations and more or less constant unbound concentrations during hypoalbuminemia. Frey and Frey demonstrated that unbound prednisolone concentrations in NS patients with normal liver function are similar to individuals with normal albumin levels.[137] Their findings were confirmed in subsequent reports.[138,142,141]

One small study by Miller and colleagues reported decreased total as well as unbound prednisolone clearance in children with active NS compared to healthy adults with normal plasma protein levels, after an intravenous dose of prednisolone.[143] These results were not in line with the findings by Frey and Frey and subsequent studies.[137,144,142,141] The study by Miller *et al* had several weaknesses; prednisolone doses and albumin levels varied considerably among the 11 patients with nephrotic syndrome, and the control group (n=4) was much older. Furthermore, Miller *et al* ignored the previously mentioned studies in their discussion; they speculated that decreased clearance of prednisolone in nephrotic children was due to impaired liver function, which is unlikely.

Studies evaluating prednisolone PK in relation to clinical outcome in children with NS

The alterations in prednisolone pharmacokinetics due to hypoalbuminemia are restricted to the active phase of NS (Figure 5)[141] and are not likely to be of use in predicting subsequent clinical course as they represent a state rather than individual traits. Accordingly, it would be more interesting to relate intrinsic interindividual differences in the metabolism of prednisolone to clinical outcome without interference of the hypoalbuminemic state, during remission.

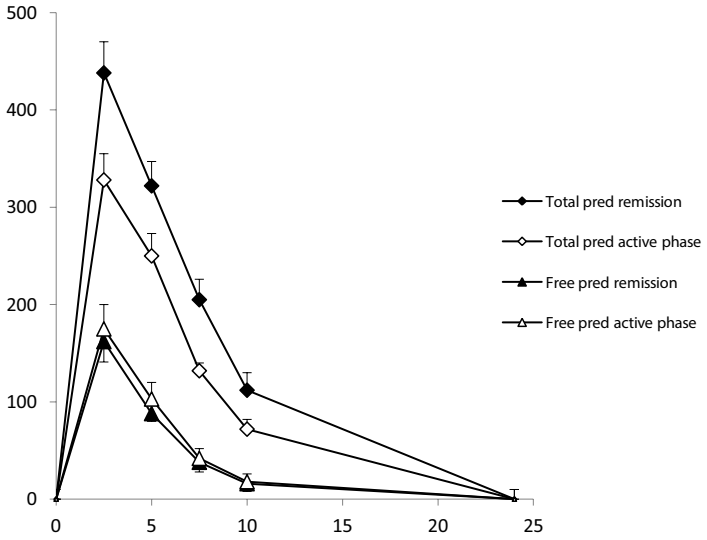

Figure 5. Levels of free and total prednisolone during the active phase of nephrotic syndrome (open symbols) and in remission (closed symbols). Values represent the means ± s.e. mean. Adapted from Gatti *et al.*[141]

Unfortunately, studies involving children with NS that indeed aimed for a correlation between pharmacokinetics of prednisolone and clinical outcome were mostly conducted in children during the active phase of NS.[145,139] Baron and colleagues found considerable inter-individual variability in prednisolone pharmacokinetics among 14 children with NS. AUCs of total prednisolone tended to be lower in patients with frequent relapses, yet this difference was not significant. Glucocorticoid side-effects were found more often in children with higher AUCs. Only 4 out of the 14 children in this study had normal albumin levels.[145] Rostin *et. al.* conducted a pharmacokinetic study in 13 children with NS, of whom 11 were steroid sensitive. They found no differences in pharmacokinetic parameters between children who were classified as either steroid sensitive, steroid dependent or steroid resistant. However, measurements were done only during the active phase of NS and the follow up period covered only six months. Side effects were not analyzed.[139]

Interconversion of prednisolone and prednisone: prereceptor ligand metabolism

Constant interconversion occurs between active prednisolone and inactive prednisone, in favour of the active metabolite.[146] The intracellular enzyme type one 11β-hydroxysteroid dehydrogenase (11β-HSD-1) is present in tissues throughout the body[130,146] and plays a crucial role in converting inactive cortisone into active cortisol. By converting prednisone to prednisolone in the same way, 11β-HSD-1

facilitates access of prednisolone to the glucocorticoid receptor. Conversely, type two 11β-hydroxysteroid dehydrogenase (11β-HSD-2) promotes the reverse step, which in fact physiologically prevents active glucocorticoid to interact with mineralocorticoid receptors. 11β-HSD-2 thus facilitates selective mineralocorticoid action[147,130,148] and is found at mineralocorticoid target sites, predominantly the kidney, colon, salivary glands and placenta.[130,149,146,148]

Both increased activity of 11β-HSD-1 and decreased activity of 11β-HSD-2 increase GC availability to the glucocorticoid receptor.[150] Variable activity of this enzyme and polymorphisms of the 11β-HSD-1 gene have been described in relation to the metabolic syndrome,[151] while polymorphisms of the 11β-HSD-2 gene are associated with salt-sensitive hypertension.[152] The role of 11β-HSD activity in the clinical course of NS has not been studied.

Prednisolone metabolism

Both its dose-dependent behavior as a result of non-linear binding to plasma proteins, and interconversion between prednisolone and prednisone, add to the complexity of the pharmacokinetics of prednisolone.[128] In addition, metabolic clearance may depend on individual activity of microsomal liver enzymes. Prednisolone is metabolised by enzymes of the cytochrome P450 family, mainly CYP3A4 and CYP3A5.[153,154] The rate at which these enzymes convert prednisolone into its metabolites varies among individuals.[155]

Hepatic hydroxylation leads to the formation of several metabolites, predominantly 6-β-OH-prednisolone.[130] Urinary fractional excretion of 6-β-OH-prednisolone has been put forward as a marker of microsomal liver enzyme activity[155] and displays a linear relationship with the non-renal metabolic clearance of prednisolone.[153] It is unclear whether this metabolite can be used as a marker for individual metabolism of prednisolone.[156] The very similar metabolite of cortisol, 6-β-OH-cortisol, has been used to deduce cytochrome P450 induction. Because of substantial interindividual (inter-liver) variability in metabolite concentrations and the fact that other metabolic processes contributed to 6-β-OH-cortisol levels, 6-β-OH-cortisol excretion was not considered specific enough as a marker for cytochrome P450 induction.[157]
Recently, genetic polymorphisms in genes encoding CYP3A and other factors involved in prednisolone absorption and metabolism, were associated with either variability of prednisolone absorption or metabolic clearance in Japanese renal transplant recipients[154,158] These polymorphisms have not yet been investigated in children with NS.

Barbiturates, carbamazepine, phenytoin, rifampicin[130,56] and HIV protease inhibitors[131] cause CYP3A induction and therefore increase clearance of prednisolone, while ciclosporin inhibits enzyme activity. Moreover, ciclosporin may increase absorption of prednisolone, causing both increased availability and decreased clearance.[130] The latter findings are of clinical relevance in children with NS, as they may explain the beneficial effect from prednisolone combined with ciclosporin for FRNS, which has been shown in clinical studies.[159]

P-glycoprotein affects cellular bioavailability of prednisolone

Naturally, reduced availability of glucocorticoids within cells will hinder their therapeutic effectiveness. Export of GC out of the cell is therefore considered a possible factor involved in the modification of GC-response.[160,161] Active expulsion from drugs out of the cell is considered one of the major mechanisms underlying drug resistant disease. Increased expression of the multidrug resistance gene (*ABCB1*) has been associated with diminished GC sensitivity in in vitro studies of malignant cells,[162] and colonic mucosa cells from patients with inflammatory bowel disease,[163] although clinical studies yielded contradictory results.[164-168] Multidrug resistance can arise from several cellular processes, of which excessive drug expulsion by the transmembrane efflux pump P-glycoprotein (P-gp) has been described extensively.[160] P-gp, which is encoded for by *ABCB1*, prevents intracellular toxicity from exogenous substances. It is highly expressed in the small intestine and kidneys. Increased expression of P-gp results in lowered intracellular drug concentrations and may consequently weaken treatment response. GCs are substrate of P-gp and may also induce P-gp expression. These processes are suggested to play a potential role in steroid dependence or even steroid resistance.[169,170] Because of this, inhibitors of P-gp have gained much interest, particularly in oncological studies.[171] Whether P-gp inhibitors are able to enhance GC availability in patients with NS has not been investigated.

In recent years, Wasilewska and colleagues have dedicated several studies to P-gp expression in Polish children with NS.[169,170,172,173] In their first report, they revealed that P-gp expression changes during the clinical course of NS. Before GC-treatment, children experiencing their initial episode of NS were found to have similar levels of P-gp on CD3+ T-lymphocytes compared to healthy controls. Expression levels increased in children with NS during GC treatment and remained elevated up to 12 months after ending GC treatment. P-gp expression was positively correlated with the number of nephrotic episodes and cumulative dose of prednisone.[170] In a larger study concerning children with SSNS during remission, they reported increased expression of P-gp in patients in whom the clinical course was characterized by steroid dependency

and/or frequent relapses, compared to children with infrequent relapses and healthy children.[169] It should be noted that the subgroups with FNRS/SDNS within their study population included a relatively high number of patients with FSGS, which may have influenced their results. The question remains whether enhanced p-gp expression is either the cause or the result of a less favourable clinical course in NS.

Funaki *et al*. found highly variable expression levels of *ABCB1* mRNA during the active phase of NS in 15 patients. These expression levels decreased after remission.[174] From these studies it is unclear whether increased expression of p-gp results from the disease state or GC-treatment or both.

In the last two decades, numerous single nucleotide polymorphisms (SNPs) of the *ABCB1* gene have been uncovered. Recent studies have focused on three common SNP's (1236C>T, 2677G>T, 3435C>T), as reviewed by Fung *et al*.[175] Wasilewska *et al*. investigated the frequency of these three SNP's in children with SSNS. A late initial response to prednisone (arbitrarily defined as time to remission >7 days) and frequent relapses were seen more often in children with homozygous mutations.[172] The functional and clinical relevance of these SNP's still needs further clarification.

From these reports, the role of P-gp expression in the individual's response to GCs in NS seems to be both intrinsic as well as acquired. Polymorphisms of the *ABCB1* gene may determine an individual's 'setpoint' of P-gp expression, while administration of GCs may induce upregulation of P-gp, in this way enhancing susceptibility to frequent relapses, steroid dependence or even steroid resistance.

Intracellular transport of glucocorticoids to the nucleus: the role of hsp-90
GCs are lipophilic, which enables unbound molecules to diffuse readily across the cell membrane where they interact with cytosolic glucocorticoid receptors (GR).[176] After entrance of GC into the cytoplasm, the first step in the pathway towards GC action is binding of GC to a so-called mature GR heterocomplex.[177] This complex consists of the glucocorticoid receptor and several molecular (co)chaperone proteins. Upon ligand binding, a rearrangement of the heterocomplex is induced, allowing its nuclear entry.[177] Within the nucleus, the GC-GR complex is paired with a second GC-GR complex (homodimerisation), after which binding to the DNA is introduced. Genomic actions take place through interaction with glucocorticoid response elements, specific DNA sequences which are located at the promoter regions of steroid sensitive genes.[176] The stability and integrity of GR heterocomplex are of critical significance for binding of GC to the GR and trafficking of the complex into the nucleus.[177]

Several abnormalities in the chaperone proteins have been associated with reduced or increased availability of GC to the nucleus. Heat shock protein 90 (hsp90) plays a key role in ligand binding and trafficking of the GR[178,179] and is the only chaperone to have been investigated in patients with NS. Normally, hsp90 dissociates from the GC-GR complex before the nucleus is entered.[87] Altered expression and distribution of this protein are associated with resistance to GC in asthma[180] and multiple sclerosis,[181] though the role of hsp90 in therapeutic response was unclear from studies concerning hematological malignancies.[182,183,184]

In adults with nephrotic syndrome, hsp90 expression in peripheral blood mononuclear cells was found to be higher in NS patients compared to healthy controls. Moreover, expression of hsp90 was significantly higher in GC-resistant patients compared to GC sensitive patients, while levels of GR expression were similar among the three groups. In healthy individuals and GC-sensitive patients, localization of hsp90 was mainly in the cytoplasm, while in GC resistant patients this protein was mainly found within the nucleus.[185,186] As an excess of nuclear hsp90 may interfere with genomic GR actions, as well as GR recycling,[187] this could explain the diminished treatment response in GR resistant patients. Whether altered localization of hsp90 accounts for susceptibility to relapses in GC sensitive NS patients is unknown.

2.4 Glucocorticoid sensitivity: the glucocorticoid receptor

Glucocorticoids exert their effects through genomic and non-genomic routes. Genomic actions take place only after binding of glucocorticoids to the cytosolic glucocorticoid receptor (GR) and its transport to the nucleus (Figure 6). Subsequent effects are mediated through several routes.[176,188] The GC-GR complex influences transcriptional activity by either interacting directly with genes harboring glucocorticoid response elements (GRE's), or by interaction with nuclear transcription factors. These pathways have been described in various tissues and cells, including podocytes.[118]

Interaction with the GRE's leads to recruitment of co-activator proteins, resulting in activation or repression of gene expression. These pathways are generally believed to be involved in a variety of metabolic processes including gluconeogenesis, mobilization of amino acids and fatty acids, and many other processes involved in the contractility of muscle, vascular tone, bone formation, and behaviour. Interactions between the GC-GR complex and transcription factors such as nuclear factor-κB (NF-κB) result in so-called transrepression. This process inhibits expression of pro-inflammatory genes, which results in immunosuppression.

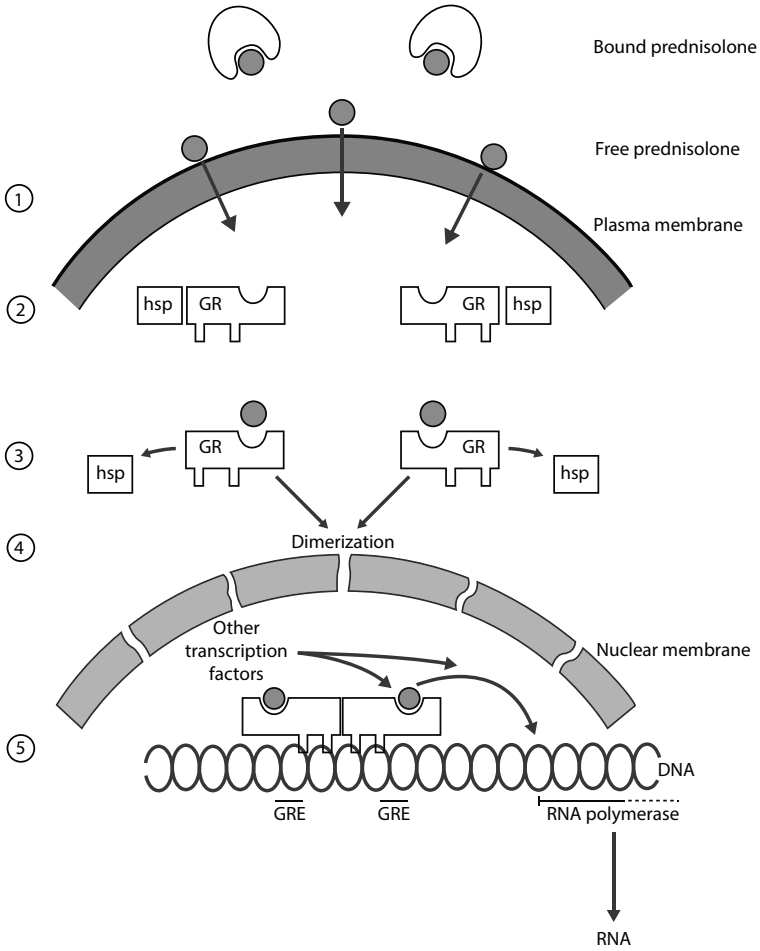

Figure 6. Interaction between prednisolone and the glucocorticoid receptor (GR), resulting in genomic actions. 1. free prednisolone readily crosses the cell membrane. 2. GRs without ligand are located in the cytoplasm attached to heat shock proteins (hsps). 3. Hsps are released upon binding of prednisolone to the GR. 4. Dimerized prednisolone-GR complexes translocate to the nuclear DNA. 5. The DNA binding domains of these complexes interact with glucocorticoid response elements (GREs) in the DNA. This results in up-regulation or repression of proteins, or interactions with transcription factors to inhibit expression of pro-inflammatory genes (transrepression). Adapted from: Nussey and Whitehead.[232]

Non-genomic actions of GC occur much more rapidly as they do not depend on the process of gene transcription. They do still rely on the expression and function of the GR, as non-genomic effects are in part mediated or modulated by this receptor.[189,190,118]

Sensitivity to glucocorticoids thus depends on both functionality and expression of the glucocorticoid receptor.[176] Glucocorticoid receptors are expressed in virtually every tissue of the body,[188] including cells of the distal convoluted tubules, collecting ducts and all subsets of human glomerular cells.[116] The GR is an intracellular receptor located in the cytoplasm as a homodimer and belongs to the nuclear hormone receptor family. The GR gene (*NR3C1*) is located on chromosome 5 (5q31). It consists of nine exons encoding for three functional domains: 1) The N-terminal harbours a transactivation domain, which accounts for transcriptional activation of GC sensitive (target) genes. 2) The central DNA-binding domain (DBD) is essential for binding to the glucocorticoid response elements (GRE). 3) The C-terminal contains a ligand-binding domain (AF-2) and is required for binding of GC, nuclear localization, receptor dimerisation and binding of hsp90.[177]

Polymorphisms of the GR gene

Although many single nucleotide polymorphisms (SNPs) of the GR gene have been revealed, only few have been associated with variability in glucocorticoid sensitivity (Figure 7).[148] Here we will discuss those polymorphisms and haplotypes that have been associated with GC sensitivity in general and in NS.

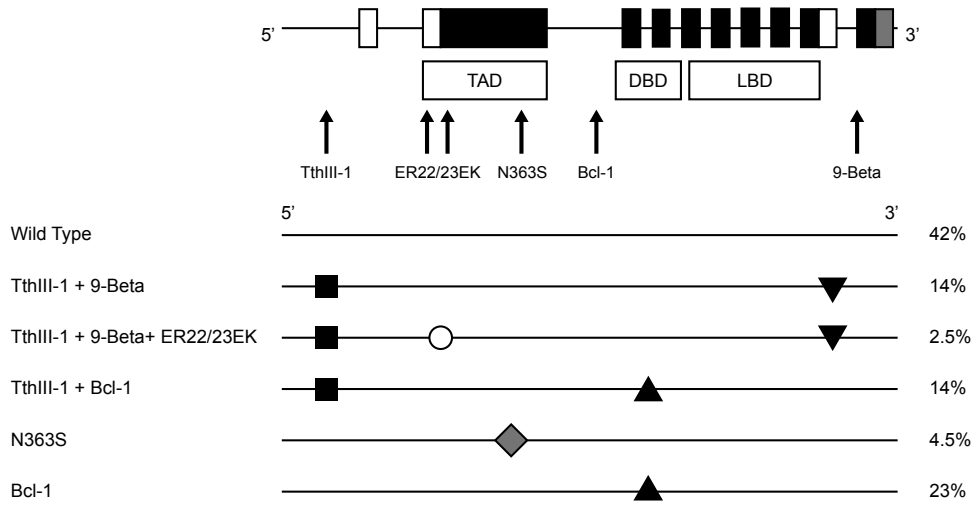

Figure 7. Glucocorticoid receptor gene haplotypes and their allelic frequencies in the general population. TAD, transactivating domain; DBD, DNA-binding domain; LBD, ligand-binding domain. The symbols represent the presence of the minor allele of the SNP indicated. Modified from Dekker *et al.*[233]

Both the Bcl I (41423247) (allele frequency 37%) and the N363S (rs6195) (allele frequency 4%) SNPs are associated with increased GC sensitivity in vivo. Individuals carrying these polymorphisms had relatively low levels of plasma cortisol following a low dose dexamethasone suppression test, which is considered an in vivo marker for GC sensitivity.[191] Interestingly, the Bcl I SNP is located in an intron, which renders the mechanism by which it increases GC sensitivity unknown. Although increased glucocorticoid treatment effect would be expected in individuals harbouring these polymorphisms, this has neither been confirmed in patients receiving glucocorticoid treatment for hematological malignancies,[192] nor in asthmatic patients.[193] Both polymorphisms predispose to a less favourable metabolic profile.[191] Stevens *et al*. studied a haplotype located in intron B of the GR gene in humans. This haplotype (Bcl I C>G, rs33389 C>T and rs33388 A>T) consists of three SNPs including the Bcl I SNP described here. Carriers of this haplotype showed lower levels of cortisol after dexamethasone suppression than non-carriers.[194] Interestingly, analysis of this haplotype in children with steroid sensitive nephrotic syndrome revealed an association between the haplotype containing the BcL I G, rs33389 T and rs33388 A SNP's and early remission at initial presentation (within 7 days), while a more abundant haplotype (Bcl I C, rs33389 C and rs33388 T), showed a tendency to slower remission. Unfortunately, correlations between these haplotypes and other parameters of clinical outcome were not reported.[195]

Individuals harboring the ER22/23EK (rs6189 en rs6190) SNP display relatively impaired GC sensitivity. This SNP is located in the transactivation domain of the GR gene. The reported allele frequency of this polymorphism in the population is around 3%. It facilitates expression of the GR subtype GR α-A, which is known to have diminished transcriptional potency.[176,196] Individuals with the ER22/23EK SNP displayed relatively high levels of cortisol after dexamethasone suppression, indicating decreased sensitivity to GC. This polymorphism is associated with increased insulin sensitivity and lowered levels of total cholesterol and low-density lipoprotein cholesterol, and a healthier metabolic profile.[197] Theoretically, the decreased GC sensitivity of this SNP would lead to impaired therapeutic effect of exogenous GC,[148] yet this remains to be proven. Results from studies concerning the relationship between the ER22/23EK polymorphism and therapeutic response are not consistent.[192,198] A haplotype holding both the ER22/23EK SNP and the TthIII 1 (rs10052957) polymorphism (the latter being located within the GR promoter) is associated with a healthy metabolic profile as well as GC resistance[199,148]. These SNP's have not been investigated in patients with NS.

The GR-9β (rs6198) polymorphism (allele frequency approximately 18%) is located at the terminal exon of the mRNA of the β isoform of the GR. It leads to increased receptor protein expression of the negative GRβ isoform (see further).[148] This polymorphism has been associated with a relative GC resistance with respect to transrepressive effects on the immune system and inflammation. These effects result in a phenotype comprising a more active immune system, a predisposition to rheumatoid arthritis[200,201] and cardiovascular disease,[202] and decreased microbial colonization.[203] Within a therapeutic context, the GR-9β SNP is associated with GC-resistance in inflammatory disease.[201] The potential clinical relevance of GR-9β in patients with NS has not been reported so far.

Ye *et al.* identified several new polymorphisms and haplotypes of the GR gene in Japanese children with NS with potential relevance to steroid response, yet due to their modest sample size, the clinical relevance of these findings could not be ascertained as yet.[204]

Although polymorphisms of the GR gene have come into view as important contributors to metabolic risk profiles, and in vivo tests indeed indicate an effect on GC sensitivity, the influence of GR gene polymorphisms on therapeutic effect of exogenous GC is still subject of debate.

GR gene mutations

Mutations of the *NR3C1* gene have been found in familial steroid resistance.[205,206] These mutations are associated with decreased functional GR expression levels and obstruction of GC binding and transactivational capacity.[205,206] The primary, generalized GC resistance syndrome is rare[207] and established mutations of the GR gene associated with GC resistance are not found in all patients presenting with this syndrome.[208] *NR3C1* mutations were also associated with acquired steroid resistance to exogenous glucocorticoids in disease, for example Cushing's disease[209] and lupus nephritis.[210] To date, no previously described mutations of the GR gene have been associated with glucocorticoid response in nephrotic syndrome. In a Chinese study describing GR gene mutations and polymorphisms in 118 children with NS, of whom 35 were classified as steroid resistant, none of these mutations were found. It should be noted that among the children with SRNS in this study, most had marked structural abnormalities on renal biopsy.[204] Their impaired steroid response might have been due to profound injury to the glomerular barrier, rather than to (generalized) glucocorticoid resistance on a genetic level.[204]

GR gene processing

After transcription of the GR gene, the exons of the GR precursor mRNA are reconnected into multiple ways through alternative splicing, which results in five isoforms of the receptor protein: GRα, -β, -γ, -A and -P.[148] The GRα isoform is generally known as being the most abundant and is crucial in facilitating GC actions. The much less expressed GRβ isoform[188] differs from GRα in its C-terminal ligand binding domain, rendering it unable to bind most GC ligands. Moreover, GRβ may operate as a negative inhibitor of GRα, in that way regulating gene expression.[188,201,211] The GRα:GRβ expression ratio differs between tissues[188] and may hypothetically contribute to individual GC sensitivity,[148] yet reports on its clinical significance are still contradictory.[188,201,212-215]

To date, isoforms of the GR have been explored in only one report considering patients with NS. Liu *et al*. described a diminished GRα:GRβ ratio in patients with steroid resistant NS compared to patients with steroid sensitive NS. Differences in clinical outcome among the group of steroid sensitive patients were not investigated.[216]

Aside from alternative splicing, alternative promoter usage may lead to differences in GC sensitivity.[217,218,219] In addition, alternative translation from the GRα mRNA leads to different subtypes of the GRα isoform, with potential influence on GC action.[220] The clinical relevance of these subtypes still needs further clarification.[176]

GR expression

Cellular response to GC is directly correlated with hormone binding capacity, and thus the level of GR expression.[221] Expression of the GR is thought to be in part regulated on a cellular level by the amount of available ligand. Administration of GC generally leads to a decrease in GR number through negative feedback routes.[222] This is a physiological phenomenon which is thought to uphold GC homeostasis within cells.[223] This may however, limit therapeutic effects in cells targeted by exogenous GC as chronic glucocorticoid treatment leads to GR downregulation.[177]

In cells of the immune system, auto-induction causes glucocorticoids to induce up-regulation of the glucocorticoid receptor, leading to highly increased sensitivity to glucocorticoids and apoptosis in T-cells. GC response in these cells is defined by the ratio of GC induced down-regulation and auto-induction. In this respect, auto-induction could be particularly relevant for hematological malignancies.[176] It is unlikely that auto-induction occurs in other cells than immune cells, such as glomerular cells.[177] Whether the phenomenon of auto-induction takes part in the therapeutic effect in NS is unknown.

Haack *et al*. assessed GR expression in mononuclear leucocytes in children with NS and found similar expression of the receptor during active NS and remission. Expression of GR was also similar in steroid responsive children compared to children with steroid resistant NS.[224] This was confirmed in a study by Carlotti *et al*.[225] Wasilewska *et al*. did find a temporary decrease of GR number in lymphocytes and monocytes during glucocorticoid treatment in children with steroid sensitive NS, which resolved spontaneously. They did not investigate a relationship between GR expression and clinical outcome.[226]

In children with NS, expression of GR has not been assessed in glomerular cells. In Korean adults with minimal change NS, a correlation was found between GR mRNA expression in glomerular cells and time to achieve remission during GC treatment.[227] From these results, it was speculated that GR expression in glomerular cells rather than immune cells may serve as an indicator for therapeutic response in nephrotic syndrome.[227] Since the last two decades however, renal biopsy is no longer considered a standard procedure in children presenting with steroid sensitive NS. Assessment of expression of GR in glomerular cells therefore is not a realistic biomarker for GC response and subsequent clinical course in children with SSNS.

Posttranslational modifications

The transcriptional acitivity of the GR can be modulated by various posttranslational processes, which are of vital importance to the receptor's subcellular distribution, protein turnover, and transcriptional activities.[228] While phosphorylation and sumoylatoin both influence the subcellular localization of the GR and modulate transcription of target genes, degradation of the GR protein takes place via the ubiquitin/proteasome-dependent protein degradation pathway. To our knowledge, these processes have not been studied in children with NS.

AIMS OF THIS THESIS

Understanding which factors influence relapse patterns in childhood nephrotic syndrome is clinically very relevant and could aid in developing new treatment strategies. Clinicians are continuously challenged to reduce relapse rates and at the same time to avoid glucocorticoid toxicity. Both intrinsic and environmental factors may take part in the underlying pathophysiological process and its impact on clinical course. Though glucocorticoids are the cornerstone of treatment, little is known about how individual handling of these drugs may affect the clinical course in children with nephrotic syndrome. Possible explanations include the complicated methods required to evaluate these factors and the low incidence of the disease.

The general aim of this thesis is to provide better understanding of the variability in the clinical course of childhood nephrotic syndrome. Within this context, special attention is given to the role of glucocorticoids in terms of treatment duration, metabolism and sensitivity. The following aspects are evaluated:

- What is the effect of extending duration of glucocorticoid treatment on clinical outcome in childhood nephrotic syndrome, without a concomitant increase in cumulative dose?
- Low birth weight was previously related to unfavourable outcome in studies with small sample sizes. Does re-assessment of this aspect with a meta-analysis provide more solid evidence for this association?
- Unbound - biologically active - concentrations of prednisolone in blood may be clinically relevant, but measurement of these concentrations poses several challenges. How can we work towards more feasible measurement and more convenient sampling methods?
- Are variations in the genes involved in prednisolone metabolism related to prednisolone pharmacokinetics in children with nephrotic syndrome?
- How variable is prednisolone exposure among children with nephrotic syndrome that are in remission? Does this variability correlate to relapse patterns and/or side effects?
- Sensitivity to glucocorticoids depends on expression and functionality of the glucocorticoid receptor. Do variations of the glucocorticoid receptor gene show any association with clinical outcome in children with nephrotic syndrome?
- Can assessment of in vivo glucocorticoid sensitivity explain (part of) the variability in clinical course in childhood nephrotic syndrome?

OUTLINE OF THIS THESIS

Part I describes the efforts made to explain relapse patterns in childhood nephrotic syndrome and those factors that still need exploration. Chapter 1 reviews demographic, clinical and biological factors in relation to clinical outcome. Chapter 2 focuses on the past and current role of glucocorticoid treatment in childhood nephrotic syndrome. In addition, this chapter illustrates a variety of aspects relevant to glucocorticoid metabolism and sensitivity.

In **Part II**, the hypothesis that prolonged treatment improves clinical outcome in children with nephrotic syndrome is re-evaluated in a randomised, placebo-controlled, double-blind clinical trial. Chapter 3 describes the results of this national multicentre study, in which different treatment durations are assessed while maintaining an equal cumulative dose.

Part III emphasizes the lack of feasible methods for assessment of prednisolone drug exposure, and strategies to overcome this. In Chapter 4, separation of total and non-protein bound concentrations of cortisol, prednisone and prednisolone in blood is described, followed by a validation process concerning measurement of these components in blood and saliva. Chapter 5 explores the relationship between total, free and salivary predniso(lo)ne concentrations in healthy adults.

Part IV concentrates on factors that may explain the variability in clinical outcome in childhood nephrotic syndrome. In Chapter 6, the previously suggested association between low birth weight and clinical outcome of children with nephrotic is evaluated in a meta-analysis. In Chapter 7 and 8, we investigate several aspects of glucocorticoid metabolism and sensitivity in a well defined, prospective cohort of children with nephrotic syndrome in the Netherlands.

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PART II

REVISING THE PROLONGED GLUCOCORTICOID TREATMENT HYPOTHESIS

CHAPTER 3

Extending prednisolone treatment does not reduce relapses in childhood nephrotic syndrome

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ABSTRACT

Prolonged prednisolone treatment for the initial episode of childhood nephrotic syndrome may reduce relapse rate, but whether this results from the increased duration of treatment or a higher cumulative dose remains unclear. We conducted a randomized, double-blind, placebo-controlled trial in 69 hospitals in The Netherlands. We randomly assigned 150 children (9 months to 17 years) presenting with nephrotic syndrome to either 3 months of prednisolone followed by 3 months of placebo (n=74) or 6 months of prednisolone (n=76), and median follow-up was 47 months. Both groups received equal cumulative doses of prednisolone (approximately 3360 mg/m^2). Among the 126 children who started trial medication, relapses occurred in 48 (77%) of 62 patients who received 3 months of prednisolone and 51 (80%) of 64 patients who received 6 months of prednisolone. Frequent relapses, according to international criteria, occurred with similar frequency between groups as well (45% versus 50%). In addition, there were no statistically significant differences between groups with respect to the eventual initiation of prednisolone maintenance and/or other immunosuppressive therapy (50% versus 59%), steroid dependence, or adverse effects. In conclusion, in this trial, extending initial prednisolone treatment from 3 to 6 months without increasing cumulative dose did not benefit clinical outcome in children with nephrotic syndrome. Previous findings indicating that prolonged treatment regimens reduce relapses most likely resulted from increased cumulative dose rather than the treatment duration.

INTRODUCTION

Nephrotic syndrome (NS) is the most common manifestation of glomerular disease in childhood. Despite its relatively low incidence of one to seven in 100.000 children,[1,2] NS poses recurring challenges to many clinicians.

Corticosteroids induce remission of proteinuria in 90-95% of patients.[3,4,5,6] Despite this high initial response rate, relapses occur in 60%-90% of the initial responders.[6,7] The disease progresses to frequent relapses, often accompanied by steroid dependence, in around 20%-60% of patients. Recurrent or continuous corticosteroid therapy in these patients frequently results in corticosteroid toxicity.[1] This finding calls for the improvement of existing treatment regimens, for which no international consensus currently exists.[8]

The present treatment modalities for initial childhood NS are mostly based on reports by the International Study of Kidney Disease in Children (ISKDC) and the Arbeitsgemeinschaft für Pädiatrische Nephrologie (APN). Currenty used regimens vary in dose and duration (Supplemental Table 1).[6,9,10] The regimen prescribed in the Netherlands is made up of 60 mg/m^2 prednisolone daily for six weeks followed by 40 mg/m^2 prednisolone on alternate days for six weeks.[10] The cumulative dose of this regimen is 3360 mg/m^2.

In 2000 Hodson and colleagues performed a meta-analysis of corticosteroid therapy in childhood nephrotic syndrome to evaluate the potential benefits of different corticosteroid regimens.[11] Based on the analysis of seven clinical trials in patients with an initial episode of NS, it was concluded that the risk of relapse was significantly reduced by prednisolone regimens that were both longer and more intensive. Additional analysis suggested that the benefits were more likely to be related to the increased duration of the treatment than to the higher cumulative dose. However, collinearity between treatment duration and dose prevented the work by Hodson *et al.* from drawing definite conclusions.[11] A subsequent study by Hiraoka *et al.*, comparing three months of prednisolone treatment to six months of treatment was also inconclusive. In this study, prolonged treatment reduced the relapse rate in children under the age of four; however, this intervention also consisted of a higher cumulative dose.[12] The independent effects of treatment duration and cumulative dose, thus, remained undetermined.

Based on these data, we designed a study protocol to explore the independent effect of treatment duration. In the present study, we hypothesized that prolongation of a three-month initial prednisolone treatment to six months using equal cumulative doses would reduce the occurrence of frequently relapsing NS (FRNS), without increasing adverse effects.

RESULTS

From February 2005 to December 2009, 212 patients were evaluated for eligibility. Participants and non-participants were similar in terms of gender and age at onset (Supplemental Table 2); 150 patients from 69 hospitals (60 general and nine university hospitals) were randomised to either three months prednisolone followed by three months placebo or six months prednisolone (Figure 1). In both groups, 12 patients could not start trial medication because of either steroid resistance or withdrawn consent. These patients were excluded from the analysis. Median follow-up was 47 months in the three-month group (interquartile range [IQR] 32-60) and 47 months in the six-month group (IQR 37-60).

Induction therapy and trial medication were administered within a total of 24 weeks in both groups. The prescribed cumulative dose of prednisolone in the six-month group depended on the number of days to remission, which is shown in Figure 2. Because the median number of days to remission was ten days in both groups (IQR 8-14 and 7-14 days, respectively), the median prescribed cumulative prednisolone dose was 3360 mg/m^2 in the three-month group and 3390 mg/m^2 in the six-month group. Baseline characteristics revealed no relevant differences between the two groups (Table 1); 65% of the study population was of Western European descent.

Frequently relapsing nephrotic syndrome (FRNS) was scored and analysed according to strict definitions (strict FRNS) as well as a broader, clinically relevant definition (clinical FRNS), as explained in the methods section.

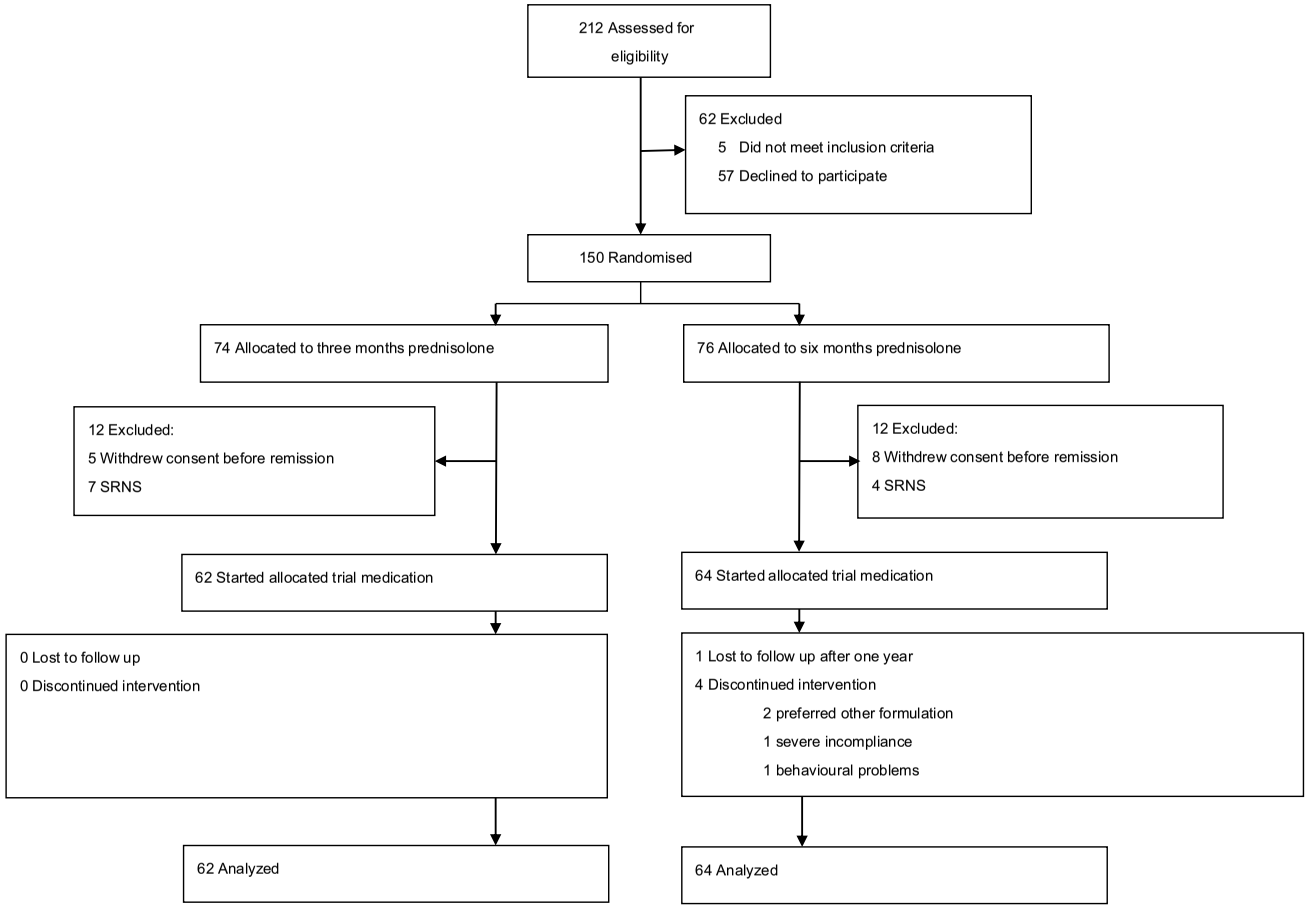


Figure 1. Trial profile. Patients were analysed in a modified intention to treat analysis. All patients that started trial medication were analysed according to their allocated treatment. SRNS, steroid resistant nephrotic syndrome.

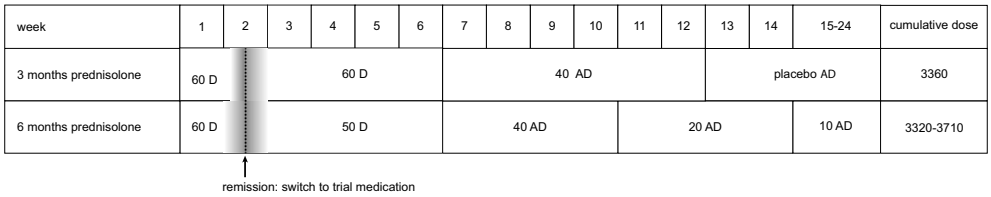


Figure 2. Treatment regimens were built up of comparable cumulative doses of prednisolone. The dotted line represents the median number of days to remission, the grey area represent the IQR. Doses are in mg/m^2. AD, on alternate days; D, daily.

Table 1. Baseline characteristics

	Overall (n=126)	**3 months prednisolone (n=62)**	**6 months prednisolone (n=64)**
Male, n (%)	86 (68)	39 (63)	47(73)
Age, years; median (IQR)	4.2 (3.2-6.2)	4·7 (3.2-5.8)	3.8 (3.2-6.4)
Blood Pressure[a]			
- Systolic, Z-value; mean ± SD	1.7 ± 1.3[b]	1.7 ± 1.3[c]	1.6 ± 1.3[d]
- Diastolic, Z-value; mean ± SD	1.6 ± 1.1[b]	1.7 ± 1.3[c]	1.6 ± 1.0[d]
Serum albumin, g/L; median (IQR)	14.0 (10.0-16.2)	14.0 (10.0-17.0)	13.4 (10.0-16.0)
Microscopic hematuria[e], n (%)	40 (33)[f]	19 (32)[g]	21 (34)[h]
Hospital, (%)			
- University	14 (11.1)	5 (8.0)	9 (14.1)
- General	112 (88.9)	57 (92.0)	55 (85.9)
Descent, n (%)			
- Western European	83 (65.9)	46 (74.2)	37 (57.8)
- Non-western European	16 (12.7)	6 (9.7)	10 (15.6)
- Mixed	13 (10.3)	3 (4.8)	10 (15.6)
- Not reported	14 (11.1)	7 (11.3)	7 (10.9)
Quarterly distribution of disease onset, n (%)			
- January-March	25 (19.8)	14 (22.6)	11 (17.2)
- April-June	24 (19.0)	11 (17.7)	13 (20.3)
- July-September	40 (31.7)	19 (30.6)	21 (32.8)
- October-December	37 (29.4)	18 (29.0)	19 (29.7)

[a] Lowest blood pressure reported in patient's chart at diagnosis. Z-values are adjusted for gender, age and height[33] [b]data available for 123/126 patients; [c]data available for 61/62 patients; [d]data available for 62/64 patients; [e]defined as > 5 erythrocytes/field; if cell count not available: ≥ + on dipstick analysis. [f]data available for 121/126 patients [g]data available for 59/62 patients; [h]data available for 62/64 patients; IQR: inter quartile range.

The cumulative incidences of FRNS did not reveal a benefit of the six-month regime, regardless of the definition used (Table 2). Strict FRNS was found in 28 out of 62 children (45%) in the three-month group and 32 out of 64 children (50%) in the six month group, logrank test: p=0.91 (Figure 3A and Table 3). Three patients in the three-

month group and six patients in the six-month group did not meet the strict criteria for FRNS, yet were characterized as having clinical FRNS (Supplemental Table 3B). Accordingly, clinical FRNS occurred in 31 out of 62 children (50%) in the three month group versus 38 out of 64 children (59%) in the six-month group, logrank test: p=0.76 (Figure 3B).

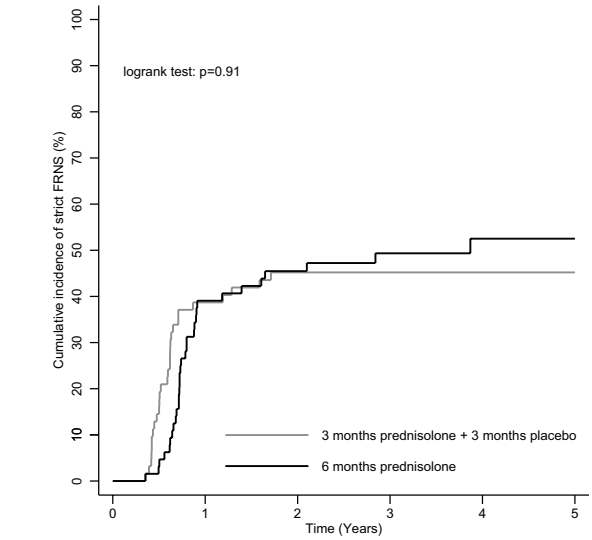
The cumulative incidences of first relapses were similar in the two treatment groups. At least one relapse occurred in 48 out of 62 children (77%) in the three-month group and in 51 out of 64 children (80%) in the six-month group. Median survival time from randomisation to the first relapse was six months (95% confidence interval [CI] 4-8) in the three-month group and eight months (95% CI 6-10) in the six-month group (logrank test: p=0.69) (Figure 3C).

Table 2. Kaplan Meier estimates of the cumulative incidences of strict and clinical FRNS

	3 month-group (n=62)	6 month-group (n=64)	Difference, % (95% CI)	Log-rank test
Strict FRNS, %				
6 months	14.5 ± 4.5	3.1 ± 2.2	-11.4 (-21.2, -1.6)	
1 year	38.7 ± 6.2	39.1 ± 6.1	0.4 (-16.6, 17.4)	
2 years	45.2 ± 6.3	45.6 ± 6.2	0.4 (-16.9, 17.7)	
3 years	45.2 ± 6.3	49.3 ± 6.4	4.1 (-13.5, 21.7)	
4 years	45.2 ± 6.3	52.5 ± 6.7	7.3 (-10.7, 25.3)	
5 years	45.2 ± 6.3	52.5 ± 6.7	7.3 (-10.7, 25.3)	p=0.91
Clinical FRNS, %				
6 months	17.7 ± 4.9	10.9 ± 3.9	-6.8 (-19.1, 5.5)	
1 year	41.9 ± 6.3	46.9 ± 6.2	5.0 (-9.1, 19.1)	
2 years	50.1 ± 6.4	53.3 ± 6.3	3.2 (-14.4, 20.8)	
3 years	50.1 ± 6.4	59.4 ± 6.4	9.3 (-8.4, 27.0)	
4 years	50.1 ± 6.4	59.4 ± 6.4	12.2 (-5.7, 30.1)	
5 years	50.1 ± 6.4	62.3 ± 6.5	12.2 (-5.7, 30.1)	p=0.76

Data are expressed as percentages ± standard errors at 6 months and yearly afterwards. Between-group differences are expressed as percentages with 95% confidence intervals. Log-rank tests were performed on all available data at the the end of follow up. Strict FRNS: Frequently relapsing nephrotic syndrome based on ≥2 relapses within 6 months after initial treatment or 4 relapses within any 12 months. Clinical FRNS: Frequently relapsing nephrotic syndrome according to the definition of strict FRNS or other indications for additional treatment measures (e.g. prednisolone maintenance therapy, ciclosporin, etc).

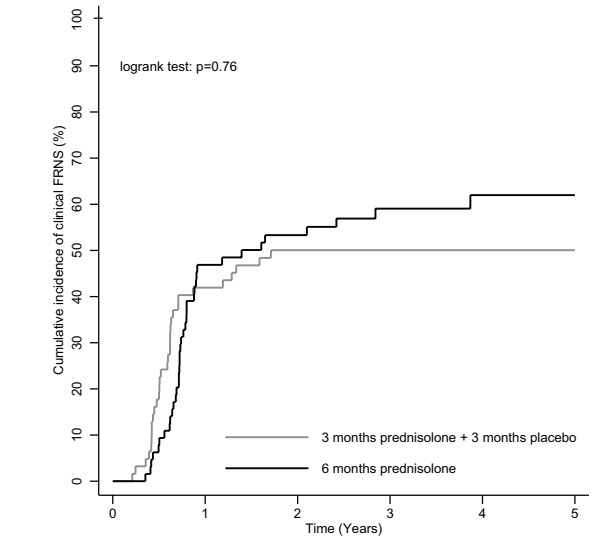
A



Number at risk

3 months	62	38	29	23	13	7
6 months	64	39	31	23	12	8

B



Number at risk

3 months	62	36	26	20	12	7
6 months	64	34	26	19	10	6

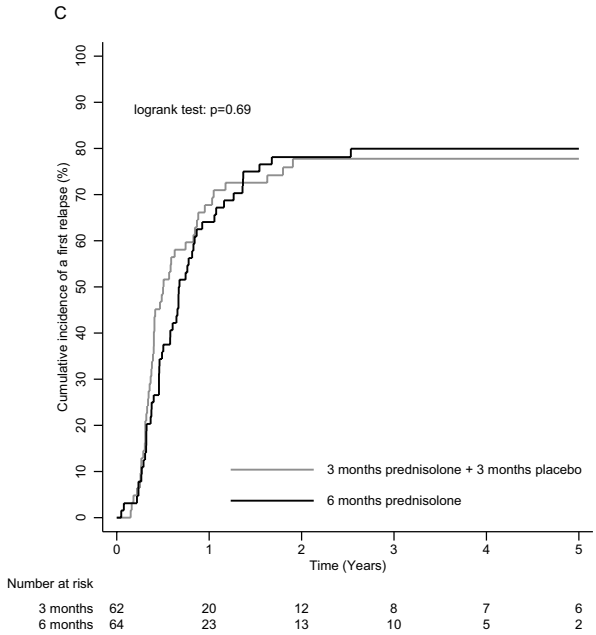


Figure 3. Initial prednisolone treatment of 3 and 6 months resulted in similar therapeutic outcome. Kaplan-Meier curves represent cumulative incidences of (A) strict FNRS - frequentlyrelapsing nephrotic syndrome based on ≥2 relapses within 6 months after initial treatment or 4 relapses within any 12 months, (B) clinical FRNS - frequently relapsing nephrotic syndrome either according to the definition of strict FRNS or a clinical indication for additional treatment (e.g. prednisolone maintenance therapy, cyclophosphamide, etc) (B), and (C) cumulative incidence of a first relapse.

Table 3. Distribution of patients according to three criteria for FRNS

	3 month-group (n=62)	**6 month-group (n=64)**
A. 2 relapses within 6 months after ending first treatment, n	23 (11 SD)	18 (11 SD)
B. 4 relapses within any period of 12 months, n	5 (3 SD)	14 (10 SD)
C. Need for additional treatment for other reasons than A or B, n	3 (1 SD)	6 (3 SD)
Strict FRNS (A or B)	28 (45%)	32 (50%)
Clinical FRNS (A, B, or C)	31 (50%)	38 (59%)

Patients fulfilling criteria A or B were characterized as strict FRNS; Patients fulfilling criteria A, B or C were characterized as clinical FRNS. Numbers of patients that also fulfilled criteria for steroid dependence are shown between parentheses. FRNS, frequently relapsing nephrotic syndrome; SD, steroid dependence. Detailed information on patients fulfilling only criterion C is presented in Supplemental Table 3.

Children allocated to the six-month group experienced more relapses during follow up compared with the three-month group, although differences were not statistically significant. The median total number of relapses during follow up was 2.5 (IQR 1.0-5.0) in the three-month group and 4.0 (IQR 1.0-6.0) in the six-month group (p = 0.13). The median number of relapses per year of follow up was 0.6 (IQR 0.2-1.4) and 1.0 (IQR 0.3-1.6) respectively (p = 0.16). Simultaneous evaluation (performed with Poisson regression) of relapse rates in relation to treatment, gender, age category and follow-up period (I, II and III) showed no significant difference between treatments. The adjusted overall relative relapse rate (RRR) for the three-month group compared to the six month-group was 0.81 (95%CI 0.60-1.09; p=0.16). The RRR was highest in the period between six and 12 months after diagnosis (1.5, p=0.008). The effect of treatment did not differ between the three follow-up periods (p=0.46).

Steroid dependence was noted less often in the three-month group: 15 out of 62 children (24%) versus 24 out of 64 children (38%) in the six-month group (Table 3). The difference did not reach statistical significance: logrank test p=0.10.

Cox regression analysis revealed that boys tended to develop FRNS more often than girls, though differences were not statistically significant. For strict FRNS, the male vs. female hazard ratio (HR) was 1.68 (95% CI 0.92-3.01; p=0.092); a similar HR was found for clinical FRNS: HR 1.72 (0.98-3.03; p =0.057) (Table 4). Interaction between gender and treatment group was not significant, indicating neither boys nor girls benefitted more from one treatment over the other. During follow up, boys tended to have higher relapse rates than girls (RRR 1.4, p=0.052). Gender was not associated with the incidences of a first relapse or steroid dependence (Table 4). Age at onset (< four years or ≥ four years) had no effect on any of the therapeutic outcome events; the same was true for the number of days to remission (Table 4). Hematuria and blood pressure were not related to development of any of the therapeutic outcome events (data not shown). Interestingly, five patients achieved remission after more than four weeks of daily prednisolone treatment. Of these, four had only one relapse and one had no relapses at all during follow-up.
Secondary steroid resistance was noted in two patients allocated to the three month-regimen and in one allocated to the six month regimen.

Table 4. Adjusted multivariate analysis of treatment group, gender, age and time to remission

	Hazard Ratio (95% CI)			
	First Relapse	**Strict FRNS**	**Clinical FRNS**	**SDNS**
Treatment				
3 months vs. 6 months	1.11 (0.74-1.64)	1.08 (0.65-1.80)	0.97 (0.60-1.56)	0.62 (0.32-1.18)
Gender				
male vs. female	1.19 (0.77-1.84)	1.68 (0.92-3.06)	1.77 (0.98-3.03)	1.96 (0.90-4.28)
Age				
< 4 yrs vs. ≥ 4 yrs	1.22 (0.82-1.82)	0.97 (0.59-1.62)	0.97 (0.60-1.56)	1.30 (0.69-2.44)
Time to remission				
(per day)	1.01 (0.99-1.04)	0.96 (0.92-1.01)	0.98 (0.95-1.02)	0.98 (0.93-1.03)

CI, confidence interval, FRNS: frequently relapsing nephrotic syndrome; SDNS, steroid dependent nephrotic syndrome.

Adverse effects were mostly transient and were similar between the two groups (Table 5). Evaluation of height SD scores showed a significant decrease of growth at three months follow-up compared with baseline ($p<0.01$), which was restored within one year after start of initial treatment. Growth did not differ between treatment groups ($p=0.58$) (Supplemental Figure 1). Overall height SD scores at baseline were lower than anticipated (-0.35 ± 0.90). This observation was irrespective of descent ($p=0.83$).

No effect of treatment was observed in the behavioural visual analogue scales at any time. Compared to baseline, children scored significantly higher on eating, overactive behaviour, and aggressive behaviour at three months follow-up (all p-values <0.01). These scores returned to baseline within one year in both groups. Scores for happiness temporarily dropped in the first six months, while those for sleeping remained relatively stable over the whole observation period.

Bone mineral density (BMD) at six months was not different from baseline in both groups. Mean change in Z-scores of lumbar spine BMD was +0.09 (-0.17-0.36) and +0.33 (-0.06-0.71) in the three month-group (n=17) and six-month group (n=19) respectively; p=0.35. Mean change in Bone Health Index SD scores was -0.10 (-0.35-0.14) in the three-month group (n=33) and -0.03 SDS (-0.16-0.11) in the six-month group (n=30); p=0.56.

Table 5. Adverse effects

	3 months prednisolone	6 months prednisolone	p-value
Blood Pressure ≥ P95			
- At diagnosis	36/61 (59%)	28/62 (45%)	0.15
- At 3 months FU	12/57 (21%)	7/60 (12%)	0.21
- At 6 months FU	8/55 (14%)	10/52 (19%)	0.61
Cushingoid Appearance			
At 6 months FU			
- Cushing (moon face)	14/59 (23.7%)	21/58 (36.2%)	0.14
- Striae	3/58 (5.2%)	4/60 (6.7%)	1.00
Ophtalmological abnormalities			
At 6 months FU			
- Glaucoma	0/51 (0.0%)	0/45 (0.0%)	-
- Cataract	1/53 (1.9%)*	0/46 (0.0%)	1.00
Severe infections			
- Pneumonia	1/62 (1.6%)	6/64 (9.4%)	0.16
- Meningitis	0/62 (0.0%)	0/64 (0.0%)	-
- Osteomyelitis	0/62 (0.0%)	0/64 (0.0%)	-
- VZV-reactivation	2/62 (3.2%)	1/64 (1.6%)	0.62
- Whooping cough	0/62 (0.0%)	2/64 (3.1%)	0.50
- Miscellaneous**	3/62 (4.8%)	1/64 (1.6%)	0.36
- Overall	6/62 (9.7%)	10/64 (15.6%)	0.42
Dyspepsia	1/62 (1.6%)	2/64 (3.1%)	1.00
Thrombosis	0/62 (0.0%)	0/64 (0.0%)	-

Data are expressed as number of events/number analysed (percentages).*mild cataract, which was absent at diagnosis; **3 month group: n=1 cellulitis, n=1 muscle abscess, n=1 intracranial abscess; six month group: n=1 appendicitis. FU, follow up; VZV, Varicella Zoster virus.

DISCUSSION

Our study demonstrates that prolongation of initial prednisolone treatment from three to six months, while maintaining an equal cumulative dose, does not reduce the risk of frequent relapses in childhood nephrotic syndrome. This finding challenges the previous assumption that prolonged treatment duration improves clinical outcome.

The high relapse rate in childhood NS initiated research aimed at improving prednisolone treatment regimens. A Cochrane meta-analysis of seven clinical trials by Hodson *et al.* last updated in 2007, showed that prednisolone regimens with both higher cumulative doses and longer treatment durations (up to seven months and 5235 mg/m^2) resulted in a reduction of relapses compared to a standard two-month regimen (2240 mg/m^2). The works by Hodson *et al.* assumed longer duration of treatment to be of greater importance than increased dose and suggested at least three months

prednisolone should be given for the first episode of NS.[11,7] Unfortunately, the existing studies have not led to international consensus. Two matters still deserved attention. First, the independent effects of treatment duration and dose remained unproven. Second, studies comparing three month-regimens with longer regimens were of limited methodological quality. The present study addresses both issues for the first time.

The main strength of our study is its design. To review our results in the context of other reports, we searched for studies comparing three to (approximately) six months prednisolone for the initial episode of NS. Four studies had been reported by Hodson *et al*.[7] We found one additional study by Mishra *et al*.[13] Characterisics of the five previous studies revealed several limitations (Supplemental Table 4). None of the studies included a placebo or blinding in their design;[12-16] allocation concealment was inadequate or not reported in three studies.[14,16,13] In at least one study, patients who did not complete study medication were excluded from the analysis after randomisation.[13] Interestingly, two studies were never fully published. Prior to our study, the Japanese trial by Hiraoka *et al.* was the only published study reporting adequate concealment of allocation. They found a therapeutic benefit of their six-month regimen only in a small subgroup of children aged less than four years; overall relapse rate and FRNS did not differ significantly between the two groups.[12] We evaluated the occurrence of FRNS in a meta-analysis, of which the results are shown in figure 4A-B. Four studies, including our study, reported FRNS. Overall analysis revealed no significant benefit of long versus short regimens; however, significant heterogeneity was present (figure 4A). Heterogeneity was no longer significant when only fully published studies and our study were included (figure 4B). Nonetheless, these studies are still quite different from each other with respect to administered dose, design, definitions and observation time, therefore overall results of this meta-analysis should be interpreted with caution.

The incidences of both strict and clinical FRNS in our study population were higher than anticipated: 60/126 (48%) and 69/126 (55%) respectively. In previous studies, FRNS was reported in 32-78% of patients who received two-month prednisolone treatment (2240 mg/m^2)[10,17-21] and in 18-44% of patients who received prednisolone for three months (3360 mg/m^2).[12,10,20] This variation may in part be explained by regional differences, or by variations in definitions of FRNS, length of observation, and relapse treatments.

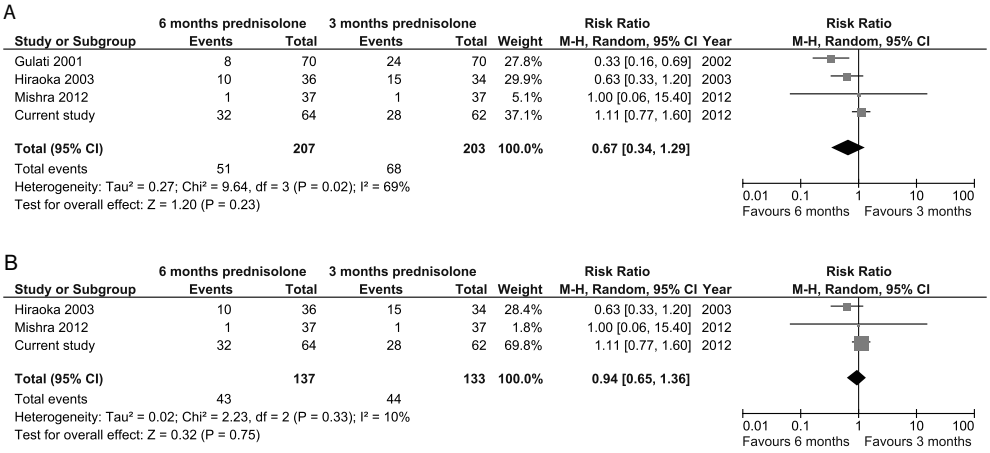


Figure 4. Meta-analyses of four studies comparing 3 months prednisolone with 6 months prednisolone do not reveal a benefit of prolonged treatment duration. (A) All four available studies (B) Two fully published studies and the current study. In both analyses, numbers of FRNS of the current study correspond with numbers of strict FRNS. Analyses were performed with Review Manager (RevMan) version 5.1 for Windows (Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2011).

Based on our data, a benefit of the six-month regimen cannot be excluded if the study had been perfomed with larger sample sizes. However, the confidence intervals we found for the difference in FRNS between the two groups exclude a clinically relevant difference in favor of the experimental regimen (Table 2). At five years, the difference between the two groups for strict FRNS was 7.3% with a 95% CI ranging from -10.7 to 25.3%. At best, the experimental six-month treatment was 10.7 percent points better than the standard three-month treatment. For clinical FRNS, which in our opinion represents an even more relevant group for clinicians, this difference was 12.2%, with a 95% CI ranging from -5.7 to 30.1%. Accordingly, applying the six-month regimen would gain 5.7 percent points at most. The cumulative incidences of steroid dependence at five years further illustrate these statements, as these were 24.9% ± 5.6 and 40.1 ± 6.8 respectively, corresponding with a between-group difference of 15.2 % (95% CI -2.1% to 32.5%). Based on these results, we are confident that a clinically relevant difference in favour of the six-month regimen is unlikely.

Previous studies have differed in observing and reporting (frequent) relapses from either the start or the end of initial therapy. We chose a transitional type of observation, to make a fair comparison yet still include early relapses during treatment. We did verify that observing strictly from the end of inital treatment did not lead to differences between the two treatments (data not shown).

Analysis of covariates in our study revealed findings of clinical interest, though not supported by statistical significance. Boys tended to have worse outcome than girls in terms of frequent relapses and relative relapse rate. In the few studies that observed an effect of gender on the clinical course of NS, males were at a disadvantage.[20,22] It would be interesting to further explore whether boys and girls benefit from different treatment regimens in studies with larger sample sizes. We found no effect of age at onset. The influence of age at onset is still debated, as several studies have reported young age to be associated with FRNS and/or steroid dependence,[3,20,22,23] while others did not find an effect of age on the clinical course of NS.[24,25,21]

Side effects were equally distributed over the two treatment groups. Cushingoid side-effects, high blood pressure and behavioural changes were clearly present, yet transient in the vast majority of patients. Ophtalmological complications were rare in our study. Cataract and glaucoma have previously most often been reported in Japanese patients;[26,12] in general, these complications are rare.[7] Our findings indicate that there is no need for standard ophtalmological screening in children with NS at an early stage. The same applies to measurements of bone mineral density, which remained stable over the first six months. We found severe infections in a clinically relevant proportion of both treatment groups. This observation is consistent with previous reports[7] and justifies awareness of and early therapeutic intervention in children with NS facing infectious diseases.

Our prospective growth data noticeably illustrated how growth velocity significantly dropped in the first months -during highly dosed prednisolone treatment- subsequently returning to its baseline within one year. Though this study was not designed to assess a causal relationship, this temporary effect corresponds with previous retrospective studies that describe a dose-dependent effect of corticosteroids on growth in children with NS.[27,28,29] It is unclear why baseline height SDS was relatively low in our study population. A similar observation was reported by Schärer *et al.*,[30] while others described normal height SDS at diagnosis of NS.[27]

In countries where a two-month prednisolone regimen is applied for the first episode of NS, children who do not achieve remission within four weeks of daily prednisolone are generally characterized as steroid resistant. Steroid resistance is associated with increased risk of renal failure and entails more aggressive immunosuppressive therapy.[1] Intriguingly, all five patients in our study who achieved remission after four to six weeks of prednisolone treatment subsequently experienced a mild clinical course. As argued by Erich *et al.*,[31] this finding suggests that patients who do not respond within four

weeks of daily prednisolone should be offered at least another two weeks of daily prednisolone to prevent late responders from undergoing unnecessary and potentially harmful interventions.

A limitation of our study is the fact that participants were observed and treated at their local hospital. Adverse effects were scored by multiple observers and ophtalmological and radiological assessments were not available for all patients. A more centralized approach could have prevented these issues to some extent; however, the setting we chose made participation feasible throughout the country. We were able to include at least one half of all newly diagnosed patients with NS in the Netherlands.[2] By including patients in a nation-wide setting, we believe we have sufficiently avoided selection bias.[7]

Frequent relapses remain a major challenge in the treatment of childhood NS. In our opinion, FRNS, rather than the occurrence of relapses in general, should be the focus of ongoing research. Broader, uniform definitions for FRNS that take into account other clinically relevant aspects besides relapse frequency per se should be considered, in order to facilitatie a more evidence based approach towards both treatment and research. A possible effect of higher cumulative prednisolone dose during initial treatment needs further exploration, since this may explain better outcomes in some of the reported prolonged treatment regimens.[7]

In contrast to what was previously assumed yet unproven, the present study shows that extending initial prednisolone treatment from three to six months, while maintaining an equal cumulative dose, does not improve clinical outcome in children with nephrotic syndrome. We believe our results offer an important contribution towards more evidence based treatment of this disease.

CONCISE METHODS

Trial design

A double blind, randomised, placebo-controlled, parallel-group trial was carried out in 84 of 87 (97%) general hospitals in the Netherlands along with one Belgian and all eight Dutch university hospitals. The trial was approved by the medical ethics committee of Erasmus MC University Medical Centre in Rotterdam and registered at The Netherlands Trial Register, www.trialregister.nl, registration number NTR255. Detailed information regarding median inclusion rates per hospital and reasons for not participating can be found in Supplemental Table 5a and 5b respectively.

Participants

Children with a first episode of nephrotic syndrome aged nine months to 17 years were assessed for eligibility. Nephrotic syndrome was defined as > 200 mg protein/mmol creatinine in urine and albumin < 25 g/L in serum. Renal biopsy was not required to establish the diagnosis, as it is generally not indicated at this stage of childhood NS.[1] Patients with underlying disease such as Henoch-Schönlein purpura or post-infectious glomerulonephritis were excluded. Remission was defined as urinary protein excretion < 20 mg/L or negative/trace on dipstick analysis on three consecutive days. Patients who did not achieve remission within six weeks of 60 mg/m^2 daily prednisolone were characterized as steroid resistant. Relapse was defined as proteinuria ≥ ++ on dipstick analysis or > 200 mg protein/mmol creatinine for three consecutive days after previously achieved remission. When milder proteinuria was present, pediatricians were instructed to hold off corticosteroid treatment, particularly when signs of mild infection were present. In these patients, relapse treatment was indicated when spontaneous remission became unlikely: continued proteinuria for more than ten days, marked edema or a decrease of serum albumin to less than 30 g/L. Relapses were treated with prednisolone 60 mg/m^2/day until remission, followed by prednisolone 40 mg/m^2 on alternate days for four weeks.

For our study, the definition of frequently relapsing nephrotic syndrome (FRNS) was originally restricted to commonly used criteria:

A. Two or more relapses within six months after completing initial treatment, or
B. Four relapses within any period of 12 months, including relapses during initial treatment

However, during the blinded data collection phase, it became clear that the use of this definition posed difficulties in some cases. Five patients displayed secondary steroid resistance and/or steroid dependency within three to six months after diagnosis. Consequently, they experienced their first relapse(s) before the end of trial therapy; additional treatment measures were taken before these patients could even meet criterion A or B. Four additional patients experienced several relapses within short periods of time, yet did not fulfill criterion A or B. The high burden of multiple relapses within a relatively short period of time, the prospect of experiencing another relapse in the near future, and several signs of steroid toxicity resulted in a clinical indication for additional measures in these patients. As we found all these patients to be clinically relevant, we decided to add a third criterion:

C. FRNS based on a clinical decision that included additional treatment: prednisolone maintenance therapy (> three months) or other immuno-suppressive agents

Detailed information on patients characterized as FRNS based on criterion C can be found in Supplemental Table 3A. We analysed both modalities of FRNS: 'strict FRNS' (criterion A or B) in order to facilitate comparison with other studies, and 'clinical FRNS' (criterion A, B or C) in order to report all clinically relevant outcome.

Steroid dependence was defined as two or more consecutive relapses either during or within two weeks after cessation of prednisolone. All patients were diagnosed and treated according to the study protocol at their local hospital by their own pediatrician. Participants' descent was obtained from self-reported countries of birth of parents and grandparents.

Procedures

A statistician provided the central trial pharmacy with a computer-generated random number table. Allocation to three months prednisolone plus three months placebo (further referred to as the three-month group) or six months prednisolone was stratified for type of hospital (general or university) and balanced with a ratio of 1:1 in fixed blocks of four patients. The central trial pharmacy fabricated trial medication, controlled allocation concealment, allocated patients, and distributed trial medication after informed consent was obtained. Participants, healthcare providers, data collectors and researchers were blinded to group allocation. Trial medication was sent pre-packaged to local pharmacies and consisted of identical, tasteless capsules containing either prednisolone or placebo. Trial medication was dispensed in five containers, each with a fixed, blinded dose and a preset time frame. While doses of the containers differed between treatment groups, container time frames were exactly the same. Container #1 was used from remission through week six, #2 weeks seven through ten, #3 weeks 11 en 12, #4 weeks 13 en 14 en #5 weeks 15-24. The first patient was randomized in February 2005, the last patient in December 2009. Follow up started at diagnosis and was truncated either at five years after diagnosis, or at July 2011, at which time the last enrolled patients had a minimum follow up of 18 months. The randomization code was subsequently broken in September 2011.

All children diagnosed with nephrotic syndrome started induction therapy of 60 mg/m^2 oral prednisolone once daily. Participants switched to trial medication only after remission was achieved. If remission was not achieved within six weeks of 60 mg/m^2 daily prednisolone, patients were characterized as steroid resistant and trial medication was not started. Both treatment regimens are shown in detail in figure 2. In both groups, induction therapy and trial medication were administered within a total of 24 weeks.

The prescribed cumulative dose of prednisolone in the three-month group was 3360 mg/m^2. Depending on the number of days to remission, the prescribed cumulative dose of prednisolone in the six-month group was 3320-3710 mg/m^2, corresponding with 99%-110% of the cumulative dose in the three-month group.

Prescribed cumulative doses did not include potential relapse treatments during trial medication, as the occurrence of a relapse and the total dose administered for that particular relapse could not be anticipated. In the event of a relapse occurring during the period of trial medication, relapse treatment temporarily replaced trial medication in order to maintain a 24-week schedule duration.

Outcomes

The primary outcome event was frequently relapsing nephrotic syndrome (FRNS). Secondary outcome parameters were cumulative incidences of a first relapse, steroid dependence, the number of relapses per patient per year, and adverse effects. Height standard deviation scores (SDS), blood pressure, Cushingoid appearance (moon face, striae), dyspepsia, thrombosis, severe infections, and behaviour were noted at diagnosis and after three months, six months, one year, and two years. Height SDS was calculated with Dutch pediatric reference data.[32] High blood pressure was defined as systolic and/or diastolic blood pressure ≥ the 95th percentile for gender, age and height.[33] Severe infections were defined as non-self-limiting infections requiring hospital admission. Behaviour was scored by parents on visual analogue scales (VAS) for over active and aggressive behaviour, happiness, eating, and sleeping. At diagnosis and after six months, participants were screened for cataract and glaucoma by an ophthalmologist; at the same time points, bone mineral density (BMD) was assessed. Using dual energy X-ray absorptiometry (DEXA), Z-scores of lumbar spine BMD were calculated according to local reference data. Changes in individual Z-scores over time were calculated from paired measurements. As an additional indicator of BMD, Bone Health Index SDS from hand X-rays was calculated with BoneXpert.[34]

Statistical analysis

Primary outcome events were originally defined as the cumulative incidences of first relapses and FRNS. Subsequently, while the study was still blinded, FRNS was chosen as the sole primary outcome, as we considered FRNS to be the most relevant parameter. Incidence of a first relapse became secondary outcome. For the cumulative incidence of FRNS to decrease by 20% points, 72 patients per treatment arm were sufficient (80% power, α=0.05).

A modified intention to treat principle was applied in such a way that all patients who started trial medication were included in the analysis. Participants who were subsequently lost to follow-up or in whom trial medication was stopped prematurely were analysed according to their allocated groups.

Cumulative event rates are expressed as Kaplan Meier estimates with standard errors. Treatment group, gender, age at onset and the number of days to remission were included as covariates in the Cox regression analysis. Age at onset was stratified as < four years and ≥ four years.[23]

For comparison of relapses within time intervals between treatments, follow-up was categorized into three periods (period I: zero to six months; period II: six to 12 months and period III: >12 months after randomisation), and within each period the number of relapses was counted. Poisson regression was used to evaluate relapse rates in relation to treatment, gender, age category and period. Calculations were done using Generalized Estimation Equations with a log-link. Longitudinal data concerning height SDS and behaviour were analyzed with linear mixed models that included treatment, age strata, gender, time, baseline values, and interaction between time and treatment as fixed effects. For the remaining variables, continuous outcome was analysed with either the Student's T-test or the Mann Whitney test and categorical outcome was analysed with either the Pearson's X^2-test or the Fisher's exact test. P-values < 0.05 were considered statistically significant. All analyses were performed with SPSS (version 17.0).

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See related editorial, "Corticosteroid Therapy for Steroid-Sensitive Nephrotic Syndrome in Children: Dose or Duration?," on pages 93 - 98.

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Supplemental Table 1. Currently reported prednisolone regimens for the first episode of childhood nephrotic syndrome.

Initial prednisolone regimen	Duration	Cumulative dose	Reported use
ISKDC: 4 weeks 60 mg/m^2 daily 4 weeks 40 mg/m^2 on 3 out of 7 days or on alternate days	8 weeks	2240 mg/m^2	Canada,[1] Nigeria,[2] South-Korea,[3] UK,[4] USA[5]
BAPN: 60 mg/m^2 daily until remission 4 weeks 40 mg/m^2 on 3 out of 7 days or on alternate days	Maximum 8 weeks	Maximum 2240 mg/m^2	UK[6]
APN: 6 weeks 60 mg/m^2 daily 6 weeks 40 mg/m^2 on alternate days	12 weeks	3360 mg/m^2	Canada,[1] Germany,[7] Japan,[8] The Netherlands,[9] Spain,[10] USA5
SNP: 4 weeks 60 mg/m^2 daily 8 weeks 60 mg/m^2 on alternate days 2 weeks 45 mg/m^2 on alternate days 2 weeks 30 mg/m^2 on alternate days 2 weeks 15 mg/m^2 on alternate days	18 weeks	3990 mg/ m^2	France[11]
Modifications of one or more of the regimens described above	4-24 weeks	uncertain	Australia,[12] Brazil,[13] Egypt,[14] India,[15] Poland,[16] Taiwan,[17] Turkey,[18] USA[5]

ISKDC, International Society of Kidney Disease in Children; APN, Arbeitsgemeinschaft für Pädiatrische Nephrologie; BAPN, British Association for Pediatric Nephrology; SNP, Société de Néphrologie Pédiatrique.

Supplemental Table 2. Comparison of baseline characteristics between randomized and not randomised patients.

Variable	Randomized, S (n=126)	Randomized, WC (n=13)	Randomized, SRNS (n=11)	Not Randomized (n=57)	p
Male, n (%)	86 (68)	11 (85)	5 (45)	35 (62)	0.193
Age at diagnosis; median (IQR)	4.2 (3.2-6.2)	3.0 (2.5-4.7)	4.1 (3.1-9.3)	4.6 (2.7-8.4)	0.218
Hospital, University (%)	14 (11)	4 (31)	2 (18)	5 (9)	0.136

S, started trial medication; WC, did not start trial medication because of withdrawn consent; SRNS, did not start trial medication because of steroid resistance.

Supplemental Table 3a. Nine patients fulfilling clinical yet not strict FRNS.

Study ID	Treatment group	Indication	Diagnosis	End of randomized treatment	Relapses and additional treatment (indicated with →)	Crit A	Crit B	Crit C	SD
28	6 months	very early onset SDNS + secondary SRNS	2 -11-2005	19-04-2006	28-2-2006[a] 31-3-2006[a] No remission after 6 wks pred: secondary SRNS → ciclosporin 26-1-2009	No	No	Yes	Yes
52	6 months	3 relapses within a time frame of 9 months	07-04-06	22-09-06	5-6-2007 22-11-2007 26-3-2008 16-8 -2008 → cyclophosphamide	No	No	Yes	No
90	3 months	secondary SRNS	11-1 -2007	05-04-2007	18-3-2007[a] No remission after 6 wks of pred: secondary SRNS → mycophenolate mofetil 15-5-2008 → ciclosporin	No	No	Yes	No
119	3 months	3 relapses within a time frame of 8 months + behavioural problems + high BP	17-9 -2007	10-12-2007	3-5-2008 17-11-2008 6-1-2009 → cyclophosphamide 7 -7 -2009 13-4 -2010 → levamisole	No	No	Yes	No
129	3 months	very early onset SDNS + secondary SRNS	31-12-2007	24-03-2008	28-2-2008[a] 17-3-2008[a] No remission after 6 wks of pred: secondary SRNS → ciclosporin 16-8-2011	No	No	Yes	Yes
135	6 months	very early onset SDNS	28-2 -2008	14-08-2008	13-6-2008[a] 29-7-2008[a] 21-10-2008[a] →cyclophosphamide 9 -2 -2011	No	No	Yes	Yes
142	6 months	very early onset SDNS + partial secondary steroid resistance	5 -4 -2008	20-09-2008	30-6-2008[a] Partial remission after 6 wks oral prednisolone → IV prednisolone: remission 15-10-2008[a] → IV prednisolone + ciclosporin 17-8-2009 6 -11-2009[a] 22-12-2009[a] → mycophenolate mofetil 5 -4 -2011[a]	No	No	Yes	Yes
150	6 months	2 relapses within a time frame of 3 months + behavioural problems at high doses	8 -8 -2008	23-01-2009	22-1-2009[a] 12-4-2009 → prednisolone maintanance	No	No	Yes	No
153	6 months	2 relapses within a time frame of 3 months	21-8 -2008	05-02-2009	14-1-2009[a] 30-3-2009 → prednisolone maintanance	No	No	Yes	No

[a]During or within two weeks after cessation of prednisolone. Very early onset SDNS, the first two relapses occurred during or within 2 weeks after cessation of prednisolone; FRNS, frequently relapsing nephrotic syndrome; SDNS, steroid dependent nephrotic syndrome. BP, blood pressure.

Supplemental Table 3b. Occurrence of a first relapse and clinical FRNS according to each criterion.

Time (months)	Relapse	NAR (relapse)	Clinical FRNS	NAR (Clinical FRNS)	A	B	C
			3 month-group				
0	0	62	0	62			
3	1	61	2	60			2
6	29	32	9	51	9		
9	6	26	14	37	14		
12	6	20	1	36		1	
15	3	17	1	35		1	
18	0	17	2	33		1	1
21	1	15	2	30		2	
24	2	12	0	26			
>24	0	<12	0	<26			
Total	**48**		**31**		**23**	**5**	**3**
			6 month-group				
0	0	64	0	64			
3	5	59	0	64			
6	18	41	5	59		2	3
9	11	30	15	44	12	3	
12	7	23	10	34	6	2	2
15	3	20	1	32		1	
18	4	16	1	31		1	
21	2	14	2	29		2	
24	0	13	0	26			
>24	1	<13	4	<26		3	1
Total	**51**		**38**		**18**	**14**	**6**

Data other than NAR represent numbers of patients with an event within time periods of 3 months. A: ≥ 2 relapses within six months after completing initial treatment; B: ≥ 4 relapses within any period of 12 months, including relapses during initial treatment; C: clinical decision that included additional intervention: prednisolone maintenance therapy (> three months) or other immunosuprressive agents. NAR, number at risk; FRNS, frequently relapsing nephrotic syndrome.

Supplemental Table 4. Characteristics of studies comparing 3 to 6 months prednisolone for the first episode of childhood nephrotic syndrome.

Study	Publication Status	Design and Setting	Inclusion Criteria
Ksiazek 1995[19]	Fully published	Single centre, renal centre, Poland Inadequate concealment of allocation No blinding: parents chose regimen Loss of follow up: 0% Intention to treat analysis	First episode of NS Age 13 months – 11 years Remission within 4 weeks of daily prednisolone
Gulati 2001[20]	Abstract only	Single centre, renal centre, India* Adequate concealment of allocation No blinding Loss of follow up: 4% No intention to treat analysis	First episode of NS
Hiraoka 20038	Fully published	Multicentre, renal centres, Japan Adequate concealment of allocation No blinding Loss of follow up: 3% Modified intention to treat analysis	First episode of NS
Pecoraro 2004[21]	Abstract only	Single centre, renal centre, Italy* Inadequate concealment of allocation No blinding Loss of follow up: not stated No intention to treat analysis	First episode of NS
Mishra 2012[22]	Fully published	Single centre, renal centre, India Unclear concealment of allocation No blinding Loss of follow up: 3% No intention to treat analysis	First episode of NS Age 1-10 years No underlying disease remission within 4 weeks of daily prednisolone
Current Study	Fully published	Multicentre, general and university centres, Netherlands (1 Belgian) Adequate concealment of allocation Double blinding Loss of follow up: 1% Modified intention to treat analysis	First episode of NS Age 9 months - 17 years No underlying disease

Search strategy: we searched Medline and abstract books from the International Pediatric Nephrology Association and the European Society for Pediatric Nephrology for studies comparing three months prednisolone therapy to longer prednisolone regimens for the first episode of childhood NS, published since the last updated Cochrane meta-analysis.[23] We searched between Jan 1st 2007 and May 31st 2012. Search terms included “nephrotic”, “syndrome”, and “prednisolone” or “prednisone”. e, estimated; * not stated in the original article/abstract, yet taken from reference[23]; **stated as strict FRNS.

Definition of FRNS	Short Regimen	Long Regimen	Relapse Treatment	Follow-up
≥ 2 relapses within 6 months after remission or 4 relapses within any 12 months *	3 months 2530 mg/m² (e) n=68	6 months 3070 mg/m² (e) n=72	Within 6 months after completing initial regime: 1 mg/kg daily until remission + 1 mg/kg on alternate days for 4 weeks > 6 months after completing initial regime: according to the long regimen	27 months and 30 months respectively
>2 relapses within any 6 months or > 6 relapses within any 18 months*	3 months 3360 mg/m² n=70	6 months 4200 mg/m² n=70	Not stated	15 months and 18 months respectively*
≥ 2 relapses within any 6 months after completing the previous regimen	3 months 3360 mg/m² n=34	6 months 4620 mg/m² n=36	6o mg/m² daily until remission + 40 mg/m² on alternate days for 4 weeks	Median 34 months (range 15-48)
Not stated	3 months 3094 mg/m² (e) n=16	6 months 5235 mg/m² (e) n=16	Not stated	No median or minimum stated; maximum 21 months
Not stated	3 months 3360 mg/m² n=37	5 months 3990 mg/m² (e) N=37	2 mg/kg daily until remission + 1.5 mg/kg for 4 weeks	12 months
≥ 2 relapses within 6 months after completing initial regime or 4 relapses within any 12 months**	3 months 3360 mg/m² n=62	6 months 3390 mg/m² n=64	6o mg/m² daily until remission + 40 mg/m² on alternate days for 4 weeks	Median 47 months (IQR 35-60)

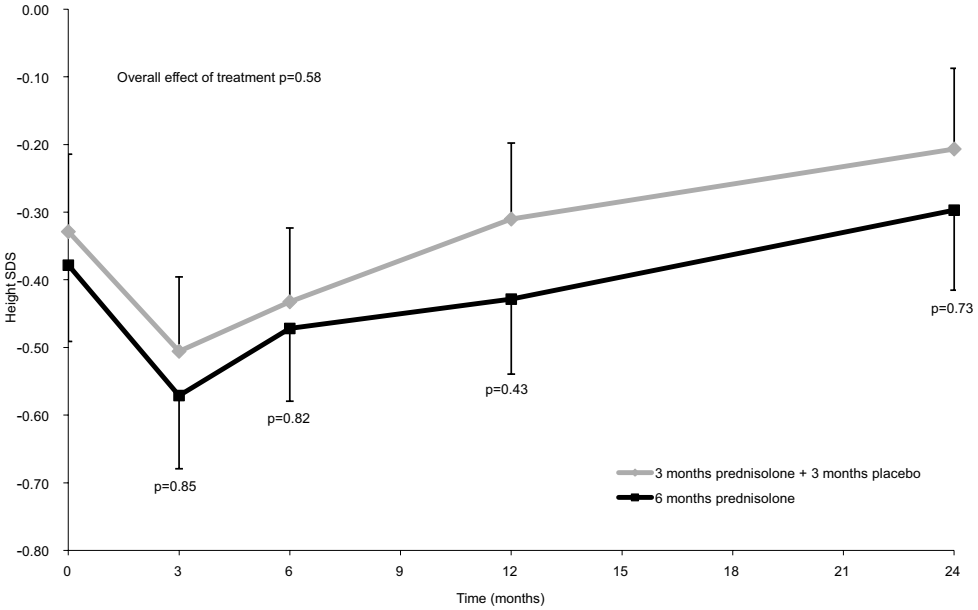
Supplemental Table 5a. Numbers of patients reported and enrolled per hospital.

Number of patients per site	Reported, number of hospitals	Enrolled, number of hospitals	Not Enrolled, number of hospitals	Enrollment Ratio	
				Quartiles	Number of hospitals
1	28	29	31	0 - 0.25	12
2	20	19	7	0.26 - 0.5	11
3	15	12	3	0.51 - 0.75	15
4	8	8	0	0.76 - 1.0	44
5	7	3	0		
6	1	0	0		
7	2	0	0		
8	0	0	1		
12	1	0	0		
Total (hospitals)	82	71	42		
Total (patients)	212	150	62		82
	Reported per hospital	Enrolled per hospital	Not enrolled per hospital		
Median number of patients (IQR)	2 (1-3)	2 (1-3)	1 (0-1)	Median	
				0.8 (0.5-1.0)	

This table shows the numbers of hospitals reporting, enrolling, or not enrolling a certain number of patients. Example: Eight hospitals have each reported four patients, 12 hospitals have each enrolled three patients. In total, 93 hospitals (84 general and 9 university hospitals) participated in the study. 82 hospitals reported patients for assessment of eligibility (n=212). 71 hospitals (due to reorganization currently 69 hospitals) enrolled patients (n=150). The majority of these hospitals enrolled one to three patients; The median enrollment ratio was 0.8. IQR, inter quartile range.

Supplemental Table 5b. Reasons for not participating (more than one reason is possible)

Reason	Number of cases
Fear of blinding/placebo	7
Insufficient understanding of the study protocol due to:	
- Language	10
- Intelligence	3
Fear of research settings in general	9
Burden considered too high:	
- Six months study medication considered too long	14
- Parental distress at the time of diagnosis	4
- Child considered too young to participate	4
- Additional testing/questionnaires	3
- Child would get too much negative attention	1
- Co-morbidity of the child	2
- Follow up period considered too long	2
Complex social situations	
- Child in foster care	1
- Parents' divorce	3
- Psychiatric disorder in one of the parents	1
Patient (≥ 12 years of age) refuses participation	2
Negative previous experiences with participation in research	5
Planned long-term emigration	1
Unknown	9



Supplemental Figure 1. Height standard deviation scores during two years follow up in 126 children with steroid sensitive nephrotic syndrome. Data represent means and error bars. SDS, standard deviation score.

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Corticosteroid Therapy for Steroid-Sensitive Nephrotic Syndrome in Children: Dose or Duration?

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Idiopathic nephrotic syndrome, although a rare disease, is the most common primary glomerular disease among children. It causes substantial morbidity because it typically runs a relapsing course punctuated with prolonged periods of corticosteroids and other immunosuppressive medication. It affects about 2 children per 100,000 aged <16 years in Europe and North America,[1] with higher rates reported among children from the Indian subcontinent.[2]

Approximately 80% of children achieve complete remission with 4 weeks of corticosteroid therapy after their first presentation and are considered to have steroid-sensitive nephrotic syndrome (SSNS),[3,4] but a similar proportion relapse ≥1 times.[3,4] Among children who relapse, about 50% will relapse frequently (defined by the International Study of Kidney Disease in Children [ISKDC] as ≥2 relapses within 6 months of initial response, or ≥4 relapses in any 12-month period[5]) or will have a steroid-dependent disease (defined by Arbetsgemeinschaft für Pädiatrische Nephrologie [APN] as ≥2 consecutive relapses either during corticosteroid therapy or within 2 weeks of ceasing it[6]). Despite relapses, most children continue to be steroid responsive, maintain normal kidney function, and ultimately, will be cured as they age into adolescence and early adult life.[4]

Over 40 years ago, the ISKDC proposed a regimen for the initial episode of SSNS, which comprised 60 mg/m^2 per day of prednisolone for 4 weeks followed by 40 mg/m^2 admin- istered on 3 of 7 days[5] for a further 4 weeks. Subsequently, a randomized trial coordinated by the APN demonstrated that alternate-day prednisolone was more effective in maintaining remission than prednisolone given on consecutive days.[7] Most pediatric nephrologists adopted a regimen of daily prednisolone for 4 weeks followed by 4 weeks of alternate-day prednisolone as their standard regimen for the treatment of the first episode of SSNS.

Because of the high relapse rate with this regimen, several trials have evaluated whether extending the duration of prednisolone therapy would result in fewer children relapsing and developing frequently relapsing nephrotic syndrome (FRNS). In a systematic review, data from six randomized controlled trials (RCTs) show that compared with 8 weeks of initial therapy, increasing the duration of prednisolone to ≥3 months reduced the risk of relapse over the following 12–24 months by 30% (relative risk [RR], 0.70; 95% confidence intervals [CI], 0.58–0.84) and the number of children with FRNS by 37% (RR, 0.63; 95% CI, 0.46–0.84).[8] A meta-analysis of four RCTs demonstrates that compared with 3 months, 6 months of prednisolone reduced the risk of relapse by 12–24 months by 43% (RR, 0.57; 95% CI, 0.45–0.71) and the number of children

with FRNS by 45% (RR, 0.55; 95% CI, 0.39–0.80). However, increased duration of prednisolone also resulted in an increased total dose of prednisolone, so it remained unclear whether the benefit resulted from the increased duration or the total dose of prednisolone. Regression analysis suggested that an increased duration, rather than dose, was the most influential variable; however, because it was a nonrandomized comparison, the potential existed for confounding by design.

In this issue of JASN, Teeninga et al.[9] report the results of a placebo-controlled, parallel group trial in which 150 children aged between 9 months and 17 years with their first episode of idiopathic nephrotic syndrome were randomized at diagnosis to receive 12 weeks of prednisolone followed by 12 weeks of placebo (74 children) or 24 weeks of prednisolone (76 children), with the dosage regimens designed to provide the same total dose of prednisolone in both groups. The primary outcome was the number of children who developed FRNS, with the secondary outcomes being the number with relapse and the adverse events seen. Twenty-four children (12 children from each treatment group) were excluded from the analysis because of primary steroid resistance (11 children) or withdrawal of consent for the study (13 children). There was no significant difference in the number of children who developed FRNS between treatment groups, whether FRNS was defined according to strict ISKDC criteria (45% versus 50%) or using clinical extended criteria (50% versus 59%). Similarly, there was no significant difference in the number of children with any relapse (77% versus 80%). Adverse effects (hypertension, ophthalmologic complications, moon face, striae, viral and bacterial infections), growth rates, bone mineral densities, and behavioral scores did not differ significantly between treatment groups. The authors conclude that extending the duration of prednisolone therapy without increasing the total dose did not improve outcomes in children with their first episode of SSNS.

The major strength of this study is its methodologic rigor. Participants were recruited from 60 general hospitals and 9 tertiary centers and represented about half of all children diagnosed with idiopathic nephrotic syndrome in the Netherlands during the study period. Participants were enrolled and followed-up using processes that limited selection, performance, detection, attrition, and selective reporting bias. In contrast, among the 10 RCTs included in meta-analyses examining extended duration or increased dose regimens,[8] 5 studies did not demonstrate adequate allocation concealment, none were blinded, and follow-up was incomplete or participants were inappropriately withdrawn from analysis in 7 studies. Inadequate allocation concealment and lack of blinding are typically associated with overestimation of the benefit of an intervention.[10]

Possible weaknesses of this study relate to the definition of the primary outcome, to the postrandomization withdrawals, and to inadequate power. The definition of the primary outcome event of FRNS was based initially on the ISKDC definition (strict FRNS), which is difficult to apply during extended-duration prednisolone regimens, because it does not account for relapses during the initial course of therapy. Consequently, the authors added a third criterion in which FRNS was diagnosed based on the clinical decision to use additional immunosuppressive therapy (clinical FRNS). However, analyses using either the ISKDC definition or the extended definition found no significant differences in the incidence of FRNS between treatment groups indicating that different outcome definitions did not influence the results. Twenty-four children were withdrawn after randomization because of steroid resistance (7%) or withdrawal of consent (9%). This may have been prevented with randomization occurring once remission had been achieved. However, given that such postrandomization exclusions were nondifferential, it is unlikely that such exclusions would bias the study; rather, they would just reduce power. Based on 80% power to detect a 20% reduction in the cumulative incidence of FRNS, enrollment and analysis of 72 children in each study arm were required. However, fewer children were enrolled and the study demonstrated no significant differences in the outcome of clinical FRNS (difference at 1 year, 5.0%; 95% CI, 29.1, 19.1). Nevertheless, the authors reasonably conclude that a significant benefit of the 24-week regimen over the 12-week regimen was unlikely because using the 24-week reg- imen would provide at best only a 9.1% benefit at 1 year and a 5.7% benefit at 5 years based on the 95% CIs around between- group differences.

Although this trial has demonstrated no benefit of extended duration of prednisolone using the same total dose, controversy remains over the most effective duration and dose of prednisolone for the initial episode of SSNS. Recent guidelines suggest 12 weeks,[11,12] ≥12 weeks,[13] or 18 weeks[14] of prednisolone with total doses of prednisolone exceeding that given in the 8-week regimen. Searches of clinical trial registries identified that two well designed placebo-controlled trials comparing extended duration prednisolone (with a higher total prednisolone dose) with short duration are in progress. In the Prednisolone in Nephrotic Syndrome (PREDNOS) trial in the United Kingdom (EudraCT number 2010-022489-29), which commenced in 2011, children are randomized after achieving remission with 4 weeks of daily prednisolone to receive either 4 weeks of alternate-day prednisolone followed by 12 weeks of placebo or to receive 16 weeks of alternate-day prednisolone with tapering of the dose. Participants are being followed for 24 months. In the second trial in India (CTRI/2010/091/ 001095), which commenced in 2010, children are randomized to 12 weeks of prednisolone (6 weeks daily, 6 weeks al- ternate days) followed by placebo for 12 weeks, or to 12 weeks of prednisolone

followed by 12 weeks of tapering doses of prednisolone. Participants are followed for 12 months from the end of therapy. These studies should determine whether increasing the total dose of prednisolone results in improved outcomes in the initial episode of SSNS.

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PART III

METABOLISM OF PREDNISOLONE: TOWARDS FEASIBLE MEASUREMENT

CHAPTER 4

Determination of unbound prednisolone, prednisone and cortisol in human serum and saliva by on-line solid phase extraction liquid chromatography tandem mass spectrometry

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ABSTRACT

Prednisolone (PLN) and prednisone (PN) are widely used glucocorticoids. Drug monitoring of PLN and PN is not routinely done due to the need of multiple blood sampling and challenging measurement of unbound PLN and PN in blood. Here we present a robust method for quantification of cortisol, PLN and PN in serum, ultrafiltrate and saliva by on-line solid phase extraction LC-MS/MS. The method is linear for the three analytes over the range of 6–1400 nmol/L for serum and 2–450 nmol/L for ultrafiltrate and saliva. Within-run precision of all three analytes was < 10% and total precision was < 15%. This method was applied to create time concentration profiles of cortisol, PLN and PN after an oral dose of prednisolone in a healthy volunteer. Salivary levels of PLN correlated well with ultrafiltrate levels ($p<0.01$), while this correlation was only marginal for PN ($p=0.052$). The PN/PLN ratio was significantly higher in saliva than in ultrafiltrate and serum ($p<0.01$). Addition sums of both metabolites in saliva showed excellent correlation with those of ultrafiltrate ($p<0.01$). These findings have not been presented before and may have important implications for future studies concerning drug monitoring of PLN and PN in saliva.

INTRODUCTION

For over six decades, glucocorticoids have played a crucial role in the treatment of a variety of diseases. Unfortunately, toxicity poses limitations to their use. Side effects include obesity, hypertension, osteoporosis, muscle weakness, peptic ulcers, cataract and depression. In children, impaired growth and changes in behaviour are added to the aforementioned problems. Both therapeutic effect and toxicity highly vary among individuals. Interindividual differences in elimination are more pronounced than intraindividual differences.[1] Monitoring these drugs could therefore aid in providing more tailored treatment regimens, yet is hampered by several practical difficulties.

Prednisolone and its inactive metabolite prednisone are widely used synthetic glucocorticoids. In blood, both endogenous and exogenous glucocorticoids are bound to corticosteroid binding globulin (CBG) and albumin.[2] It is generally accepted that only the unbound fraction is biologically and pharmacologically active, as unbound molecules can pass trough capillary walls and diffuse freely across cell membranes.[1] Unbound blood levels of prednisolone reflect glucocorticoid bioavailability more accurate than total prednisolone levels.[3,4]

Unfortunately, measurement of unbound prednisolone and prednisone in blood is complicated by several challenges, since the unbound fraction must be separated from the bound fraction first. Separation can be accomplished by several techniques including direct or indirect (tracer dilution) equilibrium dialysis and ultrafiltration. Although equilibrium dialysis is described as an acceptable method for separating plasma protein-bound and free glucocorticoids, ultrafiltration is recommended because of the gain of time, feasibility, good reproducibility and little chance of technical failure.[5,6,7]

As an alternative to these separation techniques, unbound fractions of prednisolone in blood can be calculated from equations incorporating total plasma concentrations, the amount of transport proteins and their binding characteristics.[8,9,10] These calculation methods appear attractive, yet for the purpose of drug monitoring, still multiple blood samples are needed to calculate free drug concentrations from total drug concentrations in order to obtain a representative time concentration profile. Furthermore, measurement of CBG is not accessible in an automated assay.[11]

Drug monitoring for prednisone and prednisolone is not done regularly as it apparently relies on rather invasive and cumbersome methods. This prompted us to search for more feasible alternatives. Although plasma or serum are the standard media for monitoring drugs, the use of saliva has been reported as a patient friendly method for measurement of cortisol and several lipid-soluble drugs as well.[12,13,11,14] Prednisolone and cortisol share similarities in both molecular structure and protein binding profile. Interestingly, a well defined relationship between blood and saliva concentrations has not yet been confirmed for prednisolone.[15,16,17] Furthermore, the ratio of prednisone and prednisolone within this correlation has been paid no attention so far, whereas the ratio of cortisol and cortisone considerably differs between blood and saliva.[18] Filling these lacks could thus offer new insights in drug monitoring of prednisolone and prednisone.

Nowadays liquid chromatography-tandem mass spectrometry (LC-MS/MS) has become a routine technique in endocrine laboratories. The use of this technique for measurement of prednisolone is preferred over immunoassays, as cross-reactivity with cortisol can be avoided. A LC-MS/MS method for simultaneous determination of unbound prednisolone, prednisone and cortisol in human plasma was recently validated by Ionita et al.[19] McWhinney et al. recently published a promising method for simultaneous determination of several glucocorticoids including cortisol, prednisone and prednisolone in both plasma, urine and saliva by ultra high performance LC-MS/MS. This method offered several excellent clinical applications. However, results of their measurements of prednisolone and prednisone in saliva were not shown in this study and the correlation between salivary and (unbound) plasma levels of prednisolone and prednisone was not addressed.[18]

In the present study, we describe a robust and feasible on-line solid phase extraction LC-MS/MS (XLC-MS/MS) method for quantification of cortisol, prednisolone and prednisone in both serum, ultrafiltrate and saliva. We applied this method to create time concentration profiles of these compounds in a healthy volunteer. This method can be used for further assessment of the correlation between the unbound serum and salivary concentrations of prednisolone and prednisone and may have important implications for future pharmacokinetic studies in patients treated with prednisolone.

MATERIALS AND METHODS

Chemicals and Reagents

Cortisol, prednisone, prednisolone and ammonium acetate were obtained from Sigma-Aldrich (St. Louis, MO, USA). 9,11,12,12-d_4-cortisol was purchased from Cambridge Isotope Laboratories, Inc (Andover, MA, USA). Acetonitrile, formic acid, ethanol, ammonium hydroxide and activated charcoal were obtained from Merck (Darmstadt, Germany). HPLC grade water was from the MilliQ® device (Millipore, Billerca, MA, USA).

Standards and controls

Stock solutions (3 mmol/L) of cortisol, 9,11,12,12-d_4-cortisol, prednisone and prednisolone were prepared in ethanol. The stock solutions of cortisol, prednisone and prednisolone were diluted with MilliQ® water to a combined standard of 450 nmol/L of each steroid for the analysis in saliva and ultrafiltrate. For the plasma analysis the stock solutions were diluted with steroid free plasma to a combined standard with a concentration of 1400 nmol/L. Both standards were further diluted in the appropriate medium to standards with concentrations of 150, 50, 16.7, 5.6, 1.8 and 467, 156, 51.9, 17.3 and 5.8 nmol/L respectively. The steroid free plasma for the dilution of the standards for the plasma analysis was made by charcoal treatment (stripping) of pooled plasma samples. The stock solution of 9,11,12,12-d_4-cortisol was diluted with MilliQ® water to an internal standard (IS) working solution with a concentration of 7 µmol/L for the plasma method and 1.4 µmol/L for the saliva and ultrafiltrate analysis. Quality control samples at two levels were made from plasma and saliva spiked with cortisol, prednisone and prednisolone.

Sample collection

For assessment of time concentration profiles of cortisol, prednisone and prednisolone in a healthy volunteer, blood and saliva samples were collected before and after 1, 2, 3, 4½, 6 and 12 hours of oral administration of 80 mg prednisolone. Blood samples were drawn from an indwelling peripheral venous canula. Serum was separated from the cells and stored at -20°C until analysis. Saliva was collected with Salivette polyesther swabs (Sarstedt AG&Co, Numbrecht, Germany) and stored at -20°C until analysis.

Sample preparation

Ultrafiltrate from serum was obtained by centrifuging 1 ml in Centrifree YM-30 centrifugal filter units (Millipore Ireland BV, County Cork, Ireland) with a 30 kDa molecular weight cut-off filter at 2000g for 30 minutes at 37°C. 240 µl of ultrafiltrate,

saliva, standards and quality controls were pipetted into a deep well plate and mixed with 10 µL IS working solution (1.4 µmol/L).
For the analysis of the serum samples 240 µL of the standards, quality control samples and serum were mixed with 10 µL IS working solution (7 µmol/L) and deproteinized with 1 mL of acetonitrile. After centrifugation at 1500g for 5 minutes the supernatant was evaporated to dryness under a stream of nitrogen. Samples were redissolved in 250 µL water and pipetted into a deep well plate.

XLC-MS/MS Equipment and Conditions

On-line solid phase extraction was performed on a Symbiosis Pharma system (Spark Holland, Emmen, The Netherlands). An OASIS® HLB extraction cartridge (1x10 mm, 30 µm, Waters , Millford, MA, USA) was preconditioned with 1 mL of acetonitrile followed by 1 mL of water. 20 µL extracted serum or 100 µL saliva or ultrafiltrate was applied to the cartridge together with 1 mL of water. After washing with 1 mL of 2% ammonium hydroxide followed by 1 mL of 2% formic acid/5% acetonitril the cartridge was placed automatically into the chromatographic system. The analytes were eluted from the cartridge by applying a chromatographic gradient for 2 minutes. Separation of cortisol, prednisone and prednisolone was achieved on a Zorbax SB-Phenyl analytical column (2.1x100 mm, 3.5 µm, Agilent Technologies, USA) at 30 °C with a flow of 0.2 mL/min. The gradient started with 80% 2 mmol/L ammonium acetate / 20% acetonitril and was adjusted to 69% / 31% in 2 minutes and held for 8 minutes. After a column wash with 90% acetonitrile for one minute the column was re-equilibrated with the starting conditions for 3.5 minutes. Detection was performed on a Waters Quattro Premier XE mass spectrometer (Waters, Millford, MA, USA) under positive electrospray ionization conditions (3 kV). The following multiple-reaction monitoring (MRM) ion transitions were used: m/z 363.1 -> 120.9 for cortisol, m/z 367.0 -> 120.9 for 9,11,12,12-d_4-cortisol, m/z 359.0 -> 341.1 for prednisone and m/z 361.1 -> 343.2 for prednisolone. Cone voltages and collision energies were 30 V and 30 eV for cortisol, 25 V and 20 eV for d_4-cortisol, 20 V and 15 eV for prednisone and 15 V and 10 eV for prednisolone. Data acquisition and calculations were accomplished using the MassLynx Software version 4.1 (Waters, Millford, MA, USA).

Method validation

Ion-suppression was tested by injecting blank saliva, deproteinized serum and ultrafiltrated serum while a constant flow of 10 µL/min of the 3 analytes and internal standard was infused into the mass spectrometer. The concentration of the infused solution was 2500 nmol/L for all components.

We established linearity and precision based upon the EP6 and EP5 protocols of the CLSI (Clinical and Laboratory Standards Institute) of the USA using the EP Evaluator 9 software (D.G. Rhoads Associates, Kennett Square, PA,USA).

For our purpose a minimal value for report of 10 nmol/L for cortisol, prednisone and prednisolone in plasma was satisfactory. For saliva and ultrafiltrate minimal values for report of 5 nmol/L for prednisone and prednisolone and of 2 nmol/L for cortisol were sufficient. Instead of measuring the limit of detection and the limit of quantification, we assessed whether these concentrations could be measured with a total imprecision of less than 20% by analyzing samples with approximately these concentrations in duplicate over 5 cycles.

Carry-over was tested using the carry-over protocol from the EP Evaluator 9 software. Specimens with high results were followed by specimens with low results. If the results for the high-low sequences were statistically identical to the results for the low-low sequences, the experiment passed the carry-over test.

46 patient plasma samples from our laboratory that were analyzed for cortisol with a chemiluminescense immunoassay (Immulite 2000, Siemens Healthcare Diagnostics, Deerfield, IL, USA) were also measured in the XLC-MS/MS method. The results of the two methods were compared using the EP9 protocol from the EP Evaluator 9 software. We analyzed the calibrators of the Immulite 2000 in the XLC-MS/MS method. To verify the concentration of the stock solution of cortisol it was diluted 50 times with methanol and absorbance was measured with a spectrophotometer at 239 nm. The concentration was calculated using Lambert Beer's law.

Serum concentrations of CBG and albumin

CBG was in serum was determined by a CBG-RIA-CT kit (Diasource Immuno Assays S.A., Nivelles, Belgium). Albumin in serum was determined by a spectrofotometric assay.

Abbreviations

CBG: corticosteroid binding globulin
IS: internal standard
MRM: multiple-reaction monitoring
PN: prednisone
PLN: prednisolone
(X)LC-MS/MS: (On line solid phase extraction) Liquid Chromatography Tandem Mass Spectrometry

Conversion of units

prednisolone: [nmol/L] X 0.0360 → [µg/dL]
prednisone: [nmol/L] X 0.0358 → [µg/dL]
cortisol: [nmol/L] X 0.0363 → [µg/dL]

RESULTS AND DISCUSSION

XLC-MS/MS

The ion-suppression tests did not show any suppression or enhancement in the signal at the retention times of cortisol, 9,11,12,12-d_4-cortisol, prednisone or prednisolone (Fig. 1). We therefore conclude that ion-suppression is not an issue in this method. As described by Ionita et al,[19] chromatographic separation of cortisol and prednisolone is necessary because of the naturally occuring M + 2 isotope of prednisolone which interferes with the most abundant isotope of cortisol. This was accomplished by using a Zorbax SB-Phenyl analytical column. Chromatograms of serum, saliva and ultrafiltrate samples are shown in figure 2. Separation of cortisol and prednisolone was complete. Cortisone is a glucocorticoid with an equal molecular mass as prednisolone and therefore, to avoid interference, separation of cortisone and prednisolone is needed. Cortisone and cortisol should also be separated because of the possible interference of the M + 2 isotope of cortisone with the cortisol signal. This is shown in figure 3.

Validation

The plasma standard curve was tested over five days and showed linearity for the three analytes over the range of 6–1400 nmol/L within an allowable systematic error of 6%. The saliva and ultrafiltrate standard curve showed linearity over the range of 2- 450 nmol/L within an allowable systematic error of 6%. Within-run and total imprecision were measured in duplicate over 9-16 days in two human plasma samples and two saliva samples spiked with cortisol, prednisone and prednisolone. All components were measured with a within precision < 10% CV and a total precision < 15% CV. In plasma a concentration of 9 nmol/L of the three analytes was measured with a total imprecision < 10%. The minimal value for report of 10 nmol/L therefore is valid. In saliva and ultrafiltrate the minimal value for report of cortisol was determined at 2 nmol/L and for prednisone and prednisolone at 6 nmol/L (table 1).
For all components carry-over was lower than the error limit (3 times SD of the low-low results). The carry-over tests thus passed for all 3 components (table 2).

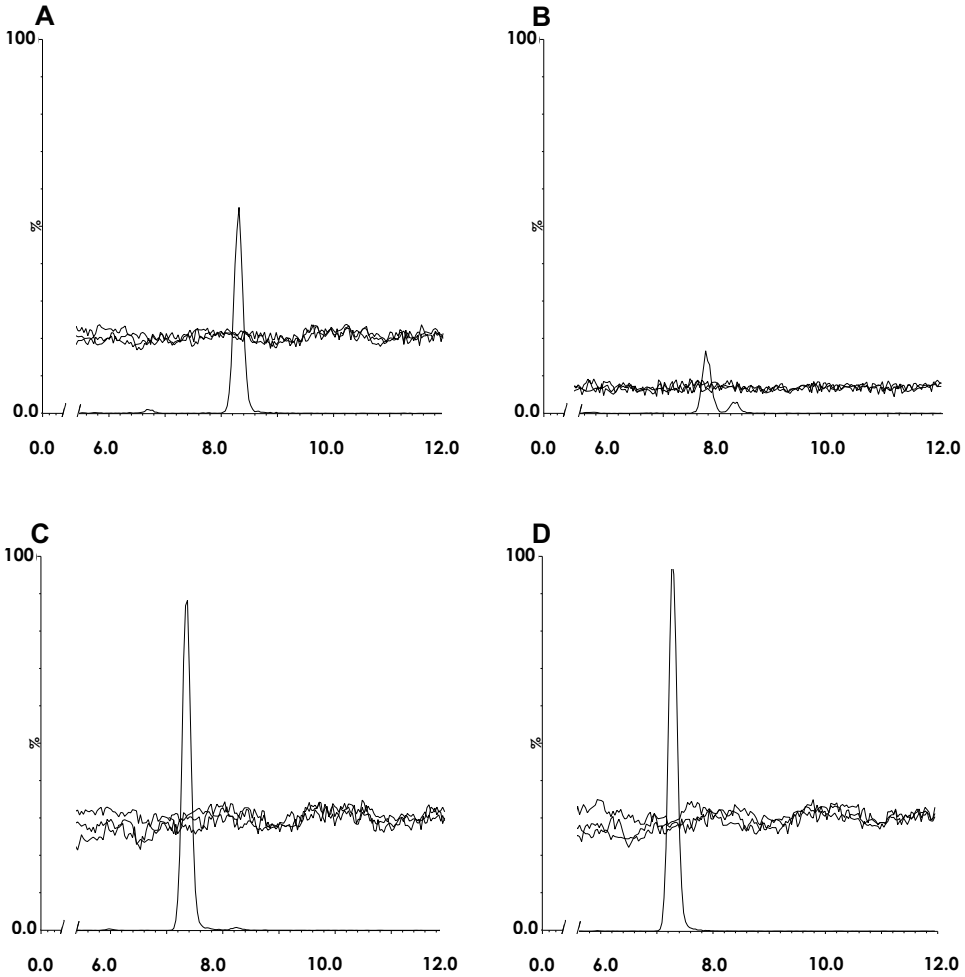

Figure 1. Combined chromatograms of blank serum, ultrafiltrate of blank serum and blank saliva. A:MRM ion transition m/z 359.0 -> 341.1 (prednisone), B: 361.0 -> 343.2 (prednisolone), C: 363.1 -> 120.9 (cortisol) and D: 367.0 -> 120.9 (d_4-cortisol). x-axis: time in minutes, y-axis: intensity in arbitrary units; 100% = $2x10^5$.

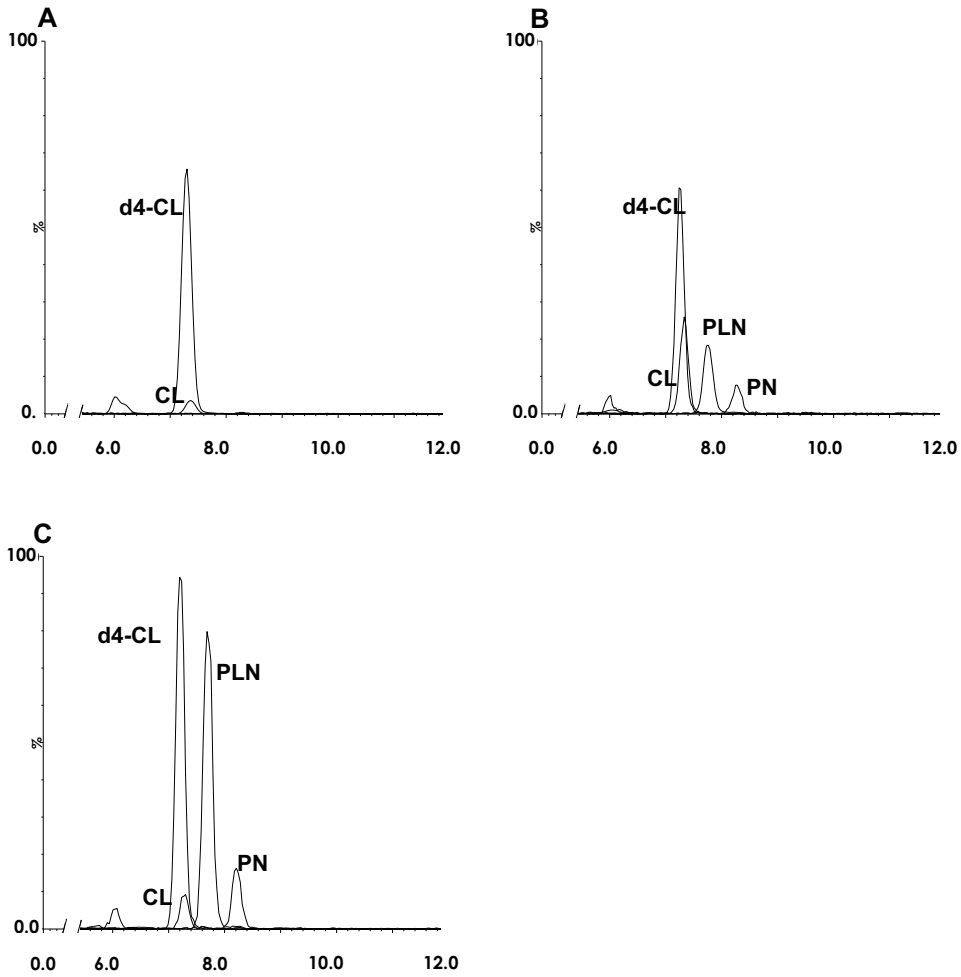

Figure 2. MRM chromatograms of saliva (A, 100% =1.5x10^5), ultrafiltrate of serum (B, 100% =1.5x10^5) and serum (C, 100% =1.0x10^5). x-axis: time in minutes, y-axis: intensity in arbitrary units. CL=cortisol, d4CL=d_4-cortisol, PN=prednisone, PLN= prednisolone.

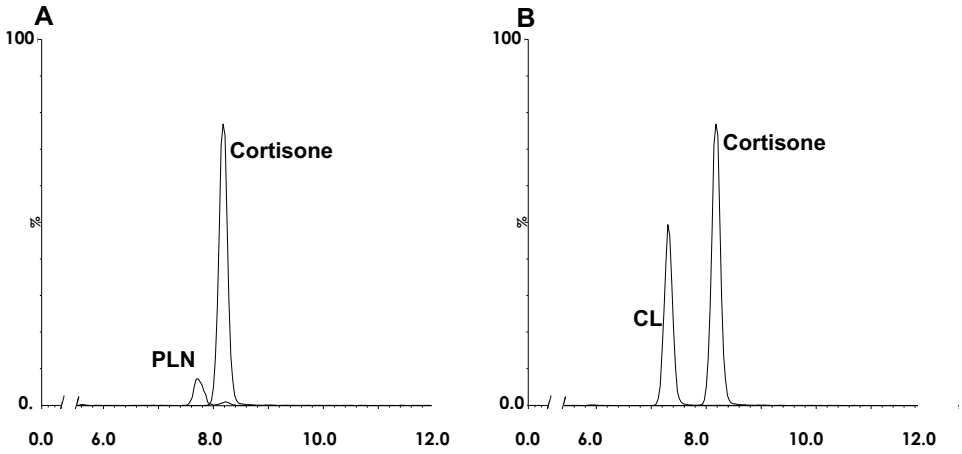

Figure 3. MRM chromatograms of cortisone and prednisolone (A) and cortisone and cortisol (B). x-axis: time in minutes, y-axis: intensity in arbitrary units; 100% = $4x10^5$. CL=cortisol, PLN= prednisolone.

Table 1. Validation results for precision and minimal value for report.

	cortisol			**prednisone**			**prednisolone**		
	mean	within-run	total	mean	within-run	total	mean	within-run	total
	nmol/L	%CV	%CV	nmol/L	%CV	%CV	nmol/L	%CV	%CV
minimal value for report *n=5*									
plasma	8.8	6.0	6.0	8.7	4.1	5.2	9.2	3.9	5.8
saliva/ultrafiltrate	2.2	5.2	9.8	4.8	2.7	11.4	6.0	2.7	9.4
precision									
plasma 1 *n=13*	159	2.8	5.2	140	3.1	5.2	113	4.6	10.5
plasma 2 *n=13*	481	2.7	2.9	361	4.5	4.5	315	4.2	5.4
saliva 1 *n=16*	13.9	2.9	2.9	209	1.4	4.7	408	4.8	7.9
saliva 2 *n=16*	6.9	3.1	3.1	79.3	2.1	5.2	124	5.9	6.1
ultrafiltrate 1 *n=15*	6.1	3.5	13.1	16.3	3.3	9.3	5.7	6.2	14.5
ultrafiltrate 2 *n=15*	61.9	2.4	6.8	190	3.1	6.3	73.1	5.1	8.0

Table 2. Carry-over results.

	cortisol		prednisone		prednisolone	
	mean nmol/L	sd	mean nmol/L	sd	mean nmol/L	sd
plasma						
high-low	42.2	1.5	39.0	1.6	52.6	2.3
low-low	41.2	1.0	37.3	2.1	49.1	3.1
carry over	1.0		1.7		3.6	
error limit	2.9		6.4		9.4	
saliva/ultrafiltrate						
high-low	3.3	0.1	8.5	0.3	7.0	0.6
low-low	3.4	0.1	8.1	0.3	6.8	0.5
carry over	-0.1		0.3		0.1	
error limit	0.4		0.9		1.5	

Comparison

We compared the results for cortisol in serum obtained by XLC-MS/MS with those of an automated immunoluminometric assay (Immulite 2000) in 46 anonymised human serum samples. Prednisone and prednisolone concentrations in these samples, measured with XLC-MS/MS, were below 10 nmol/L. The linear regression equation for cortisol was: XLC-MS/MS results = 0.76 (± 0.04) Immulite 2000 result + 28.5 (± 22.5), R = 0.9667. The two calibrators of the Immulite measured with XLC-MS/MS gave lower results: 92 and 90% (34 and 1097 nmol/L measured with XLC-MS/MS vs. 37 and 1224 nmol/L with the Immulite 2000). To exclude an incorrect value for the XLC-MS/MS standard, verification of the stock solution for cortisol was performed with the spectrophotometer and found to be 98% of the expected value. In literature, it is a well known phenomenon that cross-reactivity with other corticosteroids gives higher results for cortisol in immunoassay methods.[11] A similar difference as the one we found was reported by Kushnir et al (using LC-MS/MS with atmospheric pressure photoionization)[20] and McWhinney et al (using UHPLC-MS/MS with positive ESI).[18] In addition, Tai and Welch found that immunoassays used routinely in clinical laboratories gave higher results than their LC/MS-ESI method, which was confirmed by results of international quality control schemes (i.e. UKNEQAS).[21] Our XLC-MS/MS thus offers a more accurate method for the quantification of cortisol in plasma over a wide concentration range than the Immulite 2000. The ability to separate different steroid components and measure them in one run is a major advantage of XLC-MS/MS.

Concentrations in plasma, ultrafiltrate and saliva after a single dose of prednisolone

We combined ultrafiltration and on-line solid phase extraction LC-MS/MS techniques as described to simultaneously measure concentrations of cortisol, prednisone and

prednisolone in serum, ultrafiltrate and saliva samples of a healthy volunteer after oral administration of 80 mg prednisolone. Results are presented in figure 4 a-c.

Total prednisolone concentrations in serum reached a peak within two hours after ingestion at 1713 nmol/l. Unbound prednisolone serum concentrations, measured after ultrafiltration, revealed a peak concentration within two hours of administration at 627 nmol/L. These results are in accordance with previous findings.[3] As becomes clear from figure 4b and c, concentrations of the inactive metabolite prednisone were found to be considerably lower than those of the active metabolite prednisolone in serum and ultrafiltrate. This too is in keeping with earlier results, as interconversion between prednisolone and prednisone in blood constantly occurs in an equilibrium in favour of the active metabolite.[3,1]

As we expected, serum cortisol levels rapidly declined after administration of prednisolone (fig. 4a). This is thought to occur due to both increased clearance of cortisol in the context of competitive protein binding of prednisolone and direct suppression of adrenal cortisol production from negative feedback mechanisms within the hypothalamus-pituitary-adrenal axis.[22,23]

It is generally accepted that only the unbound fractions of cortisol and prednisolone are biologically or pharmacologically active, as only free molecules pass through the capillary wall. Consequently, only free molecules can reach the intracellular glucocorticoid receptor and interact with glucocorticoid responsive elements within DNA.[24] The unbound fraction therefore is thought to account for the vast majority of glucocorticoid effects. It is well established that cortisol levels in saliva represent unbound cortisol in blood. Therefore, salivary cortisol can be applied as a surrogate marker of biologically active cortisol in blood in clinical settings[25], for example Cushing's disease. In addition, concentrations of several drugs (theophylline, digoxin and diazepam) in saliva offer good representations of their unbound (biologically active) concentrations in blood.[12,13,11,14] Despite the fact that cortisol and prednisolone share structural and functional characteristics, there is a lack of evidence for an analogous relationship between prednisolone levels in saliva and blood. [17,15,16] In addition, the ratio of prednisone and prednisolone in saliva within this context has been paid no attention so far, whereas the ratio of cortisol and cortisone considerably differs between blood and saliva[18].

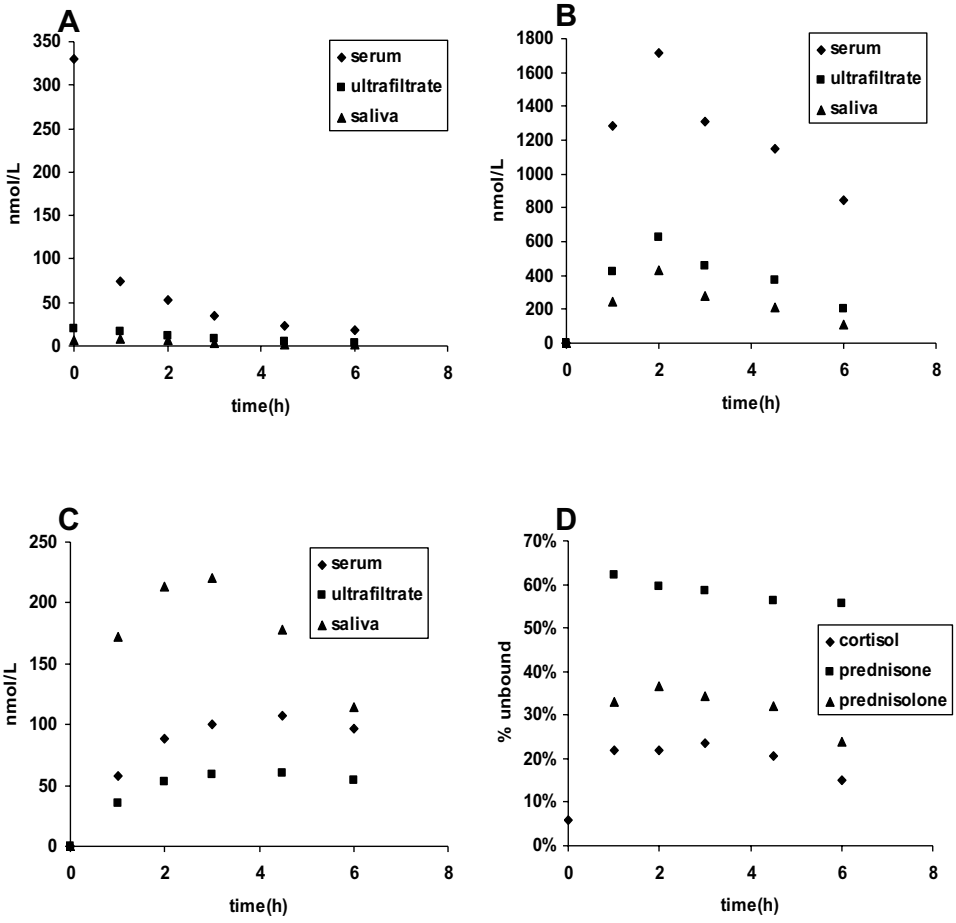

Figure 4. A-C: concentrations of cortisol (A), prednisolone (B) and prednisone (C) after oral administration of 80 mg prednisolone at timepoint 0. D: Percentage unbound cortisol, prednisone and prednisolone in serum derived from the concentrations in ultrafiltrate and serum.

To explore these relationships, we aimed to determine prednisolone and prednisone in saliva and the unbound fractions of prednisone and prednisolone in serum. Several techniques for separating bound from unbound glucocorticoids in serum or plasma have been described.[11] In equilibrium dialysis, unbound molecules freely diffuse through a semipermeable membrane from one compartment containing serum to another compartment containing a buffer solution without proteins. Equilibrium is reached when the concentrations of free molecules are equal in both compartments, after which the concentration of free glucocorticoid in serum can be approximated through (indirect) measurement of free glucocorticoid in the dialysate. The process of dialysis is time-consuming and involves continuous displacement of the glucocorticoid-

protein equilibrium, leading to stripping of proteins as well as dilution of the free concentration. Several corrections are needed, particularly when compounds with rather large free fractions are concerned.[26,27] In ultrafiltration, a centrifugal force is used to obtain ultrafiltrate from serum or plasma through a semipermeable membrane. During this process, the original glucocorticoid-protein equilibrium is also inevitably disturbed to some extent, yet this is limited since the dilution effect is minimal and the process is considerably shorter[26]. Therefore, we chose ultrafiltration as the separation technique in this study.

Protein binding of prednisolone and prednisone displays a non-lineair decrease with increasing dose. The maximum percentage of prednisolone bound to protein was reported around 90%, which decreased with increasing total concentrations to a plateau of around 65% when total concentrations reach approximately 2200 nmol/l or more.[28,29,23] In our healthy volunteer, CBG concentration was 44 mg/L and albumin concentration was 47 g/L, both within the normal reference range. The percentage unbound prednisolone in serum, reflected by concentrations in ultrafiltrate, peaked at 36,6% two hours after administration and gradually decreased to 14,5% 12 hours after administration (figure 4d). The percentage unbound prednisone only slightly decreased from 62% to 50%. Similar results were reported in previous studies.[3,23,30]
Prednisolone in saliva correlated well with prednisolone in ultrafiltrate (Fig. 5A, Spearmans's correlation; $p<0.01$). For prednisone, the correlation between saliva and ultrafiltrate did not reach statistical significance (Fig. 5B, Spearmans's correlation; $p = 0.052$.). To our knowledge, this is the first study reporting these correlations for measured concentrations of prednisolone and prednisone between saliva and ultrafiltrate.

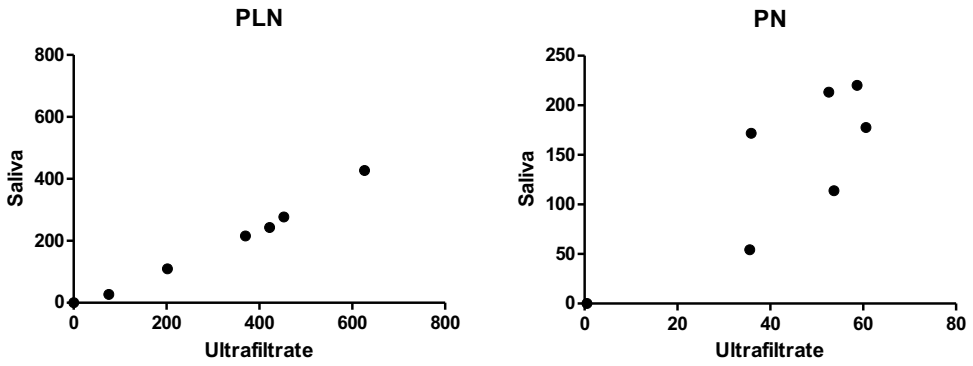

Figure 5. Prednisolone (A) and prednisone (B) concentrations in saliva vs ultrafiltrate (nmol/L) in a volunteer after oral administration of 80 mg prednisolone.

The median prednisone/prednisolone ratio was 0.08 in serum (range 0.05-0.16) and 0.2 in ultrafiltrate (range 0.1-0.3) (Fig. 6). This ratio remained relatively constant over time in both serum and ultrafiltrate with a slight increase over time. In contrast, the median salivary prednisone/prednisolone ratio (0.8, range 0.5-2.97) was significantly higher compared to the mean ratio in ultrafiltrate (Wilcoxon rank test; $p = 0.028$) and serum (Wilcoxon rank test; $p = 0.028$. Overall test for all three media: Kruskal-Wallis test; $p < 0.01$). The ratio in saliva showed considerable variation ranging from 0.5 at two hours after administration to 3.0 at twelve hours after administration. The most likely explanation for this remarkable difference is a relatively high amount of the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD-2) in salivary glands. 11β-HSD-2 converts prednisolone into prednisone, while its counterpart 11β-HSD-1 performs the reverse step. 11β-HSD-1 facilitates GC-access to the glucocorticoidreceptor and is present in tissues throughout the body [24,31]. In contrast, 11β-HSD-2 is found predominantly at mineralocorticoid target sites, such as the kidney, colon, placenta and salivary glands. [24,32,31,33] A crucial finding here was that when concentrations of prednisolone and prednisone in saliva were added up, they showed excellent correlation with the addition sum of prednisolone and prednisone in ultrafiltrate (Fig. 7, Spearman's correlation; $p < 0.01$). This suggests that although ratios of prednisone and prednisolone significantly differ between saliva and ultrafiltrate, levels of both metabolites together in saliva are in constant equilibrium with levels of both metabolites together in ultrafiltrate.

Free concentrations calculated with Coolens' equation for cortisol offer good comparison with cortisol measured after ultrafiltration.[5,7] For free prednisolone, Miller et al. and Shibasaki et al. reported equations correcting for plasma albumin levels.[8,10] Only Shibasaki et al. also corrected for total prednisolone levels. CBG was neither determined nor included in their equations. Both equations yielded lower unbound prednisolone concentrations than our ultrafiltrate results (Wilcoxon rank test; $p = 0.03$ for both comparisons). Rohatagi et al. developed a mathematical model for calculation of free prednisolone in plasma in which total prednisolone, cortisol, plasma albumin and CBG levels and protein-binding properties were considered. Interestingly, none of the equations included concentrations of prednisone.[9]

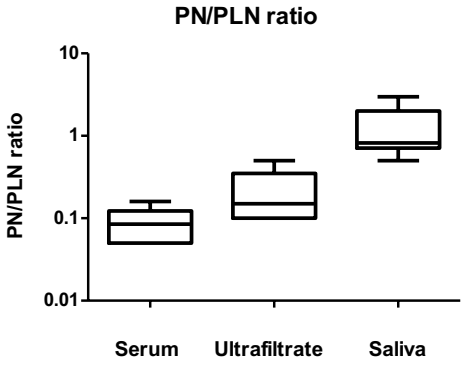

Figure 6. Prednisone/prednisolone ratios in serum, ultrafiltrate and saliva in a volunteer after oral administration of 80 mg prednisolone. The boxes depict median ratio and range.

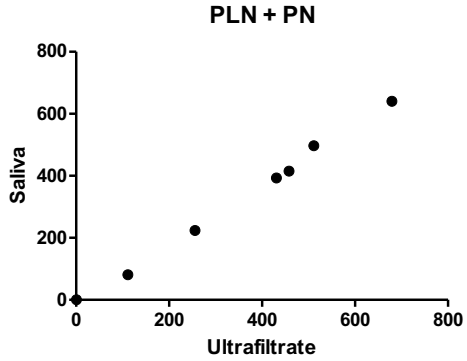

Figure 7. Addition sum of prednisolone and prednisone in saliva vs ultrafiltrate (nmol/L) in a volunteer after oral administration of 80 mg prednisolone.

In summary, we suggest that salivary measurement of prednisolone and prednisone by our on-line solid phase extraction LC-MS/MS is a useful tool to deduce unbound prednisolone and prednisone in blood. From our observations, we suggest that measurement of prednisolone in saliva be accompanied by measurement of prednisone to obtain a good indication of the absolute unbound concentrations of these drugs in blood. Further studies with larger series are needed to confirm these results.

Conclusions

The combination of ultrafiltration and an on-line solid phase extraction LC-MS/MS technique described in this study provides a robust and feasible method for the determination of cortisol, prednisone and prednisolone in serum, ultrafiltrate and saliva. The results of the measurements in a healthy volunteer indicate that prednisolone levels in saliva correlate well with prednisolone levels in ultrafiltrate. We found that the ratio

of prednisone and prednisolone is significantly higher in saliva than in ultrafiltrate and serum. This may well be a reflection of the ratio of 11-βHSD type 1 and 2 activity, which varies between tissues. To our knowledge, this is the first study reporting measurement of prednisone and prednisolone in saliva with (on line solid phase extraction) LC-MS/MS and describing de correlations of these glucocorticoids between saliva and ultrafiltrate. Addition sums of both metabolites in saliva showed excellent correlation with those in ultrafiltrate. These findings may have important implications for the use of saliva in drug monitoring of prednisone and prednisolone. Further research with larger series is needed to confirm these observations. The techniques described here provide a practical method to further explore the possibility for drug monitoring of prednisone and prednisolone in saliva.

Acknowledgements
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CHAPTER 5

Monitoring prednisolone and prednisone in saliva: a population pharmacokinetic approach in healthy volunteers

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ABSTRACT

Background

Prednisolone is a widely used corticosteroid in a variety of immune mediated diseases. Treatment regimes generally consist of empirically derived treatment doses, while therapeutic response among patients is highly variable. Drug monitoring of serum prednisolone levels might support a more rational approach to dose selection, yet is invasive and laborious. In analogy to cortisol, salivary prednisolone may offer a good alternative for serum prednisolone, being a representative approximation of biologically active free serum prednisolone. The aims of this study were to evaluate the correlation between free serum and salivary prednisolone levels and to quantify this relationship within a population pharmacokinetic model.

Methods

Prednisolone and prednisone concentrations were measured in 396 samples from 19 healthy volunteers after oral ingestion of 80 mg prednisolone. Measurements in serum, ultrafiltrate and saliva were performed with a recently validated LC-MS/MS method. Population pharmacokinetic analysis was performed with nonlinear mixed effect modeling using NONMEM.

Results

Salivary prednisolone levels correlated well with free serum prednisolone levels ($r=0.931$, $p<0.01$). A weaker correlation was found for prednisone ($r=0.318$ $p<0.01$), which may be explained by the finding that salivary prednisone levels mainly appeared to originate from prednisolone enzymatically converted to prednisone. Total and free serum prednisolone concentrations decreased over time after drug administration and showed a non-linear mutual relationship, consistent with concentration-dependent protein binding. Modeled prednisolone pharmacokinetics corresponded with previous reports. Low to moderate inter-individual variability was found for V/F and CL/F (coefficients of variation were 13.8% and 14.6% respectively). Free and salivary prednisolone showed a non-linear relationship with total prednisolone. An equation predicting free serum levels from salivary levels was successfully derived from the data.

Conclusion

This study is the first to describe the relationship between salivary and (free) serum prednisolone using a population pharmacokinetic model. Salivary prednisolone was found to be a reliable predictor of free and total serum prednisolone in healthy volunteers. Our results encourage further exploration of the use of saliva as a non-invasive and feasible method for drug monitoring of prednisolone.

INTRODUCTION

Prednisolone (PLN) and its prodrug prednisone (PN) are widely used corticosteroids in the treatment of inflammatory and autoimmune diseases in adults and children. Patients show considerable variability in therapeutic response and side-effects to standard, empirically based, treatment regimens.[1,2] This is explained in part by inter-individual variability in pharmacokinetics of predniso(lo)ne.[3,4] It is increasingly common to monitor drug concentrations and adjust dosages based on these measurements for other immunosuppressant agents such as ciclosporin, mycophenolate mofetil and tacrolimus. For predniso(lo)ne, drug monitoring is hardly used in clinical practice. Monitoring drug concentrations and individualizing predniso(lo)ne treatment in order to minimize glucocorticoid side effects is a relevant yet overlooked subject as it requires rather cumbersome methods.

Understanding the pharmacokinetics of P(L)N poses several challenges. After administration, a dynamic equilibrium exists between the active drug, PLN and the inactive drug, PN, which is strongly in favor of the former.[5,6,7] In addition, concentration-dependent binding of PLN and PN to transport proteins, mainly corticosteroid binding globulin (CBG) and albumin, results in a non-linear relationship between total and free PLN and PN.[1,8]

PLN shares great similarity with endogenous cortisol, which shows a comparable protein binding profile. In analogy to cortisol, the unbound or free fraction of PLN is biologically active; only unbound molecules can readily diffuse into target tissues and cells and are therefore considered clinically most relevant.[1,2,8]

Measurement of unbound PLN in blood is time consuming as it requires separation of the unbound and bound fractions. One way to overcome this problem might be the measurement of PLN levels in saliva, as concentrations in saliva may represent free PLN levels in blood. For cortisol, the relationship between salivary levels and free serum levels is well established.[9,10,11] Saliva sampling is a patient friendly method, which is now increasingly applied in clinical practice and may offer new possibilities for monitoring other corticosteroids. Unfortunately, the sparse evidence concerning the correlation between predniso(lo)ne in blood and saliva is based on obsolete methods and conflicting results.[12,13,14,15]

Recently, we have reported a method for simultaneous measurement of PLN and PN in saliva, serum and ultrafiltrate that demonstrated promising results with respect

to the relationship between free serum prednisolone concentrations and salivary prednisolone concentrations. This validation report included a preliminary application in one healthy subject.[16] In the present study, the relationship between salivary and (free) serum predniso(lo)ne levels is further explored in a population of healthy volunteers after administration of a single oral dose of PLN. Pharmacokinetic parameters of predniso(lo)ne and their inter-individual variability are described from a population pharmacokinetic model. The effects of several covariates on the pharmacokinetic parameters is explored.

MATERIALS AND METHODS

Participants

Healthy volunteers aged 18-65 years were asked to participate. Participants were excluded if they used contraceptives containing estrogens, if they were pregnant or breast feeding, had a history of kidney, liver or gum disease, a history of hyperthyroidism, inflammatory bowel disease, hypercortisolism or diabetes mellitus or the use of medication known to interact with the pharmacokinetics on predniso(lo)ne: rifampicin, phenytoin, barbiturates, ketoconazole, local or systemic corticosteroids. This study was approved by the medical ethics committee of the Erasmus Medical Centre in Rotterdam. Participants gave written informed consent.

Drug administration and Sampling

Following an overnight fast, an intravenous canula was placed and baseline sampling of blood and saliva was performed in each participant. Prednisolone (20 mg tablets, Pharmachemie B.V. Haarlem, the Netherlands) was then administered orally in a single dose of 80 mg. All intact tablets were swallowed at once with water. Blood samples were collected in plastic tubes containing a polymer gel for serum separation before and at t=60, 120, 180, 240, 300 and 360 minutes after ingestion. Unstimulated whole saliva was collected with Salivette (Sarstedt, Nümbrecht, Germany) polyester swabs simultaneously.[17,18] Participants were instructed not to brush their teeth within 30 minutes before baseline sampling and to refrain from eating or drinking at least 30 minutes before each subsequent sampling, to avoid contamination of saliva samples with blood from the oral mucosa. Serum and saliva were stored at -20 °C until further analysis.

Tandem-mass spectrometry analysis (LC-MS/MS)

This method was described in detail in our previous report.[16] In brief, we obtained ultrafiltrate for the separation of unbound analytes using Centrifree YM-30 centrifugal filtration units (30,000 MW cut-off; Millipore Ireland BV, Country Cork, Ireland). Predniso(lo)ne and cortisol concentrations in serum (total), ultrafiltrate (unbound) and saliva were determined with an on-line solid phase extraction liquid chromatography system (Symbiosis Pharma, Spark Holland, Emmen, The Netherlands) combined with a Waters Quattro Premier XE mass spectrometer (Waters, Millford MA, USA). Complete separation of cortisol and PLN was achieved by use of a Zorbax SB-Phenyl analytical column. This method was linear for cortisol, PLN and PN over a range of 6-1400 nmol/L in serum. In ultrafiltrate and saliva, linearity was achieved over a range of 2-450 nmol/L. Within-run precision and total precision were <10% and <15% respectively, for all analytes in all media. For all analytes in serum, the minimum reported concentrations were 9 nmol/L. For ultrafiltrate and saliva, this was 2 nmol/L for cortisol and 6 nmol/L for PN and PLN. The following conversion of units was used: prednisolone: [nmol/L] × 0.360 → [ng/ml], prednisone: [nmol/L] × 0.358 → [ng/ml], cortisol: [nmol/L] × 0.363 → [ng/ml].

Measurement of transport proteins

Corticosteroid binding globulin in serum was determined with a CBG-RIA-CT kit (Diasource Immuno Assays S.A., Nivelles, Belgium). Serum albumin was measured with a standard spectrophotometric assay.

DATA ANALYSIS AND PK MODEL EVALUATION

Correlations between observed concentrations of salivary and (free) serum predniso(lo)ne

Correlations between observed concentrations in different media were explored. Data displaying a non-normal distribution were log transformed and subsequently analyzed with a two-tailed Pearson's X^2-test, using SPSS 17.0.

Population Pharmacokinetic Model Analysis

Population pharmacokinetic analysis was performed by nonlinear mixed effect modeling using NONMEM version 7.2.0 (Icon Development Solutions, Ellicott City, MD, USA). First order conditional estimation with interaction (FOCEI) was used throughout, with an additive and/or a proportional residual error model. Log-normal distributions were used to approximate the inter-individual variability of model parameters. The statistical software environment R version 2.12.0 (R Foundation for Statistical Computing, Vienna,

Austria, 2010) was used for input file preparation and processing (tables and graphs) of the model results, and for simulations. Simulations were performed by implementing the identified models and the estimated parameters in R using the function lsoda from the deSolve library (version 1.8.1) and the function mvrnorm from the MASS library (version 7.3-8).

Structural Model

One- and two-compartment models with first-order absorption were used to fit the pharmacokinetic profile of total prednisolone and its metabolite prednisone in serum. Model development was guided by comparing an objective function value (OFV) based on -2× log likelihood (-2LL) of increasingly more complex models and standard goodness of fit plots. The absolute fraction metabolized and the relative bioavailability (F) of both metabolites could not be obtained.

Relationship between modeled salivary and free serum prednisolone

Based on the PK model of total prednisolone concentrations in serum, linear and/or exponential relationships were explored to predict free serum prednisolone and salivary prednisolone. Subsequently, the relationship between salivary and free prednisolone was mathematically derived from the two former relationships.

Covariate Model

The influence of relevant covariates on the model parameters was explored by forward inclusion of covariates into the base model (one extra parameter per covariate) and comparing two sequential models using a likelihood ratio test. The difference in -2LL was compared with a chi squared distribution, and a difference of 3.84 or more was interpreted as being significant (assuming that this difference is chi squared distributed, with the degrees of freedom set to the difference in number of estimated parameters, and $p<0.05$). Tested covariates included gender, age, weight, height, body surface area, lean body mass, serum albumin and serum CBG.

Model Qualification

The identified models were qualified by evaluating standard goodness of fit plots and parameter uncertainty. A visual prediction check[19] (VPC) based on 1000 simulated subjects was performed to evaluate the median predictions and the 95% prediction interval and to compare these with the observed concentrations.

RESULTS

We obtained 396 samples (132 serum, 133 ultrafiltrate and 131 saliva samples) from 19 healthy volunteers. Baseline characteristics of the participants are presented in Table 1.

Table 1. Participant characteristics.

Gender, n M/F	8/11
Age, years; median (IQR)	28 (23-50)
Weight, kg	74.0 ± 12.1
Height, cm	176 ± 8.5
Body surface area, m^2	1.90 ± 0.18
Lean body mass, kg	54.9 ± 8.1
Serum albumin, g/L (reference range)	46 ± 3.0 (35-55)
Serum CBG, g/L (reference range)	48 ± 6.8 (30-54)
Serum cortisol, nmol/L (reference range)	392 ± 102 (200-800)

Data are presented as mean ± SD values unless otherwise specified. Body surface area and lean body mass were calculated according to the methods of James[33] and Mosteller[34] respectively. IQR: interquartile range; CBG: corticosteroid binding globulin.

Observed Concentrations of predniso(lo)ne in serum, ultrafiltrate and saliva

The mean concentration-time profiles for serum total, free and salivary PLN and PN are illustrated in Figure 1. Salivary concentrations of prednisolone correlated well with free serum prednisolone concentrations ($r= 0.931$, $p<0.001$). Total serum concentrations of prednisolone reached a mean maximum concentration (C_{max}) of 992 ± 246 ng/ml after 1.5 ± 0.6 hours. The mean C_{max} of prednisolone in ultrafiltrate at 1.5 (± 0.6) hours was 401 ± 96 ng/ml, corresponding to a mean free fraction of 41 ± 8% at maximum concentration levels. This fraction gradually decreased over time to 26 ± 4% at six hours after administration. The mean C_{max} of PLN in saliva was 381 ± 114 ng/ml.

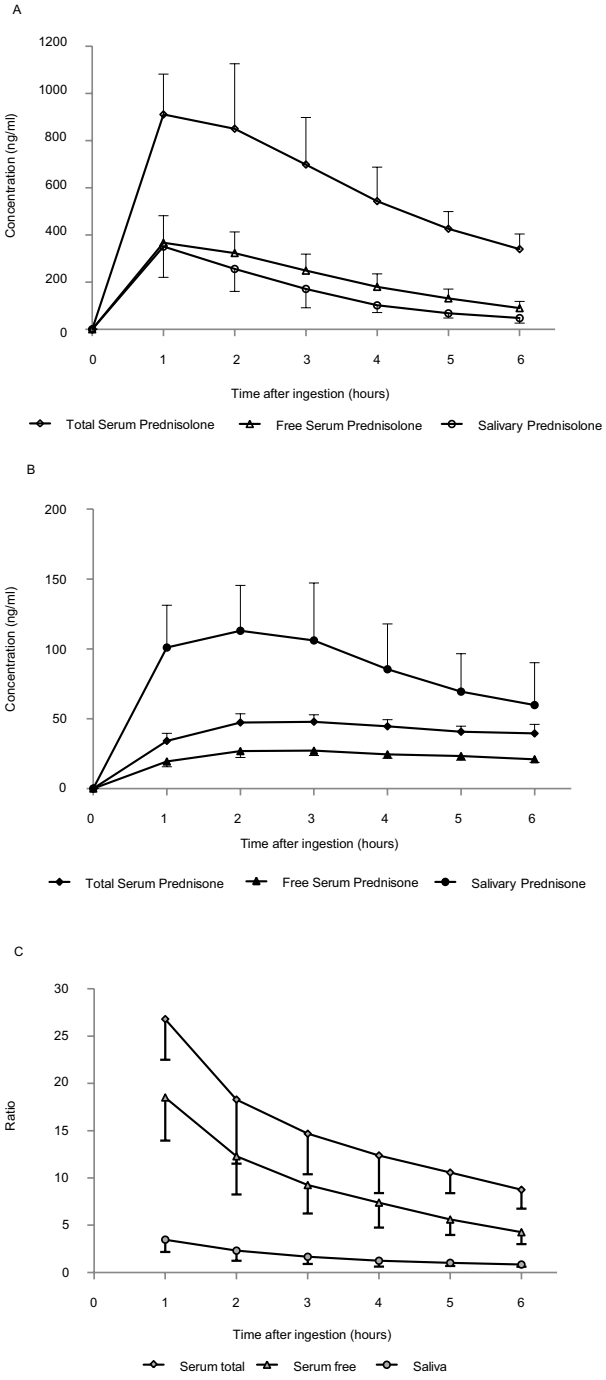

Figure 1. Time-concentration curves of total serum, free serum and salivary prednisolone (a) and prednisone (b) and prednisolone to prednisone ratios (c) in different media after a single oral dose of 80 mg prednisolone.

Prednisone displayed a weaker correlation between salivary and free serum levels (r= 0.318, p<0.01). The mean C_{max} of total and free serum prednisone were 50 ± 6 ng/ml and 28 ± 4 ng/ml respectively, consistent with a mean free fraction of 57 ± 7%. This fraction remained stable during six hours after administration. The mean C_{max} of PN in saliva was 122 ± 40 ng/ml.

Salivary levels of prednisone considerably exceeded free prednisone levels in serum, indicating that salivary PN mostly consisted of PN directly converted from the PLN influx. For this reason, we explored the correlation between the addition sum of PLN and PN in saliva and free prednisolone. Although a good correlation was found (r=0.904, p<0.01), it was not superior to the above described association between salivary and free serum PLN concentrations.

The equilibrium of the two metabolites in serum and ultrafiltrate was in favour of the active metabolite prednisolone at all times; however, the proportion of prednisolone gradually decreased from 95 ± 1% at maximum concentration levels to 89 ± 2% at six hours after ingestion in serum and from 93 ± 2% to 80 ± 4% in ultrafiltrate. In saliva, the equilibrium reversed in favour of the inactive metabolite prednisone, as the percentage of prednisolone decreased from 75 ± 5% to 45 ± 8%.

As expected, total and free cortisol levels in serum decreased rapidly after prednisolone administration (data not shown).

Population Pharmacokinetic Model

A one-compartment structural model with first-order absorption and first-order elimination was used to describe the total serum prednisolone pharmacokinetics from concentration-time profiles, while a two-compartment model was selected for the inactive metabolite prednisone (Figure 2). The VPC showed that most of the data fell within the 95% prediction interval and were symmetrically distributed around the median (Figure 3).

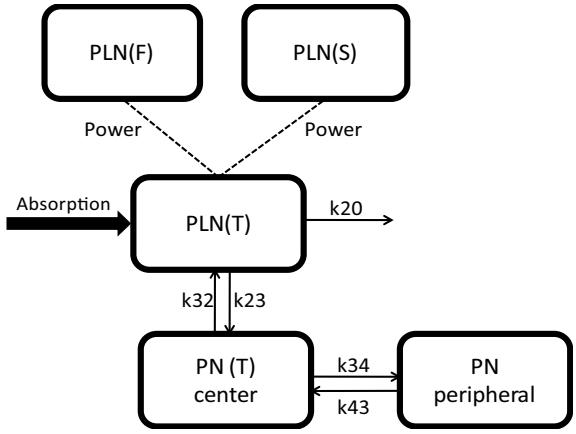

Figure 2. Model structure. PLN (F): free serum prednisolone; PLN (S): salivary prednisolone; PLN (T): total serum prednisolone; PN(T): total serum prednisone; PN: prednisone; k's represent rate constants between compartments.

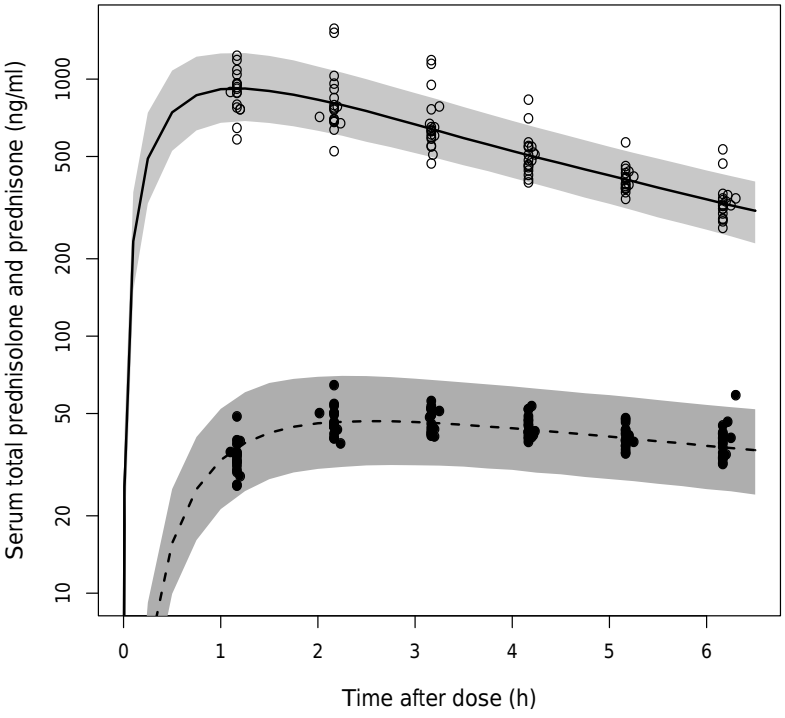

Figure 3. Visual predictive check of total serum prednisolone and prednisone concentration-time profiles in healthy volunteers following a single oral dose of 80 mg prednisolone. Open circles (prednisolone) and solid circles (prednisone) represent observations, lines (solid line: prednisolone, dashed line: prednisone) and grey area represent predicted median and 95% prediction interval respectively.

Covariate Model

The relationship of the model parameters and several covariates was explored by adding them to the models as scaling terms, and applying a likelihood ratio test to compare the model with and without the covariate influence. The ΔOFV of weight, height, body surface area (BSA) and lean body mass (LBM) were significant at $p<0.05$. Since all four covariates were strongly correlated and LBM was considered to be most clinically relevant,[20] this scaling parameter was kept in the final model. Gender, age, albumin and CBG did not improve the structural model and were therefore not maintained. After incorporating LBM in the model, the estimate of apparent volume of distribution changed according to Equation 1:

$$V/F = 61.6 \times (1 + 0.0144 \times (LBM - 55)) \quad \text{(Equation 1)}$$

In this formula V/F represents population predicted apparent distribution volume adjusted for individual lean body mass and 55 kg is the reference LBM.

Pharmacokinetic parameters of the final model and their inter-individual variability (IIV), both estimated with NONMEM, are shown in Table 2. Since the subjects only received an oral dose, the absolute fraction metabolized and the relative bioavailability (F) could not be determined. Therefore, clearance (CL) and distribution volume (V) of PLN correspond with the ratios CL/F (apparent clearance) and V/F (apparent volume of distribution) respectively. Inter individual variability of the absorption rate constant, V/F and CL/F was described by log-normal distributions. The estimates of $AUC_{0-\infty}$ and T1/2 were derived from V/F and CL/F.

Table 2. Estimates of the final model parameters for total serum prednisolone and prednisone, their relative standard error and estimated inter-individual variability, after including the effect of lean body mass.

Parameter	Units	Estimate	RSE (%)	IIV(%)
ka	1/h	2.01	22.6	--
k20	1/h	0.21	9.56	17.3
V/F	L	61.6	6.36	13.8
CL/F	L/h	12.9	6.35	14.6
θ_{LBM}	1/kg	0.0144	31.9	--
$AUC_{0-\infty}$	ng·h/ml	6180	6.35	14.6
T 1/2	h	3.30	9.56	17.3
k23	1/h	0.102	20.8	18.3
k32	1/h	0.936	35.7	--
k34	1/h	1.28	36.8	--
k43	1/h	0.385	44.1	--

θ_{LBM} is the covariate coefficient of lean body mass on V/F (the structure of the model is shown in equation 1). The values of $AUC_{0-\infty}$ and T 1/2 were derived from the estimates of V/F and CL/F. ka: absorption rate constant; k20: elimination rate constant; V/F: apparent distribution rate; CL/F: apparent clearance; k23: rate constant from prednisolone to prednisone central compartment; k32: rate constant from prednisone central compartment to prednisolone; k34: rate constant from prednisone central compartment to peripheral compartment; k43: rate constant from prednisone peripheral compartment to central compartment. RSE: Relative standard error = 100 × standard error/ estimate; IIV= inter-individual variability expressed as the coefficient of variation for a log-normal distribution: = $IIV = 100 \cdot \sqrt{\exp(\omega^2) - 1}$, where w^2 is the estimated variance.

Relationship between modeled salivary and free serum prednisolone

The relationship between salivary prednisolone and free serum prednisolone was constructed after exploring functions that predicted salivary and free serum prednisolone from total serum prednisolone (Equations 2, 3 and 4). Exponential functions yielded better prediction of free serum and salivary prednisolone from total serum prednisolone than linear relations, since the latter showed considerable bias in the low concentration range. Data were fitted according to the following equations:

$$PLN(F) = Slope_1 \times PLN(T)^{\gamma_1} \quad \text{(Equation 2)}$$

$$PLN(S) = Slope_2 \times PLN(T)^{\gamma_2} \quad \text{(Equation 3)}$$

$$PLN(F) = Slope_1 \times (PLN(S) / Slope_2)^{\gamma_1/\gamma_2} \quad \text{(Equation 4)}$$

In these equations PLN (F) and PLN (S) represent free serum and salivary PLN and PLN (T) represents total serum PLN. Values of the slopes were 0.0255 (RSE 29.9%, IIV 9.43%) and 0.000427 (RSE 48.3%, IIV 21.0%) respectively. The two powers γ_1 and γ_2 were 1.40 (RSE 3.44%) and 1.98 (RSE 3.86 %). A visual predictive check was performed to verify the model performance (Figure 4a-b).
The median $AUC_{0-\infty}$ derived from the model for free serum PLN and salivary PLN were 1469 (interquartile range 1366-1790) ng.h/ml and 1049 (908-1327) ng.h/ml respectively. The mean ratio of the AUC of free serum PLN to the AUC of salivary PLN was 1.5 ± 0.23.

We explored whether the addition sum of PLN and PN would yield a more accurate prediction of observed free serum PLN. As this step resulted in a higher residual error in the higher concentration ranges, it was not preferred over the established equation (Figure 4c-d).
Exploratory analysis of the covariates was also performed on the models concerning the relationships between free serum prednisolone and total prednisolone and salivary and total prednisolone (Table 3). Among the tested covariates, transport proteins (albumin and CBG) appeared to influence the inter-subject variability of slope 1, while weight, BSA, LBM, and age were associated with the inter-subject variability of slope 2. The findings of this exploratory analysis suggest these covariates might play a role in the relationship between serum and salivary prednisolone; these results should however be interpreted with caution, as confirmation is needed in larger study populations.

Table 3. Change in objective function value (OFV) of covariates on the structural pharmacokinetic model of total serum prednisolone and on the models predicting free serum prednisolone (slope 1) and salivary prednisolone (slope 2) from total serum prednisolone.

Covariates	ΔOFV on V/F	ΔOFV on k20	ΔOFV on Slope 1	ΔOFV on Slope 2
Gender	2.534	-0.376	-0.178	3.476
Age	-0.439	-0.229	0.031	4.313
Weight	5.251	-0.142	0.0100	4.600
Height	13.188	2.203	0.0100	3.201
BSA	8.877	0.376	0.00500	4.945
LBM	11.133	0.086	0.025	5.078
Albumin	1.006	-0.253	8.541	0.585
CBG	-0.284	0.334	7.177	0.736

LBM: lean body mass; BSA: body surface area; CBG: corticosteroid binding globulin

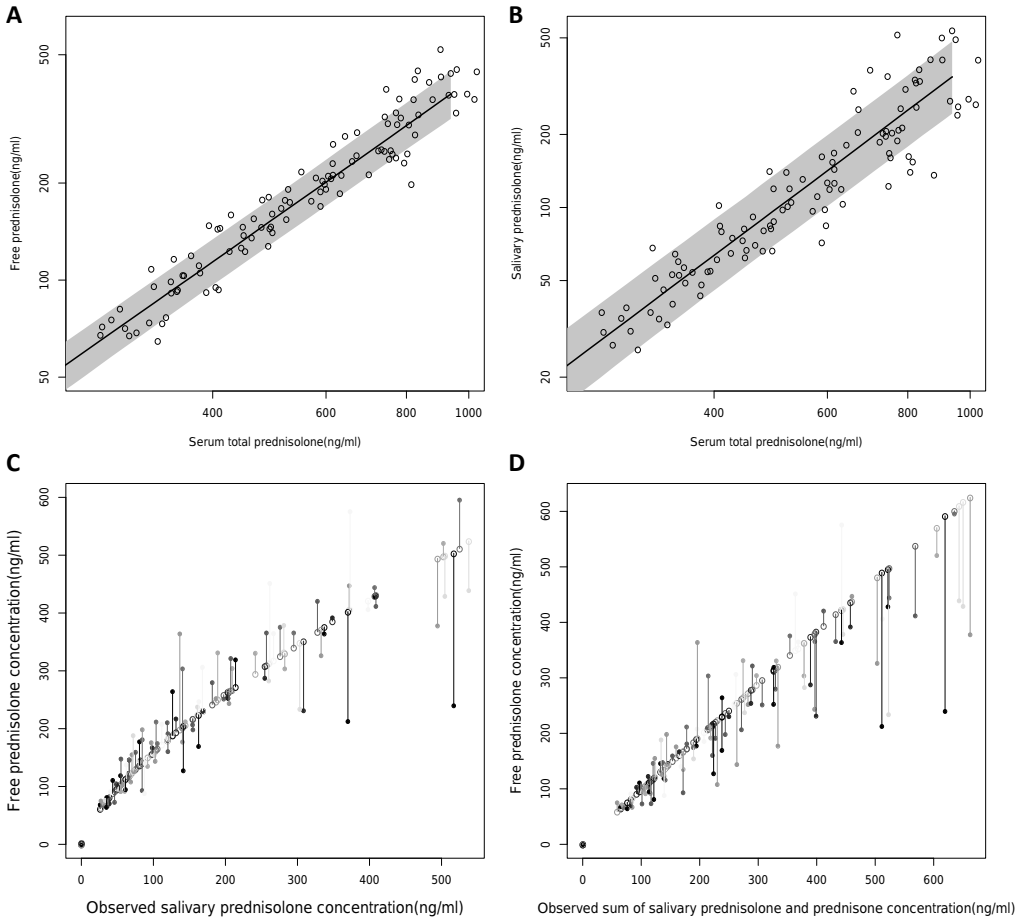

Figure 4.

a-b: Visual predictive check of total serum prednisolone versus free serum prednisolone (a) and salivary prednisolone (b). Circles represent observations, solid line and grey area represent predicted median and 95% prediction interval respectively.

c. Salivary versus free serum prednisolone as derived from the modeled relationships in a and b. Solid circles represent observed values, open circles represent predicted values.

d. Addition sum of salivary prednisolone and prednisone versus free serum prednisolone. Solid circles represent observed values, open circles represent predicted values.

DISCUSSION

In the present study, an excellent quantitative relationship between salivary and free serum levels and between salivary and total serum levels of prednisolone was found. In spite of the previously well established relationship between salivary and (free) serum cortisol levels and the analogies between prednisolone and cortisol in terms of molecular structure and protein binding profile, a good relationship between salivary and (free) serum prednisolone levels was yet to be confirmed. The novel findings described in this study offer interesting possibilities for the development of non-invasive drug monitoring strategies for patients receiving predniso(lo)ne treatment.

The relationship between prednisolone levels in plasma and saliva was addressed in a few small studies in the early 1980's. Most studies reported variable ratios of salivary and total plasma prednisolone over time. These data were considered to be a major drawback for the use of saliva for drug monitoring of prednisolone.[15,14,13] One study reported good agreement between (calculated) free plasma and salivary levels of prednisolone, yet did not advise the use of saliva for drug monitoring since the then available assays lacked sensitivity in the low salivary concentration ranges.[12] Free serum prednisolone concentrations, which represent the biologically active proportion of the administered drug, were either not included in these studies, or calculated instead of measured directly. Remarkably, concentrations of prednisone were not reported in any of these studies.[15,12,14,13] All of the former issues are dealt with in our study, since we used a robust and sensitive method for direct measurement of prednisolone (PLN) and prednisone (PN) in saliva and both free and total concentrations of these steroids in serum.

We found a free fraction of 40% for PLN at maximum concentration levels, which decreased with lower concentrations. As a result, non-linearity was present in both the relationship between total and free prednisolone and between total and salivary prednisolone, which is consistent with previous studies reporting concentration-dependent plasma protein binding of PLN.[1,8,21-24] While the free fraction of PLN clearly decreased over time, the free fraction of PN remained relatively stable in our study. This may be explained by differences in affinity for CBG between the two steroids. Both PLN and PN can bind to CBG, which displays low capacity yet high affinity, or to albumin, harboring high capacity yet low affinity.[1] Since the affinity for CBG is around ten-fold higher for the active steroid, PLN causes displacement of PN from CBG binding sites. Following saturation of CBG, protein binding of PLN will shift to albumin with low affinity, causing the free fraction of PLN to increase instantly. Between the two

steroids no competition exists for albumin, which results in PN being bound primarily to non-saturated albumin in a stable fashion, regardless of PLN concentration.[24,23] Similar observations have been described for cortisol and cortisone.[25] Thus, free PN fractions remain unaffected by changes in concentration, while protein binding and the free fraction of PLN are highly concentration-dependent.

The differences in the protein binding profile as well as differences in concentration ranges between the two steroids most likely account for the fact that prednisolone disappeared more rapidly than prednisone. This caused the serum PLN/PN ratio to gradually decrease over time. The PLN/PN ratio was substantially lower in saliva than in ultrafiltrate and serum, for which a rationale is found in analogous findings reported for cortisol and cortisone. Conversion from the active into the inactive steroids is performed by the enzyme 11-β-dehydrogenase type two (11-βHSD-2). While type one 11-βHSD promotes the reverse step in virtually all tissues,[1] type two is particularly expressed in certain tissues, including the salivary glands.[1] Physiological salivary cortisol to cortisone ratios are highest when the capacity of 11-βHSD-2 is challenged; this typically occurs in the morning, when plasma levels reach their peak and the salivary influx of cortisol is high. At lower levels, 11-βHSD-2 is able to convert a higher proportion of the active into the inactive metabolite, resulting in lower cortisol to cortisone ratios.[26] The presence and capacity of 11-βHSD-2 in salivary glands may very well account for the relatively low PLN/PN ratios in saliva compared to those in serum and ultrafiltrate.

Even though free steroids are known to rapidly enter saliva from the blood as no active transport mechanisms are involved, free levels are not necessarily identical to salivary levels.[27] Indeed, we found lower levels of prednisolone in saliva than in ultrafiltrate. The fact that we found relatively high levels of PN in saliva offers a rationale for this finding. Since PN levels in saliva considerably exceeded those in ultrafiltrate, our results indicate that salivary PN concentrations mainly consisted of PN enzymatically converted from PLN. Accordingly, the relationships between total and free prednisolone levels and the relationship between total and salivary prednisolone levels were not equivalent in our study. This finding also explains why the correlation between PN in saliva and ultrafiltrate was weaker compared to PLN. Despite the fact that PLN was partially converted to PN in saliva, a sound relationship between salivary and free serum PLN was found, suggesting that salivary prednisolone alone will suffice as an indicator of free - biologically active - serum prednisolone.

Our study is the first to consider serum total, free and salivary levels of prednisolone using a population pharmacokinetic approach. Model based estimates of pharmacokinetic parameters of total serum prednisolone were consistent with previous reports.[1,6,28] The concentration upswing of prednisolone was rapid, followed by a slower washout phase. Parameters reflecting body size influenced volume of distribution and clearance, as reported previously.[22,29] Equilibrium of prednisolone between different media was reached rapidly as there was no observable delay, consistent with fast binding of prednisolone to transport proteins.[24,27,23]

Prednisolone pharmacokinetics displayed low to moderate inter-individual variability in this rather homogeneous group of healthy volunteers, which supports previous findings in similar study populations.[12,28,30-32] Similar or larger values of IIV were reported in more distinct populations that included adults and/or children with immune mediated diseases.[3,7,22] It should be noted that different approaches were used to calculate and express IIV in these studies.
Limitations of this study were the small sample size and the use of a single fixed dose. The predictive performance of the model should be evaluated in studies with larger sample sizes and various doses. Naturally, prednisolone pharmacokinetics and their IIV could not be evaluated in relation to clinical response, as the study population consisted of healthy volunteers. The clinical applicability of this model in terms of exposure-effect relationships should be assessed in therapeutic settings and under circumstances that may affect the PK of prednisolone, such as hypoalbuminemia and impaired renal function.[1,5]

Comparison of observed and simulated concentrations resulted in accurate predictions of free serum and salivary prednisolone from total serum prednisolone. Serum albumin, age and body size (LBM) appeared to improve the accuracy of these predictions. This study is the first to assess effects of covariates on the relationship between salivary and (free) serum prednisolone concentrations. The clinical significance of these covariates should be confirmed in future studies.

The model described here provides an equation from which free serum prednisolone can be predicted from salivary prednisolone. We believe these results encourage further development of limited sampling strategies for modeling the AUC of biologically active prednisolone in serum from salivary concentrations. This introduces a whole new approach towards evaluation of prednisolone PK in relation to therapeutic outcome in clinical studies, as the model does not require invasive or time consuming methods. Saliva samples are easily obtained in a non-invasive manner and need little preparation

prior to the quantification process. Both the promising results of this study and the feasibility of saliva sampling can aid in the translation of this method from healthy adults to specific patient populations. This may especially apply to children, for whom blood repeated sampling is often problematic.

CONCLUSION

Predniso(lo)ne is a frequently and intensively used drug with a wide range of applications. Despite its extensive use, clinicians are challenged with the variability in therapeutic response and tolerance of predniso(lo)ne. Drug monitoring of biologically active, non-protein bound serum prednisolone levels is possible, but requires a rather invasive and restrictive clinical setting. The model described in the present study supports the use of salivary levels of prednisolone as a reliable predictor of free levels in serum, offering new possibilities for sampling outside of controlled clinical settings. It would be interesting to further explore these findings and the possibility of limited sampling in specific patient populations, particularly in those where invasive sampling is undesirable, such as children.

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PART IV

EXPLAINING VARIETY IN CLINICAL OUTCOME OF CHILDHOOD NEPHROTIC SYNDROME

CHAPTER 6

Low birth weight in relation to clinical outcome of childhood nephrotic syndrome: a meta-analysis

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ABSTRACT

Background

Low birth weight (LBW) has been shown to lead to a low nephron endowment with subsequent glomerular hyperfiltration. Additional renal disease can therefore be expected to have a more severe course. Minimal change nephrotic syndrome (MCNS) is a common chronic illness in childhood. As it is important to be able to predict prognosis in MCNS, we set out to study the effect of LBW on MCNS in a cohort of patients from our University Medical Center, and performed a meta-analysis.

Methods

A retrospective chart review of children with MCNS treated at the VU University Medical Center was performed, identifying 55 patients of which 4 had LBW. The meta-analysis was performed using Review Manager (The Cochrane Collaboration).

Results

The meta-analysis consisted of 201 patients (25 LBW, 176 normal birth weight). More LBW patients were classified as steroid resistant [odds ratio (OR) 6.97 (95% confidence interval [CI] 2.02-24.04), p=0.002]. The number of relapses per year of follow-up was significantly higher in the LBW patients with MCNS [weighted mean difference 0.93 (95% CI 0.71-1.15) relapse per year, p<0.0001]. MCNS patients with LBW were significantly more likely to be treated with ciclosporin [OR 4.4 (95% CI 1.7-11.8), p=0.003] or cytotoxic agents [OR 4.2 (95% CI 1.8-10.2), p=0.001] during the course of their disease, and they had a higher chance of developing several complications during the follow-up period, including hypertension.

Conclusions

This meta-analysis provides support for an adverse effect of LBW on the course and prognosis of MCNS in children, which can aid clinicians and parents in assessing the expected clinical course.

INTRODUCTION

Nephrotic syndrome (NS), defined as the combination of heavy proteinuria, hypoalbuminemia and oedema, is considered a common chronic illness in childhood. Several diseases are associated with NS, but minimal change NS (MCNS) is the most common idiopathic form. About 60-80% of children with MCNS will show relapses, and about 40% will have more than 5 relapses. Corticosteroids are used for treatment of the initial episode and subsequent relapses. Complications of NS and its therapy may be serious, especially if the clinical course shows frequent relapses with the need for prolonged periods of corticosteroid use. Recent investigations have focussed on determining potential risk factors that could predict such an unfavourable course of NS in children, but no suitable prediction is possible yet.

Intrauterine growth retardation (IUGR), based on low birth weight (LBW), has been shown to increase the risk of developing diseases later in life, like obesity, insulin resistance, cardiovascular disease and hypertension.[1] The kidneys appear to be extremely susceptible to IUGR and are often found small in proportion to body weight. Several studies in animals and humans have described a reduced number of nephrons after IUGR.[2,3,4,5,6] The reduced number of nephrons results in an inborn decreased glomerular filtration surface area, while renal blood flow per glomerulus is increased in an attempt to maintain a normal overall glomerular filtration rate. According to the hyperfiltration-hypothesis as put forward by Brenner and co-workers, this leads to glomerular hypertension and hypertrophy, which causes systemic hypertension and glomerular damage resulting in albuminuria and glomerulosclerosis.[7,8,9,10] Therefore, IUGR can eventually lead to impairment of renal function.[11]

Additional renal disease in hyperfiltrating kidneys can be expected to have a protracted course and poorer prognosis. LBW has been found to be of influence on IgA nephropathy,[12] membranous nephropathy,[13] diabetic nephropathy,[14] and MCNS in children.[15,16,17] It has been proposed that the hemodynamic changes occurring in IUGR kidneys, leading to glomerulosclerosis, have a negative effect on podocyte function.[10] Podocytes are affected in (childhood) NS and are thought to be in part responsible for the excessive protein loss.[18] Even though all reports on MCNS in children show a deleterious effect of IUGR, the described results differ among these 3 studies.[15,16,17] A possible explanation can be the low number of patients included, especially with LBW (only 5 to 8 per study).

In order to shed more light on the effect of LBW on the presentation and clinical course of NS in children, we present data on a cohort of children with MCNS from our center, and perform a meta-analysis on the previously published studies.[15,16,17]

SUBJECTS AND METHODS

To study the effect of LBW on NS in children, we performed a retrospective chart review of children with MCNS, referred to the Department of Pediatric Nephrology of the VU University Medical Center between May 1992 and January 2005. The diagnosis of MCNS was based on clinical findings in combination with the response to corticosteroid treatment, or was based on renal biopsy.

MCNS was defined as proteinuria > 40 mg/m²/hr, plasma albumin < 25 g/L, and the presence of edema. Renal biopsy was performed in children who did not respond adequately to initial corticosteroid treatment and/or who had frequent relapses. Remission was defined as the absence of proteinuria for at least 3 consecutive days. A relapse was scored when proteinuria reoccurred during 3 consecutive days. Frequent relapses were defined as two or more relapses within 6 months of initial response or four or more relapses within any 12-month period. A patient was considered to be corticosteroid dependent when relapses occurred while the corticosteroid dose was decreased, or within two weeks of corticosteroid cessation. Corticosteroid resistance was characterized by proteinuria continuing for over 8 weeks, in spite of full dose administration.

The initial episode was treated with oral prednisolone 60 mg per m² of body surface area daily for 6 weeks, followed by prednisolone 40 mg/m² on alternate days for another 6 weeks. Relapses were treated with prednisolone 40 - 60 mg/m² daily until remission, followed by a gradually tapered dose, administered on alternate days. In some of the steroid dependent children, the prednisolone dose was maintained at a low dose on alternate days in-between relapses. Additional therapy was necessary with some children to manage oedema and high blood pressure, and consisted of diuretics, beta-blockers, ACE inhibitors and calcium antagonists. If the clinical course of NS was considered to respond inadequately to initial therapy or steroid usage led to too many side-effects, additional treatment with cytotoxic agents (cyclophosphamide) or ciclosporin was started. Complications of immunosuppressive therapy, i.e. urinary tract infection, peritonitis, sepsis and pneumonia were scored.

Birth history, including birth weight and gestational age, were obtained from interviews with the parents, which has been shown to be reliable.[19,20] LBW was defined as birth weight below the tenth percentile for gestational age, gender, and maternal parity.[21] A recent consensus statement suggests that IUGR should be reserved for children with a birth weight and/or birth length at least 2 SD below the mean for gestational age.[22] However, in order to use the same definition as previous reports on the influence of IUGR on MCNS,[15,16,17] we decided to use this classification but use LBW instead of IUGR. Children were excluded from the study if birth weight and/or gestational age were unavailable. Only single births were included. Patients with a follow up period less than one year were also excluded.

The group of patients from our center consisted of 55 children, diagnosed with MCNS at the median age of 3.2 (interquartile range (IQR) 2.4 – 5.2) years. The study group consisted of 41 boys and 14 girls.

Statistical analysis

As the cohort of LBW patients with MCNS only consisted of 4 children, no statistical analysis was performed apart from the meta-analysis. Parameters with a normal distribution are expressed as means (standard deviation) and parameters that did not have a normal distribution are presented as median (IQR). SPSS was used as a statistical analysis system.

Statistical analysis for the meta-analysis was performed with Review Manager (RevMan) version 4.2 for Windows (Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2003), which enables the calculation of a pooled effect size of weighted mean differences for continuous data, and odds ratio (OR) for dichotomous data of the included studies. The weight (%) is based on study size and variation of the data (SD). Heterogeneity of the combined studies was assessed with this computer program and considered significant if $p < 0.1$. Other statistical differences were considered significant if $p < 0.05$ (two-tailed).

RESULTS

Patients from VU University Medical Center

Among the 55 children with MCNS included in this study, 4 had LBW, with a median birth weight of 2,825 (2,550 – 2,925) g. Fifty-one children had a normal birth weight [controls: median 3,200 (3,000 – 3,500) g, $p<0.001$ vs. LBW] and all children were

born at term. Children with LBW were of similar age at the onset of MCNS as children with normal birth weights [median 3.0 (2.9 – 3.6) years and 3.3 (2.4 – 5.2) years, respectively]. Data concerning the clinical course are shown in Table 1.

Table 1. Clinical course of 55 children with minimal change nephrotic syndrome treated at the VU University Medical Center with normal and low birth weights.

	Low birth weight (n = 4)	Normal birth weight (n = 51)
Follow up (yr)	6.4 (4.2 – 8.5)	4.1 (2.7 – 7.3)
Days to remission	7 (7 – 20)	9 (7 – 14)
≤ 9 days to remission	2/3 (67%)	24/45 (53%)
Occurrence of relapse(s)	3/4 (75%)	47/51 (92%)
Days to first relapse	129 (110 – 210)	163 (92 – 231)
Number of relapses in first 6 months*	1.5 (1.3 – 1.8)	1.0 (1.0 – 2.0)
Number of relapses during follow up*	13.0 (8.5 – 15.0)	5.0 (4.0 – 9.5)
Number of relapses per year*	1.4 (1.1 – 2.1)	1.6 (0.9 – 2.3)
Corticosteroid dependent	3/4 (75%)	41/51 (80%)
Corticosteroid resistant	0	2/51 (4%)
Additional form of medication	3/4 (75%)	29/51 (57%)
Cyclophosphamide	1/4 (25%)	23/51 (45%)
Ciclosporin	3/4 (75%)	9/51 (18%)
Biopsy	3/4 (75%)	41/51 (80%)

Values expressed as median (inter-quartile range) or number (percentage). *only in patients that showed relapsing nephrotic syndrome.

Meta-analysis

A meta-analysis was performed, using the data from our center together with published data from Zidar et al.[15] (5 IUGR and 35 control patients), Sheu et al.[16] (8 IUGR and 42 control patients), and Na et al. [17] (8 IUGR and 48 control patients). As is shown in Table 2, no differences in the age at onset, or the duration of follow-up was present between the LBW and control groups. As there was significant heterogeneity between the studies (p=0.04), no meta-analysis of serum albumin at diagnosis could be performed. Serum cholesterol was significantly higher in the LBW group at diagnosis (Table 2).

Table 2. Meta-analysis of various continuous data, comparing low birth weight and control children with minimal change nephrotic syndrome.

	Low birth weight		Controls		Weighted mean difference		p for overall effect
	n	Mean	n	Mean	Mean difference	(95% CI)	
Age at onset (yr)	25	4.46	176	4.90	-0.63	(-1.67 – 0.41)	0.23
At diagnosis							
-Albumin (g/l)	18	14.0	133	15.2	*	*	*
-Cholesterol (mg/dl)	20	526.3	141	456.6	53.0	(8.7 – 97.3)	0.02
Period of follow-up (yr)	17	7.24	128	6.29	0.56	(-0.44 – 1.57)	0.27

* not applicable due to significant heterogeneity.

No significant differences were seen in the number of patients without any relapses (Table 3). More LBW patients were classified as steroid resistant, but no differences in steroid dependence could be determined due to significant heterogeneity (p=0.02). As can be seen in Table 4, both the absolute number of relapses as well as the number of relapses per year of follow-up were significantly higher in the LBW patients with MCNS. Even though the latter showed significant heterogeneity, this provides support for an adverse effect of LBW on the course of MCNS in children.

Table 3. Meta-analysis of various dichotomous data, comparing low birth weight and control children with minimal change nephrotic syndrome.

	Low birth weight	Controls			p for overall effect
	n/N	n/N	Odds ratio	(95% CI)	
No relapses	2/17	29/134	0.51	(0.13 – 2.01)	0.34
Steroid resistance	5/25	8/174	6.97	(2.02 – 24.04)	0.002
Steroid dependence	15/25	66/174	*	*	*
Renal biopsy taken	20/25	94/176	3.84	(1.40 – 10.49)	0.009
Complications					
-Hematuria	9/25	35/176	2.30	(0.95 – 5.56)	0.07
-Hypertension	11/25	40/176	3.30	(1.34 – 8.13)	0.009
-Pneumonia	7/25	20/176	3.48	(1.26 – 9.63)	0.02
-Sepsis	2/20	2/141	7.95	(1.41 – 44.72)	0.02

*not applicable due to significant heterogeneity.

Table 4a. Absolute number of relapses in low birth weight and control children with minimal change nephrotic syndrome.

		Low birth weight		Controls		Weight (%)	Weighted mean difference	
Study	Year	n	Mean ± SD	n	Mean ± SD		Mean difference	(95% CI)
Zidar [15]	1998	5	10.4 ± 6.2	35	3.3 ± 3.5	16.4	7.1	(1.5 – 12.7)
Sheu [16]	2001	8	13.0 ± 3.5	42	3.4 ± 3.0	75.4	9.6	(7.0 – 12.2)
Present study	2007	3	11.3 ± 6.7	47	7.3 ± 7.5	8.2	4.1	(-3.8 – 11.9)
Pooled data		16		124			8.7	(6.5 – 11.0)

Test for heterogeneity, p=0.34. Test for overall effect, p<0.0001.

Table 4b. Number of relapses per year in low birth weight and control children with minimal change nephrotic syndrome.

		Low birth weight		Controls		Weight (%)	Weighted mean difference	
Study	Year	n	Mean ± SD	n	Mean ± SD		Mean difference	(95% CI)
Sheu [16]	2001	8	1.6 ± 0.3	42	0.5 ± 0.4	85.1	1.1	(0.9 – 1.3)
Na [17]	2002	8	0.8 ± 0.9	48	0.8 ± 0.9	11.6	-0.0	(-0.7 – 0.6)
Present study	2007	3	1.7 ± 1.1	47	1.7 ± 1.0	3.3	-0.0	(-1.2 – 1.2)
Pooled data		19		137			0.93	(0.71 – 1.15)

Test for heterogeneity, p=0.002. Test for overall effect, p<0.0001.

Table 5 shows that MCNS patients with LBW were significantly more likely to be treated with ciclosporin (OR 4.42, p=0.003) or cytotoxic agents (OR 4.24, p=0.001) during the course of their disease. This may explain the fact that LBW patients underwent a renal biopsy significantly more often (OR 3.84, p=0.009; Table 3). Table 3 also shows the higher chance of developing several complications during the follow-up period for the LBW MCNS patients, including hypertension. No differences in the incidence of urinary tract infections or peritonitis was found (data not shown).

Table 5a. Ciclosporin use in low birth weight and control children with minimal change nephrotic syndrome.

Study	Year	Low birth weight n/N	Controls n/N	Weight (%)	Odds ratio	(95% CI)
Zidar [15]	1998	2/5	2/35	8.3	11.0	(1.1 – 108.5)
Sheu [16]	2001	2/8	0/42	3.5	32.7	(1.4 – 760)
Na [17]	2002	4/8	20/48	79.2	1.4	(0.3 – 6.3)
Present study	2007	3/4	9/51	9.1	14.0	(1.3 – 151)
Pooled data		11/25	31/176		4.4	(1.7 – 11.8)

Test for heterogeneity, p=0.15. Test for overall effect, p=0.003.

Table 5b. Use of cytotoxic agents use in low birth weight and control children with minimal change nephrotic syndrome.

Study	Year	Low birth weight n/N	Controls n/N	Weight (%)	Odds ratio	(95% CI)
Zidar [15]	1998	4/5	10/35	10.2	10.0	(1.0 – 100.8)
Sheu [16]	2001	6/8	11/42	18.0	8.5	(1.5 – 48.3)
Na [17]	2002	6/8	14/48	20.5	7.3	(1.3 – 40.6)
Present study	2007	1/4	23/51	51.3	0.41	(0.04 – 4.2)
Pooled data		17/25	58/176		4.2	(1.8 – 10.2)

Test for heterogeneity, p=0.14. Test for overall effect, p=0.001.

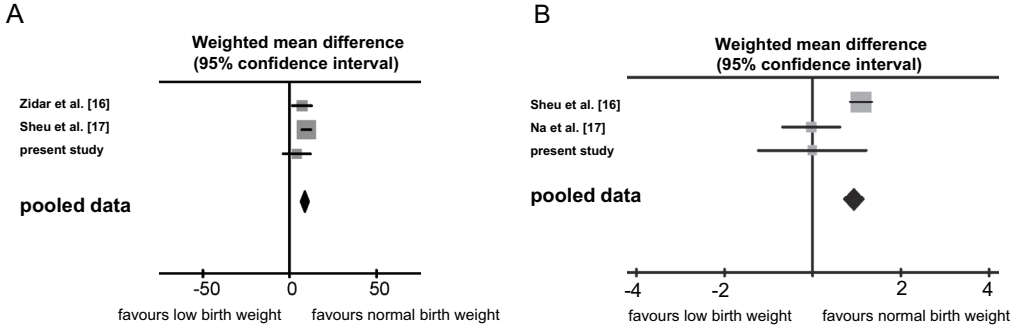

Figure 1. Weighted mean difference with 95% confidence interval of the separate studies and pooled data for the absolute number of relapses during follow-up (**a**) and the number of relapses per year during follow-up (**b**) between minimal change nephrotic syndrome patients with low birth weight and normal birth weight.

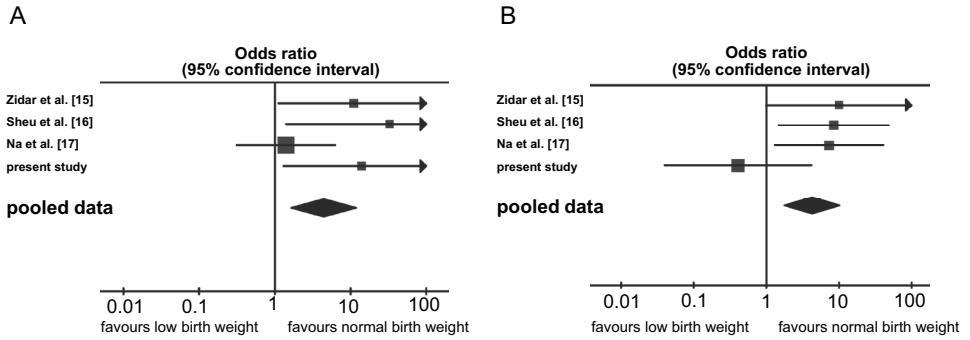

Figure 2. Odds ratios with 95% confidence interval of the separate studies and pooled data for the number of patients that used ciclosporin during follow-up (**a**) and the number of patients that used cytotoxic agents during follow-up (**b**), comparing minimal change nephrotic syndrome patients with low birth weight and normal birth weight.

DISCUSSION

We conclude that LBW has an adverse effect on the clinical course of NS in children. Our results are based on a meta-analysis of patients from our center and all previously published studies on the effect of birth weight on the course of MCNS.[15,16,17] A possible explanation may be that IUGR leads to an inborn deficit of nephrons.[2-6] The remaining nephrons are subject to hyperfiltration,[7,8,9,10] which has a negative effect on glomerular podocytes,[10] known key roleplayers in the pathophysiology of NS in children.[18]

NS in children can have serious implications for a child's health if the disease takes on a severe form, with frequent relapses and a necessity for high doses and prolonged courses of corticosteroids.[18] It is therefore important to identify factors that can be helpful to predict the clinical course of NS. However, only few clinical and laboratory parameters have been found to be of value: predictive factors of a favourable course are age at onset between 4 and 8 years, good responsiveness to steroids, and a low number of relapses within the first six months of onset.[18] Based on our meta-analysis, LBW is another non-immunologic factor that influences the course of MCNS. However, the pathways that cause MCNS to be adversely affected by LBW are not yet clear. A recent study in IUGR rats with an induced acute mesangioproliferative glomerulonephritis showed a higher expression of inflammatory and profibrotic markers, leading to more glomerulosclerosis and more extracellular matrix deposition.[23] This anti-Thy-1.1 glomerulonephritis normally shows spontanuous resolution within 2 weeks. Therefore, the authors suggest that the histological findings may be indicative of a transition from

an acute form into a chronic-progressive form of glomerulonephritis.[23] A possible explanation for the poorer course of MCNS in LBW children may also be that it has turned into a chronic disease with glomerulosclerosis, based on more structural damage. However, studies into the pathophysiology of MCNS after IUGR are necessary to shed more light on this issue.

Age under 4 years has been shown to be associated with more relapses.[18] In our meta-analysis, we observed no differences in the mean age at onset of MCNS between the groups. No data on the number of relapses within the first 6 months could be substracted from the previous reports,[15,16,17] so this factor was unavailable for meta-analysis. In our cohort, no statistical significant difference in the number of relapses within the first 6 months were noted. The time from onset to first relapse was shorter in LBW children, but this did not reach statistical significance either. However, this may indicate that they have a tendency to relapse earlier, which may explain the significantly higher number of relapses during the follow-up period that is indicative of an unfavourable course.

Considering an inadequate reaction to corticosteroids to indicate worse outcome of NS in children, we hypothesized that the LBW group would show a longer period of time to remission, and more corticosteroid dependency and resistancy. No differences were found between the two groups regarding steroid response. However, a significantly higher percentage of steroid resistance was found in the LBW group. Also, LBW children with MCNS were more often treated with additional medication, like ciclosporin and cytotoxic drugs. In addition, a higher incidence of renal biopsy in LBW children with MCNS was found, even though a previous study has shown that steroid sensitivity rather than the histological picture is the major determinant of the prognosis in childhood NS.[24] Differences in presentation and clinical course of NS thus result from other causes, as they cannot be explained by underlying histology.

Because MCNS is treated with high doses of corticosteroids, children with MCNS are at risk for complications that occur as a result of a suppressed immune system.[18] Children experiencing frequent relapses or having poor responses to corticosteroids will probably be exposed to prolonged courses of steroids or have higher cumulative doses. As LBW children fall in this category, we hypothesized that they could be at a higher risk for occurence of complications of treatment. Our meta-analysis supports this hypothesis in that pneumonia and sepsis were seen more frequently in LBW patients, even though no differences were noted in peritonitis or urinary tract infections.

IUGR is associated with hypertension in the general adult population.[1] A possible explanation for the association can be found in the low nephron endowment that is associated with IUGR[2-6] and has been described in patients with hypertension.[25] An increased blood pressure could therefore become more apparent in individuals with LBW and additional renal disease. Zidar et al. demonstrated an increased risk for arterial hypertension in IUGR children with IgA glomerulonephritis.[12] In our meta-analysis, hypertension did indeed occur more often in LBW children.

A limitation of our study population could be that it is a somewhat selected one, since the majority of children were referred to our (tertiary) Pediatric Renal Center as a result of frequent relapses or inadequate response to corticosteroids. In our study, 32 of 55 (58%) patients with MCNS needed some form of additional therapy, which is higher than expected in an average group of children with MCNS.[18] Also, our method of obtaining information about birth history differed from previous reports,[15,16,17] i.e. by interviewing the parents instead of acquiring this information from medical records. In The Netherlands, a large proportion of children are born at home and no national registry with birth data was available. However, previous studies have established that parental recall of birth weight and gestational age is sufficiently accurate for clinical and epidemiological use.[19,20]
In conclusion, we have shown that LBW has an adverse effect on the course and prognosis of MCNS in children, which can aid clinicians and parents in assessing the expected clinical course.

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CHAPTER 7

Population pharmacokinetics of prednisolone in relation to clinical outcome in children with nephrotic syndrome

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CHAPTER 8

Genetic and in vivo determinants of glucocorticoid sensitivity in relation to clinical outcome of childhood nephrotic syndrome

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PART V

REFLECTION

CHAPTER 9

General Discussion

9.1. Childhood nephrotic syndrome: 'mild' but unkind
9.2. The prolonged treatment hypothesis re-evaluated
    9.2.1. Extending initial prednisolone treatment without increasing cumulative dose
    9.2.2. More prednisolone, fewer relapses?
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    9.4.4. Sensitivity to glucocorticoids in relation to clinical outcome
9.5. Glucocorticoid treatment for childhood nephrotic syndrome: weighing the cornerstone
    9.5.1 Directions for future research

The studies in this thesis aim to increase our understanding of the clinical variability observed in childhood nephrotic syndrome and to offer new insights for improving current therapy. Particular attention is given to treatment duration, metabolism of prednisolone, and sensitivity to glucocorticoids. Here, the results of these studies are discussed and reviewed in light of the current literature. In addition, directions for future research are given.

9.1. Childhood nephrotic syndrome: 'mild' but unkind

As becomes clear from our prospective cohort study (Chapter 3), children with nephrotic syndrome (NS) are at high risk of relapsing disease. Though steroid sensitive childhood NS is generally considered relatively benign in terms of renal function,[1] the high burden and morbidity accompanying recurrent relapses should not be underestimated. Steroid resistant NS is clearly distinguished from steroid sensitive NS in terms of treatment and prognosis; throughout this text, NS therefore refers to steroid sensitive NS unless described otherwise.

Our prospective cohort, which was closely followed for a median of four years, clearly shows a clinical spectrum ranging from no relapses at all to frequent relapses with steroid dependence. Unfortunately, the lion's share of patients arrives at the 'bad end' of this spectrum: only 20% of children remain free of relapses following treatment for the first episode. Half of all patients experience frequent relapses, over half of these being steroid dependent (Figure 1). Though most adverse events are transient, Cushingoid appearance, high blood pressure, behavioural changes, and severe infections are seen in a substantial proportion of patients. This hard reality urges improvement of current treatment strategies based on reliable, good quality trials.

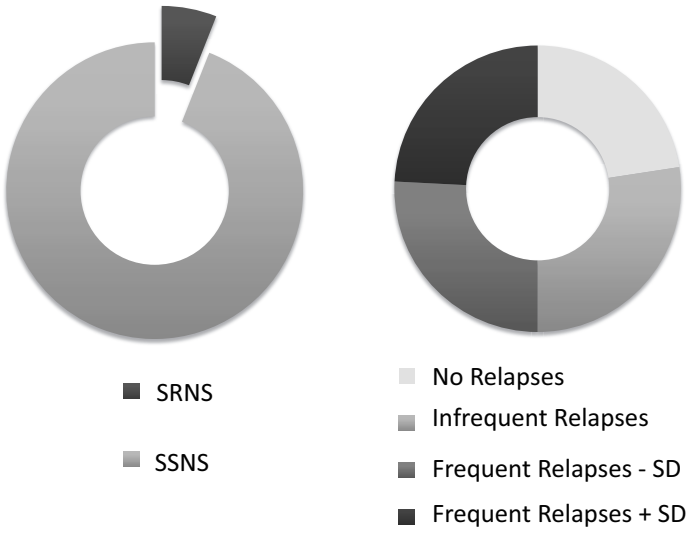

Figure 1. Therapeutic outcome in children with nephrotic syndrome receiving standard three- month prednisolone treatment (3360 mg/m^2). Left: distribution of steroid sensitive and steroid resistant patients. Right: clinical outcome in steroid sensitive patients. SRNS, steroid resistant nephrotic syndrome; SD steroid dependence.

Each time a relapse occurs, induction therapy (60 mg/m^2 prednisolone) is restarted until remission is achieved, generally followed by four weeks of 40 mg/m^2 on alternate days. One can imagine the large cumulative amount of prednisolone administered to patients with frequent relapses. Other immunosuppressive agents are considered when frequent relapses with or without steroid dependence occur, to limit the use of prednisolone. Cyclophosphamide, chlorambucil, mycophenolate mofetil, ciclosporin, tacrolimus, and levamisole can be used as first additional agents. Each of these has limited efficacy and additional side effects.[2] The choice for the additional agent largely depends on the experience of the patients' pediatric nephrologist. Recent guidelines state that based on the currently available evidence no conclusions can be drawn on which steroid sparing agent should be used first.[2]

Recurrent relapses and concomitant steroid exposure put children at risk of severe complications. A dose-dependent increase in the risk of infection is seen in patients with systemic glucocorticoid therapy.[3] During follow up, 13% of the children in our study population had severe infections, including pneumonia, VZV-reactivation, whooping cough and in one case, a cerebral abcess. Though bacterial peritonitis and/or cellulitis were rare in our cohort, these potentially dangerous infections have been reported in 2-6% of patients.[1] This justifies awareness of (less common) infectious diseases and early therapeutic intervention in children with NS.

Ophtalmological side effects were rare in our study. Cataract and glaucoma have previously most often been reported in Japanese patients;[4,5] in general, these complications are unusual.[6] Our findings indicate that there is no need for standard ophtalmological screening in children with NS at an early stage.

Glucocorticoids reduce proliferation of chondrocytes, causing impaired bone growth in children receiving highly dosed or prolonged glucocorticoid therapy. Catch-up growth is often observed after cessation of glucocorticoid therapy, while reduced adult height can be seen after intensive, sustained treatment.[7] Our prospective growth data illustrated how growth velocity significantly dropped in the first months -during highly dosed prednisolone treatment- subsequently returning to its baseline within one year (Chapter 3). Though this study was not designed to assess a causal relationship, this temporary effect corresponds with previous retrospective studies that describe a dose-dependent effect of corticosteroids on growth in children with NS.[8,9,10] Nevertheless, monitoring growth remains an important aspect in the follow up of children with NS, particularly in those who are steroid dependent. It would be interesting to assess (adult) height in our cohort in relation to cumulative dose and duration of steroid exposure in the long term.

Bone mineral density remained stable over the first six months in our study, indicating screening for osteoporosis is not needed early in the disease course. However, paired dual-energy X-ray absorptiometry (DEXA or DXA) scans were available in a limited number of children, due to the fact that these scans could not be performed in all centres at the time patients were enrolled. Today, an increasing number of hospitals have radiological facilities and reference data required to adequately perform DEXA scans in children. Prospective data covering longer periods are needed to evaluate whether (certain) children benefit from BMD measurements in the long term.

In our experience, behavioural changes are among the most common complaints reported by parents having a child with NS. Surprisingly, psychological side effects of glucocorticoids have not been studied extensively in children with nephrotic syndrome.[11] We explored short-term behavioural changes using a simple format consisting of five visual analogue scales. At three months, a significant increase in scores for increased appetite, overactive behaviour, and aggressive behaviour was observed, while scores for happiness temporarily dropped in the first six months, and those for sleeping remained relatively stable. Long term effects of glucocorticoid treatment on behaviour, psychosocial adjustment and quality of life in children with nephrotic syndrome may not solely depend on disease-related factors such as cumulative steroid dose,[12,13,14] but also on family-related factors. In a Swiss study, family climate, particularly maternal distress, had a negative impact on both behaviour/psychosocial adjustment and quality of life.[15]

Perceived stress, the occurrence of relapses, changes in behaviour and family coping strategies may interact with each other in the clinical course of children with NS. Perceived stress has been related to relapses in other relapsing-remitting diseases such as inflammatory bowel disease[16] and systemic lupus erythematosus (SLE).[17] A retrospective Japanese study reported stressful events as potential triggers of relapses in children with nephrotic syndrome.[18] We need more prospective studies specifically designed to assess changes in these children's behaviour, learning performance, quality of life, perceived stress and coping strategies of the child and his or her parents. Insight in the impact of nephrotic syndrome on families and the effect of stress on the occurrence of relapses could aid in developing coping strategies.

9.2. The prolonged treatment hypothesis re-evaluated

For more than twenty years, it has been suggested that prolonging initial glucocorticoid treatment reduces the risk of relapses in childhood nephrotic syndrome.[19,6] In fact, this idea had already been put forward in the 1950s, during the early hours of glucocorticoid treatment. Since short regimens (days to weeks) of adrenocorticotropic hormone (ACTH) and/or cortisone were followed by rapid occurrence of relapses, prolonged regimens -up to more than one year of 300-400 mg cortisone on intermittent days- were promoted at the time.[20] But the sometimes detrimental adverse effects accommodating these intensive regimes,[21] lead the International Study of Kidney Diseases in Children (ISKDC) to decide on a standard two-month regime in 1966.[22] Afterwards, no real consensus existed, since this regime was adopted but also adapted by many pediatric nephrologists (Figure 2).[23,24]

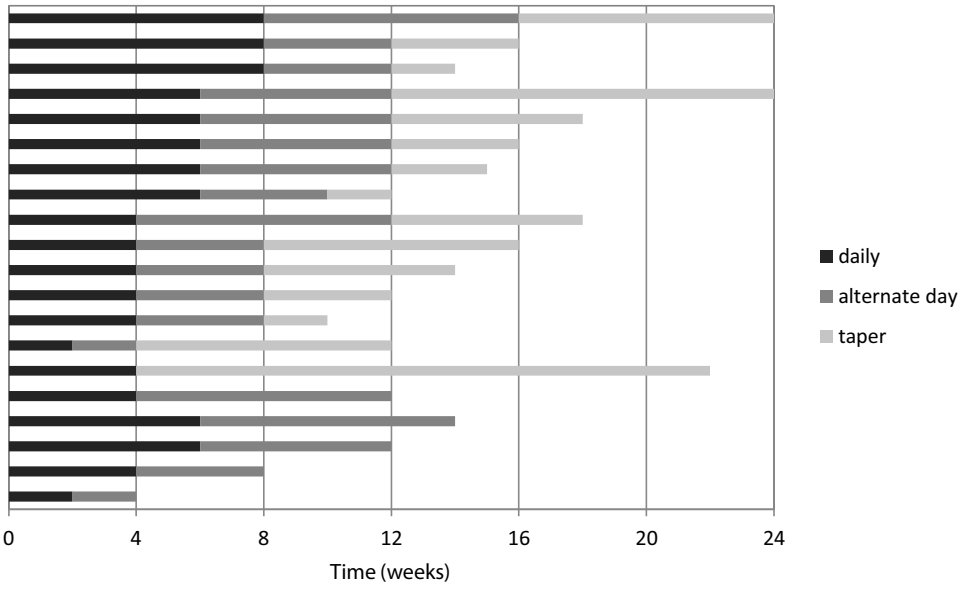

Figure 2. Initial predniso(lo)ne schedules used worldwide for childhood nephrotic syndrome.[5,24,26,89-103]

Around twenty years later, prolonging initial treatment gained new interest when the Arbeitsgemeinschaft für Pädiatrische Nephrologie (APN) demonstrated that shortening initial treatment from two to one month increased the risk of relapses.[19] At the same time, results from a small Japanese study suggested that prolonged tapering of prednisolone following the ISKDC schedule successfully reduced the incidence of (frequent) relapses.[25] Several other studies investigating the effect of prolonged, tapered treatment followed in the 1990s.[26,27,28,29] In 2000, the first meta-analysis summarizing

the results of previous studies was published by Hodson and colleagues,[30] and several sequels appeared in the Cochrane Database of Systematic Reviews.[6]

From the work by Hodson *et al*., it became clear that many of the existing studies showed methodological weaknesses, particularly those comparing three month-treatment to longer treatment regimes.[6] In addition, no sufficient evidence ascribing the beneficial effect to either prolonged treatment duration or a higher cumulative dose of glucocorticoids existed.

Considering the quality level of current evidence, it is hardly surprising that no worldwide consensus exists on the duration and dose of prednisolone treatment for childhood NS. Many different schedules are used across countries and even within countries. Regional guidelines are largely based on expert opinions. While some believe in prolonged, more intensified treatment schedules, others are reluctant to expose their patients to large amounts of steroids. The lack of high quality evidence concerning initial glucocorticoid treatment of childhood NS was again confirmed in the recently published Kidney Disease: Improving Global Outcomes (KDIGO) guidelines (Box 1).

Box 1. Treatment of the initial episode of steroid sensitive NS according to the KDIGO Clinical Practice Guideline for Glomerulonephritis, 2012.

"3.1: Treatment of the initial episode of SSNS

3.1.1:
We recommend that corticosteroid therapy (prednisone or prednisolone) be given for at least 12 weeks. (1B)

3.1.1.1:
We recommend that oral prednisone be administered as a single daily dose (1B) starting at 60 mg/m2/day or 2 mg/kg/day to a maximum 60 mg/day. (1D)

3.1.1.2:
We recommend that daily oral prednisone be given for 4–6 weeks (1C) followed by alternate-day medication as a single daily dose starting at 40 mg/m2 or 1.5 mg/kg (maximum 40 mg on alternate days) (1D) and continued for 2–5 months with tapering of the dose. (1B)."

Grade	Quality of the evidence	Meaning
A	High	We are confident that the true effect lies close to that of the estimate of the effect
B	Moderate	The true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different
C	Low	The true effect may be substantially different from the estimate of the effect
D	Very low	The estimate of the effect is very uncertain, and often will be far from the truth

KDIGO, Kidney Disease: Improving Global Outcomes; SSNS, steroid sensitive nephrotic syndrome.

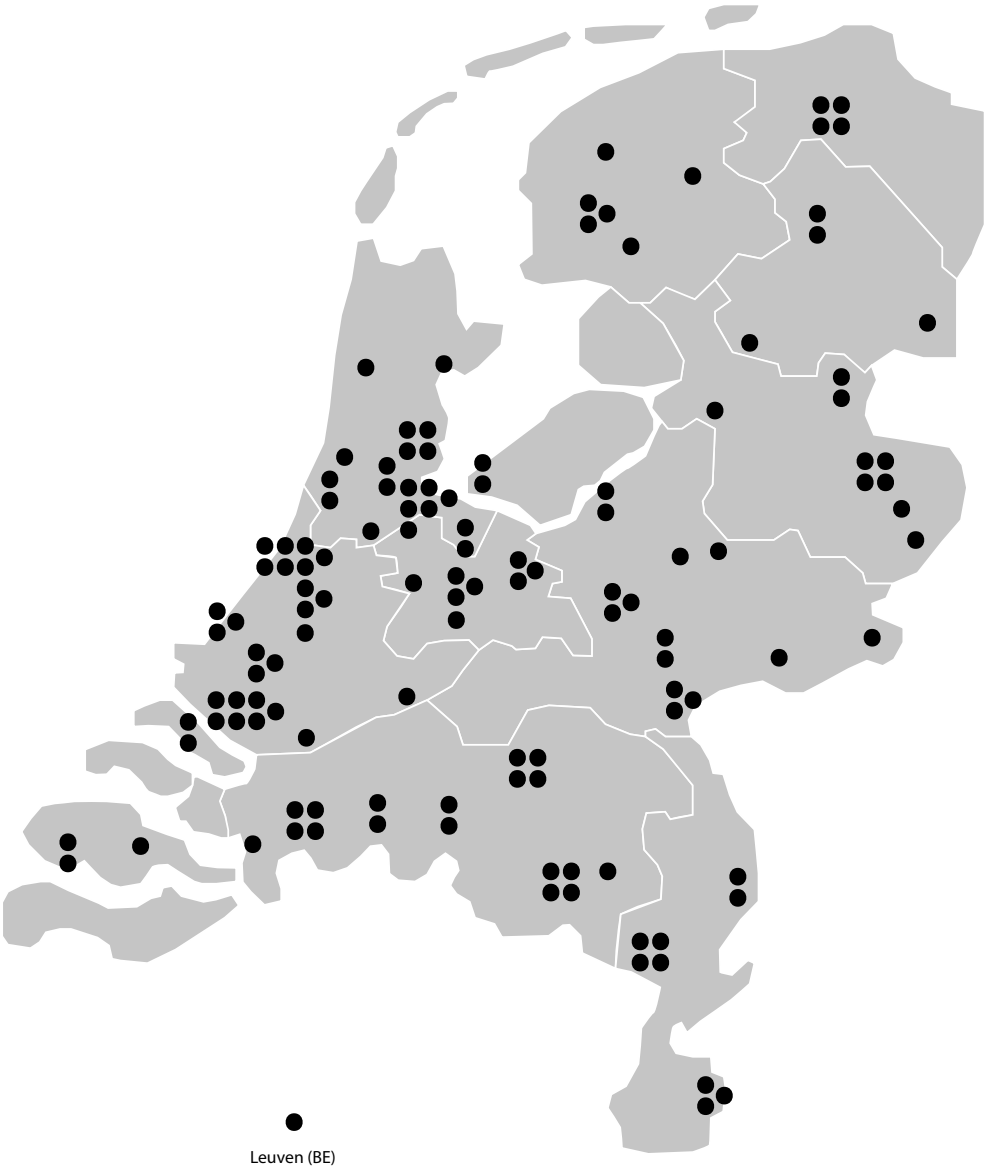

Figure 3. Geographical distribution of 126 children with steroid sensitive nephrotic syndrome.

9.2.1. Extending initial prednisolone treatment without increasing cumulative dose

We found no favourable effect of six months prednisolone treatment compared to three-month treatment, using equally high cumulative doses (Chapter 3). This new finding challenges the previous assumption that prolonged treatment duration improves clinical outcome. Our randomized controlled trial had a strong design including adequate concealment of allocation and blinding of all involved. Follow up was considerably longer than in all previous studies. Furthermore, by including patients from all over the Netherlands, a representative study population was available, reducing selection bias to a minimum (Figure 3). Analysis of the results was thorough and adequate. Altogether, we are confident that prolonging prednisolone treatment without increasing cumulative dose will not be of any use to children with a first episode of nephrotic syndrome.

Though childhood nephrotic syndrome may in part be considered a chronic disease, our findings do not justify long-term prednisolone maintenance therapy following first induction. This is further illustrated by the fact that 21 out of 64 children (33%) in the six-month group had already relapsed before initial treatment ended. Of these, 15 developed a first relapse while receiving 10 mg/m^2 on alternate days. Intriguingly, one of the few similarities across currently used initial treatment regimes is that they all extend several weeks or months beyond the moment of achieving first remission. In the majority of patients, remission occurs within two weeks of daily prednisolone treatment.[1] In our study cohort, patients receiving standard three-month treatment achieved remission well before the end of the 12-week schedule (median 10 days, interquartile range 8-14 days). Previous work by the APN showed that reducing initial treatment duration to one month resulted in rapid occurrence of relapses.[19] It is therefore generally assumed that some form of 'consolidation therapy' within the initial regime beyond one month is needed to prevent patients from early relapses. It appears that if duration of this 'consolidation' phase is important, it is limited to an optimum, since long-term tapering of prednisolone after remission did not prove to be beneficial either.

9.2.2. More prednisolone, fewer relapses?

Studies comparing different cumulative doses while maintaining the same treatment duration are scarce, small and inconclusive.[6] In 2000, Hiraoka and colleagues compared a standard 3-month regime (3360 mg/m^2) to a 3-month regime (n=30) with a lower induction dose (40 mg/m^2/day) and a concomitant cumulative dose of 2520 mg/m^2 (n=30). In the group receiving more prednisolone, the proportions of patients with relapses and frequent relapses were lower; however, the former did not apply to girls and the latter did not reach statistical significance. Follow up covered only 12 months.[4] The study by Pecoraro *et al.* was published in abstract form only. Two of the

three regimes studied both covered 26 weeks, with estimated cumulative dosages of 5240 mg/m^2 (n=16) and 4160 mg/m^2 (n=16) respectively. The proportion of patients with relapses was highest in the group receiving the lower dose; no information on frequent relapses was given. Allocation concealment was inadequate, and follow up time was not clearly stated.[6,31]

From the work by Hodson and colleagues, there is still a very good possibility that increased cumulative doses of prednisolone are more effective in reducing relapse rates. The group repeatedly and systematically reviewed reports concerning glucocorticoid treatment in childhood nephrotic syndrome. Results of these meta-analyses implied better outcome for prolonged initial treatment regimes, which all included a higher cumulative dose.[6] In our opinion, it is unlikely that extending the period over which these doses are given is the key. In light of the results from our RCT, we do believe the findings by Hodson *et al.* should be interpreted as in that higher cumulative dosages seem more effective.

9.3. Drug monitoring of prednisolone – towards feasible methods for children

Therapeutic drug monitoring, together with pharmacogenomics and sparse sampling strategies, may lead to safer and more effective treatment as it allows for personalised therapy.[32,33,34] The fact that patients, regardless of their condition, differ considerably in their response to protocolised doses of prednisolone, suggests that drug monitoring of prednisolone may offer the possibility of tailored treatment.

Though prednisolone is one of the most widely used drugs, monitoring this drug is currently not part of clinical practice. This may have several reasons. Drug monitoring is essential for drugs with small therapeutic indexes,[34] which does not apply to prednisolone. In addition, pharmacokinetics of the drug are complex since prednisolone is a protein-bound drug, which is continuously being converted into its inactive metabolite prednisone and back.[35] Furthermore, measuring prednisolone, particularly unbound prednisolone, poses several challenges which are hard to tackle in routine laboratories. Measurement of unbound - biologically active - concentrations in blood is relevant when monitoring prednisolone, especially at low to intermediate concentrations, as clearance is highly influenced by protein binding.[36] Lastly, multiple blood sampling is unfavourable in studies that concern children.

In Chapter 4 and 5, we described several steps towards making drug monitoring of prednisolone more readily available. A method was validated for separation and measurement of the three very similar compounds prednisolone, prednisone and

cortisol in serum and saliva with LC/MS-MS (Chapter 4). As the naturally occurring M+2 isotope of prednisolone can interfere with the most abundant isotope of cortisol,[37] adequate chromatographic separation of prednisolone and cortisol is warranted; this was accomplished in our study.

By means of ultrafiltration, we were able to assess unbound compounds in addition to total concentrations. Though we did experiment with equilibrium dialysis (data not shown), we eventually chose ultrafiltration as the technique for separating bound from unbound glucocorticoids. During ultrafiltration, the original glucocorticoid-protein equilibrium is least disturbed, no corrections are needed afterwards, and the process is considerably shorter than equilibrium dialysis.[38,39] Still, measurement of prednisolone and similar compounds in ultrafiltrate and serum requires considerable pre-treatment of samples.

We wondered whether prednisolone in saliva would be a good indicator of the free drug in blood, as this relation had previously been established for cortisol. Prior to our work, there was a lack of evidence for a relationship between prednisolone levels in saliva and blood.[40,41,42] It is well established that salivary cortisol can be applied as a surrogate marker of unbound - biologically active - cortisol in blood in clinical settings.[43] In addition, concentrations of several drugs (theophylline, digoxin and diazepam) in saliva offer good representations of their unbound concentrations in blood.[44-47] In Chapter 4, preliminary analysis suggested a good correlation between prednisolone in saliva and ultrafiltrate. Taking into account prednisone concentrations seemed to improve the relationship between salivary levels and those in ultrafiltrate even more. The latter was not confirmed in our larger study with healthy adults (Chapter 5), in which we found that salivary prednisolone by itself is a good index of (free) serum prednisolone at the concentration ranges that were studied.

The validated quantification method described in Chapter 4 enabled us to study the pharmacokinetics of prednisolone and prednisone in a group of healthy volunteers (Chapter 5); a population pharmacokinetic approach was used to assess prednisolone clearance and to derive relationships between prednisolone concentrations in saliva, ultrafiltrate and serum.

The ratio of prednisolone to prednisone was considerably lower in saliva than in ultrafiltrate and serum; this may well be a reflection of the ratio of 11-βHSD type 1 and 2 activity, which is known to vary between tissues.[35,48] We also found that free prednisone fractions remained relatively unaffected by changes in concentration,

while those of prednisolone were concentration-dependent. This may be explained by differences in affinity for corticosteroid binding globulin (CBG), which is ten-fold higher for prednisolone, in addition to the lower concentrations of prednisone. Prednisolone causes displacement of prednisone from CBG binding sites, which results in prednisone being bound primarily to non-saturated albumin with low affinity in a stable fashion, regardless of prednisolone concentration.[49,50]

For the first time, a good relationship between salivary prednisolone levels and both free and total levels of prednisolone was established. This new finding could be of particular relevance to children receiving prednisolone, as it encourages new studies aimed at drug monitoring with non-invasive methods. Collecting saliva is painless and can be adequately performed from a young age; using good, easy instructions, parents can even collect samples at home. Compared to ultrafiltrate and serum, measurement of prednisolone in saliva requires little preparation and time.[51] Though confirmation of our results is needed for lower dosages, these promising results justify further development of the use of saliva for drug monitoring of prednisolone.

9.4. Explaining variety in clinical outcome of childhood nephrotic syndrome

9.4.1 Pathophysiology

Nephrotic syndrome very likely originates from multifactorial pathways, which could offer a good explanation for the observed variety in clinical course. Current theories are discussed in detail in several excellent reviews[52-55] and will only be discussed briefly here. Historical and current evidence points towards an immune-mediated process, though the exact plasma factors and actual target cell(s) are still unknown. NS is currently put forward as a podocytopathy, which may either be triggered by alterations in the immune system or vice versa. Research aimed at uncovering these mysteries took flight in the past fifteen years; several research groups currently focus on pathophysiologic mechanisms. In addition to improved animal models, innovative techniques that enable studying human podocytes in vitro have been developed. Plans directed at genome wide association studies are made at this time. In other words, the pathophysiology of nephrotic syndrome is 'hot' and research may produce some promising results in the near future.

9.4.2 Clinical parameters

Research aimed at uncovering clinical predictors of clinical outcome in childhood nephrotic syndrome reported contradictory results. One aspect, low birth weight, was related to unfavourable outcome in a few small studies. In Chapter 6, this was re-

evaluated in a meta-analysis. Results showed increased odds ratio's for children with low birth weight (below the 10th percentile for gestational age, gender and maternal parity) for primary steroid resistance and hypertension. Birth weight was not related to the occurrence of relapses in general. Though frequent relapses and steroid dependence could not be analysed due to significant heterogeneity, the need for additional treatment with ciclosporin or cyclophosphamide was more common in children with low birth weight, suggesting an unfavourable clinical course in these children. A subsequent retrospective study by Plank *et al*. confirmed the association between low birth weight (below -1.5 SD) and steroid resistance, and reported increased use of anti-hypertensive agents in children with nephrotic syndrome and low birth weight. However, low birth weight was not related to relapse rate, steroid dependence, or the need for additional therapy.[56]

A causative mechanism for an effect of low birth weight on relapse patterns in childhood NS is currently not at hand. Intrauterine growth retardation is associated with a reduced number of nephrons at birth[57,58] According to the hyperfiltration hypothesis put forward by Brenner and colleagues, a reduced nephron number leads to glomerular hypertension and hypertrophy. This may result in systemic hypertension and glomerular damage followed by albuminuria and glomerulosclerosis.[59,60] This might offer an explanation for the higher risk of hypertension found in our meta-analysis. Whether the remaining nephrons are more prone to the conformational changes seen in NS is not fully understood. Hyperfiltration can lead to detachment of podocytes,[61] but this process is irreversible and is more likely to result in slowly progressive proteinuria than the relapsing-remitting type seen in NS.

The definition of low birth weight below the 10th percentile together with the low incidence of NS inevitably results in small studies. Pooling results in meta-analyses can partly resolve this, but due to heterogeneity in definitions, interpretation should be done with caution.

Analysis of other covariates in our study revealed findings of clinical interest, though not supported by statistical significance. Boys tended to have worse outcome than girls in terms of frequent relapses and relative relapse rate. In the few studies that observed an effect of gender on the clinical course of NS, males were at a disadvantage.[62,63] It would be interesting to further explore whether boys and girls benefit from different treatment regimens in studies with larger sample sizes. We found no effect of age at onset. The influence of age at onset is still debated, as several studies have reported young age to be associated with FRNS and/or steroid dependence,[64,62,65,63] while others

did not find an effect of age on the clinical course of NS.[66,67,68] The same applies to the number of days to remission.[62,66,68-72]

As reported earlier by the ISKDC,[73,68] the early clinical course appears to be an important indicator of subsequent outcome. This particularly applied to patients with either zero or ≥ three relapses within the first six months. In our RCT cohort, the occurrence of at least one relapse in the first six months of follow up correlated strongly with subsequent occurrence of FRNS and SDNS, regardless of initial treatment. The same applied when only relapses in the first six months after cessation of initial treatment were considered.

9.4.3 Metabolism of prednisolone in relation to clinical outcome

It is striking that although every child with nephrotic syndrome starts out with an equal dose of prednisolone, such a wide variety exists in subsequent relapse patterns and side effects. In Chapter 7, we aimed to explain this clinical variability from between-subject variability in drug clearance and exposure (AUC). We used a unique approach that combined non-invasive (sparse) saliva sampling, the previously established population pharmacokinetic model from Chapter 5, and allometric scaling. This way, individually predicted values of clearance, volume of distribution and AUC (area under the concentration curve) of (free) prednisolone could be derived from sparse salivary samples.

The between-subject variability in prednisolone pharmacokinetics in our study population was found to be low to moderate. The remaining variability could not be explained from certain genetic polymorphisms in genes thought to be involved in prednisolone metabolism. The latter however requires further evaluation in larger study populations. At present, the influence of pharmacogenetic factors on glucocorticoid pharmacokinetics and pharmacodynamics is not well understood.[74]

We did not find an association between model-derived pharmacokinetics of prednisolone during initial treatment and clinical outcome. As explained in Chapter 7, previous studies directed at correlating prednisolone pharmacokinetics to clinical outcome in children with nephrotic syndrome had contrasting results.[75,76] However, characterization of these relationships has been limited due to small subject numbers and failure to consider free drug exposure. These issues were largely dealt with in our study through accurate measurement of salivary prednisolone, together with a population pharmacokinetic method to approximate free drug AUCs in a relatively large study population. It is therefore unlikely that pharmacokinetics of prednisolone during initial treatment are one of the major determinants of the clinical course in

children with nephrotic syndrome. For this particular goal, evaluating prednisolone pharmacokinetics is not recommended. Pharmacokinetic assessment of prednisolone in these children may still be useful for children receiving maintenance therapy at lower dosages, for resolving future questions concerning drug interactions, or to evaluate patient compliance.

Ideally, we would have used a model based on a relationship between salivary and free prednisolone in children instead of adults. Drugs can be studied either in affected or healthy adults, or in affected children. Though we did consider several alternative designs that involved children using prednisolone for medical reasons, these had several practical implications, such as the use of co-medication, uncertainty of the time of dosing in the outpatient ward, and the simple fact that the group of patients available would be small and heterogeneous. We decided that a controlled setting with healthy adults would be the next best thing.

The findings and methods described in Chapters 5 and 7 may well prove to be relevant to other patients populations. In solid organ transplant recipients, there is evidence of a relationship between prednisolone pharmacokinetics and the incidence of acute rejection and adverse cardiovascular and metabolic events.[74] Since confirmation of these associations is still required in larger, prospective studies, research considering these patients may benefit from the methods and possibilities brought in Chapters 5 and 7.

The possibility of providing personalised treatment for children with NS is attractive, yet needs to be built on improved knowledge concerning pathophysiology of the disorder as well as pharmacokinetics and dynamics of glucocorticoids. Since the effects of prednisolone in children with nephrotic syndrome may be seen long after the drug has been stopped, treatment effect may depend more on indirect, delayed effects than on measured concentrations of the drug. Future studies may clarify the role of pharmacokinetic factors in the pharmacodynamic effects of prednisolone at the tissue and receptor levels. To shed more light on these processes, mechanism-based pharmacokinetic/pharmacodynamic models of glucocorticoids may be helpful.[74]

9.4.4 Sensitivity to glucocorticoids in relation to clinical outcome

The results in Chapter 7 indicate that pharmacokinetics of prednisolone (during initial treatment) are not one of the major determinants of the clinical course in childhood NS. Diversity in clinical course may therefore rely on processes that take place at the tissue or cellular level, rather than on extracellular bioavailability of prednisolone,

or on disease-related factors. Pharmacodynamic effects of glucocorticoids depend on the efficiency of numerous pathways, giving rise to inter-individual differences in glucocorticoid sensitivity.

In Chapter 7, we observed a lower incidence of all therapeutic endpoints in patients exhibiting early signs of Cushing. It should be noted that significance was only reached for the association between Cushingoid appearance and steroid dependence. Though this assessment is explorative, it supports the theory that inter-individual differences in pharmacodynamics of glucocorticoids determine part of the clinical outcome.

As described in Chapter 2, many of the pleiotropic glucocorticoid effects occur through interactions with the genome and thus take time to become manifest.[35] This explains why transient glucocorticoid (side) effects remain present for days, weeks, or even months after cessation of prednisolone treatment.[74] Intriguingly, the extent to which wanted and unwanted glucocorticoid effects are seen differs greatly among individuals. The relevance of glucocorticoid sensitivity is increasingly recognized in patients with various diseases such as asthma,[77] rheumatoid arthritis,[78,79] multiple sclerosis,[80] and neuropsychiatric disorders.[81]

Previously, partial adrenal suppression tests have been put forward as an in vivo index of glucocorticoid sensitivity.[82] We found no relation between a very low dose dexamethasone suppression test and clinical outcome in children with NS (Chapter 8). Low dose dexamethasone suppression tests are popular in the fields of psychology and psychiatry;[83] it is possible these tests better reflect glucocorticoid sensitivity of certain tissues than others. Results of the test depend on several factors, such as timely measurements of cortisol as well as dose and metabolism of dexamethasone.[82] Unfortunately, the latter could not be included in our analyses due to immeasurable amounts of dexamethasone in many samples. Another plausible explanation for the lack of association between the VLD-DST and clinical response could be that the high glucocorticoid doses given to these patients overruled subtle inter-individual differences in glucocorticoid sensitivity.

In Chapter 8, we found a new and interesting association between the GR-9β haplotype of the glucocorticoid receptor gene and unfavourable clinical outcome in children with NS. The GR-9β polymorphism affects the functionality of the most abundant glucocorticoid receptor isoform. The functionality of this receptor determines the magnitude of the individual clinical response to glucocorticoids.[74] The GR-9β haplotype was previously related to diminished transrepressive effects, which

account for glucocorticoid-induced suppression of the immune system. Carriers of GR-9β generally have a more active immune system and a predisposition to a pro-inflammatory status.[84,85,79,86] In our study, carriers of the GR-9β haplotype showed increased incidences of a first relapse, frequent relapses and steroid dependence compared to non-carriers. Though it is currently unknown which mechanisms underlie these observations, we hypothesise a more active immune system in carriers of GR-9β, and/or reduced sensitivity to glucocorticoids may predispose to relapses and steroid dependence in patients with NS.

Since well-defined markers for clinical outcome of childhood nephrotic syndrome are currently not at hand, the findings described in Chapter 8 could be very relevant to children with this disease. However, implementation of genetic analysis of the GR gene in the treatment of these children would be premature at this moment. Because evidence on the association between the GR-9β haplotype and efficacy of exogenous glucocorticoids has only just begun to emerge,[87] this association needs to be confirmed in future cohorts of children with NS. The next step towards the goal of more personalised treatment could then be to evaluate whether GR-9β carriers benefit from either higher glucocorticoid doses, or earlier introduction of other agents may prove to be more effective in these patients.

9.5. Glucocorticoid treatment for childhood nephrotic syndrome: weighing the cornerstone

The short-term advantages of prednisolone for childhood NS are obvious: remission is seen within days or weeks in most cases and in two out of ten patients, no further treatment is needed after the first prednisolone course. Understandably, its position as first line treatment has been sturdy and virtually unquestioned until now.

Though the benefits of prednisolone with regard to morbidity and mortality in childhood nephrotic syndrome were clearly recognized shortly after the discovery of this drug, idle progress has been made since. Glucocorticoid treatment regimes for nephrotic syndrome are still barely evidence based. Though new agents have made their way into the treatment of frequent relapses and steroid dependence in the last decades, it is highly unsatisfactory that so many patients develop frequent relapses. That said, much work needs to be done before current empirical glucocorticoid therapy can be actually replaced by better treatment strategies. For the time being, glucocorticoids remain important within the treatment of childhood nephrotic syndrome.

The studies in this thesis have addressed several aspects of glucocorticoid therapy in children with nephrotic syndrome. One of the major findings is that extending

glucocorticoid treatment does not reduce the risk of frequent relapses. We believe these results, being based on strong methodology, could really cause a turnaround in current thinking about the treatment of this disease. An additional finding of particular interest is that salivary prednisolone can be used as an index of free, biologically active prednisolone in blood. Though pharmacokinetics of prednisolone did not prove to be the silver bullet in explaining clinical variability in childhood nephrotic syndrome, the promise of non-invasive drug measurement could be highly relevant to other pediatric populations. Finally, we have found evidence to support a relationship between a common variation in the glucocorticoid receptor gene and clinical outcome in children with nephrotic syndrome. We identified opportunities to optimize the cornerstone treatment in the future. Altogether, this thesis has brought exciting, new information to the field of pediatric nephrology.

9.5.1 Directions for future research

Find the cause to find the cure?

Both the pathogenesis and the pathophysiology of steroid sensitive nephrotic syndrome are unknown. A better understanding of these processes could provide specific targets for treatment of the initial presentation and prevention of relapses. Although a number of ultrastructural components of the glomerular protein barrier have been identified, it is unclear which mechanism disrupts the integrity of this barrier in SSNS. Historical and current evidence points towards an immune-mediated process, which intriguingly seems to affect only the kidney. Various circulating plasma factors and local glomerular factors are suggested to play a role, but no firm conclusions are made at this time. Research aimed at uncovering these mysteries took flight in the past fifteen years and may produce promising results in the future. But for the time being, 'finding the cure' will largely depend on empirical clinical studies, like the ones reported in this thesis.

Optimising treatment

In this thesis we focus on the prevention of frequently relapsing NS. Treatment of FRNS is beyond the scope of this thesis. The development of better treatment of the early stages of NS will require rigorous testing in multicenter collaborations.

An interesting concept for future research may be that the first blow is half of the battle in childhood nephrotic syndrome. Previous research suggests intensified regimes are more effective. Several options for immunomodulation, alternative to the current protocols, could be suggested. Here we present some examples.

Using the current prednisolone schedules as a starting point, we consider it useful to differentiate between the induction phase - up to remission - and the consolidation phase of initial treatment.

- Induction

The generally accepted prednisolone dosage of 60 mg/m^2/day successfully induces remission in 90-95% of patients. Challenging this dosage would not be our prime focus of research: 'Never change a winning horse'. On the other hand, the dosage has been chosen arbitrarily over fifty years ago. It is unknown whether a much higher initial dosage (similar to schedules used for induction in SLE) might have a more lasting effect.

- Consolidation

The term 'consolidation phase' of treatment has been introduced to indicate all prednisolone administered, after remission has been achieved. According to our standard protocol this is 60 mg/m^2 daily until six weeks after presentation, followed by six weeks of 40 mg/m^2 on alternate days. This may postpone relapses to some extent, but it cannot prevent the occurrence of relapses and frequent relapses in 80% and 50% of patients respectively. We should aim at redefining initial treatment, particularly the consolidation phase.

Our interpretation of the meta-analysis by Hodson *et al*. suggests that an even higher cumulative dosage of prednisolone in this consolidation phase will reduce the risk of relapses. A higher dosage of prednisolone will obviously come at a cost, an increased risk of side effects. We suggest to study as alternative consolidation treatment:

- Increased cumulative dosage of prednisolone by extension of current protocols. Two large, well-designed RCTs are currently undertaken in the UK and in India.
- Increased cumulative dosage of prednisolone by increasing daily dosage, without increasing the total duration.
- Adding an alternative immunosuppressive agent to initial treatment, preferably one that is generally well-tolerated in the treatment of relapsing NS, such as mycophenolate mofetil or levamisole.[2]

- Relapse treatment

The current prednisolone schedule for relapses is generally accepted and successful in inducing remission in the majority of cases. Little evidence exists on alternative treatment schedules to prevent subsequent relapses. Since 65% of relapsing patients develop a frequently relapsing pattern, we suggest to evaluate alternative schedules that include intensification of consolidation, such as:

- Intensification of the prednisolone consolidation phase, or
- Addition of other immunosuppressive agents to the consolidation phase of relapse therapy

- Prevention of relapses

How relapses are triggered is largely unknown. Increasing evidence concerning the prevention of relapses, either by improving (initial) treatment or by learning to know and how to avoid triggering events is of key importance. Confirmation is needed of a previous suggestion that modest doses of prednisolone during (viral) infections reduce the risk of relapse. Another lead might be the observation that relapsing disease usually subsides before adulthood is reached. This suggests that maturational factors of the immune, endocrine or other systems might be involved in triggering or preventing relapses. Of particular interest could be the association between psychological stress and the development of relapses. This is currently being studied prospectively in a Dutch cohort of patients to document whether stress can indeed induce relapses and to asses family coping strategies.

Individualising treatment

The fact that both treatment effects and side effects differ greatly among patients with SSNS offers a rationale for research aimed at individualising treatment. This might imply individualised dosage of prednisolone and/or personalised switch to alternative treatment modalities.

It proved however difficult to identify factors that could direct such individualised treatment. In our studies we were unable to identify pharmacokinetic profiles or in vivo sensitivity to glucocorticoids as risk indicators of frequent relapses. The GR-9β haplotype of the glucocorticoid receptor gene and an early Cushingoid habitus did show associations with clinical outcome. If confirmed by others, these factors might be a starting point for individualised treatment. Future research at a more basic level could explore the pharmacodynamic and cellular basis of the variable response to prednisolone in SSNS.

Suggestions for future study designs

- Defining clinically relevant endpoints

Research aimed at comparing initial treatment regimes should include relapse frequency and/or steroid dependence, or simply the need for additional treatment measures, as primary outcome. Most studies focus on the number of patients with at least one relapse, a group which is very heterogeneous and has limited clinical relevance.
There is more to FRNS than just relapse frequency, as we learned that some patients do need additional therapy well before fulfilling currently used FRNS-criteria. The existing definition of frequently relapsing NS can be problematic when comparing initial regimes. Within such trials, the need for additional treatment measures should be well-defined and include early steroid dependence and severe steroid toxicity in addition to relapse frequency.

- Cooperation

To provide dynamic and high quality research in children with nephrotic syndrome, both national and international cooperation is needed in the future. We have learned that it is possible to perform a nationwide study, and we were fortunate to have so many pediatricians contributing to this study at no direct benefit to themselves. We have also learned that realizing this study involved many challenges, including getting local approval from all medical ethical committees. Ensuring contact with families and pediatricians from almost 70 centres during enrolment and follow up and collecting data from all sites took time and effort. During this process, it became clear that the treatment of FRNS and SDNS still differs considerably between centres in the Netherlands. Central registration, centralisation of research visits, and further standardisation of treatment could optimise future national trials in the Netherlands.
Similar national networks can provide the foundation of international cooperation. As was discovered from internationally cooperative registries involving children with malignant disease, central registration and follow up allows for powerful prospective studies and regular improvement of treatment strategies. In 2010, an international online registry was launched by the European Working group on Idiopathic Nephrotic Syndrome and NephcEurope,[88] which may offer a platform for future international trials. Solid international cooperation requires an open mind, excellent coordination, funding, and above all dedication.

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CHAPTER 10

Summary / Samenvatting

The studies in this thesis aim to increase our understanding of the clinical variability observed in children with nephrotic syndrome and to offer new insights for improving current therapy. Particular attention is given to treatment duration, metabolism of prednisolone, and sensitivity to glucocorticoids.

SUMMARY

PART I – INTRODUCTION

Nephrotic syndrome is a common manifestation of renal disease in childhood, characterized by massive loss of proteins via the urine (proteinuria) and retention of salt and water. This proteinuria results from leakage through the capillary sieve in the kidney. The underlying mechanisms are still largely unknown. Ever since their discovery, glucocorticoids have been the cornerstone of treatment in childhood nephrotic syndrome. These drugs prevent life-threatening complications, as most children show an adequate response to a first course of prednisolone. However, the clinical course thereafter is highly variable, with multiple relapses in the majority of patients. Children experiencing frequent relapses receive large amounts of prednisolone and are at risk of developing numerous side effects (such as an altered appearance, high blood pressure, impaired growth, behavioural changes) and severe complications. There is a great need for improvement of current treatment regimes.

Part I reviews research aimed at predicting or explaining variety in the clinical course of children with nephrotic syndrome. At present, there are no undisputed associations between demographic, clinical, or biological factors and clinical course (**Chapter 1**). **Chapter 2** focuses on the historical and present role of glucocorticoids within the treatment of nephrotic syndrome and current views on how glucocorticoids may exert therapeutic effects in this disease. In addition, several processes involved in prednisolone metabolism and glucocorticoid sensitivity are reviewed.

PART II – IS PROLONGED TREATMENT MORE EFFECTIVE?

For years, researchers have hypothesised that prolonging prednisolone treatment results in improved clinical outcome compared to standard (two or) three-month treatment. Independent effects of treatment duration and dose however remain unclear. Another concern is the limited methodological quality of most trials. **Chapter 3** describes the results of a nationwide, placebo-controlled, double-blind study. A total of 150 newly diagnosed children with nephrotic syndrome from 69 hospitals are randomised. We compare a standard three-month treatment schedule to an experimental six month-treatment with the same cumulative amount of prednisolone. This study convincingly demonstrates that prolongation without increasing dose is no more efficient than standard treatment.

This prospective study has substantial follow up, which for the first time enables a detailed clinical picture of childhood nephrotic syndrome in the Netherlands. The nationwide setting, involving many peripheral centres, allowed for a well-defined and representative study population. As much as 80% of patients have one or more relapses, more than half need additional medication because of frequently occurring relapses, and around one third develop steroid dependence. These findings underscore the need for studies evaluating alternative treatment strategies.

PART III – MEASURING PREDNISOLONE: TOWARDS FEASIBLE METHODS

For certain drugs, measurement of the amount available to the body following administration can help in tailoring treatment. Measurement of biologically active prednisolone however is challenging. Part of the drug is bound to proteins in blood, and the degree to which this occurs is concentration-dependent. Only free prednisolone molecules can actually enter cells. Part III describes strategies to get around these issues in the future. In **Chapter 4**, we describe a method that enables adequate separation of prednisolone and other, chemically similar molecules. Subsequently we describe validated measurement of prednisolone in saliva and total as well as free prednisolone levels in blood.

Measurement of prednisolone in saliva is more feasible and convenient than measurement of free prednisolone in blood. **Chapter 5** illustrates concentrations of total and free prednisolone concentrations in blood in relation to prednisolone in saliva in a group of healthy volunteers. This study evidenced that prednisolone in saliva is an index of free prednisolone concentrations in blood. This may have important consequences for future studies that aim for non-invasive measurement of prednisolone clearance. Collecting saliva is painless and can be done at home; the findings in this chapter therefore are of particular interest to the field of pediatrics.

PART IV – WHICH FACTORS AFFECT CLINICAL OUTCOME?

This part takes us back to children with nephrotic syndrome and the search for factors predicting or explaining relapse patterns in these patients. Over the years, age, gender, time to first remission, early relapses, and several biological markers have been put forward as potential predictors. These associations could not be confirmed in several studies and may have been based on coincidence or selection of patients.

In the past, several small retrospective studies have reported a relationship between birth weight and clinical outcome in children with nephrotic syndrome. **Chapter 6** summarises the results of previous research and a Dutch retrospective cohort in a meta-analysis. In these studies, steroid resistance and high blood pressure are found more often in children with low birth weight. Assessment of the relationship between birth weight and frequent relapses or steroid dependence is not possible due to large variety among studies. Causality between birth weight and relapse patterns remains unclear. We conclude that this should be re-evaluated in prospective studies.

It is well known that individuals differ with respect to drug metabolism. In **Chapter 7**, we evaluate the calculated amount of prednisolone in blood after a given dose (area under the curve or AUC) in relation to relapse patterns and side effects. In addition, we explore the potential effect of variations in genes involved in prednisolone metabolism. The findings described in Part III allowed us to estimate the AUC of (free) prednisolone in blood using just a few salivary samples per child. It appears the genetic variations investigated do not influence the clearance of prednisolone, though additional studies in larger samples are needed to confirm this. In addition, the results do not reveal a relationship between the AUC and (frequent) relapses, steroid dependence, or side effects. Based on these results, extracellular metabolism of prednisolone is unlikely to be a major determinant of the variability in clinical course in children with nephrotic syndrome. It is therefore important that future studies concentrate on other factors, such as intrinsic sensitivity to prednisolone and factors underlying pathophysiology of the disease.

Chapter 8 addresses several aspects of glucocorticoid sensitivity in relation to relapse patterns and side effects in children with nephrotic syndrome. Results of a low dose dexamethasone suppression test are not correlated to relapse patterns. Possible explanations include the test may not represent glucocorticoid sensitivity in the currently unknown 'target cells' in nephrotic syndrome. In addition, we evaluate variations of the glucocorticoid receptor gene associated with either reduced or increased sensitivity to glucocorticoids. The genetic variation 'GR-9beta' shows an association with unfavourable clinical outcome. This new finding suggests a more active disease process and/or reduced sensitivity to glucocorticoids in children with nephrotic syndrome harbouring this variation. Confirmation of this association in other study populations may therefore have important clinical implications.

PART V – REFLECTION

In **Chapter 9** we discuss the studies in this thesis, in light of current literature. In addition, considerations for future studies are described.
One of the key findings is that extending glucocorticoid treatment does not reduce the risk of frequent relapses. We believe our observations, being based on strong methodology, could really cause a turnaround in current thinking about the treatment of this disease. We should look for possibilities to refine and redefine treatment for nephrotic syndrome.

Based on previous research, intensifying initial treatment is likely to increase efficacy. Current initial treatment consists of an induction and a 'consolidation' phase. The efficacy of prednisolone as an inducing agent is clearly recognized. Increasing prednisolone dosage as well as the possibility of personalised glucocorticoid treatment should be subjects of future research. The efficacy and safety of intensifying the 'consolidation' phase of initial treatment (after remission is achieved), by adding other immunomodulatory drugs, should be studied as well.

Though pharmacokinetics of prednisolone did not prove to be the silver bullet in explaining clinical variability in childhood nephrotic syndrome, the promise of non-invasive drug measurement could be highly relevant to other pediatric populations.

We have found new evidence to support a relationship between a common variation in the glucocorticoid receptor gene and unfavourable clinical outcome in children with nephrotic syndrome. Confirmation of this finding in other cohorts may have important implications.

Important future directions include warranting the quality of research, a call for studies based on national and international cooperation, and appropriate use of definitions. This will provide efficient and reliable research aimed at improving clinical outcome for children with nephrotic syndrome.

SAMENVATTING

De onderzoeken in dit proefschrift hebben als doel het beter begrijpen van de klinische variabiliteit bij kinderen met nefrotisch syndroom en het bieden van aanknopingspunten om de huidige therapie te verbeteren. De nadruk ligt hierbij op de behandelduur van prednisolon, alsmede het metabolisme van en gevoeligheid voor glucocorticoïden.

DEEL I – INTRODUCTIE

Nefrotisch syndroom is één van de meest voorkomende nieraandoeningen op de kinderleeftijd en wordt gekenmerkt door verlies van eiwitten via de urine en het vasthouden van vocht. Alhoewel duidelijk is dat de lekkage van eiwitten voortkomt uit een verstoorde filterfunctie van de nier, is de onderliggende oorzaak nog grotendeels onbekend. Onbehandeld is deze aandoening levensbedreigend. Sinds de ontdekking van glucocorticoïden in de jaren 50 is de prognose voor kinderen met nefrotisch syndroom enorm verbeterd. Deze medicijnen worden dan ook beschouwd als de 'hoeksteen' van de behandeling. Vrijwel alle kinderen vertonen een goede respons op de eerste behandeling met prednisolon, een veel voorgeschreven glucocorticoïd. Het klinisch beloop daarna is echter sterk variabel, waarbij de grote meerderheid één of meer recidieven (terugvallen) krijgt. Het is onduidelijk waar deze variabiliteit vandaan komt. Kinderen waarbij recidieven frequent optreden krijgen grote hoeveelheden prednisolon, met de nodige bijwerkingen en risico's tot gevolg. Voorbeelden hiervan zijn een veranderd uiterlijk, hoge bloeddruk, groeivertraging, gedragsveranderingen en een verhoogde kans op infecties. Er is daarom grote behoefte aan het verbeteren van huidige therapieschema's. Deel I van dit proefschrift geeft een overzicht van het onderzoek dat tot nu toe verricht is om het klinisch beloop bij kinderen met nefrotisch syndroom te voorspellen, dan wel de variatie hierin te verklaren. In **Hoofdstuk 1** komt duidelijk naar voren dat voorgaand onderzoek niet heeft geleid tot duidelijke relaties tussen demografische, klinische, of biologische factoren en klinisch beloop. **Hoofdstuk 2** richt zich op de historische en huidige rol van glucocorticoïden in de behandeling van nefrotisch syndroom. Daarnaast komen verschillende aspecten aan bod welke relevant zijn voor de verwerking van prednisolon door het lichaam alsmede de gevoeligheid van het lichaam voor glucocorticoïden.

DEEL II – IS VERLENGDE BEHANDELING BETER?

Al jarenlang bestaat de hypothese dat verlengde behandeling met prednisolon betere uitkomsten geeft dan een standaard behandeling van (twee of) drie maanden. Het is hierbij altijd onduidelijk gebleven of het gunstige effect het gevolg was van verlengde behandelduur of de hierbij gepaard gaande verhoging in cumulatieve dosering. Nog belangrijker is het gebrek aan grote en kwalitatief goede studies. **Hoofdstuk 3** beschrijft de resultaten van een landelijke, placebo-gecontroleerde, dubbelblinde studie. 150 nieuw gediagnosticeerde kinderen vanuit 69 ziekenhuizen werden gerandomiseerd. De Nederlandse standaardbehandeling van drie maanden prednisolon werd vergeleken met een experimentele behandeling van zes maanden, met dezelfde cumulatieve dosering. De studie toont overtuigend aan dat verlenging van de initiële behandeling in deze vorm geen voordeel heeft boven de standaard behandeling.

Deze prospectieve studie, waarbij kinderen langdurig zijn gevolgd, brengt voor het eerst het klinisch beloop van kinderen met nefrotisch syndroom in Nederland grondig in kaart. Doordat zowel academische als perifere ziekenhuizen vanuit het hele land participeerden, kon een representatief beeld worden geschetst van de variatie in het klinisch beeld. Maarliefst 80% van de kinderen krijgt één of meer recidieven. Ruim de helft heeft aanvullende therapie nodig in verband met frequente recidieven. Ongeveer een derde blijkt afhankelijk van prednisolon. Deze bevindingen benadrukken de noodzaak van studies naar alternatieve behandelstrategieën.

DEEL III – METING VAN PREDNISOLON: VAN MOEIZAAM NAAR BRUIKBAAR

Het meten van de hoeveelheid medicijn wat het lichaam 'ziet' na toediening wordt regelmatig toegepast om een therapie meer toe te spitsen op de patiënt. Voor prednisolon is dit echter niet eenvoudig. Zo is een deel van dit medicijn gebonden aan eiwitten in bloed en de mate van binding concentratie-afhankelijk. Alleen vrije prednisolonmoleculen kunnen daadwerkelijk tot de lichaamscellen doordringen en zorgen dus voor het biologisch effect. Deel III richt zich op strategieën om het benaderen van de niet-eiwit gebonden prednisolon concentratie makkelijker te maken. In **Hoofdstuk 4** wordt een methode beschreven die scheiding tussen prednisolon en stoffen die hier veel gelijkenis mee vertonen mogelijk maakt. Vervolgens wordt de bepaling van prednisolon in speeksel en (zowel vrij als totaal) in bloed beschreven.

Meting van prednisolon in speeksel is eenvoudiger dan meting van vrij prednisolon in bloed. **Hoofdstuk 5** beschrijft de relatie tussen concentraties van (vrij) prednisolon in bloed en speeksel in een groep gezonde volwassenen. Uit dit onderzoek komt naar

voren dat het meten van prednisolon in speeksel een goede weerspiegeling is van vrije concentraties in bloed. Deze bevinding kan belangrijke gevolgen hebben voor toekomstige studies waarbij men op een niet-invasieve manier informatie wil verkrijgen over de afbraak (klaring) van prednisolon. Het afnemen van speeksel is niet belastend en kan in de thuissituatie plaatsvinden. De beschreven bevindingen zijn hierdoor met name voor kinderen veelbelovend.

DEEL IV – WELKE FACTOREN ZIJN BEPALEND VOOR HET KLINISCH BELOOP?

Dit deel neemt ons terug naar kinderen met nefrotisch syndroom en de zoektocht naar verklaringen voor het variabele recidiefpatroon bij deze kinderen. Zoals beschreven in Hoofdstuk 1 wordt al lange tijd gezocht naar factoren die het klinisch beloop kunnen voorspellen. Door de jaren heen werden leeftijd, geslacht, tijd tot eerste remissie, vroege recidieven en biologische markers naar voren geschoven als mogelijke voorspellers. Deze associaties werden echter in andere studies niet of slechts deels bevestigd en berusten daarom mogelijk op toeval of op selectie van patiënten.
In het verleden werd in verschillende kleine retrospectieve onderzoeken gekeken naar een verband tussen geboortegewicht en klinisch beloop bij kinderen met nefrotisch syndroom. **Hoofdstuk 6** vat de resultaten van deze onderzoeken en de bevindingen in een Nederlandse groep kinderen samen in een meta-analyse. In deze studies wordt bij de kinderen met een laag geboortegewicht vaker een hoge bloeddruk waargenomen. Een relatie met het daadwerkelijk optreden van frequente recidieven en afhankelijkheid van prednisolon kan echter niet goed worden onderzocht omdat de studies te verschillend zijn. Ook is de oorzakelijkheid van de verbanden nog niet aangetoond. Dit moet in prospectieve studies nader worden onderzocht.
Het is bekend dat er variatie bestaat tussen mensen als het gaat om de opname, verdeling en uitscheiding (farmacokinetiek) van geneesmiddelen door het lichaam. In **Hoofdstuk 7** wordt de relatie tussen de berekende hoeveelheid prednisolon in bloed na een gegeven dosering (area under the curve ofwel AUC) en klinisch beloop van kinderen met nefrotisch syndroom geëvalueerd. Hierbij wordt tevens gekeken naar een mogelijk effect van variaties in genen die betrokken zijn bij de farmacokinetiek van prednisolon. Dankzij de bevindingen beschreven in Deel III kan met enkele speekselmonsters per kind een inschatting worden gemaakt van de hoeveelheid (vrij) prednisolon die in het bloed aanwezig is. Er lijkt geen invloed te zijn van de onderzochte genetische variaties op de klaring van prednisolon, alhoewel dit in grotere studiepopulaties nader zal moeten worden onderzocht. De resultaten laten ook geen verband zien tussen de

berekende hoeveelheid prednisolon in bloed en (frequente) recidieven, afhankelijkheid van prednisolon, of bijwerkingen. De variatie in klinisch beloop lijkt op grond hiervan niet (direct) verklaard te worden door de hoeveelheid prednisolon die beschikbaar is in de bloedbaan. Het is daarom van belang dat toekomstige onderzoeken zich richten op andere factoren, zoals de gevoeligheid van het lichaam voor glucocorticoïden en factoren die aan de ziekte ten grondslag liggen.
In **Hoofdstuk 8** wordt ingegaan op verschillende aspecten van glucocorticoïdgevoeligheid in relatie tot klinische uitkomsten bij kinderen met nefrotisch syndroom. De uitslagen van een 'low dose' dexamethason-suppressietest blijken niet gecorreleerd aan klinische uitkomsten. Hiervoor zijn verschillende verklaringen mogelijk, bijvoorbeeld dat de test mogelijk niet geheel representatief is voor gevoeligheid van de nog onbekende 'target cel(len)' bij nefrotisch syndroom. Daarnaast worden genetische variaties van het glucocorticoïdrecepor-gen onderzocht, waarvan bekend is dat deze gepaard gaan met verminderde dan wel toegenomen gevoeligheid voor glucocorticoïden. De genetische variatie 'GR-9beta' komt vaker voor bij kinderen met een ongunstig beloop van nefrotisch syndroom. Deze nieuwe bevinding wijst mogelijk op een actiever ziekteproces en/of verminderde gevoeligheid voor glucocorticoïden bij kinderen met nefrotisch syndroom die deze genetische variatie hebben. Indien deze associatie wordt bevestigd in andere studies, kan dit belangrijke gevolgen hebben voor de klinische praktijk.

DEEL V – REFLECTIE

In **Hoofdstuk 9** wordt teruggekeken op de studies in dit proefschrift, mede in het licht van nieuwe literatuur. Daarnaast worden mogelijkheden voor toekomstig onderzoek beschreven.

Eén van de belangrijkste bevindingen is dat het verlengen van de eerste behandeling met prednisolon het risico op recidieven niet verlaagt. Vanwege de sterke methodologische opzet van de studie kan dit belangrijke gevolgen hebben binnen het denken over de behandeling van deze ziekte. Toekomstige studies moeten zich richten op mogelijkheden om de behandeling verder te verfijnen en opnieuw vorm te geven.

Afgaand op eerder onderzoek lijkt intensiveren van de eerste behandeling te leiden tot grotere effectiviteit. De huidige behandeling bestaat uit een 'inductiefase', om het eiwitverlies te stoppen en een 'consolidatie'-fase, om dit effect vast te houden. Het staat vast dat prednisolon effectief is binnen de inductiefase. Het intensiveren van de

‘consolidatiefase’ van de eerste behandeling zou bijvoorbeeld kunnen bestaan uit het verhogen van de dosis prednisolon zonder verlenging van de behandelduur, of door toevoegen van aanvullende medicatie. De effectiviteit en veiligheid hiervan zal in toekomstige studies moeten worden onderzocht.

Belangrijke aandachtspunten voor toekomstig onderzoek zijn het waarborgen van de kwaliteit van onderzoek, goed gebruik van definities en de noodzaak tot nationale en internationale samenwerking. Zo kan op een efficiënte en betrouwbare manier worden gestreefd naar betere uitkomsten voor kinderen met nefrotisch syndroom.

Appendices

Dankwoord

Curriculum Vitae

List of Publications

PhD Portfolio

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A

Het klinkt als een cliché, maar wat is het toch een potje fijn om familie en vrienden om je heen te hebben. Ik heb menig ei bij jullie kwijt gekund, ook in tijden dat ik mezelf even moest onderdompelen in het onderzoek (lang leve de updates via what's app). Ik noem een paar namen in het bijzonder:

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Nynke

CURRICULUM VITAE

Nynke Teeninga werd geboren op 23 september 1981 te Nes, Ameland. In 2000 behaalde zij haar VWO diploma aan het Lauwers College in Buitenpost. Daarna studeerde ze een jaar pedagogische wetenschappen aan de Rijksuniversiteit Groningen. In 2001 startte ze met de studie geneeskunde aan de Vrije Universiteit in Amsterdam. De voorkeur voor het vak kindergeneeskunde was al snel duidelijk, wat resulteerde in de keuze voor het afstudeerprofiel Kind en Jeugd. Tijdens haar studie werkte ze in de particuliere thuiszorg en als student-docent voor de vakken anatomie en probleemgestuurd onderwijs. Ook nam ze met veel plezier plaats in verschillende commissies van de medische faculteitsvereniging, waaronder Toneelgroep Geneeskunde (TonG). In 2004 volgde een bijzondere klinische stage in The Apostolic Hospital in Banga Bakundu, Kameroen. Haar interesse voor wetenschappelijk onderzoek werd gewekt tijdens een wetenschappelijke stage in 2005, getiteld 'Influence of Intrauterine Growth Retardation on Nephrotic Syndrome', begeleid door dr. M.F. Schreuder. Daarna droeg zij tijdens haar keuze-coschap kinderendocrinologie bij aan het project 'Brain development after prenatal growth retardation; effects of growth hormone treatment' onder begeleiding van dr. H.M.A. de Bie. Na het artsexamen in 2007 startte ze met een promotietraject in het Erasmus Medisch Centrum - Sophia Kinderziekenhuis en het Leids Universitair Medisch Centrum. Onder supervisie van dr. J.E. Kist-van Holthe, dr. J. Nauta, prof. dr. A.J. van der Heijden en prof. dr. T. Van Gelder werkte zij gedurende vier jaar (2008-2009 en 2011-2012) aan dit landelijke onderzoek met als onderwerp het optimaliseren van de glucocorticoïd behandeling van nefrotisch syndroom bij kinderen. Ze werkte vanaf 2010 ruim een jaar als arts-assistent kindergeneeskunde in het Sophia Kinderziekenhuis. Per juli 2013 is zij in opleiding tot kinderarts in het Gelre Ziekenhuis te Apeldoorn (dr. B.T. Van Maldegem) en het Universitair Medisch Centrum Utrecht – Wilhelmina Kinderziekenhuis (dr. J. Frenkel, prof.dr. E.E.S. Nieuwenhuis). In haar vrije tijd houdt ze van reizen en optreden met het Concilium Hilaricum Paediatricum en a capella vocal group SingerG. Nynke woont samen met Jeroen Oomens in Amsterdam.

LIST OF PUBLICATIONS

Teeninga N, Schreuder MF, Bökenkamp A, Delemarre-van de Waal HA, van Wijk JA. Influence oflow birth weight on minimal change nephrotic syndrome in children, including a meta-analysis. *Nephrol Dial Transplant* (2008) 23: 1615–1620

Teeninga N, Op de Coul ME, Wolf BHM. Plotseling bleek en geel zien. *Praktische Pediatrie* (2008) 2: 131-135

Teeninga N, Willemze AJ, Emonts M, Appel IM. Acute illness following chicken pox: spleen infarction as a complication of varicella zoster infection. *Ned Tijdschr Geneeskd*. (2011) 155:A2987

Ruiter AFC, **Teeninga N**, Nauta J, Endert E, Ackermans MT. Determination of unbound prednisolone and cortisol in human serum and saliva by on-line solid-phase extraction liquid chromatography tandem mass spectrometry and potential implications for drug monitoring of prednisolone and prednisone in saliva. *Biomed Chromatogr* (2012) 26(7):789-96.

Teeninga N, Kist-van Holthe JE, van Rijswijk N, de Mos NI, Hop WCJ, Wetzels JFM, van der Heijden AF, Nauta J. Extending prednisolone treatment does not reduce relapses in childhood nephrotic syndrome. *J Am Soc Nephrol* (2013) 24 (1): 149-59.

Teeninga N, Guan Z, Freijer J, Ruiter AFC, Ackermans MT, Kist-van Holthe JE, van Gelder T, Nauta J. Monitoring prednisolone and prednisone in saliva: a population pharmacokinetic approach in healthy volunteers. *Ther Drug Monit, in press*

Teeninga N, Kist-van Holthe JE, van den Akker ELT, Kersten MC, Boersma E, Krabbe JG, Knoers NV, van der Heijden AF, Koper JW, Nauta J. Genetic and in vivo determinants of glucocorticoid sensitivity in relation to clinical outcome of childhood nephrotic syndrome. (submitted)

Teeninga N, Guan Z, Freijer J, Kist-van Holthe JE, Ackermans MT, van der Heijden AF, van Schaik RHN, van Gelder T, Nauta J. Population pharmacokinetics of prednisolone in children with nephrotic syndrome. (ready for submission)

A

PHD PORTFOLIO

Summary of PhD training and teaching

Erasmus MC Department	Pediatric Nephrology
PhD period	Jan 2008 – Dec 2009 and Feb 2010 – Feb 2012
Promotors	Prof.dr. A.J. van der Heijden
	Prof.dr. T. van Gelder
Co-promotors	Dr. J. Nauta
	Dr. J. Kist-van Holthe (LUMC/VUmc)

	Year	Workload (ECTS)
General academic courses		
Scientific Writing in English *Expertisecentrum Academisch Engels, Universiteit Leiden*	2008	2.0
Basic Methods and Reasoning in Biostatistics *Boerhaave Instituut, LUMC Leiden*	2008	1.4
Good Clinical Practice course (BROK) *ErasmusMC*	2009	1.0
In-depth courses and research meetings		
Annual Working Group Nephrotic Syndrome (WINS) meeting	2008-2012	0.5
Winterschool *Nierstichting Nederland*	2009	1.2
Regression Analysis *Boerhaave Instituut, LUMC Leiden*	2009	1.4
Repeated Measurements *Boerhaave Instituut, LUMC Leiden*	2009	1.4
Survival Analysis *Boerhaave Instituut, LUMC Leiden*	2009	1.4
Evidence based medicine sessions *ErasmusMC/Sophia*	2010	1.0
Weekly research meeting Pediatric Nephrology *ErasmusMC/Sophia*	2011-2012	3.5
Monthly Glucocorticoid Receptor research meeting *ErasmusMC*	2011-2012	1.1
Research Integrity *ErasmusMC*	2012	2.0
Principles of Clinical Pharmacology *National Institutes of Health Center (NIH), USA*	2011-2012	2.0

(Inter)national conferences		
42nd Annual ESPN (European Society of Pediatric Nephrology) meeting *Lyon (poster presentation)*	2008	1.4
Jonge Onderzoekersdag NVK (Nederlandse Vereniging voor Kindergeneeskunde) *Veldhoven (poster presentation)*	2008	0.6
Pediatric Nephrology Spring Meeting GPN (Gesellschaft für Pädiatrische Nephrologie) *Amsterdam (oral presentation)*	2009	0.6
15th Annual Congress meeting IPNA (International Pediatric Nephrology Association) *New York (poster presentation)*	2010	1.4
45th Annual ESPN meeting *Krakow (oral presentation and poster presentation)*	2012	1.4
34e Congres Kindergeneeskunde NVK *Veldhoven (oral presentation)*	2012	1.0
Symposia & seminars		
Congres Kindergeneeskunde *NVK*	2008-2012	2.0
Pediatrics reseach day *LUMC (poster presentation)*	2008	0.5
Nederlandse Nefrologiedagen	2008	0.5
Annual pediatrics research day *ErasmusMC/Sophia (oral presentation)*	2008	0.6
NWO Talentdag *NWO (Nederlandse Organisatie voor Wetenschappelijk Onderzoek)*	2009	0.3
TULIPS (Training Upcoming Leaders in Pediatrics and Science) open program	2009, 2011	0.4
Annual PhD day *ErasmusMC*	2011-2012	0.6
NephcEurope patient day *(oral presentation)*	2011	0.3
SOV (Sophia Onderzoekers Vertegenwoordiging) Meeting *ErasmusMC/ Sophia*	2012	0.1
Renal Seminar *Royal Free Hospital/GOSH London (oral presentation)*	2012	1.0
Teaching		
Pediatrics curriculum course *LUMC*	2008	0.6
Supervising Master's thesis Marie C. Kersten: *"Glucocorticoid sensitivity in Children with Nephrotic Syndrome"*	2011	4.0
Guest teacher IMC Weekendschool Rotterdam	2012	0.3
Other		
Peer review		
- Archives of Diseases in Childhood	2009	0.3
- Therapeutic Drug Monitoring	2013	0.5