Pediatric Graduate School
Hospital for Children and Adolescents
Helsinki University Central Hospital
University of Helsinki
Helsinki, Finland

Circulating Glucocorticoid Bioactivity and Serum Glucocorticoid-Responsive Biomarkers during Steroid Therapy in Children and Adolescents with Inflammatory Bowel Disease

Marianne Sidoroff

ACADEMIC DISSERTATION

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SUPERVISED BY

Docent Kaija-Leena Kolho, MD, PhD
Hospital for Children and Adolescents
Helsinki University Central Hospital
University of Helsinki
Helsinki, Finland

Docent Taneli Raivio, MD, PhD
Hospital for Children and Adolescents
Helsinki University Central Hospital
University of Helsinki
Helsinki, Finland

Institute of Biomedicine/Physiology
Biomedicum Helsinki
University of Helsinki
Helsinki, Finland

REVIEWED BY

Docent Timo Sane, MD, PhD
Department of Endocrinology
Helsinki University Central Hospital
University of Helsinki
Helsinki, Finland

Docent Juhani Grönlund, MD, PhD
Department of Pediatrics
Turku University Hospital
University of Turku
Turku, Finland

OFFICIAL OPPONENT

Docent Harri Niinikoski, MD, PhD
Department of Pediatrics
Turku University Hospital
University of Turku
Turku, Finland

Cover design & layout: Mirkka Hietanen

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ABSTRACT

Glucocorticoids (GC) have been used in a wide variety of inflammatory and immune conditions for over fifty years. In inflammatory bowel disease (IBD), ulcerative colitis (UC) and Crohn's disease (CD), they are an essential component of the treatment of acute flare-ups of the disease. The care of children with IBD follows the same principles than that of adults. Upon disease activation, standard treatment strategy is to use doses of 1-2 mg/kg/day of prednisone equivalents for 4-6 weeks and then taper. After 3 months, approximately 80% of the patients have responded positively to the therapy, however, the development of steroid dependency and resistance as well as treatment-induced side-effects are common. The aim of this study was to evaluate whether a new bioassay measuring GC bioactivity (GBA) directly from a small amount of patient serum could be used alone or in combination with GC-responsive biomarkers to optimize steroid therapy in paediatric IBD patients in such a way that the children would get the best possible benefit from the treatment with the least possible side-effects.

Sixty-nine paediatric IBD patients from the Paediatric Outpatient Clinics of the University Hospitals of Helsinki and Tampere participated in the studies. Control patients included 41 disease controls in remission and 101 non-IBD patients. Blood samples were withdrawn before the GC treatment was started, at 2-4 weeks after the onset of the steroid and at 1-month intervals thereafter. The development of GC-related side effects was carefully registered. GBA was measured with a COS-1 cell bioassay in which the cells were transfected with the GC receptor (GR) gene and the luciferase reporter gene. The cells were incubated with the patients' 10 µl serum sample, and the relative luciferase activity was read from the standard curve. The GC-responsive biomarkers analyzed included adipocyte-derived adiponectin and leptin, bone turnover-related collagen markers amino-terminal type I procollagen propeptide (PINP) and carboxyterminal telopeptide of type I collagen (ICTP) as well as insulin-like growth factor 1 (IGF-1) and sex hormone binding globulin (SHBG), and inflammatory marker high-sensitivity C-reactive protein (hs-CRP).

In the first study, an upper limit for endogenous serum GBA (118 nM cortisol equivalents, mean + 2SD) was established by measuring GBA in the 142 control children. In patients treated with systemic GCs, serum GBA showed a 4-fold increase after two weeks of therapy. In contrast, in patients treated with budesonide, only a 1.8-fold increase was seen after four weeks of treatment. In all patients, the GBA levels remained above the upper limit for endogenous serum GBA until the withdrawal of the steroid. GBA levels in patients less than 10 years of age were similar to the older patients, even though the young patients received higher weight-adjusted doses of prednisolone (1.3 vs. 0.79 mg/kg, $P<0.05$). The GBA levels were not related to the development of GC-related side-effects or to therapeutic response.

In the second study, the changes in the GBA levels and adipose tissue-derived hormones leptin and adiponectin were evaluated during GC therapy. Steroid treatment induced serum adiponectin (from 11.9 ± 1.5 μg/ml to 18.7 ± 1.8 μg/ml) and leptin (from 4.4 ± 0.9 μg/l to 7.7 ± 1.5 μg/l). Notably, after 2 to 4 weeks of therapy, the adiponectin levels were higher in 7 patients who developed acute GC-related side-effects (rounding of the cheeks) than in the 11 patients who did not (22.9 ± 2.6 μg/ml vs. 16.0 ± 2.1 μg/ml, $P<0.05$). Serum leptin levels indicated a similar trend. GBA levels were not related to adiponectin or leptin levels.

The third study examined GBA and bone turnover-related markers in children receiving systemic steroid therapy. Before GC treatment, PINP and IGF-1 levels were lower in children with active IBD compared with control children with IBD in remission. After 2 weeks of steroid therapy, serum IGF-I had increased from 23 to 37 nmol/l ($P<0.001$). In contrast, serum PINP levels had further declined, from 271 μg/l to 163 μg/l ($P<0.001$), ICTP from 14.2 μg/l to 9.6 μg/l ($P<0.001$) and SHBG from 54 to 35 nmol/l ($P<0.001$), respectively. After the withdrawal of the GC, all bone markers restored to levels similar to the controls. Serum GBA, response to GC treatment or the appearance of GC-related side-effects did not correlate with the markers of bone metabolism.

Finally, in the fourth study, the levels of the inflammatory marker hs-CRP were evaluated during GC therapy and in patients undergoing colonoscopy. The median pre-treatment CRP level of the 22 IBD patients starting peroral GC therapy was 0.6 mg/L (0.01-39), which decreased to 0.08 mg/L (0.004-60, $P<0.05$) after 2-4 weeks of treatment. The hs-CRP levels did not differ between patients that responded to steroid therapy and non-responders. The development of GC-related side-effects did not associate with serum CRP. Hs-CRP was not able to distinguish the patients with different disease activities.

Based on these findings, it can be concluded that even though the GBA levels rose in paediatric IBD patients receiving systemic GC treatment, they did not associate with the clinical response to GCs or with GC-related side-effects. In the light of these results therefore, the GBA measurement cannot guide the GC therapy in these patients. Of the biomarkers investigated, adipocyte-derived adiponectin associated with GC therapy-induced side-effects, and was the most promising marker of GC sensitivity. Markers of bone turnover and inflammation changed in response to the GC therapy; however, they did not correlate with the clinical response or GC therapy-related adverse events.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications that are referred to in the text by their roman numerals:

I **Vihinen MK**, Raivio T, Verkasalo M, Jänne OA, Kolho KL. Circulating glucocorticoid bioactivity during peroral glucocorticoid treatment in children and adolescents with inflammatory bowel disease. *Journal of Clinical Gastroenterology* 2008 42 1017-1024

II **Vihinen MK**, Kolho K-L, Janne OA, Andersson S, Raivio T. Circulating adiponectin as a marker for glucocorticoid-related side effects in children and adolescents with inflammatory bowel disease. *Journal of Pediatric Gastroenterology and Nutrition* 2009 48 504-506

III **Vihinen MK**, Kolho KL, Ashorn M, Verkasalo M, Raivio T. Bone turnover and metabolism in paediatric patients with inflammatory bowel disease treated with systemic glucocorticoids. *European Journal of Endocrinology* 2008 159 693-698

IV **Sidoroff M,** Karikoski R, Raivio T, Savilahti E, Kolho K-L. High-sensitivity C-reactive protein in paediatric inflammatory bowel disease. *World Journal of Gastroenterology* 2010 16 2901-2906

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ABBREVIATIONS

aa	amino acids
AAT	α1-antitrypsin
ACTH	adrenocorticotropic hormone
AF1	activation function 1
AF2	activation function 2
AP1	activating protein 1
ARIP3	androgen receptor interacting protein 3
5-ASA	5-aminosalicylate
BMD	bone mineral density
BMI	body mass index
CBG	cortisol-binding globulin
CD	Crohn's disease
cDNA	complentary deoxyribonucleic acid
CRH	corticotropin-releasing hormone
CRP	C-reactive protein
C.V.	coefficient of variation
DBD	DNA-binding domain
DDD	defined daily dose
DHPLC	denaturing high-performance liquid chromatography
DNA	deoxyribonucleic acid
DST	dexamethasone suppression test
ENaC	epithelial sodium channel
ELISA	enzyme-linked immunosorbent assay
ESR	erythrocyte sedimentation rate
FOXP3	forkhead P3
GC	glucocorticoid
GH	growth hormone
$GHbA_{1C}$	glycosylated haemoglobin
GITR	glucocorticoid-induced tumour necrosis factor receptor
GR	glucocorticoid receptor
GRE	glucocorticoid response element
hGR	human glucocorticoid receptor
HLA	human leukocyte antigen
HOMA-IR	homeostatic model assessment – insulin resistance
HPA	hypothalamic-pituitary-adrenal
HR	hinge region
hs-CRP	high-sensitivity C-reactive protein

hsp	heat shock protein
5-HT	hydroxytryptamin (serotonin)
I_m	maximum inhibition
IκBα	inhibitor of NFκB
IA	immunoassay
IBD	inflammatory bowel disease
IC	indeterminate colitis
ICTP	carboxyterminal telopeptide of type I collagen
IGF-1	insulin-like growth factor 1
IGFBP	insulin-like growth factor binding protein
K_d	dissociation constant
LBD	ligand-binding domain
LPS	lipopolysaccharide
MDR1	multi-drug resistance gene
mRNA	messenger ribonucleic acid
nd	no data
NFκB	nuclear factor κB
NLS1	nuclear localization signal 1
NLS2	nuclear localization signal 2
NSAID	non-steroidal anti-inflammatory drug
NTD	N-terminal domain
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PCR-RFLP	PCR-restriction fragment length polymorphism
PGA	physician's global assessment
PEPCK	phosphoenolpyruvate carboxykinase
PINP	amino-terminal type I procollagen propeptide
POMC	pro-opiomelanocortin
RIA	radioimmunoassay
RT-PCR	reverse transcription polymerase chain reaction
sgk	serum- and glucocorticoid-regulated kinase
SHBG	sex hormone-binding globulin
Th1	T helper 1
Th2	T helper 2
TNFα	tumour necrosis factor α
UC	ulcerative colitis
WBC	white blood cell count

INTRODUCTION

Glucocorticoids (GCs) are steroid hormones that originate from the adrenal cortex. The endogenous GCs, cortisone and corticosterone, are essential for life and have a large array of functions in the maintenance of body homeostasis during stress response, in protein, lipid and carbohydrate metabolism, and in the regulation of inflammatory and immune reactions. GCs exert their effects through binding to the intracellular glucocorticoid receptor (GR) that then affects the expression of GC-responsive genes by a number of different mechanisms.

Pharmacological GC therapy started with the successful treatment of rheumatoid arthritis in the 1940s, using cortisone acetate. Since then, the use of steroids has expanded, and today they form part of the treatment of various diseases in practically every organ system. In gastroenterology, inflammatory bowel disease is a central target.

Inflammatory bowel disease (IBD) encompassing Crohn's disease (CD) and ulcerative colitis (CD) is a chronic, relapsing and remitting inflammatory disorder of the gastrointestinal tract. Its cause is not known, but the current view is that it results from an exaggerated and inappropriate immune response to environmental antigens in a genetically susceptible host. Approximately 20% of the cases manifest during childhood, and the disease has a profound effect on the physical and psychological development of the growing child. No cure exists, but with the aid of nutritional, pharmacological, surgical and psychological treatment and support the patients can be ensured an as near-to-normal life as possible.

In IBD, GCs come into play upon disease activation when the patient complains of frequent, often bloody diarrhea, abdominal pain, weight loss and fever. In children, active inflammation may associate with growth failure. The therapy is initiated with a daily dose of 1-2 mg/kg of prednisone equivalents for 4-6 weeks and then tapered off in small steps. After three months, approximately 80% of the patients have obtained a positive clinical response. Unfortunately however, clinical improvement does not often associate with endoscopical and histological remission. In addition, the development of GC-related side-effects and steroid dependency and resistance are common.

In current clinical use, we do not have any assay or biomarker with which to predict the individual's response to exogenous GC therapy in advance. This would be vital in order to save this effective but potentially toxic treatment for patients who could actually benefit from it. The aim of this study was to thus evaluate whether a new bioassay measuring circulating GC bioactivity (GBA) directly from a small amount of human serum could be used alone, or together with GC-responsive biomarkers, to improve the benefit-to-risk ratio of paediatric IBD patients receiving systemic GC therapy.

REVIEW OF THE LITERATURE

1. Glucocorticoids – basic

GCs are steroid hormones that are synthesized from cholesterol in the zona fasciculata of the adrenal cortex in a circadian and stress-related fashion. The name *glucocorticoid* originally refers to the actions these hormones have on glucose metabolism, however the effects of GCs are now known to be far more widespread and essential for life (Guyton and Hall 2006). In DNA microarray analysis, 20% of the expressed human leukocyte genome was found to be affected by GCs, and the current view is that GCs are involved in nearly every molecular, cellular and physiologic network of the organism (Chrousos and Kino 2005).

1.1 Glucocorticoids

Cholesterol needed for steroid hormone synthesis derives mainly from circulating low-density lipoproteins. They enter the adrenocortical cells through coated pits and liberate the cholesterol which is then transported to mitochondria and endoplasmic reticulum to be processed further (Guyton and Hall 2006). Specific enzymes guide every step of the pathway that in addition to GCs results in the synthesis of aldosterone and sex steroids (Figure 1).

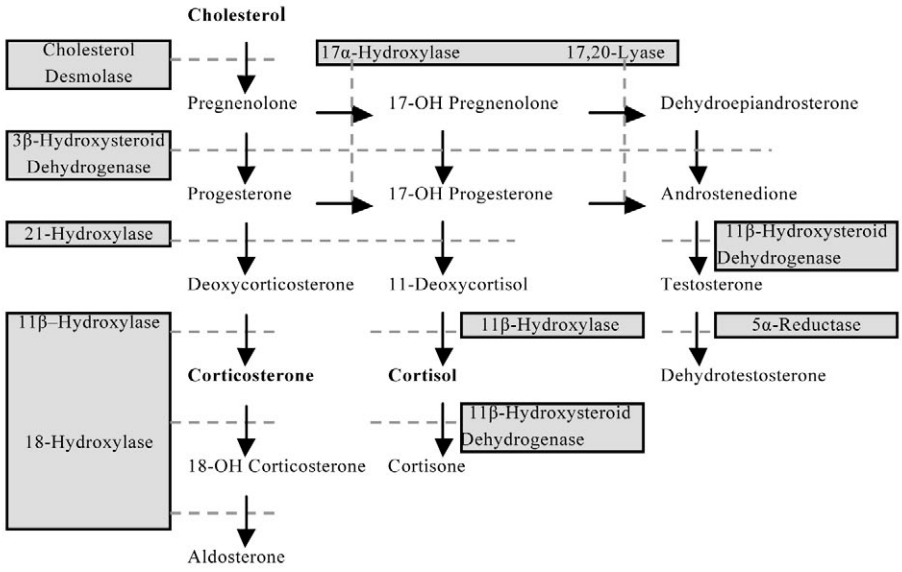

Figure 1 Steroid biosynthesis in the adrenal cortex (modified from Nimkarn et al. 2007)

The main endogenous GC hormone **cortisol** (hydrocortisone, Figure 2) accounts for 95 per cent of the GC activity of the body. The rest of the activity is divided between the much less potent **corticosterone** (4 per cent) and the possible circulating synthetic GCs. Over 90 per cent of the circulating cortisol is bound to cortisol-binding globulin (CBG), and to a lesser extent and affinity to albumin. Plasma protein-bound steroids might serve as a reservoir to reduce the rapid fluctuations of free hormone concentrations that would occur for example during stress and episodic adrenocorticotropic hormone (ACTH) secretion (Guyton and Hall 2006).

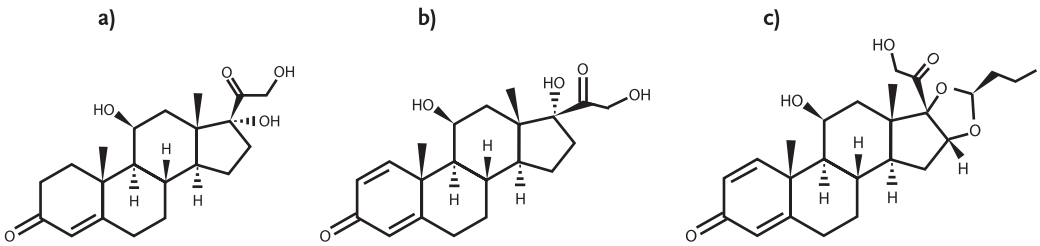

Figure 2 The structures of cortisol (a), prednisolone (b) and budesonide (c). Hydroxyl group at carbon 11 is necessary for the GH activity of the molecule. Therefore 11-keto compounds cortisone and prednisone need to be converted to their respective 11-hydroxyl counterparts to be operative (Axelrod 2003).

1.1.1 Cortisone-cortisol shuttle

11β-hydroxysteroid dehydrogenases (11β-HSDs) interconvert inactive cortisone and cortisol. 11β-HSD1 is expressed in many tissues including the liver, bone and adipose tissue and possesses both reductase and dehydrogenase activities. 11β-HSD2, in contrast, is expressed in mineralocorticoid target tissues, predominantly in the kidney, and catalyzes the conversion of cortisol to cortisone (Tomlinson et al. 2004). These enzymes are now considered to be significant regulators of hormone action at tissue level. Altered 11β-HSD1 activity has been implicated in the metabolic syndrome and both enzymes in modulating the functions of the immune system (Cooper and Stewart 2009). 11β-HSD2 expression has been reported in the macrophages and synovial tissue of patients with rheumatoid arthritis, which might render these tissues less sensitive to GC therapy (Rabbitt et al. 2008, Haas et al. 2007). On the contrary, increased 11β-HSD1 and decreased 11β-HSD2 expression has been found in both human patients with UC and rats with induced colitis (Žbánková et al. 2007).

1.1.2 Function in humans

GCs have effects on carbohydrate, protein and lipid metabolism of the organism, as well as on immune and inflammatory functions. In addition to the basal pulsatile circadian secretion, the main inducer of GC release from the adrenal cortex is stress, psychological or physical. Stress activates the hypothalamic-pituitary-adrenal (HPA) axis where the secretion of corticotropin-releasing hormone (CRH) from the hypothalamus activates ACTH secretion in the anterior pituitary. ACTH in turn increases the production and secretion of cortisol that exerts negative feedback at the brain, thus controlling its own secretion (Guyton and Hall 2006).

The net effect of GCs on glucose metabolism is an increase of blood glucose concentration due to increased gluconeogenesis by the liver and decreased glucose utilization by most cells of the body. Cellular protein stores diminish, with the exception of liver whose intake of amino acids (aa) and protein synthesis increase. The mobilization of fatty acids from the adipose tissue also augments, and their elevated concentration in the plasma enhances their utilization for energy (Guyton and Hall 2006).

The use of GCs in immunological and inflammatory conditions is based on their anti-inflammatory properties. At cellular level, GCs stabilize lysosomal membranes, decrease the permeability of capillaries and reduce the migration of white blood cells into inflamed areas (Guyton and Hall 2006). At molecular level, they favour innate immunological responses and Th2-driven humoral immunity, and suppress Th1 cellular immunity (Franchimont 2004).

1.2 Glucocorticoid receptor

GCs exert their functions through the GC receptor (GR, *NR3C1*) that belongs to the superfamily of nuclear receptors (Mangelsdorf et al. 1995). Nuclear receptors are intracellular transcription factors that together with cognate ligands and other proteins regulate the expression of target genes. The members of the family bear functional and structural similarity, and GR belongs to the subfamily 3 together with receptors for estrogen, progesterone, androgen and mineralocorticoids (Nuclear Receptors Nomenclature Committee 1999).

1.2.1 Glucocorticoid receptor gene

The human GR (hGR) gene is localized in the long arm of chromosome 5q31-32 (Francke and Foellmer 1989, Theriault et al. 1989). The gene has at least 13 different splice variants that derive from 9 different exon 1s (Figure 3). The transcripts contain 9 exons, the first of which is 5' untranslated region (Figure 3) (Encío and Detera-Wadleigh 1991, Breslin et al. 2001, Turner and Muller 2005, Presul et al. 2007). The alternative exon 1 variants are spliced to a common acceptor site at the beginning of exon 2, and do not alter the amino acid sequence of GR (McCormick et al. 2000, Turner and Muller 2005). However, the tissue expression profiles of the transcripts differ and it has been postulated that exon 1 variants with their own putative promoters might be a significant tissue, cell, and stimuli specific control mechanism of the transcription of hGR (Turner and Muller 2005, Turner et al. 2006).

Translation initiation codon is localized in exon 2. Exon 2 also contains the coding sequence for activation function 1 (AF1) that is essential for the receptor's transactivation capacity (Figure 3). Exons 3 and 4 each encode for their respective zinc finger motifs that constitute the DNA-binding domain (DBD) of the receptor. Exons 5 to 9 contain the sequence for the ligand-binding domain (LBD) in which the activation function 2 (AF2) is embedded (Encío and Detera-Wadleigh 1991).

The two GR isoforms, GRα and GRβ, arise from the alternative splicing of the exon 9 (Encío and Detera-Wadleigh 1991). GRα is considered the "classical" GR of 777 aa. Its shorter counterpart GRβ was originally thought to be unable to bind ligand, and considered merely as a dominant negative inhibitor of GRα-dependent transactivation (De Castro et al. 1996). However, more recent studies have shown that GRβ might be able to interact with GRα partial agonist/antagonist RU-486 and is able to regulate gene expression even in the absence of GRα (Kino et al. 2009a). The ability of GRβ to antagonize the effects of GRα led to the idea that GRβ may be involved in the tissue-specific sensitivity to GCs, and GRβ expression has been shown to be increased in GC-resistant asthmatics and patients with IBD (Hamid et al. 1999, Orii et al. 2002).

In 2005, Lu *et al.* reported that at least 8 different translation initiation sites exist for hGRα. These GRα isoforms were termed GRα-A, -B, -C1, -C2, -C3, -D1, -D2 and –D3 (Figure 3) (Lu et al. 2005). The generation of the different isoforms is caused either by ribosomal leaky scanning or ribosomal shunting, where the ribosome initiates translation of the mRNA from alternative translation initiation sites located in the exon 2 (Lu et al. 2005). Thus, the generated proteins differ from their amino-terminal region but have similar DNA- and ligand-binding domains. The expression levels of these GRα isoforms varied between different tissues, as did their transactivation capacity on synthetic GC response element (GRE) -driven promoter (Lu et al. 2005). In addition, every isoform had its own target-gene expression profile when cDNA microarray data were studied (Lu et al. 2005). It has been hypothesized that similar N-terminal receptor isoforms would also exist for GRβ, which would increase the amount of different N-terminal GR isoforms to at least 16 (Chrousos and Kino 2005). The discovery of these multiple hGRα and putative GRβ isoforms further increases the capacity of the GR to modulate its signal transduction.

Other GR isoforms identified until now include the GR-P (lacking aa encoded by exons 8 and 9) and GR-A (lacking aa from exons 5, 6 and 7) that have been detected at high levels in GC-resistant myeloma patients (Figure 3) (Krett et al. 1995, Moalli et al. 1993). GRγ, in contrast, is a receptor variant found for example in childhood lymphoblastic leukemia that has an insertion of an additional arginine in the intron between exons 3 and 4 (Rivers et al. 1999, Beger et al. 2003). The insertion of the arginine between the two zinc fingers of the DBD decreases the transactivation potential of the receptor by 50% (Beger et al. 2003, Ray et al. 1996).

In addition to the receptor isoforms, several polymorphisms have been found in the GR gene. Two of these, ER22/23EK and N363S, reside in the exon 2 and are reported to associate with relative GC resistance and sensitivity respectively (van Rossum and Lamberts 2004). The third polymorphism called BclI lies in the intron between exons 2 and 3. The data on BclI polymorphism has been controversial; at present however, the majority of the studies promote the idea that BclI is also linked to increased GC sensitivity (van Rossum and Lamberts 2004). In a small group of IBD patients, the frequency of the BclI polymorphism has been shown to be increased in patients with CD (Decorti et al. 2006). The fourth polymorphism, GR-9β, is a polymorphism in the exon 9β 3' nontranslated region. *In vitro* data indicate that the nucleotide substitution in this polymorphism causes the hGRβ mRNA to be more stable, which might lead to a relative GR resistance (Derijk et al. 2001).

1.2.2 Structure

Similar to other steroid receptors, the structure of hGR is modular, and its three different domains have distinct functions (Mangelsdorf et al. 1995).

The N-terminal domain (NTD, aa 1-420) is the least conserved and varies even between different hGR isoforms (Figure 3) (Kumar and Thompson 2005). NTD contains the AF1 domain that is responsible for much of the receptor's transcriptional activity. AF1 has been shown to be able to interact with the coregulatory proteins and general transcription machinery, and to significantly enhance the receptor's transcriptional capacity (Kumar and Thompson 2005). NTD also contains the GRs major phosphorylation sites that can further modulate the receptor's ability to affect target-gene transcription (Duma et al. 2006).

The median domain DBD (aa 421-526) is highly conserved and harbours the two zinc finger motifs that interact with the GREs on target genes (Duma et al. 2006). Beside the DBD lies the hinge region (HR) that allows the receptor to bend and change conformation (Tsai and O'Malley 1993). Upon ligand binding, the conformation of the receptor changes and uncovers the nuclear localization signal (NLS1) that is necessary for the recognition by the transport machinery of the cell (Hager et al. 2000).

The LBD (aa 527-777) is the site of hormone binding. The amino acids of this segment form a hydrophobic loop that closes when the ligand is bound, and the freshly shaped molecule presents new surface for coregulator binding. LBD contains the second nuclear localisation signal (NLS2) and activation function 2 (AF2) (Duma et al. 2006).

GR is a subject of post-translational covalent modifications that can further affect the receptor's activity (Figure 3). Phosphorylation of specific serine residues can either activate or repress the receptor's functions, whereas ubiquitination regulates the GC signalling pathway via controlling the degradation rates of GR (Wallace and Cidlowski 2001, Duma et al. 2006). Sumoylation of the receptor affects the GR in a promoter-context dependent fashion, either activating or repressing its function (Tian et al. 2002). Acetylation and methylation have also been shown to affect GR; however, their effect seems not to be direct but occurs through modulating the functions of core histones and coregulatory proteins (Duma et al. 2006).

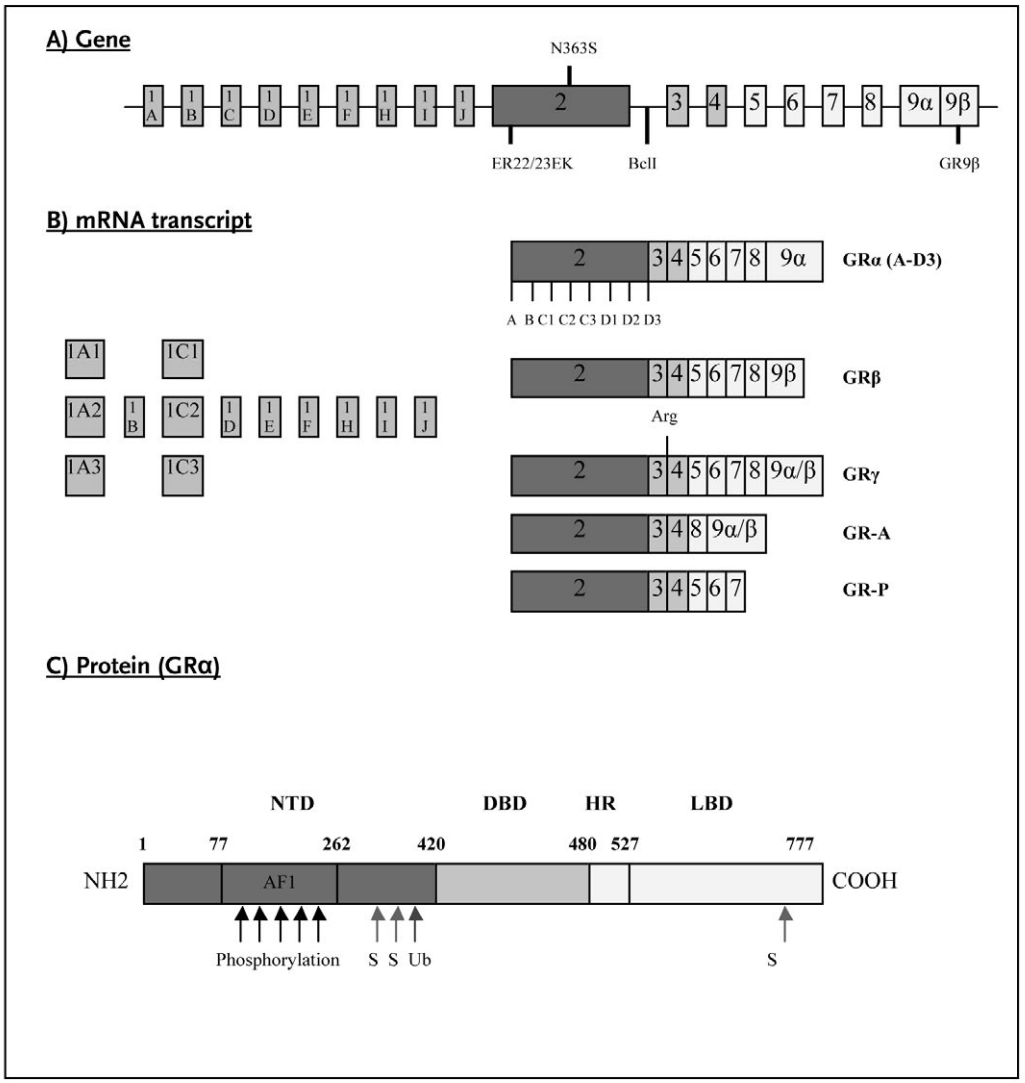

Figure 3 The structure of the GC receptor (GR): A) The GR gene consists of at least 9 alternative exon 1s (A-J) and 8 other exons. The polymorphisms of the GR are presented at their respective positions in the gene. B) The mRNA transcript variants of the GR gene. For GRα the presence of the different translation initiation sites for A-D3 isoforms is schematically presented. Arg, arginine. C) The full-length GRα, its main functional domains and the sites of post-translational covalent modifications: S, sumoylation; Ub, ubiquitination.

1.3 Mechanism of action

1.3.1 Genomic actions

Both natural and synthetic GCs are lipophilic proteins that enter the cells via diffusion. The GR resides mainly in the cytoplasm as a part of a multiprotein complex, with heat shock protein (hsp) 90s, hsp70, immunophilins, Cyp-40 and p23. Upon ligand binding, GR dissociates from the protein complex, partly homodimerizes and translocates to nucleus (Duma et al. 2006). The translocation through nuclear pores is an active process mediated by the NLS1 and NLS2 (Chrousos and Kino 2005). Once inside the nucleus, the ligand-receptor dimer transactivates or represses the expression of the target genes by binding directly to the positive or negative GREs on promoter regions. The AF1 and AF2 domains mediate the interaction of GR with nuclear receptor coregulator and chromatin remodeling complexes that ultimately affect the RNA II polymerase activity and thereby the transcription velocity of the GC responsive genes. The ligand-activated GRα monomers, in contrast, modulate the gene transcription by interacting with other transcription factors [activating protein 1 (AP1), nuclear factor κB (NFκB), p53 and others] and influencing their activity on their own target genes (Chrousos and Kino 2005).

1.3.2 Nongenomic actions

In addition to these classical genomic actions of GCs that require the modulation of gene expression and take from minutes to hours to be operative, other so-called nongenomic effects of GR are being investigated. Characteristic of the nongenomic actions is that they occur rapidly within seconds to minutes, and do not require the classical pathway of ligand-binding, translocalization to nucleus and binding to DNA target sequences (Stellato 2004). At present, the nongenomic effects of GR are divided into three categories: 1) nongenomic effects without receptor involvement, 2) nongenomic actions via classical intracellular receptors, 3) nongenomic actions via nonclassical receptors (Falkenstein et al. 2000). An example of the first category is that steroids have been shown capable of diffusing easily in the lipid membranes, thus possibly interacting with the ion channels and receptors within the membranes with no contact to their intracellular cognate receptors. The second category necessitates contact between ligand and receptor. However, treatment with transcription inhibitor does not abolish these effects, thus they are probably not mediated through traditional genomic ways but could for example activate different kinase pathways. The third category calls in the newly identified membrane-bound GR, the functions of which remain elusive as yet (Löwenberg et al. 2008).

2. Pharmacological glucocorticoid therapy

Pharmacological GC therapy was initiated with the first successful injection of cortisone acetate to patients with rheumatoid arthritis in 1948. Today, GCs are the most commonly prescribed anti-inflammatory preparations and exceed many other drugs in terms of the variety of applications and the number of patients treated (Schäcke et al. 2002). It is estimated that between 1% and 3% of the adult population worldwide use long-term GC therapy (Walsh et al. 1996, Van Staa et al. 2000). Statistics on drug consumption in Finland and other Nordic countries reveal that the use of steroids is still increasing and that more people are exposed to the effects of systemic GC therapy (Figure 4).

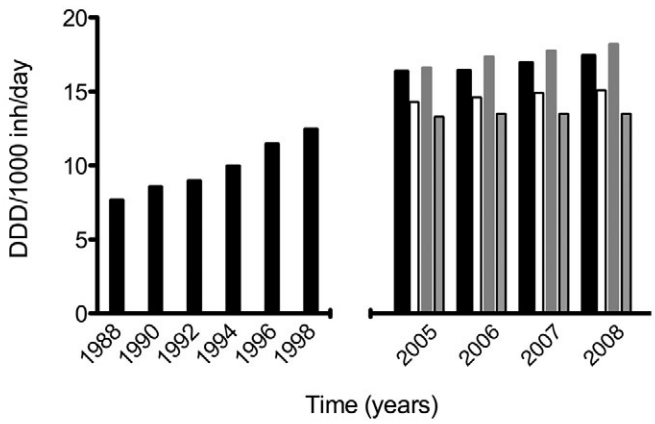

Figure 4 The use of systemic GCs in Finland (black bars), Sweden (white bars), Norway (grey bars) and Denmark (grey and black bars) between the years 1988-2008 expressed as defined daily doses per 1000 inhabitants per day (National Agency for Medicines, Apoteket AB/ Statistiksenheten, Nasjonalt folkehelseinstitutt, The Danish Medicines Agency).

Steroid therapy is utilized in inflammatory, autoimmune, collagen, renal, gastrointestinal, respiratory, nervous, hematologic, dermatologic and ophthalmic diseases, transplantations, neoplastic disorders, medical emergencies and infections. In addition, GCs are used in replacement therapy for patients with primary or secondary adrenal failure as well as in suppression therapy in congenital adrenal hyperplasia. The large array of applications reflect the wide range of effects that GCs have from inhibiting cell-mediated immune function to altering metabolic conversion of non-steroidal hormones from inactive to active forms (Stewart 2008).

Synthetic GCs are derivatives of the natural GC skeleton where the basic steroid nucleus or its side chains have been altered (Figure 2). These changes affect the pharmacokinetic properties of these compounds (absorption, metabolism, protein binding and tissue distribution) as well as their GC and mineralocorticoid potencies (Stewart 2008, Axelrod 2003). The characteristics of commonly used GC agents are presented in Table 1.

Table 1 The properties of synthetic GCs using cortisol as a standard (adapted from Rang et al. 2007). [1] Human foetal lung cells, [2] Budesonide data obtained from Edsbäcker et al. 2004.

Compound	Relative affinity for GC receptor[1]	Approximate relative potency in clinical use		Duration of action after oral dose
		Anti-inflammatory	Sodium retaining	
Cortisol	1	1	1	short (8-12)
Prednisolone	2.2	4	0.8	short (8-12)
Methylprednisolone	11.9	5	minimal	intermediate
Triamcinolone	1.9	5	none	intermediate
Dexamethasone	7.1	30	minimal	long
Betamethasone	5.4	30	negligible	long
Budesonide[2]	195	nd	nd	very short (2-4.5)
Aldosterone	0.38	none	500	-

2.1 Side-effects

GC therapy is associated with several side-effects (Figure 5) (Schäcke et al. 2002, Fardet et al. 2007, McDonough et al. 2008, Rhodes et al. 2008). Of patients treated with conventional doses of GCs, as many as 80-90% report at least one side-effect during therapy (Curtis et al. 2006). The adverse events vary in terms of severity from cosmetic (Cushingoid appearance) to life-threatening (gastric haemorrhage) (Schäcke et al. 2002). However, especially in children and adolescents, the less serious, "merely cosmetic" side-effects can be a cause for great anxiety, and the more severe events (osteoporosis, cataracts) go initially unrecognized (Huscher et al. 2009). Also, we have no information as to whether the appearance of the frequently observed, GC treatment-related "mild" side-effects early on during therapy could be a sign of underlying GC sensitivity, and possibly predict either the treatment success and/or the appearance of more severe adverse events.

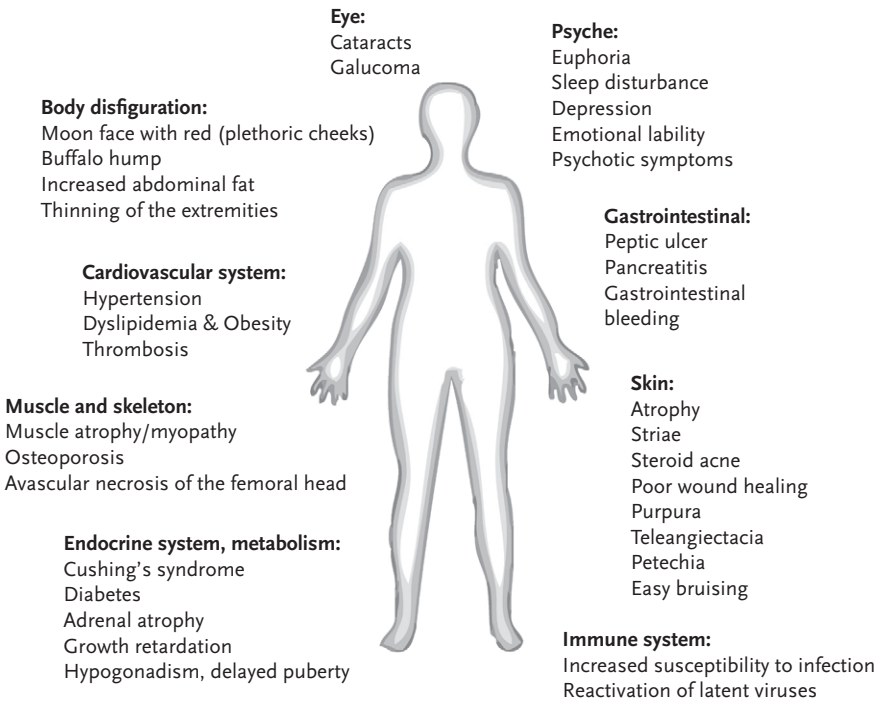

Figure 5 GC-related side-effects (Schäcke et al. 2002, Fardet et al. 2007, McDonough et al. 2008, Rhodes et al. 2008).

2.1.1 Dose and duration

The frequency of the reported adverse events increases with dosage, or when the treatment period extends while the dose remains constant (Huscher et al. 2009, Curtis et al. 2006). The early days of GC treatment saw a myriad of GC-related adverse events linked to high dose steroid therapy. Since then, the principle of *primum non nocere* in GC therapy has translated into the attempt to treat patients with as low effective dose as possible. Historically, doses less than 7.5-10 mg/day of prednisone equivalents have been considered low due to decreased rates of adverse events (Da Silva et al. 2005). There has been some controversy regarding the true incidence of the GC-related side-effects, especially in this low dose group, due to limitations in the study design as well as the confounding effects of the underlying disease (Da Silva et al. 2005). However, a number of studies do support the concept that even doses less than 10 mg/day of prednisone equivalents are associated with increased frequency of fractures, cataracts, skin bruising, acne, weight gain and cushingoid phenotype and increased risk of infections and fractures, especially if the treatment is long (Curtis et al. 2006, Wolfe et al. 2006, Vestergaard et al. 2008, Huscher et al. 2009).

2.1.2 Side-effects in paediatric patients

Children may be even more sensitive to the GC-related side-effects than adults. In a Japanese report, paediatric patients with UC had higher incidence of osteoporosis, glaucoma and cataracts after steroid therapy than adults, even when adjusted to weight-based dosing (Uchida et al. 2005). In addition, in children major GC-related side-effects occurred at a significantly lower preoperative dosing of steroids (Uchida et al. 2005). A special concern in the paediatric population receiving steroid therapy is growth. In children with CD, 19% of the patients receiving low-dose prednisone (0.1 – 0.4 mg/kg/day) treatment were found to have growth suppression, even though their growth velocity before GC therapy was normal. Also, fracture risk in paediatric patients requiring 4 or more courses of oral GCs or using 30 mg prednisolone or more each day has been shown to be increased (Van Staa 2003).

2.1.3 Mechanisms of the side-effects

Today, the commonly agreed view is that the desired anti-inflammatory effect of GC therapy is mediated through transrepression, and that the majority of the unwanted events come via transactivation or non-genomic repression (Schäcke et al. 2002, Buttgereit et al. 2005).

However, at present the exact molecular mechanisms for most of the GC effects remain unclear (Table 2).

Table 2 Common GC therapy induced side-effects and the possible mechanisms behind them (adapted from Schäcke et al. 2002). x, mechanism mediating the GR effect; s, suspected mechanism of the GR effect.

		Mechanism		
		DNA-dependent		DNA-independent
Side-effect	Molecule	Transactivation	Transrepression	Repression
Osteoporosis	Osteoblast/ osteocyte apoptosis	x		
	Osteocalcin		x	
	Type I collagen			s
Skin atrophy	Type I collagen			s
	Type III collagen		s	s
Decreased wound healing	Down-regulation of proinflammatory genes			x
Muscle atrophy/ myopathy	Components of the ubiquitin-proteasome pathway	s		
Suppression of HPA axis	CRH			x
	POMC/ACTH		x	
Psyche	5-HT_{1A}receptor			x
Glaucoma	Fibronectin	s		
	Type IV collagen	s		
	Type I collagen	s		
Hypertension	αEnaC	x		
	sgk	x		
Diabetes mellitus induction	AAT PEPCK	x x		

3. Glucocorticoids in paediatric inflammatory bowel disease

3.1 Paediatric inflammatory bowel disease

Inflammatory bowel disease (IBD) comprising ulcerative colitis (UC) and Crohn's disease (CD) is an immune-mediated chronic disorder that results in relapsing inflammation of the gastrointestinal tract. Although the exact pathogenetic mechanism behind the onset of

IBD remains unknown, the generally accepted hypothesis is that it occurs in a genetically susceptible host as a result of an inappropriate and exaggerated mucosal immune response to ubiquitous environmental antigens including commensal microflora.

Approximately 25% of IBD cases manifest during childhood, the mean age at diagnosis in paediatric patients being around 13-14 years (Griffths 2004, Turunen et al. 2006). The incidence of paediatric IBD is increasing; in Finland between the years 1987 and 2003 the incidence almost doubled to 7.0/100 000 (Turunen et al. 2006). The cause for this rise in the incidence rates in the Western world remains obscure; however, the role of some environmental and other risk factors as potential contributors to the onset of the disease has been extensively discussed (Table 3).

Table 3 The role of environmental and other risk factors that have been associated with the incidence of IBD (Saeed and Kugathasan 2008).

Risk factor	Ulcerative colitis	Crohn's disease
Smoking	Protective	Predisposing
Specific infectious agents	Unclear	Unclear
Intestinal commensal flora	Predisposing	Predisposing
Appendectomy	Protective	Predisposing
Breast feeding	Unclear	Unclear
Dietary factors	Unclear	Unclear
Drugs Oral contraceptives NSAIDs	 nd Predisposing	 Predisposing Predisposing
Sedentary lifestyle	Predisposing	Predisposing

3.1.1 Pathogenesis

As reported above, the aetiological trigger behind the onset of paediatric IBD is still unknown. However, the heterogeneity of the disease phenotypes does imply that IBD is a polygenic disorder in which the disease susceptibility loci and other disease-modifying genes together with environmental factors customize the specific disease subtype of an individual patient.

A number of genes have been associated with the development and/or severity of IBD. In Crohn's disease, one of the most studied candidates is the NOD2/CARD15 gene located in the IBD1 locus on chromosome 16 (McGreal and Cho 2008). NOD proteins NOD1 and NOD2 are intracellular pattern recognition receptors that serve the innate

immune system as bacterial sensing molecules (McGreal and Cho 2008). Carriage of the NOD2/CARD15 mutations increases the susceptibility of developing CD, and in children has been associated with ileal disease location and increased risk of small bowel surgery (McGreal and Cho 2008). 20 to 65% of the children with ileal Crohn's disease have been estimated to carry at least one NOD2/CARD15 mutation (McGreal and Cho 2008). Another actively investigated gene is the interleukin 23 receptor (IL23R) gene on chromosome 1. An uncommon coding variant of the IL23R gene has been shown to be protective against both UC and CD; however, the exact role of the IL-23 pathway in the inflammatory pathogenesis is still unclear (McGreal and Cho 2008). Other genes linked to IBD are for example the SLC22A4/A5 variants, DLG5 gene variants, polymorphisms in the HLA type, multi-drug resistance (MDR1) gene variants and polymorphisms in the NFκB1 promoter (McGreal and Cho 2008).

The gastrointestinal tract harbours the most numerous collections of immune cells in the body. Its antigenic load of food, commensal flora and microbial pathogens is massive, and since it is the body's largest interface with the external milieu, the intestinal mucosa has to orchestrate the pro- and anti-inflammatory responses in order to protect the organism from harmful bacteria and at the same time regulate itself to avoid uncontrolled immunological activation. By nature, these functions are complex and interrelated and it is somewhere along this pathway that IBD develops.

At least three different immunity-related mechanisms have been proposed as being involved in the development of IBD. Epithelial barrier – the continuous single layer of epithelial cells lining the gastrointestinal tract sealed by tight junctions – has been shown to be abnormally permeable in patients with CD and their first degree relatives (Faubion and Fiocchi 2008). This might lead to an excessive antigen stimulation of the submucosal immune cells and eventually to mucosal inflammation. In addition, multiple lines of evidence suggest that the innate immune system is dysfunctioning in IBD patients. One of the most discussed theories is the existence of the above-mentioned NOD2/CARD15 mutations that might lead to defective responsiveness to enteral bacteria, possibly promoting chronic inflammation (Faubion and Fiocchi 2008). Also adaptive immunity plays a role in the development of IBD, supported merely by the fact that established and emerging IBD therapy is directed against Th1-type cytokines and effector T cells (Ardizzone et al. 2005).

3.1.2 Clinical presentation

Common clinical features in children with IBD include abdominal pain, weight loss, rectal bleeding and diarrhoea. However, the symptom patterns differ between UC, CD and IC (Table 4). Importantly, the presence of arthritis, fever, non-specific rash, weight loss or perirectal abscess can be the only findings of paediatric IBD (Blank and Keljo 2008).

Table 4 Symptom frequency (%) in newly diagnosed patients with inflammatory bowel disease (adapted from Sawczenko and Sandhu 2003).* Data obtained from Langholtz et al.

Symptom	Crohn's disease (n=379)	Ulcerative colitis (n=172)	Indeterminate colitis (n=72)
Abdominal pain	72	72	75
Diarrhea	56	74	78
Rectal bleeding	22	84	68
Weight loss	58	31	35
Fatigue	27	12	14
Anorexia	25	6	13
Fever*	48*	34*	nd
Arthritis	7.5	6	4
Nausea / Vomiting	6	0.5	1
Constipation / Soiling	1		
Anal fistula	4.5		
Growth failure / Delayed puberty	4		1
Anal abscess, ulcer	2		
Erythema nodosum, rash	1.5	0.5	
Liver disease	0.8	3	3
Toxic megacolon		0.5	

3.1.3 Clinical course

At present, there is no cure for IBD and the long term health outcome of the affected child is dependent on the disease activity, response to treatment, the possible side-effects brought about by the used therapeutic modality and the patients and their families ability to develop adaptation and coping strategies.

Paediatric IBD differs from adult-onset disease in that the disease is often more extensive, presenting as pancolitis in UC and colitis or ileocolitis in CD (Heyman and Gupta 2008, Vernier-Massouille et al. 2008). In paediatric CD, isolated ileal disease is rare (~10%) (Vernier-Massouille et al. 2008). Risk factors for surgery in CD include stricturing disease phenotype at diagnosis and GC treatment; the overall risk being around 30-40% at 5-8

years from diagnosis (Vernier-Massouille 2008, Turunen et al. 2009). In paediatric UC, the risk of surgery is significantly higher than in adults. In Finland, 24% of the young adults diagnosed with pediatric onset UC a median of 8 years earlier have undergone surgery (Turunen et al. 2009). Other studies report similar figures (Gower-Rousseau et al. 2009).

The onset of a lifelong debilitating illness in adolescence, during a critical developmental period, is a major psychosocial stress factor. Therefore, paediatric patients with IBD are at risk of social isolation, depression and anxiety (Karwowski et al. 2009). Their parents report that the patients have more emotional and social problems and lower competence than their population-based peers (Väistö et al. 2010). Young adults diagnosed with IBD a median of 8 years previously report a decreased quality of life (Turunen et al. 2009). Strategies to improve the well-being of the patients include psychosocial interventions and psychotherapy, social support, education and self-management (Karwowski et al. 2009).

3.1.4 Therapeutic options

Goals for the therapy in paediatric IBD are the induction and maintenance of remission, prevention of cancer of the affected bowel, facilitation of normal growth and development and improvement of the quality of life (Carvalho et al. 2007). The choice of treatment depends on disease subtype, localization and associating presenting features such as weight loss and short stature (Sandhu et al. 2010). In moderate to severe IBD, the current therapeutic strategy may involve 5-aminosalicylate (5-ASA) preparations, systemic and topical GCs, immunomodulators (e.g. 6-mercaptopurine, azathioprine, methotrexate and cyclosporine), nutritional interventions, biologic therapy and surgery (Markowitz et al. 2008).

5-ASA preparations sulfasalazine and mesalamine are a widely used first-line treatment of paediatric IBD (Sutherland et al. 2006a, Sutherland et al. 2006b, Moyer 2008). The precise mechanism of action of these drugs is as yet unclear; however, one of the most important effects is probably the inhibition of the cyclo-oxygenase that impedes the production of pro-inflammatory prostaglandins and leukotrienes (Moyer 2008). In UC, 5-ASA preparations are used in the induction and maintenance of remission (Sandhu et al. 2010). In CD, aminosalicylates are employed in the induction of remission; however, their role in the maintenance of remission is still unclear (Wilson et al. 2010).

Nutritional management of children with IBD is essential and the nutritional status of the patient should be addressed at each control visit (El Matary and Zachos 2008). In addition, exclusive enteral feeding with polymeric or elemental formulas may be used in the induction of remission in CD patients, especially if the patients present with growth failure (Sandhu et al. 2010).

Immunomodulators, mainly azathioprine or 6-mercaptopurine, are employed in patients with moderate to severe colitis that are refractory or unresponsive to induction therapy with salicylates and steroids, or fail to wean off GC treatment (Prefontaine et al. 2009a, Prefontaine et al. 2009b, Timmer et al. 2007, Sandhu et al. 2010). These agents are immunosuppressive and lymphocytotoxic and the measurement of the thiopurine S-methyltransferase (TPMT) alleles is encouraged before their introduction to avoid serious side effects (Cuffari 2008). Methotrexate is another option that can be considered for treatment-resistant patients (Alfadhli et al. 2004, Patel et al. 2009, Sandhu et al. 2010).

Infliximab is the most studied preparation of the new biologic pharmaceuticals and has been established in the treatment algorithm of refractory CD (Akobeng and Zachos 2004, Behm and Bickston 2008, Rutgeerts et al. 2009). Infliximab is a chimeric monoclonal antibody to TNF-α, and in adults it has been shown to induce and maintain remission (Wilson et al. 2010). However, studies in paediatric patients are still few, and with the risk of rare but serious adverse events (sepsis, increased risk for hepatosplenic T-cell lymphoma), at the moment infliximab is reserved for patients with severe disease that are unresponsive to conventional therapy and for whom surgery is not recommended (Wilson et al. 2010).

3.2 Glucocorticoid therapy in paediatric IBD

GCs have been used in the treatment of active IBD since the 1950s (Truelove and Witts 1955). Today, they are utilized in the induction of remission in moderate to severe IBD in both paediatric and adult populations (Rufo and Bousvaros 2006, Domènech 2006). Strikingly few studies have focused on the optimal dose or dosage tapering for GC treatment in adult patients (Baron et al. 1962, Rutgeerts 2001). In children, the treatment strategy has been extrapolated from the experience in adults. At present, the recommended dose for traditional GCs (prednisone, prednisolone) in paediatric patients is 1-2 mg/kg/day prednisone equivalents (max 60 mg/day) for ~2 weeks followed by a tapering period of 4-8 weeks (Rufo and Bousvaros 2006). Maintenance therapy or alternate-day therapy with steroids are generally not recommended (Rufo and Bousvaros 2006, Escher et al. 2003). A newer synthetic GC, budesonide, that has high affinity for the GR, enhanced first-pass metabolism and low systemic bioavailability has been studied in the treatment of CD (McKeage and Goa 2002, Rufo and Bousvaros 2006). A recent Cochrane review, however, stated that budesonide seems to be less effective than conventional steroids in the treatment of CD, specifically if the patients have more extensive colonic involvement or severe disease (Seow et al. 2008).

3.2.1 Response to glucocorticoid therapy

Studies investigating the response rates to oral GCs in paediatric IBD differ in their primary outcome measures and in how the response, lack of response and steroid dependency are defined. In addition, therapeutic response in a heterogenous disease such as IBD is influenced by the duration of the disease, disease behaviour and disease severity. This makes the comparison between different studies challenging. Two recent studies on newly diagnosed paediatric (<16 years of age) UC (n=97) and CD (n=109) patients found that after 3 months of treatment with conventional steroids (oral prednisone or i.v. methylprednisolone 1-2 mg/kg/day) around 85% of the patients showed inactive/mild disease or complete/partial response respectively (Hyams et al. 2006, Markowitz et al. 2006). After 1 year, 50-60% of the patients were defined as GC responsive, 30-40% as GC dependent and 5-8% had required surgery (Hyams et al. 2006, Markowitz et al. 2006). In these studies the outcome measure was clinical: physician global assessment (PGA) and/or the continuation or successful withdrawal of GC therapy. However, an older study by Beattie *et al.* showed that clinical response does not often correlate with endoscopic or histologic findings. After 8 weeks of GC therapy, 85% of the paediatric patients demonstrated complete remission in clinical disease activity, but only 40% showed complete endoscopic and 15% full histological remission (Beattie et al. 1996). In adult studies, the clinical response rates to GC therapy have been similar to children, although the number of steroid-resistant patients has been higher, possibly due to longer disease duration or more severe disease (Table 5).

Table 5 Steroid resistance in adult patients with IBD (modified from Creed et al. 2007).

Trial	No. of Pat.	Disease/ treatment	Severity	Remission	Partial response	Resistance
Truelove and Witts 1955	109	UC / oral cortisone	Mild, moderate & severe	41.3%	27.5%	31.2%
Truelove et al. 1978	87	UC / iv steroids	Severe	60%	15%	25%
Chakravarty 1993	89	UC / iv steroids	Severe	72%	-	28%
Munkholm et al. 1994	109	CD / oral steroids	Moderate & severe	48%	32%	20%
Travis et al. 1996	49	UC / iv steroids	Severe	42%	31%	27%
Lindgren et al. 1998	97	UC / iv steroids	Severe	40%	26%	34%
Faubion et al. 2001	63	UC / oral or iv steroid	Moderate & severe	54%	30%	16%
	74	CD / oral steroids	Moderate & severe	58%	26%	16%

3.3 Glucocorticoid sensitivity/resistance in IBD

Only 40-70% of the patients with active IBD enter clinical remission during GC treatment (Table 5), and around 20% show resistance to the therapeutic effects of the administered steroids. Unfortunately, the population that fails to respond to GC therapy might still suffer from the adverse effects associated with steroid treatment. Thus, GC sensitivity differs between individuals, and between cells and tissues of a single subject.

3.3.1 Ligand availability

The first possible step that could influence the patient's response to administered GCs is the absorption and/or metabolism of these agents. However, the absorption of prednisolone in paediatric IBD patients with both active and quiescent disease seems unaffected (Olivesi 1985, Faure et al. 1998, Schwab et al. 2001). Once in the circulation, GCs bind to CBG. Only the unbound, "free" GCs are capable of diffusing through the cell membranes to exert their actions, and thus the relative concentration of transcortin is another determinant of GC activity (Breuner and Orchinik 2002). Reduced levels of CBG have been found in diverse inflammatory conditions, as well as during stress and hypercortisolemia (Marques et al. 2009); however no alterations in the CBG levels of IBD patients have been reported.

The metabolism of the administered GCs by 11β-HSD enzymes could alter the ligand availability in IBD patients. However, studies on 11β-HSD1 and 11β-HSD2 expression in IBD have shown that the former enzyme is upregulated and latter downregulated in the inflamed tissues (Žbánková et al. 2007, Stegk 2009). Thus, the conversion of the GCs is predominantly from inactive to active form. Therefore, if the steroids are administered as an active form, as in our study, the variable metabolism of the GCs by the dehydrogenases should not alter the ligand availability.

3.3.2 Access to the target cells

GCs enter their target cells mostly via passive diffusion. There is, however, an active mechanism, drug-efflux pump P-glycoprotein 170 that transfers GCs and other agents out of lymphocytes and intestinal epithelial cells. The expression of the multi-drug resistance gene (MDR1) that codes for the pump protein was previously shown to be elevated in IBD patients requiring bowel resection for failed medical therapy (Farrell et al. 2000). More recently, however, the contribution of the MDR1 to GC sensitivity in IBD patients has been questioned (Annese et al. 2006).

3.3.3 Glucocorticoid receptor in IBD

No IBD patients with primary GC resistance caused by mutations in the GR gene and leading to hypertensive, hyperandrogenic disorder characterized by high serum cortisol levels have been described in the literature. However, since the late 1990s, more subtle alterations in GR number and structure that could affect the therapeutic response to GC therapy have been reported (see below).

3.3.3.1 Altered numbers of glucocorticoid receptors

The number of GRs in the peripheral blood mononuclear cells (PBMC) and colonic mucosal cells of IBD patients has been evaluated by various researchers (Table 6). The results have been conflicting. This probably reflects the different study designs applied in these studies, as well as the cells' dynamic potential to up- and downregulate the expression of the GR and its cofactors in different situations. Rogler *et al.* (1999) suggested that the GR numbers are decreased in the colonic mucosa of all IBD patients, lower in the PBMC of the steroid-treated patients, and similar to controls in the PBMC of the IBD patients not on steroids. Flood *et al.* (2001), in contrast, reported GR mRNA levels to be higher in IBD patients than in the controls. A third study stated that no difference in the expression of GR mRNA between IBD patients and the controls could be seen (Raddatz et al. 2004). In other diseases, altered GR numbers and affinity have been associated with response to GC treatment; in IBD however, more studies would be needed to answer these questions.

3.3.3.2 Altered affinity to the ligand

Another mechanism for varying responses to administered exogenous steroids could be the altered affinity of the steroid receptor to its ligand. In IBD, Shimada *et al.* (1997) found that in the PBMC of steroid-resistant patients the affinity of the GR to GCs was decreased (Table 6). Similarly, in another study on patients with mild to moderately active IBD, both steroid-treated and steroid-free IBD patients had a higher dissociation constant of GR in their PBMC than normal controls (Schottelius et al. 2000). Even though these studies are relatively small, they do promote the hypothesis that systemic inflammation present in active IBD could alter the affinity of the GR to its ligand, a phenomenon seen for example in severe asthma (Irusen et al. 2002).

Table 6 Studies evaluating GR number and affinity in peripheral blood mononuclear cells (PBMC) and colonic mucosa and their relationship to GC therapy response in adult IBD patients. Dex, dexamethasone.

	Pat.	Dis.	Objective	Method	Results
Shimada et al. 1997	11	UC	To study the number of GR and their affinity to ligand in PBMC	Whole cell [^{3}H]dexamethasone binding assay	Steroid non-responders (n=6) had higher number of GR binding sites than responders (n=5) or controls and lower affinity of GR to ligand than responders.
Rogler et al. 1999	31 18	CD UC	To study GR receptor numbers in PBMC and colonic mucosa	Cytosolic [^{3}H]dexamethasone radioassay	Mucosal GR levels were decreased in IBD patients when compared with controls. In steroid-treated IBD patients GR numbers were lower in PBMC.
Schottelius et al. 2000	22 17	UC CD	To investigate the expression of GR and their apparent dissociation constant in PBMC of IBD patients	Whole cell [^{3}H]dexamethasone binding assay	In IBD patients the K_d was constantly higher than in the healthy controls. IBD patients not on steroid therapy had higher GR levels than steroid-treated IBD patients or controls.
Flood et al. 2001	21	UC	To study GR mRNA levels in peripheral leukocytes and the effect of low-dose dex treatment on them	Quantitation of GR mRNA in solution hybridization assay	GR mRNA levels were higher in IBD patients than in the controls. The mRNA levels were the same between steroid responders (11) and non-responders (10).
Raddatz et al. 2004	33 21	UC CD	To study GR mRNA expression in PBMC and colonic mucosa of IBD patients and whether they correlate with disease activity or can predict GC treatment response	Quantitation of GR mRNA by RT-PCR, immuno-histochemistry	Systemic and local GR mRNA expression was similar to controls; however in the mucosa of UC patients with impaired GC response the levels were down-regulated. GR immunoreactivity was found in immune and epithelial cells.

3.3.3.3 Glucocorticoid receptor isoforms α and β

The expression of GR isoforms α and β has also been studied in IBD patients (Table 7). A number of reports support the concept that the GRβ receptor isoform is a possible determinant of GC sensitivity in IBD patients. GRβ expression has been shown to be increased in PBMCs as well as colonic mucosal cells of GC resistant IBD patients (Honda et al. 2000, Orii et al. 2002, Zhang et al. 2005, Towers et al. 2005, Fujishima et al. 2009). However, the largest study on the subject, of 86 IBD patients, arrived at opposing results (Hausmann et al. 2007). Although the differences in the clinical setting could explain the divergent results, the precise role of the GRβ isoform in IBD as well as in other diseases is yet to be fully clarified (Kino et al. 2009b).

Table 7 Studies assessing the association between GC therapy response and the number of GRα and GRβ in adult IBD patients.

	Pat.	Dis.	Objective	Method	Results
Honda et al. 2000	23	UC	To study whether RT-PCR analysis of hGRβ mRNA in PBMC of UC patients can predict the response to GC therapy.	GRα and GRβ analysis by mRNA RT-PCR, Western-blot	GRβ was positive in 83.3% of the GC non-responders (n=12) and in 9.1% of the GC-responsive patients (n=11).
Orii et al. 2002	34 13	UC CD	To quantify the levels and serial changes of PBMC GR mRNA in IBD patients.	Real time fluorescence monitored PCR	GRβ mRNA expression was increased in active UC. GRβ mRNA expression was also higher in GC-resistant than GC-sensitive UC.
Hori et al. 2002	17	CD	To quantify the levels of GR mRNA in PBMC.	RT-PCR	GRα and GRβ mRNA levels were lower in patients with CD than in the controls. The longer the disease duration, the lesser the amount of GRα mRNA in PBMCs of the patients.
Zhang et al. 2005	25	UC	To study the expression of GRα and GRβ in colonic mucosal cells and their correlation with response to GC therapy and inflammation.	Immuno-histochemistry	The expression of GRα was positively associated with response to GC treatment, whereas GRβ associated negatively with the response to GCs.
Towers et al. 2005	42	CD	To assess the expression of GRα and GRβ in PBMCs of CD patients and to study their relationship to the response to GC therapy.	RT-PCR using real-time PCR techniques, IL-18 levels were measured with ELISA.	GRβ mRNA expression was significantly higher in GC non-responders with active CD. In these patients, GRβ mRNA correlated directly with IL-18 levels measured from the serum.
Hausmann et al. 2007	86	IBD	To study whether GRβ expression in PBMCs of IBD patients could serve as a predictor of GC response	RT-PCR	GRβ expression was similar between GC-treated and non-GC-treated patients and between GC-responders and non-responders.
Fujishima et al. 2009	38	UC	To study the relationship between the frequency and type of infiltrating cells expressing GRα, GRβ and Foxp3 in biopsied colonic mucosa and the GC responsiveness of UC patients.	Immuno-histochemistry, RT-PCR.	GC non-responders had higher $GR\beta^{+}$cell count than GC-responders. The $Foxp3^{+}$ cell count was significantly higher in GC-responders.

3.3.3.4 Glucocorticoid receptor polymorphisms

The only GR characteristic investigated in the paediatric population in IBD patients are the single-nucleotide polymorphisms (SNPs) located within the GR gene (Table 8). The studies were conducted by an Italian group that reported the BclI polymorphism to be significantly more frequent in GC-responsive patients than in GC-dependent or unresponsive patients (De Iudicibus et al. 2007). This is one of the most promising markers of GC-sensitivity discovered so far, even though the confirmation of these findings in larger studies as well as their association with other markers reflecting steroid sensitivity is still lacking.

Table 8 Studies on GR polymorphisms in paediatric IBD patients.

	Pat.	Dis.	Objective	Method	Results
Decorti et al. 2006	23 34	UC CD	To study GR polymorphisms in IBD.	Genomic DNA extraction from PBMCs and digestion with specific restriction enzymes.	The frequency of the BclI polymorphism of the GR gene, associated with increased sensitivity to GCs, was found to be higher in patients with CD.
De Iudicibus et al. 2007	55 64	UC CD	To study the impact of genetic variations in hGR and MDR1 genes on the efficacy and individual response to GCs in IBD	PCR-RFLP	A significantly higher frequency of BclI genotype was observed in the GC-responsive patients.

4. Methods to assess glucocorticoid sensitivity

Two separate patient groups have to be considered when discussing methods to assess individual GC sensitivity. The first is the extremely small group of patients suffering from generalized GC resistance or sensitivity, conditions often caused by mutations in the GR gene (Russcher et al. 2006, Charmandari et al. 2008, Longui et al. 2009). These patients have alterations in the standard tests designed to study the HPA axis function and the diagnosis can be further confirmed by analyzing the GR gene (Charmandari et al. 2008). However, a problem is posed by the vastly larger population of otherwise healthy individuals who have more subtle alterations in their sensitivity to GCs which might manifest only as a poorer response to exogenous GC therapy (Chriguer et al. 2005). An ideal test to characterize

the alterations in the individual GC sensitivity in this large group would be cheap, simple, sensitive and relatively quick to perform. A number of mechanisms have been studied; however, at present we do not have any method in clinical use with which to predict in advance the patient's response to GCs.

4.1 Methods to assess glucocorticoid sensitivity in vivo

The dexamethasone suppression test (DST) is commonly used to study hypercortisol states in the clinical setting. In addition, it has been employed in studies assessing GC sensitivity (Flood et al. 2001, McMahon et al. 2009). Recently, a very low-dose (20 µg/m^2 body surface area) dexamethasone suppression test was suggested as an index for GC sensitivity (Faria et al. 2008). However, even though the authors were able to show a spectrum of individual responses in different age groups, the findings were not correlated to any other parameters reflecting steroid sensitivity. In addition, no cut-off value was set for impaired GC sensitivity in this or other studies. DST measures the sensitivity of the pituitary GR to GCs. Inflammation, critical illness and mental disorders have been shown to alter the HPA axis responses (Bornstein 2008, Mawdsley and Rampton 2005). As GC sensitivity varies between different tissues even in healthy individuals, it is unclear whether the results of the DST correlate with the GC sensitivity of the target tissues.

Another test often employed in clinical practice is the adrenocorticotrophic hormone (ACTH) stimulation test (Kannisto et al. 2000, Dickstein and Saiegh 2008). It assesses the cortisol release from the adrenal cortex in response to exogenously administered ACTH (Dickstein and Saiegh 2008). At the end of systemic GC treatment, the ACTH test is routinely performed to assess the degree of suppression of the cortisol production which is considered to reflect the hypothalamic-pituitary GC sensitivity of the patient. In the previously described studies evaluating the GC response in IBD patients, only one study used the ACTH test as a variable (Flood et al. 2001). It arrived at conflicting results: after GC treatment, the non-responders to GC therapy had the highest degree of cortisol suppression (Flood et al. 2001).

Assays based on skin blanching are another method for evaluating the steroid sensitivity in vivo. In these assays, topical GCs are applied on the skin for varying time periods and the vasoconstriction caused by the steroid is calculated from the intensity of the blanching (Noon et al. 1996). Previous studies showed an association between GC sensitivity and skin blanching; however, more recent reports have contradicted these findings (Brown et al. 1991, Noon et al. 1996, Wilson et al. 2003). In IBD the skin blanching tests have not been used.

4.2 Methods to assess glucocorticoid sensitivity in vitro

The progress in the field of molecular and cell biology during the past 20 years has produced a large array of applications that can be used in studying GC sensitivity. With these technologies, the structure, expression and ligand binding and affinity of the GR has been studied in IBD patients (Tables 6, 7 and 8). In addition, more indirect methods to assess the GR effect such as the study of dexamethasone inhibition of phytohaemagglutin- or lipopolysaccaride-induced peripheral lymphocytes and studying other genes, their polymorphisms and cellular markers associated with the GC response have also been employed in IBD patients (Table 9).

Table 9 Methods employed to study GC response in IBD patients.

	Pat.	Dis.	Objective	Method	Results
Hearing et al. 1999	18	UC	To study the hypothesis that GC-resistant UC patients have GC-resistant T lymphocytes.	Phytohaemagglutin stimulated peripheral blood T lymphocytes treated with dexamethasone.	Treatment failures and incomplete responders had an I_{max} of less than 60%, all complete responders had an I_{max} of more than 60%.
Franchimont et al. 1999	19	CD	To compare the corticosensitivity of CD patients to that of healthy subjects.	Dexamethasone inhibition of LPS-induced cytokine secretion by whole blood cell cultures.	CD patients had a markedly decreased dexamethasone-mediated inhibition of TNF-α secretion.
Jinno et al. 2006	30 15	UC CD	To study the lymphocytes in the colonic mucosa.	Immunohistochemistry, flow cytometry.	High labelling of $CD19^+$ Ki-67 lymphocytes was specific for active UC that was resistant to GC therapy.
Cucchiara et al. 2007	186 200	UC CD	To study the polymorphisms in the TNF-α and MDR-1 genes that could affect the patients' predisposition to IBD and response to therapy.	PCR-RFLP, DHPLC	The TNF-α promoter polymorphism -308A carries a significant reduction in response to steroid therapy.
Nakahara et al. 2008	94 94	UC CD	To study the relationship between steroid responsiveness and SLC22A4/A5 polymorphism located within IBD 5 locus.	Genotyping and haplotype analysis	The G allele on -368T>G in SLC22A5 was associated with steroid resistance in CD patients. Haplotype analysis between -446C>T and -368T>G in the SLC22A5 promoter region indicated CG allele as the risk haplotype for steroid resistance in CD patients.
Rintamäki et al. 2010	16 2 1	UC CD IC	To study whether the sera from IBD patients treated with GCs causes immunological activation in PBMCs from healthy donors.	ELISA, RT-PCR	After the onset of GC treatment, the expression of FOXP3 and GITR decreased by 33% and 24% respectively. In addition, the levels of IFNγ in cell culture supernatant decreased.

4.3 Circulating glucocorticoid bioactivity

The method of assessing GC treatment and sensitivity in this study was first published in 2002 (Raivio et al. 2002). It is a COS-1 cell based bioassay in which the cells are transiently transfected with GR, coactivator androgen receptor interacting protein 3 (ARIP-3) and luciferase and galactosidase reporter genes. 10µl serum samples are added to the culture medium and after an overnight incubation, the cells are lysed and the activities of the reporter genes are measured. The resulting GC bioactivity (GBA) can be read from the standard curve (Figure 6).

In the original study, it was shown that excess GBA (i.e. GBA not accounted for by endogenous GCs) can be measured in paediatric patients receiving inhaled steroid therapy for asthma (Raivio et al. 2002). In addition, the assay was able to differentiate between transactivation potentials of synthetic GCs, detecting lowest bioactivity appropriately for cortisol, followed in rising order by methylprednisolone and dexamethasone (Raivio et al. 2002). Mifepristone, in contrast, blocked the glucocorticoid-induced response (Raivio et al. 2002).

GBA has also been shown to be elevated in preterm infants receiving antenatal glucocorticoid treatment (Kajantie et al. 2004, Nykänen et al. 2007). In contrast, in women receiving mifepristone for emergency post-coital contraception or for the termination of pregnancy, the GBA levels were reported to be subnormal (Leminen et al. 2005, Heikinheimo et al. 2003). In paediatric renal transplant patients, GBA was suggested to associate positively with excessive weight gain (Seikku et al. 2006). Recently, in infants born small for gestational age, the GBA levels associated with an insulin-resistant state (HOMA-IR) and low adiponectin levels (Tenhola et al. 2009).

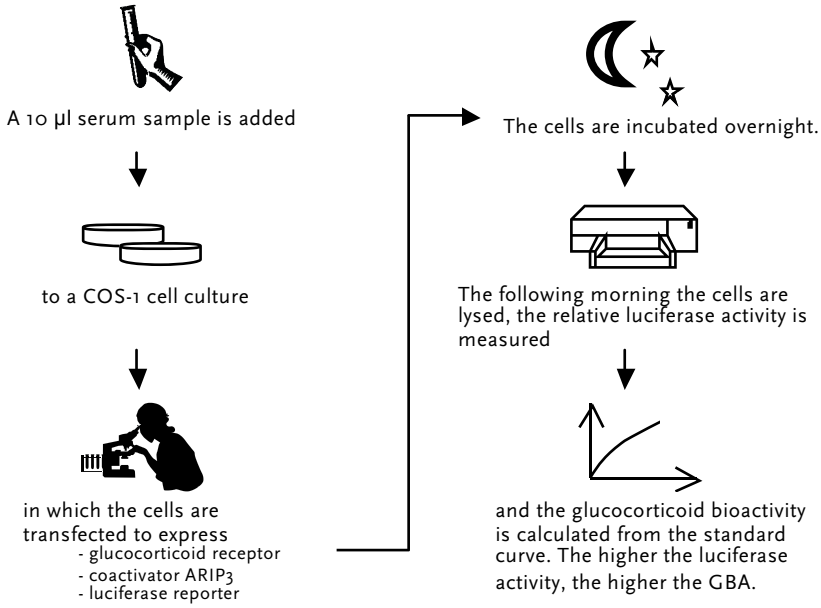

Figure 6 Schematic representation of the GBA measurement.

4.4 Glucocorticoid-sensitive biomarkers

The GC-responsive biomarkers analyzed in this study were selected to mirror GC effects in different tissues that are either involved in the development of common GC-related side effects during systemic GC therapy or reflect therapeutic response to steroid therapy in IBD patients. Therefore, biomarkers originating from adipose tissue and bone and reflecting glucose homeostasis and inflammation were measured.

4.4.1 Adiponectin

Adiponectin is a 30 kDa adipokine that is secreted mainly from adipocytes (Liu and Liu 2010). In serum, its concentration is remarkably high (10-30 µg/ml) and it circulates in three forms: in low-molecular weight trimer, hexamer and a high-molecular weight species (Liu and Liu 2010). Adiponectin increases insulin sensitivity, suppresses hepatic gluconeogenesis and stimulates fatty acid oxidation (Liu et Liu 2010). It possesses anti-inflammatory and anti-atherogenic properties and has a protective role against chronic inflammation (Fantuzzi 2005, Beltowski 2003). Factors known to control circulating adiponectin

levels are presented in Table 10. The promoter region of adiponectin gene contains GR binding sequence; however studies on GC effect on adiponectin levels have arrived at contradicting results (Takahashi et al. 2000, Halleux et al. 2001, Fasshauer et al. 2002, Uchida et al. 2006, Weigert et al. 2009, Rieth et al. 2009).

4.4.2 Leptin

Leptin is an adipose tissue-derived hormone that is secreted in direct proportion to amount body fat (Friedman et al. 1998, Kelesidis et al. 2010). The circulating leptin levels reflect the body energy reservoir and thereby control the regulation of energy homeostasis, metabolism and neuroendocrine function (Kelesidis et al. 2010). In addition, leptin has been reported to have pro-inflammatory properties (La Cava and Matarese 2004). GCs augment leptin levels, other regulating factors are presented in Table 10.

4.4.3 PINP and ICTP

Type I collagen is the main component of bone organic matrix (Calvo et al. 1996). Circulating amino-terminal type I procollagen propeptide (PINP) and carboxyterminal telopeptide of type I collagen (ICTP) measure type I collagen formation and resorption, respectively (Melkko et al. 1996, Risteli et al. 1993). Serum PINP and ICTP levels are affected by factors that control bone metabolism and their levels are related to growth velocity in healthy children and in children with growth disorders (Trivedi et al. 1991, Hyams et al. 1988).

4.4.4 SHBG

Sex hormone-binding globulin (SHBG) binds estrogen and testosterone with high affinity and is the main transporter of sex steroids in human plasma (Pugeat et al. 2010). It is synthesized in the liver where hormonal, metabolic and nutritional factors affect its expression (Table 10). SHBG has been postulated as an independent predictor of bone turnover rate (Välimäki et al. 2004). *In vivo*, GCs have been shown to decrease SHBG levels, however *in vitro* the results have been inconsistent (Pugeat et al. 2010).

4.4.5 IGF-I

Insulin-like growth factor I (IGF-I) is essential for longitudinal growth and the maintenance of adult bone mass (Gazzerro and Canalis 2006). IGF-I circulates as a part of

a 150 kDa complex associated with IGF-binding protein (IGFBP)-3 or IGFBP-5 and acid labile subunit (Canalis 2009). IGF-I acts both as a circulating hormone and as a local growth factor and its expression is regulated by GH and a number of other factors (Table 10, Gazzerro and Canalis 2006). GCs decrease the IGF-1 expression in the liver, affect the synthesis of the IGFBPs and suppress effects of IGF-1 in the local microenvironments (Canalis 2005).

4.4.6 Hs-CRP

C-reactive protein (CRP) was discovered in the 1930 (Bajpai 2009). It is an acute phase protein produced in the liver in response to numerous cytokines and is at present used as a marker of inflammation, infection and tissue injury in many diseases, including IBD. In paediatric IBD however, the standard CRP measurement fails to identify patients with active inflammation (Beattie et al. 1995). High-sensitivity CRP (hs-CRP) measures CRP levels that were previously thought to be under the detection limit (Bajpai 2009). In different conditions (cardiovascular disease, asthma) it is considered as a marker of disease risk or severity (Qian et al. 2008, Bajpai et al. 2009). In paediatric IBD, hs-CRP levels have not been investigated.

4.4.7 HbA_{1C}

Glycated haemoglobin (HbA_{1C}) is employed in the follow-up of children and adolescents with diabetes (Zeitler et al. 2009, Koenig et al. 1976, Saudek et al. 2006). It conveys information on the long-term chronic glycaemic levels in the circulation and correlates with the risk of diabetes complications (International Expert Committee). In children receiving low dose inhaled GCs, HbA_{1C} levels have been reported to be increased (Yucel et al. 2009).

Table 10 Factors that regulate circulating levels of adiponectin (Cook and Semple 2010, Galic et al. 2010, Liu and Liu 2010), leptin (Kelesidis et al. 2010), IGF-1 (Clayton and Hall 2004, Rajpathak et al.2009) and SHBG (Tiitinen 2009, Pugeat et al. 2010).

Leptin	
Overfeeding	↑
Obesity	↑
Glucose	↑
Insulin	↑
Glucocorticoids	↑
Estrogens	↑
Fasting	↓
Leanness	↓
Thyroid hormones	↓
Androgens	↓
PPARγ agonists	↓

Adiponectin	
Weight loss	↑
Thiazolidinediones	↑
Obesity	↓
Insulin resistance	↓
Inflammation	↓
Testosterone	↓
Hypoxia	↓

IGF-1	
Growth hormone	↑
Undernutrition	↓
Inflammation	↓
Glucose intolerance	↓
Age, gender, pubertal status	↔
Liver and renal function	↔
Ethnicity	↔
Hereditary factors	↔
Testosterone, estradiol	↔
Thyroid hormone	↔
Cortisol	↔

SHBG	
Hereditary factors	↑
Hyperthyreosis	↑
Estrogens	↑
Androgens	↓
Insulin resistance	↓
Monosaccharides	↓

AIMS OF THE STUDY

The major aim of this study was to evaluate whether GC treatment could be optimized by monitoring GBA and/or GC-responsive biomarkers in paediatric patients with inflammatory bowel disease in order to obtain the best possible therapeutic effect from the steroids with the least possible side-effects.

The specific aims were:

I to study circulating serum GBA during systemic glucocorticoid treatment in children and adolescents with IBD.

II to evaluate the possibility of using adipose tissue-derived circulating hormones together with serum GBA measurement in monitoring paediatric IBD patients receiving glucocorticoid therapy.

III to assess the effect of systemic glucocorticoid treatment on markers reflecting bone turnover and metabolism and contrast these findings to circulating GBA in children with active IBD.

IV to investigate whether the serum levels of high-sensitivity C-reactive protein could aid the assessment of disease activity and glucocorticoid response in paediatric patients with IBD.

PATIENTS AND METHODS

1. Study subjects

1.1 Patients with inflammatory bowel disease

The patients with inflammatory bowel disease were collected prospectively from the Outpatient Clinics for Paediatric Gastroenterology at the University Hospitals of Helsinki and Tampere. The diagnosis of IBD was based on standard clinical, endoscopical and histological criteria (Lennard-Jones 1989). A total of 69 patients (male 43, female 26) participated in the studies (Table 11). In studies I-III, all the patients were from the original cohort of 24 patients. From this original cohort, 22 patients were selected to study I, 19 patients to study II, 22 patients to study III and 16 patients to study IV. In study IV, an additional 6 patients were collected into the prospective extension of the study. In study IV, a separate group of 39 IBD patients was also analyzed retrospectively.

Table 11 Patients that participated in the studies I-IV. In **bold**, the additional 6 patients collected to study IV.

Study	UC	CD	IC	Remission controls	Other controls
I	18	3	1	41	101
II	16	2	1	-	-
III	19	3	-	22	-
IV original cohort	12	3 + **5**	1 + **1**	-	-
retrospective cohort	19	20	-	-	33

Patients that started the glucocorticoid therapy due to the exacerbation of the disease received either peroral prednisolone (1 mg/kg/d, Prednisolone, Leiras, Finland, n=21), or budesonide (9mg/d, Entocort, AstraZeneca, Sweden, n=3) according to the clinician's decision. After two weeks of treatment, tapering of the steroid dose was initiated following standard clinical practice (Rufo and Bousvaros 2006). Depending on the severity of the disease, the first clinical control was scheduled either 2 or 4 weeks after the onset of

the glucocorticoid. Thereafter, the controls took place at 2 to 4 week intervals. During the control visits, response to treatment and the appearance of glucocorticoid-related side-effects were registered, and a venous blood sample was drawn. At the time of the study, no paediatric index for the activity of UC yet existed. Therefore the response to treatment was defined as 1) physician's global assessment (PGA) of clinical improvement, and 2) decrease in the inflammatory markers faecal calprotectin and ESR (Turner et al. 2010a). The response to treatment and the appearance of glucocorticoid-related side-effects (development of moon-face, weight gain, acne or striae) were graded with two-graded scales: good/poor response, yes/no side-effects.

The 39 patients in the study IV were consecutive paediatric IBD patients that underwent colonoscopy in the Hospital for Children and Adolescents, Helsinki, Finland. Colonoscopy was performed either to confirm the diagnosis of IBD or to assess the activity of the disease. For all patients taking part in the study IV, exclusion criteria were 1) previous surgery, 2) any signs of infection during the preceding week.

All of the patients in whom diagnosis was not fresh received conventional IBD medication, depending on the disease severity. These included 5-ASA products, azathioprine, antibiotics and infliximab.

1.2 Control patients

We included two control groups: disease controls in remission (I, III), and non-IBD controls (I, IV).

1.2.1 Disease controls in remission

Remission controls were 41 paediatric patients with IBD in clinical remission (median age 14 years, age range 4.4 to 18 years). Their maintenance medication included 5-ASA products, and in some patients azathioprine. None of these patients had received glucocorticoids during the preceding month. In study I, the whole group was used as a control group. In study III, 22 age, sex, pubertal status and disease subtype-matched controls were selected from the 41 remission controls.

1.2.2 Non-IBD controls

101 paediatric patients (mean age, 8.2 years, age range 0.9 to 17 years) that visited the Outpatient Clinic in the Hospital for Children and Adolescents formed the other control

group. None of these patients had IBD, and none of these patients received glucocorticoids. In study I, all of the control patients were used. In study IV, 33 patients that matched the age and sex of the study patients were chosen from the total group of non-IBD controls to form a smaller control group.

2. Study design

The studies I, II, III and part of study IV were prospective non-randomized follow-up studies. Their design is visualized in Figure 7.

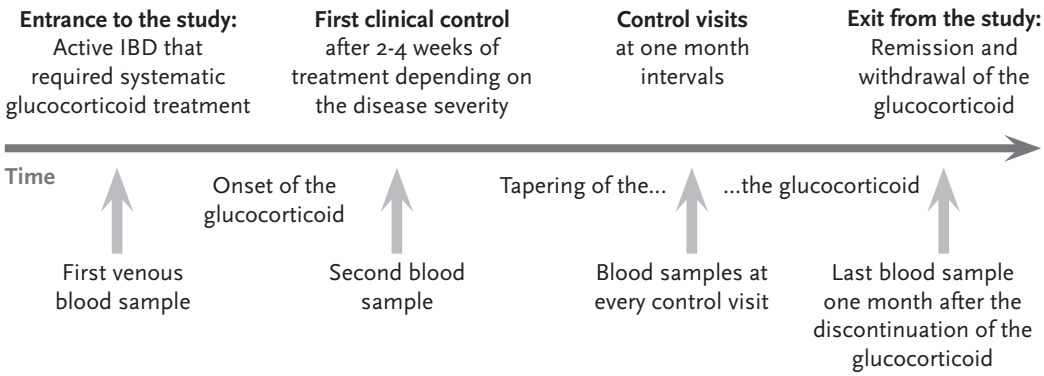

Figure 7 The design of the studies I-IV. The tapering of the steroid was started after two weeks from the onset of the glucocorticoid.

In study IV, an additional retrospective cross-sectional cohort of 39 paediatric IBD patients was analyzed at the time of the colonoscopy (Table I, IV).

3. Ethical considerations

The study protocol was approved by the Ethics Committee for the Hospital for Children and Adolescents, University of Helsinki, and the Ethics Committee of Tampere University Hospital, Tampere, Finland. The patients and their guardians were thoroughly informed about the studies, and a written informed consent was signed by both.

4. Methods

4.1 Glucocorticoid-responsive biomarkers

Table 12 GC-responsive biomarkers analyzed in the present study.

Origin	Biomarker	Type	Properties
Adipose tissue	Adiponectin	Hormone	A newly identified adipokine with anti-inflammatory, anti-atherogenic and insulin-sensitizing properties (Fantuzzi 2005, Beltowski 2003, Lihn et al. 2005).
	Leptin	Hormone	Adipose tissue-derived hormone that functions in the control of food intake and energy homeostasis (Friedman et al. 1998). Leptin has also been reported to have pro-inflammatory properties (La Cava and Matarese 2004)
Red blood cells	$GHbA_{1C}$	Glycated haemoglobin	Reflects chronic glycemic levels (The International Expert Committee 2009).
Bone	PINP	Type I collagen formation product	A marker of bone formation (Melkko et al. 1996).
	ICTP	Type I collagen resorption product	A marker of bone resorption (Risteli et al. 1993).
Liver	IGF-1	Hormone	Mediates GH actions (Walters et al. 2009).
	SHBG	Sex hormone-binding globulin	Transporter of sex hormones, has been postulated as an independent predictor of bone turnover rate (Välimäki et al. 2004)
	Hs-CRP	Acute phase protein	Measures low-grade inflammation (Kushner et al. 2006).

4.2 Laboratory analyses

Venous blood samples were collected between 11 a.m. and 3 p.m. The analyses of standard laboratory tests performed in a clinical laboratory comprised erythrocyte sedimentation rate (ESR), blood count, standard C-reactive protein (CRP) and $GHbA_{1C}$. The other serological markers used in the original publications are presented in Table 13. Faecal calprotectin was measured with an enzyme immunoassay (Phical Test, Calpro AS, Oslo, Norway).

Table 13 Serological markers used in original studies I-IV

Study	Test	Assay	Manufacturer	Intra-assay C.V.	Inter-assay C.V.	Performer
I	Cortisol	Immulite 2000 cortisol kit	Diagnostic Products Corporation, Los Angeles, CA	< 7.4%	< 9.5%	Clinical laboratory
II	Adi-ponectin	Quantikine Human Adiponectin IA	R&D Systems, Minneapolis, MN	< 4.7%	< 6.9%	Professor Sture Andersson's laboratory
II	Leptin	Linco Research Human Leptin RIA Kit	Linco Research, St Charles, MO	< 8.3%	< 8.3%	Clinical laboratory
III	PINP	UniQ RIA	Orion Diagnostica, Espoo, Finland	< 10.2%	< 10.2%	Clinical laboratory
III	ICTP	UniQ RIA	Orion Diagnostica, Espoo, Finland	< 9.4%	< 9.4%	Clinical laboratory
III	SHBG	AutoDELFIA SHBG kit	AutoDELFIA, Wallac, Turku, Finland	< 1.8%	< 10.1%	Clinical laboratory
III	IGF-1	IMMULITE 2000 analyzer	DPC, Los Angeles, CA	nd	< 4%	Clinical laboratory
IV	hs-CRP	Human C-reactive protein Instant ELISA kit	Bender MedSystems, Vienna, Austria	6.9 %	13.1%	Author

4.3 Measurement of the glucocorticoid bioactivity (GBA)

The GBA assay is described in more detail in the review of the literature. The amount of serum used by the bioassay is 10 µl, in duplicate and the results are expressed as nanomolar cortisol equivalents. Cortisol equivalent here means the concentration of cortisol in charcoal-stripped fetal calf serum that induces the same amount of reporter gene activity as a sample with unknown GBA (Raivio et al. 2002). The detection limit of the assay is 15.6 nM cortisol, within-assay coefficient of variation (C.V.) for human serum <8% and inter-assay variation 10%.

4.4 Assessment of the histological activity of colonic inflammation

In study IV, the histological activity of the colonic inflammation was assessed by an experienced paediatric gastro-intestinal pathologist using a grading system originally developed for CD (D'Haens et al. 1998, Sipponen et al. 2008).

4.5 Statistics

All statistical analyses were carried out with SPSS versions 13.0-17.0 by SPSS Inc. software. Associations between non-parametric variables were tested with Mann-Whitney's U test, Kruskal-Wallis test, Spearman's rank order correlation test and Wilcoxon's signed rank sum test. P value of <0.05 was accepted to indicate statistical significance.

1. Circulating GBA in paediatric patients with IBD (I)

1.1 GBA in the control patients – defining the reference value for GBA

The mean GBA level of the 142 control patients (41 paediatric IBD patients in remission and 101 non-IBD patients) was 61.6 nM cortisol equivalents. By adding two standard deviations (28 nM cortisol equivalents) to the mean, an upper limit of 118 nM cortisol equivalents for endogenous GBA was defined. The GBA showed strong ($r=0.712$, $P<0.001$, $n=38$) correlation with serum cortisol in the 41 paediatric IBD patients in remission (Figure 8).

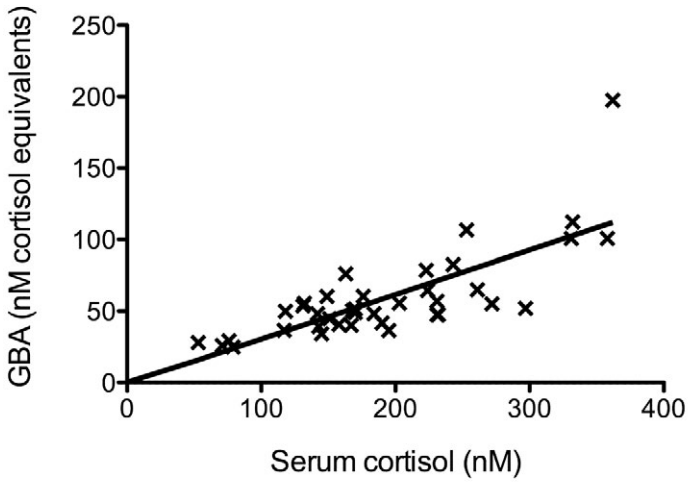

Figure 8 The correlation between serum cortisol and GBA levels in paediatric IBD patients in remission.

1.2 GBA levels in paediatric IBD during glucocorticoid treatment

Before treatment was started, the GBA levels of the 22 patients from the original cohort were similar to controls (84 ± 14 nM cortisol equivalents, mean ± SE, P=NS).

1.2.1 Patients treated with prednisolone

Patients treated with prednisolone (n=19) showed a significant increase in their GBA levels (4-fold increase at two weeks of therapy, Figure 1A, I) that persisted until the withdrawal of the steroid despite lowering doses of GC. GBA correlated with the time that had passed from the GC dose ($r=-0.618$, $P<0.001$, $n=17$) but not with the dose (Figure 1B, I). Young patients (under 10 years of age, n=4) received higher weight-adjusted doses of prednisolone than older patients (1.3 vs. 0.79 mg/kg, $P<0.05$); however, their GBA levels were similar to the older patients (Figure 3, I). After the prednisolone treatment, the GBA levels of the patients decreased to values that prevailed before therapy (51.2 ± 5.4 nM cortisol equivalents, P=NS).

1.2.2 Patients treated with budesonide

Three patients that started the therapy with budesonide showed less increase in their GBA levels after 4 weeks of treatment (151 ± 20 nM cortisol equivalents) when compared to patients treated with prednisolone (267 ± 21 nM cortisol equivalents) (Figure 9). Two of these patients were later switched to prednisolone that induced a 3-fold increase in their GBA levels (Figure 9).

1.2.3 GBA and glucocorticoid-related side effects

10/22 patients presented with acute (presenting during the first month of treatment) glucocorticoid-related side effects (rounding of the cheeks in all, steroid acne in 4). In addition, one girl developed increased ocular pressure after two months of treatment (late side-effect). The GBA levels did not predict or associate with the development of these side-effects. At the beginning of the study, parameters such as serum glycosylated haemoglobin ($GHbA_{1C}$), ACTH and blood pressure were followed-up (Figure 10). However, no change was observed in these variables during the first months of glucocorticoid treatment and their follow-up was ended. Budesonide-treated patients did not present with visible side-effects.

1.2.4 GBA and disease activity

Markers of inflammation, faecal calprotectin and ESR, decreased during the glucocorticoid therapy; however, their levels did not associate with the GBA before, during or after the therapy (I). Clinical response after 4 weeks of therapy did not associate with the GBA levels.

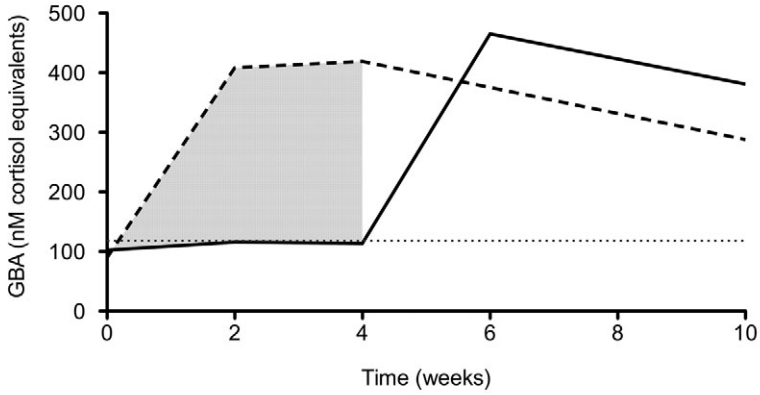

Figure 9 The GBA levels in two children with IBD treated with peroral prednisolone (dashed line) and budesonide (continuous line). The grey area underlines the difference in the GBA levels between these two GCs. After week four, the child treated with budesonide was also switched to prednisolone. Dotted line, the upper normal limit for endogenous serum GBA.

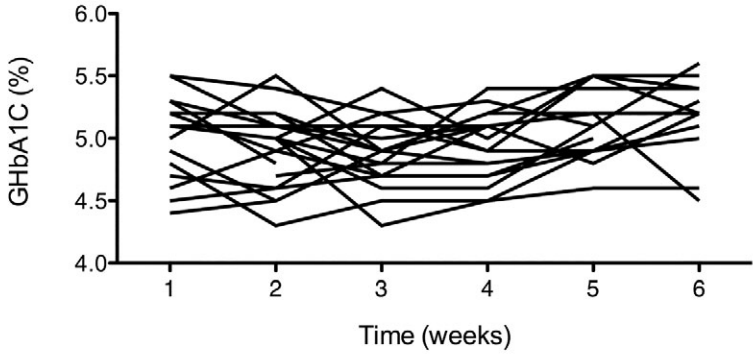

Figure 10 $GHbA_{1C}$-levels of the 22 paediatric IBD patients during the first 6 weeks of systemic glucocorticoid therapy.

2. Adipokines during glucocorticoid treatment (II)

GC treatment increased the levels of adipose tissue-derived hormones, adiponectin (from 11.9 ± 1.5 µg/ml to 18.7 ± 1.8 µg/ml, P<0.01) and leptin (from 4.4 ± 0.9 µg/l to 7.7 ± 1.5 µg/l, P<0.01) after 2 (n=15) to 4 (n=3) weeks of treatment. Circulating GBA increased accordingly (from 86 ± 16 to 288 ± 33 nM cortisol equivalents).

The adiponectin levels were higher in 7 patients (3 boys, 4 girls) that developed acute GC-related side-effects (rounding of the cheeks in all and acne in 4) than in the 11 patients who did not (22.9 ± 2.6 µg/ml vs. 16.0 ± 2.1 µg/ml, P<0.05, Figure 11) after 2 to 4 weeks of treatment. Serum leptin levels showed a similar trend; however, the difference did not reach statistical significance (11.0 ± 3.1 µg/l in patients with side-effects vs. 5.5 ± 1.2 µg/l in patients without side effects, P=0.15).

The GBA levels, weight gain and erythrocyte sedimentation rate at 2 to 4 weeks of treatment were similar between the subgroups with and without side-effects (II). The pretreatment adiponectin levels were also similar between these two groups (side-effects, 12.4 ± 1.5 µg/ml, no side-effects, 11.5 ± 2.5 µg/ml). The adiponectin levels at weeks 2 to 4 were similar between patients with different diagnosis (Crohn's disease or ulcerative colitis), treatment regimen (prednisolone or budesonide), therapeutic response, and sex (II).

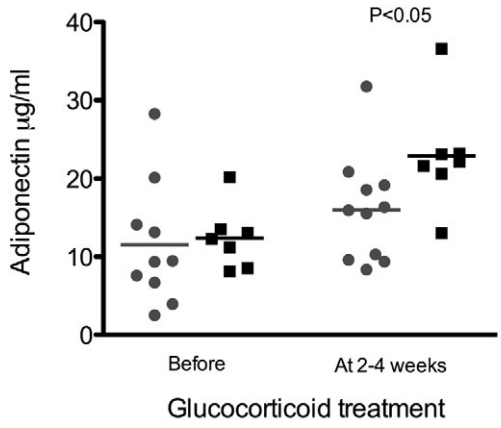

Figure 11 Serum adiponectin levels before and during GC treatment in 18 paediatric patients with IBD. Grey circles, patients without GC side-effects; black squares, patients with side-effects; horizontal line, mean (modified from II).

3. Markers of bone turnover during glucocorticoid treatment (III)

The bone markers were measured in 22 paediatric patients with active IBD; the control patients in this study were 22 age-, sex-, Tanner stage- and disease subtype-matched paediatric IBD patients in remission.

3.1 Markers reflecting bone formation (PINP) and resorption (ICTP)

Before GC therapy, the PINP levels were lower in IBD patients with active disease than in IBD patients with disease in remission ($P<0.05$). The levels of PINP and ICTP before, during, and after the steroid therapy are presented in Figure 12 and Table 14.

Before treatment, PINP correlated positively with ICTP ($r=0.459$, $n=22$, $p<0.05$) and SHBG ($r=0.438$, $n=22$, $p<0.05$), and negatively with faecal calprotectin ($r=-0.550$, $n=15$, $p<0.05$) and ESR ($r=-0.672$, $n=20$, $p<0.01$). During treatment, no association was found between PINP, ICTP, GBA or the inflammatory markers. The development of glucocorticoid-related side-effects or response to treatment were not related to the PINP, ICTP or GBA levels before, during or after the therapy nor to the change of these markers during treatment (P=NS). The PINP and ICTP levels were similar between patients at different pubertal stages or disease subtype (III).

3.2 Markers reflecting bone metabolism and GH actions (SHBG and IGF-I)

The serum IGF-I and SHBG levels before and during GC treatment can be found in Figure 12 and Table 14.

Before treatment, IGF-I correlated negatively with serum SHBG ($r=-0.585$, $n=22$, $p<0.01$) but did not associate with PINP or ICTP. Pubertal stage affected the IGF-1 levels; patients in Tanner stage IV-V had 3.5-fold higher IGF-1 levels than prepubertal patients (III). Weight, height and BMI of the patients correlated with the IGF-1 and SHBG levels; however, markers of disease activity (ESR, faecal calprotectin) were not related to their levels before or during GC therapy. Serum GBA levels, response to glucocorticoid treatment or the development of glucocorticoid-related side-effects did not correlate with serum IGF-1 or SHBG.

Table 14 The serum levels of PINP, ICTP, IGF-1 and SHBG before, during, and after glucocorticoid therapy in 22 paediatric patients with active IBD and in 22 age-, sex-, disease subtype- and pubertal stage-matched control patients with IBD in remission (III, modified from Vihinen et al. 2008). # p<0.05 (compared with controls); *p<0.05 (compared with pre-treatment levels); *** p<0.001 (compared with pre-treatment levels).

	S-PINP, µg/l		S-ICTP, µg/l		S-IGF-1, nmol/l		S-SHBG, nmol/l	
	Median	Range	Median	Range	Median	Range	Median	Range
Active IBD								
Pre-treatment	271#	88 – 849	14.2	7.6 – 26.3	23#	3 – 37	54	22 - 180
2 weeks	163***	29 – 472	9.6***	5.2 – 16.4	37***	21 – 85	35***	14 – 84
5 weeks	152***	23 – 393	8.2***	5.5 – 16.7	37***	3 – 72	28***	11 – 108
1 month after treatment	516*/NS	143 – 909	18.7*/NS	10.1 – 24.9	27*/NS	16 – 50	66/NS	22 – 179
Controls	535	111 – 1390	14.7	8.8 – 28	29	13 – 54	87	15 – 167

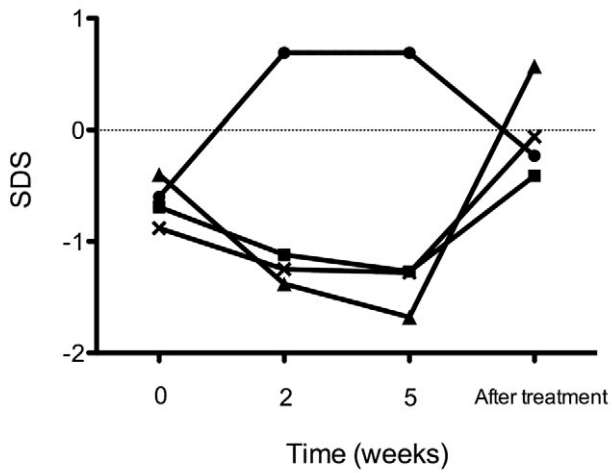

Figure 12 Serum PINP (X), ICTP (▲), IGF-1 (●) and SHBG (■) in 22 paediatric patients with active inflammatory bowel disease before, during, and after treatment with systemic glucocorticoids.

4. High sensitivity C-reactive protein (hs-CRP) in paediatric IBD (IV)

4.1 Hs-CRP during glucocorticoid treatment

59% (13/22) of the patients with active IBD presented with undetectable (<5 mg/L) standard CRP levels, and their hs-CRP levels were measured. Of these patients, 7 children showed a good response to GC therapy. However, the change in the hs-CRP levels during steroid treatment was similar between patients that responded to the therapy and in non-responders (P=0.16) (Figure 13). The pretreatment hs-CRP levels were similar between responders (median, 0.3 mg/L, range 0.05-1.7) and non-responders (0.1 mg/L, 0.01-0.6, P=NS) and did not predict the treatment outcome (*unpublished results*). The development of glucocorticoid-related side-effects did not associate with serum CRP levels.

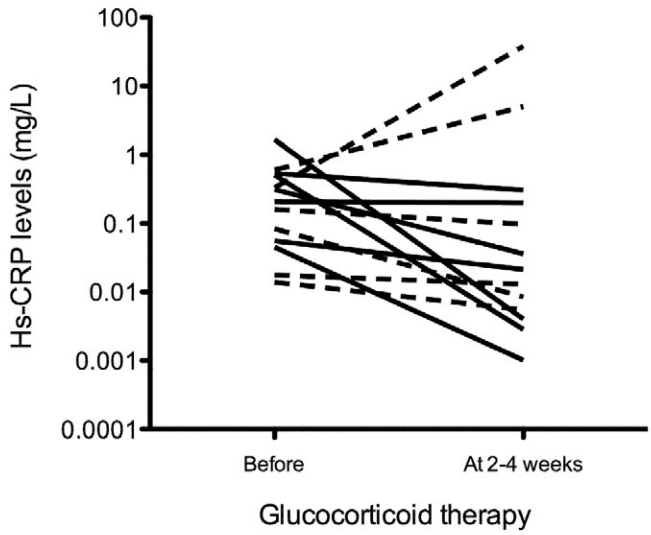

Figure 13 The hs-CRP levels in GC treatment responders (continuous line) and non-responders (dashed line) in 13 children with active IBD.

4.2 Hs-CRP levels related to intestinal inflammation

In retrospective analysis, 64% (25/39) of the paediatric IBD patients investigated at the time of the colonoscopy had undetectable standard CRP levels. The median CRP level of the patients was 0.4 mg/L (0.007 – 45 mg/L), significantly higher than in the 33 control

patients (0.03 mg/L, 0.008-0.7 mg/L, P<0.001). Hs-CRP was measurable in all cases with undetectable standard CRP (25/39) and higher than in the control patients (P<0.01, *unpublished results*), but could not sort the patients according to disease activity (active disease 0.2 mg/L, 0.007-1.37, n=17 vs. quiescent disease 0.1 mg/L, 0.01-1.89, n=8, P=NS). Children with ileocolonic CD had significantly higher CRP levels (14 mg/L, 0.06-45, n=13) than patients with CD colitis (0.18 mg/L, 0.01-9, n=7, P<0.01) or UC (0.13 mg/L, 0.007-23, P=NS). The CD patients presenting with granulomas had clearly higher CRP levels (14 mg/L, 0.5-39, n=9) than patients without granulomas (0.5 mg/L, 0.01-45, n=11, P<0.05). The levels of CRP, faecal calprotectin, ESR and WBC in these patients are presented in Table 15 according to disease activity. CRP only correlated with the histological activity of the inflammation in the ileum (IV).

Table 15 The CRP, faecal calprotectin, ESR and WBC levels in 39 paediatric patients treated with systemic GCs. * p<0.05; ** p<0.01.

	CRP, mg/L	Calprotectin, µg/g	ESR, mm/h	WBC, E9/L
Active disease				
Yes	0.7, 0.007-45	1030, 14-4400	22, 2-97	7.8, 3.2-17.4
No	0.1, 0.01–12*	180, 24-1010*	5, 4-14**	6.6, 4.3-8.6
Fresh diagnosis				
Yes	0.7, 0.02-39	1040, 26-4400	26, 2-97	8.0, 3.2-17.0
No	0.2, 0.007-45	610, 14-2010	12, 4-33**	7.0, 3.9-17.4

5. Summary of the results of GC-responsive biomarkers

Table 16 Direction of change of the GC-responsive biomarkers during GC-therapy in paediatric patients with IBD.

Origin	Biomarker	Direction of change
Adipose tissue	Adiponectin	↑
	Leptin	↑
Bone	PINP	↓
	ICTP	↓
Liver	IGF-1	↑
	SHBG	↓
	Hs-CRP	↓
Red blood cells	$GHbA_{1C}$	↔

DISCUSSION

Systemic GC therapy is used worldwide in the treatment of diverse inflammatory and immune disorders. In IBD, the majority of the patients benefit from the treatment, however around 30% of the patients are resistant to the therapy. In addition, the development of the GC treatment-related side-effects is common. A measurement that could identify the possible responders from non-responders and the patients who are susceptible to serious side-effects would be vital. At present, we have no method in clinical practice with which to predict the individual's response to treatment in advance. Therefore, the aim of this study was to identify biomarkers originating from different tissues, and evaluate the possibility of using the GBA measurement as an aid in recognizing GC-responsive patients during systemic steroid therapy in children and adolescents with IBD.

1. Glucocorticoid bioactivity during glucocorticoid treatment (I)

In the first study, the aim was to assess whether the GBA measurement could direct the steroid therapy in children and adolescents with IBD.

In this study, the GBA was measurable in all patients, and as in other studies correlated strongly with serum cortisol before the treatment was started, showing high internal validity of the assay (Raivio et al. 2002, Heikinheimo et al. 2003, Nykänen et al. 2007). Regrettably however, the GBA levels did not associate with the clinical response to GCs (therapeutic response or the development of GC-related side-effects) during systemic GC treatment in paediatric IBD patients. Thus, in the light of these results, the GBA cannot be used as a tool to optimize steroid therapy in children with IBD. The findings are in accordance with those of a recent Canadian study which reported no association between GBA and GC-response in 50 paediatric patients treated with intravenous GCs for acute severe UC (Turner et al. 2010b). However, the assay has been reported to differentiate appropriately between different biopotencies of synthetic steroids in this and other studies, and to be blocked by a GC antagonist/partial agonist mifepristone (Raivio et al. 2002, Heikinheimo et al. 2003, Leminen et al. 2005). Therefore, the assay should reliably reflect the circulating GBA, and other factors that could explain the results will be discussed.

1.1 Factors that could have affected the GBA measurement

1.1.1 GC absorption, dose and timing

Factors that could have affected the GBA results in this study are the absorption of the administered GCs, as well as the steroid dose and the time that had elapsed from the GC. As previously stated, the absorption of prednisolone in active IBD is considered to be normal (Olivesi 1985, Faure et al. 1998, Schwab et al. 2001). In this study, the steroid dose did not correlate with the GBA levels, and minor differences in the absorption of the drug that could have influenced the results cannot be ruled out. However, time that had passed from ingesting the previous GC dose correlated strongly with the GBA, and might have masked the relationship between the dose and the GBA. Due to the small sample size, we were unfortunately unable to test this hypothesis with logistic regression. However, the Canadian group which bypassed the question of variable absorption by administering the steroids intravenously arrived at similar conclusions as us (Turner et al. 2010b). They performed multivariate regression analysis adjusted for dose and the time from the last GC in a large group of patients, and confirmed our findings of no association between the GBA levels and the clinical response to GCs.

1.1.2 Assay properties

A potential technical factor within the assay that could have influenced the GBA results is the 10-fold dilution of the serum samples in cell culture medium. Dilution of this kind can break the weakest steroid-CBG complexes and affect the GBA levels (Dunn et al. 1981). The affinity of prednisolone to CBG is reported to be almost as strong as that of cortisol, and all studies that have employed the GBA assay have reported strong correlation between serum cortisol and GBA (Pugeat et al. 1981). However, the possibility that the dilution of the samples may have influenced the GBA measurement cannot be overruled.

Another explanation for the disconnection between the GBA results and clinical response to GCs stems from the general principle of the assay. The GBA assay measures GR-driven transactivation. Previously it was thought that the majority of the anti-inflammatory effects of GCs are mediated via transrepression, and the side-effects are arbitrated by transactivation. However, recent studies have reported that there are actually a number of genes with a distinct anti-inflammatory profile up-regulated by GR (De Bosscher and Haegeman 2009). The expression of IL-10 for example, an anti-inflammatory and immunosuppressive cytokine important in the development of IBD, has been recently reported to be induced by GR, possibly by transactivation (Mayer 2010, Clark et al.

2007). In addition, the inhibitor of NFκB α (IκBα), another important anti-inflammatory mediator in IBD, is up-regulated by GCs (Clark et al. 2007). Therefore, it is today considered that for a single wanted or unwanted GC effect there is probably a host of GC-induced mechanisms, positive and negative, genomic and non-genomic, that depend on the cellular environment and activation status and lead to various expression of a given effect in different situations (Clark et al. 2007). That said however, we cannot exclude the possibility that the end-points we looked at in this study (therapeutic response, the development of GC-related side-effects) were mainly mediated by some mechanism other than GR induced transactivation.

1.2 GBA and GC sensitivity

Compared to adults, the GC doses of 1-2 mg mg/kg/day of prednisolone equivalents administered to paediatric IBD patients at the beginning of the therapy are relatively high. The circulating GBA levels induced by doses like this are clearly supraphysiological, as shown in this study by the 5-fold rise in the GBA levels after 2 weeks of treatment with prednisolone. However, both we and the Canadian group were unable to show any difference between the GBA levels in the patients who responded to GCs, and in those who did not (I, Turner et al. 2010b). In adult patients with IBD, the increase of the steroid dose from 40mg to 60mg of prednisone equivalents/day has not been shown to improve the clinical response to the treatment (Baron et al. 1962). It may be that the doses used at present in the treatment of children and adolescents with moderate to severe IBD are more than sufficient to induce a therapeutic response, if this occurs. Therefore, one of the main messages of this study is that the GC sensitivity of paediatric IBD patients, defined as therapeutic response to GC therapy and/or the development of GC-related side-effects, seems not to be determined by circulating GBA measured with a transactivation assay. Rather, it is probably the net sum of the GBA together with the tissue- and cell-type specific expression of different GR isoforms and polymorphisms, their covalent modifications and interaction with other transcription factors.

2. Adiponectin as a marker of glucocorticoid sensitivity (II)

The second specific aim of the study was to assess the effect of systemic GC treatment on white adipose tissue (WAT) derived adipokines adiponectin and leptin, and their relationship to GBA and clinical response to GCs. These investigations led to one of the

most interesting findings of this study, namely the association of serum adiponectin with visible GC-treatment related side-effects at 2-4 weeks of systemic GC therapy. No such connection was found for serum leptin.

At present, great interest has been posed to adiponectin's negative association with indices of insulin resistance and inverse correlation with fat mass (Cook and Semple 2010, Tenhola et al. 2010). As GCs often impair insulin actions in humans, a negative correlation between GCs and adiponectin could be hypothesized. However, studies on GC effect on adiponectin levels have arrived at controversial results. In *in vitro* studies, steroid administration has been shown to decrease adiponectin mRNA expression and adiponectin release (Halleux et al. 2001, Fasshauer et al. 2002, Degawa-Yamauchi et al. 2005). *In vivo*, exogenous GCs have been reported to increase adiponectin levels (Uchida et al. 2006, Weigert et al. 2009, Rieth et al. 2009).

The adiponectin gene ADIPOQ contains consensus GR binding sequence on its promoter (Takahashi et al. 2000). Thus, in this study it is possible that the administered exogenous GCs affected the adiponectin levels either directly or through lowering the levels of potent adiponectin secretion inhibitors IL-6 and TNF-α (Swarbrick and Havel 2008). Further studies are needed to address this question. However, if our findings can be confirmed in larger patient groups, it is possible that adiponectin may aid the recognition of steroid sensitive patients.

3. Markers of bone turnover (III)

The third aim of this study was to evaluate the association of serum markers of bone turnover and metabolism with respect to serum GBA and GC therapy. Children with IBD may have decreased bone mineral density (BMD) already at diagnosis, and GC therapy can further compromise bone health (Sylvester 2005, Walther et al. 2006, Eastell et al. 2010). We assessed acute changes in bone turnover and metabolism by measuring serum levels of amino-terminal type I procollagen propeptide (PINP) reflecting bone formation, and carboxyterminal telopeptide of type I collagen (ICTP) reflecting bone degradation respectively (Melkko et al. 1996, Risteli et al. 1993). In addition, insulin-like growth factor 1 (IGF1) and sex hormone binding globulin (SHBG) were measured for they mediate growth hormone (GH) actions, and SHBG has been reported to independently predict bone turnover rate in adults (Yakar et al. 2002, Välimäki et al. 2004).

3.1 Markers reflecting type I collagen turnover

In this study, the marker of type I collagen formation PINP was lower in children with IBD compared with remission controls, even before GC treatment was started (III). PINP also correlated negatively with the markers of inflammation, ESR and faecal calprotectin. At the same time, the ICTP levels reflecting bone degradation were similar to controls. "Low-turnover" osteoporosis has been characterized as osteoporosis where bone resorption is normal and bone formation is reduced (Tilg et al. 2008). These findings suggest that active inflammatory process present in children and adolescents with IBD could be a driving force behind the development of this type of "low-turnover" deterioration of bone structure that can eventually lead to osteoporosis.

After two and five weeks of GC therapy, both the markers of bone formation and degradation declined. This is consistent with previous reports from animal and human studies that have shown decreased bone turnover and reductions in the age-dependent increases in trabecular bone mineral content, trabecular thickness, linear growth and cortical thickness during GC treatment (Sorva et al. 1996, Crowley et al. 1998, Ortoft et al. 1999, Ikeda et al. 2003, Ton et al. 2005). In addition, in this study during GC, the PINP and ICTP no longer correlated with each other, as they did at baseline. The processes of bone modelling and remodelling are coupled by osteoblast-derived RANKL and osteoprotegerin (OPG) (Hadjidakis and Androulakis 2006). In children treated with inhaled GCs for asthma, PINP and ICTP decreased similarly to this study, but the markers correlated even during the therapy (Sorva et al. 1996). The lack of correlation between bone formation and resorption markers during systemic GC treatment in this study can reflect the loss of the tight coordination between these two processes. Taken together, these findings do support the concept that despite the potential beneficial effects that GC treatment has on the bone structure by controlling the inflammatory process in IBD patients, it is probably still an additional risk factor for impaired bone quality (Eastell et al. 2010).

After treatment, the PINP and ICTP levels rose to a level similar to the controls, showing that the effects of inflammation and peroral steroid therapy on bone turnover were reversible. The long-term impact that systemic GC treatment in childhood has on adult BMD and height is being debated. The theory that the peak bone mass achieved in young adulthood determines bone health decades later has been questioned, and studies show that interventions to increase BMD in all age groups seem to have only transient effects (Gafni et al. 2007). In addition, GC-treatment induced increased fracture risk has been shown to return to baseline approximately 1 year after the withdrawal of the steroid (Van Staa et al. 2000, Vestergaard et al. 2008). The findings of this study thus accord, in that reasonably high-dose (1 mg/kg/day prednisone equivalents at initiation) systemic GC treatment that

lasted for a moderately long period (median 6 months) did not have a persistent effect on bone turnover markers at one month after the therapy was withdrawn.

3.2 Markers reflecting GH actions

At the beginning of the study, the IGF-I levels were lower in patients that had active disease compared with remission controls. Previous studies have yielded similar results (Eivindson et al. 2007, Sylvester et al. 2007). This finding probably reflects the inhibitory effect that circulating inflammatory cytokines such as TNF-α, IL-6 and IL-1β have on the GH-receptor and IGF-I expression in the liver, and might contribute to the decreased BMD and possible growth failure seen in patients with active IBD (Cezard et al. 2002, Sylvester et al. 2007, Theiss et al. 2004, Eivindson et al. 2007).

During GC treatment, the IGF-I levels rose in contrast to all the other markers reflecting bone turnover. In earlier studies, partial normalization of IGF-I levels during GC treatment has been seen in IBD adults (Grønbæk et al. 2002, Eivindson et al. 2007). In addition, in IBD children therapeutic interventions have been shown to increase IGF-1 levels (Thomas et al. 1993, Beattie et al. 1998). However, in the former paediatric study, the patients treated with steroids displayed lower growth velocity SD score despite higher IGF-1 levels than patients treated with elemental diet (Thomas et al. 1993). Postulated mechanisms for this controversial phenomenon are GC-induced IGF-1 resistance, low IGF-1 bioactivity and dose-dependent effects of GCs on IGF-1 expression (Thomas et al. 1993, Dong et al. 2003, Grønbæk et al. 2002).

The SHBG levels in children with active IBD were similar to controls. However, at baseline the SHBG correlated positively with the marker of bone formation, PINP. This finding, first published in young Finnish men, was very recently confirmed in a group of young Belgian men (Välimäki et al. 2004, Vanbillemont et al. 2010). The clinical significance of this result is as yet unclear, as studies on the relationship of SHBG with BMD have arrived at contradicting results (Lormeau et al. 2004, Vanbillemont et al. 2010). Similarly, also the molecular basis of this association remains elusive. However, as SHBG has been shown to be an independent predictor of bone turnover rate, the presence of a specific membrane bound receptor for SHBG has been postulated (Vanbillemont et al. 2010).

3.3 Bone turnover markers, GBA and clinical response

Regrettably, no associations were found between PINP, ICTP, IGF-1, SHBG, GBA levels, therapeutic response to GCs or the development of GC-related side-effects. Therefore,

according to the results of this study, these markers do not aid the GC treatment of paediatric IBD patients. A possible explanation might be that the GC effects on bone are mediated via mechanisms other than DNA-dependent transactivation, or the mechanisms responsible for GC-associated visible adverse effects.

4. Hs-CRP in paediatric IBD (IV)

The fourth specific aim of this study was to evaluate the possibility of using hs-CRP in children and adolescents with IBD. In detail, to assess whether it associates either with the outcome of GC therapy or is related to the clinical and/or histological disease activity in IBD patients. Disappointingly, the answer for both of these main questions was negative, and the hs-CRP was found to be no more useful in the treatment of these patients than standard CRP testing. However, the CRP levels were higher in patients with CD and associated with histological activity of the inflammation in the ileum and with the presence of granulomas. Recently, the hs-CRP levels have been reported to associate with the endoscopical activity of IBD inflammation in CD adults (Jones et al. 2008). Thus, in CD, the CRP measurement might act as an indicator of intestinal inflammation; however, further studies are needed.

In this study, it was found that the change in hs-CRP levels in children treated with GCs did not associate with the response to treatment. This result reinforces the concept that in paediatric IBD, the CRP measurement is of limited clinical value (Vermeire et al. 2006). However, the CRP levels in the IBD patients were higher than in healthy controls. Earlier this year, Dagni *et al.* reported that hs-CRP levels and carotid intima media thickness, carotid arterial stiffness, insulin resistance assessed by HOMA-IR and homocysteine levels were higher in adult IBD patients compared with healthy controls with a similar BMI. This is the first study to show that paediatric IBD patients also present with such low-grade inflammation. IBD that manifests during childhood can therefore be a possible risk factor for the development of early atherosclerosis which may increase the disease burden of the patients later on in life (Dagni et al. 2010, Papa et al. 2005, Ha et al. 2009).

5. Limitations of the study

One of the limitations of this study is the small number of patients, which is a common problem in paediatric studies. However, these studies were designed to be pilot studies

and the objective was to collect enough patients to obtain preliminary results of the large array of laboratory markers investigated. The specific findings of this thesis do need to be confirmed in larger patient groups, but this is the first study that approaches the question of GC-responsiveness by measuring GBA and biomarkers from different tissues in the clinical setting.

Another possible limitation of this study is that the clinical activity indices of UC and CD were not included; however, at the time the study was initiated, no activity index existed for paediatric UC (Turner et al. 2007). Furthermore, recent studies show that clinical indices correlate poorly with the presence of intestinal inflammation (Sipponen et al. 2008). Here the assessment of disease activity was based on PGA (Physician's Global Assessment).

Finally, the design of this study does limit the findings to paediatric patients with IBD. Therefore, the conclusions on the utility of the GBA assay cannot be generalized to other patient groups.

6. General discussion and future considerations

During recent years our knowledge of the GR signalling pathway has expanded, and a system that once might have seemed straightforward has grown into a complex web of associations which depend upon the prevailing conditions. At the moment we are in a situation where we have an enormous amount of data on newly identified GR isoforms, polymorphisms, signalling cofactors and interacting proteins, but no clear view of their significance in health and disease. Therefore, this study that combines clinical data with newly developed biomolecular assay and recently identified biomarkers from different tissues is exactly the type of "crossroads" study that we need, to step-by-step build a general picture of GR actions in different tissues.

The main finding of this study, that systemic bioactivity of GC treatment measured with a transactivation assay does not associate with clinical response to steroid therapy, raises new prospects for future research. It seems ever more likely that the response to GC treatment depends on the GC sensitivity at target cell level, not on systemic bioactivity of a given compound. However, in IBD there is local as well as systemic activation of the inflammatory pathways and defining the exact culprit cell population is, at present, impossible. Therefore, future studies on GR function should include both circulating PBMCs and colonic mucosal epithelial and inflammatory cells. An example for future research could be to study the presence of the BclI polymorphism in circulating PBMCs

in patients with IBD, and correlate the findings with GRα/β expression in the colonic mucosa and with the response to GC therapy (Labuda et al. 2010).

Another novel finding of this study, the association of serum adiponectin with GC treatment related adverse events, warrants further studies. During the past decade, adipose tissue has emerged as a new endocrine organ that is actively involved in the control of energy homeostasis, insulin sensitivity and inflammation (Galic et al. 2010). It is possible that the adiponectin levels in this study reflect indirectly the decrease in the levels of adiponectin secretion inhibitors TNF-α or IL-6. However, if these findings can be confirmed in larger studies, serum adiponectin could be used as a readily available marker of sensitivity to GC-related adverse events.

Regrettably, in this study no correlations were found between the markers of bone turnover, GBA and therapeutic response to GCs. However, the present study strengthens the view that bone health in active paediatric IBD is compromised and systemic GC therapy further disturbs the balance between bone formation and resorption. In addition, we report that after the withdrawal of the steroid, all the bone turnover markers restore to levels similar to controls, showing that good response in controlling the inflammatory process reflects to bone health. A recent report in Cell Metabolism states that GCs might suppress bone formation by interfering osteoblast differentiation via monomeric GR (Rauch et al. 2010). It is probable that monomeric GR does not mediate its functions through binding to the DNA, but affects gene transcription by interacting with other transcription factors. If this finding is confirmed in other studies and the GR monomer emerges as the predominant mediator of GC effects in bone, we need new types of bioassays to evaluate and predict the impact of GCs on bone in the clinical setting.

An intriguing novel finding of this study was to show that SHBG correlates with PINP, a marker of bone formation, before GC treatment in children with IBD. In adults, SHBG has been shown to correlate negatively with BMD, and high levels of SHBG associate with the presence of fractures (Hoppé et al. 2010). In children, the significance of the relationship between SHBG and bone turnover marker is as yet unclear. Whilst puberty affects the SHBG levels, a specific issue to be addressed in the future is the effect of age on this finding.

Finally, the value of measuring hs-CRP in paediatric IBD was evaluated. It was found that the high-sensitivity measurement of CRP does not seem to offer any advantage over the standard CRP testing, neither when measured at the time of the colonoscopy nor in monitoring GC treatment. However, the histological activity of the ileal inflammation associated with hs-CRP levels in CD patients, and patients with ileal disease did present with higher CRP levels than patients with colonic involvement only. The NOD2/CARD15

mutation has been associated with ileal disease location and it is tempting to speculate that differences in the innate immune system might alter the disease process in the ileum (McGreal and Cho 2008).

In this study, the GBA levels did not associate with the response to GCs. However, there are clinical situations where information on the bioactivity of the circulating GCs would be vital. These could include for example patients in intensive care or situations where the immunologically analyzed cortisol levels and clinical symptoms do not match.

Recently, it has been evaluated that the GR splice and translational isoforms identified until now can form up to 256 different homo- and heterodimers that all might express distinct patterns of intracellular distribution, coactivator recruitment and transactivation and -repression properties (Nicolaides et al. 2010). Consequently, as the system turns out be far more complex than expected, explaining why some patients respond to GC therapy while others develop only the side-effects seems to be fleeing further away. This study is the first attempt to open up this subject, and bring together basic and clinical research.

CONCLUSIONS

The main results and conclusions of this study can be summarized as follows:

I The GBA levels of paediatric patients with IBD receiving systemic GCs were higher than in control patients with no GC therapy. However, the GBA levels did not correlate with clinical response or the development of GC-related side-effects and therefore do not seem to aid the GC therapy in these patients.

II Adipocyte-derived adiponectin was associated with the development of GC therapy-related adverse events. This circulating adipokine could be a potential biomarker of GC-sensitivity. The adiponectin levels were not related to the GBA levels.

III Serum PINP and IGF-1 reflecting bone formation and GH actions were lower in patients with active IBD than in controls, probably because of the active inflammatory process. All the bone turnover markers changed readily in response to the onset of systemic GC treatment. After the withdrawal of the steroid, they restored to levels similar to the controls. However, the bone turnover markers were not related to the clinical response, the development of GC-related side effects or the GBA levels.

IV Serum hs-CRP levels were similar in patients that responded to GC treatment and in non-responders. In CD patients, the hs-CRP levels associated with the histological activity of the ileal inflammation.

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