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Investigating the role of potential genetic markers that can predict risk for steroid refractory inflammatory bowel disease

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Cette thèse intitulée :

Investigating the role of potential genetic markers that can predict risk for steroid refractory inflammatory bowel disease

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RÉSUMÉ

Contexte - La variation interindividuelle de la réponse aux corticostéroïdes (CS) est un problème important chez les patients atteints de maladies inflammatoires d'intestin. Ce problème est bien plus accentué chez les enfants avec la prévalence de la corticodépendance extrêmement (~40 %) élevée. La maladie réfractaire au CS a des répercussions sur le développement et le bien-être physique et psychologique des patients et impose des coûts médicaux élevés, particulièrement avec la maladie active comparativement à la maladie en rémission, le coût étant 2-3 fois plus élevé en ambulatoire et 20 fois plus élevé en hôpital. Il est ainsi primordial de déterminer les marqueurs prédictifs de la réponse aux CS. Les efforts précédents de découvrir les marqueurs cliniques et démographiques ont été équivoques, ce qui souligne davantage le besoin de marqueurs moléculaires. L'action des CS se base sur des processus complexes déterminés génétiquement. Deux gènes, le *ABCB1*, appartenant à la famille des transporteurs transmembraneaux, et le *NR3C1*, encodant le récepteur glucocorticoïde, sont des éléments importants des voies métaboliques. Nous avons postulé que les variations dans ces gènes ont un rôle dans la variabilité observée de la réponse aux CS et pourraient servir en tant que les marqueurs prédictifs.

Objectifs - Nous avons visé à: (1) examiner le fardeau de la maladie réfractaire aux CS chez les enfants avec la maladie de Crohn (MC) et le rôle des caractéristiques cliniques et démographiques potentiellement liés à la réponse; (2) étudier l'association entre les variantes d'ADN de gène *ABCB1* et la réponse aux CS; (3) étudier les associations entre les variantes d'ADN de gène *NR3C1* et la réponse aux CS.

Méthodes - Afin d'atteindre ces objectifs, nous avons mené une étude de cohorte des patients recrutés dans deux cliniques pédiatriques tertiaires de gastroentérologie à l'Ottawa (CHEO) et à Montréal (HSJ). Les patients avec la MC ont été diagnostiqués avant l'âge de 18 ans selon les critères standard radiologiques, endoscopiques et histopathologiques. La corticorésistance et la corticodépendance ont été définies en adaptant les critères reconnus. L'ADN, acquise soit du sang ou de la salive, était

génotypée pour des variations à travers de gènes *ABCB1* et *NR3C1* sélectionnées à l'aide de la méthodologie de tag-SNP. La fréquence de la corticorésistance et la corticodépendance a été estimée assumant une distribution binomiale. Les associations entre les variables cliniques/démographiques et la réponse aux CS ont été examinées en utilisant la régression logistique en ajustant pour des variables potentielles de confusion. Les associations entre variantes génétiques de *ABCB1* et *NR3C1* et la réponse aux CS ont été examinées en utilisant la régression logistique assumant différents modèles de la transmission. Les associations multimarqueurs ont été examinées en utilisant l'analyse de haplotypes. Les variantes nongénotypées ont été imputées en utilisant les données de HAPMAP et les associations avec SNPs imputés ont été examinées en utilisant des méthodes standard.

Résultats - Parmi 645 patients avec la MC, 364 (56.2%) ont reçu CS. La majorité de patients étaient des hommes (54.9 %); présentaient la maladie de l'iléocôlon (51.7%) ou la maladie inflammatoire (84.6%) au diagnostic et étaient les Caucasiens (95.6 %). Huit pourcents de patients étaient corticorésistants et 40.9% - corticodépendants. Le plus bas âge au diagnostic (OR=1.34, 95% CI: 1.03-3.01, p=0.040), la maladie cœxistante de la région digestive supérieure (OR=1.35, 95% CI: 95% CI: 1.06-3.07, p=0.031) et l'usage simultané des immunomodulateurs (OR=0.35, 95% CI: 0.16-0.75, p=0.007) ont été associés avec la corticodépendance. Un total de 27 marqueurs génotypés à travers de *ABCB1* (n=14) et *NR3C1* (n=13) ont été en l'Équilibre de Hardy-Weinberg, à l'exception d'un dans le gène *NR3C1* (rs258751, exclu).

Dans *ABCB1*, l'allèle rare de rs2032583 (OR=0.56, 95% CI: 0.34-0.95, p=0.029) et génotype hétérozygote (OR=0.52, 95% CI: 0.28-0.95 p=0.035) ont été négativement associes avec la dépendance de CS. Un haplotype à 3 marqueurs, comprenant le SNP fonctionnel rs1045642 a été associé avec la dépendance de CS (p empirique=0.004). 24 SNPs imputés introniques et six haplotypes ont été significativement associés avec la dépendance de CS. Aucune de ces associations n'a cependant maintenu la signification après des corrections pour des comparaisons multiples. Dans *NR3C1*, trois SNPs: rs10482682 (OR=1.43, 95% CI: 0.99-2.08, p=0.047), rs6196 (OR=0.55, 95% CI: 0.31-

0.95, p=0.024), et rs2963155 (OR=0.64, 95% CI: 0.42-0.98, p=0.039), ont été associés sous un modèle additif, tandis que rs4912911 (OR=0.37, 95% CI: 0.13-1.00, p=0.03) et rs2963156 (OR=0.32, 95% CI: 0.07-1.12, p=0.047) - sous un modèle récessif. Deux haplotypes incluant ces 5 SNPs (AAACA et GGGCG) ont été significativement (p=0.006 et 0.01 empiriques) associés avec la corticodépendance. 19 SNPs imputés ont été associés avec la dépendance de CS. Deux haplotypes multimarqueurs (p=0.001), incluant les SNPs génotypés et imputés, ont été associés avec la dépendance de CS.

Conclusion - Nos études suggèrent que le fardeau de la corticodépendance est élevé parmi les enfants avec le CD. Les enfants plus jeunes au diagnostic et ceux avec la maladie coexistante de la région supérieure ainsi que ceux avec des variations dans les gènes *ABCB1* et *NR3C1* étaient plus susceptibles de devenir corticodépendants.

Mots-clés : Épidémiologie génétique, étude d'association génétique, gène candidat, maladie inflammatoire de l'intestin, maladie de Crohn, réponse aux médicaments, variations interindividuelles, *ABCB1*, *NR3C1*, polymorphismes d'un nucléotide simple (SNPs).

ABSTRACT

Background - Inter-individual variation in response to treatment by corticosteroids (CS) is an important problem in the management of inflammatory bowel disease (IBD) patient's. This problem is even more prominent in children, the prevalence of steroid dependence (~40%) in whom is extremely high. Steroid refractoriness has a considerable impact on the physical and psychological development of these children, also imposing high medical costs related to treatment. Active disease, as opposed to quiescent, increases medical costs 2-3 times in ambulatory patients and 20 times in hospitalized cases. Identifying markers that could predict steroid response is therefore a high clinical priority. Previous attempts to investigate potential clinical and demographic markers have been equivocal, highlighting the need for further investigations of other predictive markers. It is well known that the action of CS entails complex processes controlled by genetic factors. Two genes, the *ABCB1* gene, which belongs to the family of trans-membrane transporters, and the *NR3C1* gene, coding for the glucocorticoid receptor, are major elements of the pathway. We postulated that inter-individual variations in these genes may play a role in the observed variability of the response to CS and could serve as potential predictors.

Objectives - We aimed to: (1) examine the burden of steroid refractoriness in children diagnosed with CD and explore the potential clinical/demographic factors related to CS response; (2) study the association between DNA variants in the *ABCB1* gene and CS response; (3) investigate the associations between DNA variants in the *NR3C1* gene and CS response.

Methods - We investigated these objectives in a cohort of CD patients recruited from two tertiary paediatric gastroenterology clinics from Ottawa (CHEO) and Montreal (HSJ). CD patients diagnosed prior to age 18 using standard clinical, radiological, endoscopic and histopathological criteria were included. Published criteria were adapted to define CS-resistance and dependence. DNA acquired from blood and/or saliva was genotyped for variations across the *ABCB1* and *NR3C1* genes selected using the tag-SNP

methodology. The frequencies of steroid resistance and dependence were estimated assuming a binomial distribution. Associations between clinical/demographic variables and steroid responses were examined using logistic regression modeling after accounting for potential confounding variables. Associations between *ABCB1* and *NR3C1* genes' variants and steroid responses were examined using logistic regression assuming different models of inheritance. Multi-marker associations were examined via haplotype analysis. Un-genotyped variants in the genes were imputed using HAPMAP data as the reference panel and associations with imputed SNPs examined using standard methods.

Results - Among 645 CD patients diagnosed at the study centers, 364 (56.2%) received corticosteroids during the first year since diagnosis. The majority of patients were male (54.9%), had inflammatory (84.6%), ileo-colonic (51.7%) disease phenotypes at diagnosis and were Caucasians (95.6%). Eight percent of patients developed CS-resistance and 40.9% became CS-dependent. Younger age at diagnosis (OR=1.34, 95% CI: 1.03-3.01, p=0.040), coexisting upper digestive tract involvement (OR=1.35, 95% CI: 1.06-3.07, p=0.031) and concomitant immunomodulators use (OR=0.35, 95% CI: 0.16-0.75, p=0.007) were significantly associated with CS-dependency in multivariate analysis. From among the 27 markers genotyped across the *ABCB1* (n=14) and *NR3C1* genes (n=13), all except one in *NR3C1* gene (rs258751, excluded) were in Hardy-Weinberg Equilibrium. For *ABCB1*, the rare allele of rs2032583 (OR=0.56, 95% CI: 0.34-0.95, p=0.029) and heterozygous genotype (OR=0.52, 95% CI: 0.28-0.95, p=0.035) conferred protection from CS dependency. A 3-marker haplotype including the functional SNP rs1045642 was associated with CS-dependence (empiric p-value=0.004). On imputation 24 intronic SNPs and six haplotypes were statistically significantly associated with CS dependence. None of these associations however maintained significance after corrections for multiple comparisons.

For the *NR3C1* gene 3 SNPs, rs10482682 (OR=1.43, 95% CI: 0.99-2.08, p=0.047), rs6196 (OR=0.55, 95% CI: 0.31-0.95, p=0.024), and rs2963155 (OR=0.64, 95% CI: 0.42-0.98, p=0.039), showed associations under an additive model whereas rs4912911 (OR=0.37, 95% CI: 0.13-1.00, p=0.03) and rs2963156 (OR=0.32, 95% CI: 0.07-1.12,

p=0.047) showed associations under a recessive model. Two haplotypes encompassing these 5 SNPs (AAACA and GGGCG) were significantly (empirical p=0.006 and 0.01 respectively) were associated with CS-dependence. On imputation 19 SNPs were associated with CS-dependence. Two multi-marker haplotypes (p-values=0.001 each) including genotyped and imputed SNPs conferred susceptibility for CS-dependency. Conclusions - Our studies suggest that the burden of steroid dependence is high among children with CD. Children diagnosed at a younger age, those with co-existent upper tract disease and with variations in the *ABCB1* and *NR3C1* genes were more likely to become CS dependent.

Keywords: Genetic epidemiology, genetic association study, gene candidate, inflammatory bowel disease, Crohn's disease, drug response, inter-individual variations, *ABCB1*, *NR3C1*, single nucleotide polymorphisms (SNPs).

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ABBREVIATIONS LIST

6-MP: 6-mercaptopurine

ABCB1: ATP-binding cassette, subfamily B, member 1

ABCG2: ATP-binding cassette, subfamily G, member 2

ACTH: Adrenocorticotropic hormone

ALL: Acute lymphoblastic leukemia

ASA: Acetyl salicylic acid

ATP: Adenosine triphosphate

AZA: Azathioprine analogs

BCCH: British Columbia Children's Hospital

CD: Crohn's disease

CEU: Utah residents with ancestry from northern and western Europe

CHEO: the Children's Hospital of Eastern Ontario

CI: Confidence interval

CRP: C reactive protein

CS: Corticosteroids

DNA: Deoxyribonucleic acid

GC: Glucocorticoid

GIT: Gastrointestinal tract

GR: Glucocorticoid receptor

GRE: Glucocorticoid response elements

GWAS: Genome Wide Association Study

HPA: Hypothalamic-pituitary-adrenal axis

HWE: Hardy-Weinberg Equilibrium

IBD: Inflammatory bowel diseases

IL: Interleukin

LD: Linkage disequilibrium

MAF: Minor allele frequency

MDR1: Multidrug resistance 1

MT: Mercaptopurine

MTX: Methotrexate

NF-κB: Nuclear factor kappa B

NR3C1: Nuclear receptor subfamily 3, group C, member 1

OR: Odds ratio

PB: Periferal blood

P-gp: P-glycoprotein

RA: Rheumatoid arthritis

RR: Relative risk

SJH: Ste-Justine Hospital

SNP: Single nucleotide polymorphism

TPMT: Thiopurine S-methyltransferase

UC: Ulcerative colitis

WGO: World Gastroenterology organisation

To my family…

To all travelers that shared this journey with me

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INTRODUCTION

Crohn's disease (CD), a type of inflammatory bowel disease (IBD), is a lifelong condition characterized by a chronic, extensive inflammation of the gastro-intestinal tract (GIT) with a relapsing and remitting clinical course. Its burden in Canada is one of the highest in the world. In particular, as in most western countries, the incidence of paediatric onset CD is increasing in Canada and Quebec [1-3]. Various lines of evidence suggest that CD's complex aetiology is due to the interplay between genetic, environmental and immunological factors. It is now well recognized that CD represents a group of heterogeneous diseases showing phenotypic variation [4-6]. Presently, given the yet unknown and most likely complex aetiology of CD, primary preventive strategies are not available, making secondary and tertiary prevention essential. Since active, as opposed to quiescent disease is associated with a 2-3-fold increase in costs for non-hospitalized cases and a 20-fold increase in costs for hospitalized cases [7], the achievement of clinical remission remains the main goal in disease management, well-warranted from the patient's and societal perspective.

Corticosteroids (CS) are the mainstay of treatment in moderate to severe CD, effectively inducing clinical remission [8]. A major issue with CS treatment is the observed large inter-individual variation in efficacy and associated side effects. For example, about 8%-20% of patients receiving CS do not respond to treatment, and, are CS resistant. Furthermore, among those who initially respond, a larger percentage (30% to 45%) subsequently relapse during dose reduction thus impeding discontinuation of treatment, or relapse shortly after the end of treatment (CS dependent) requiring additional doses of steroids.[9-14]. CS are relatively inexpensive therapeutic agents, but they come with significant long-term complications, such as osteoporosis, impaired glucose tolerance, cataracts and poor wound healing, which entail high medical costs. For example, CS dependent patients are more likely to require surgical intervention [13, 14] and additional CS requirement has been shown to be associated with disabling disease course [15]. Surgical intervention accounts for 40% of inpatient medical costs

[16-18]. The consequences of surgical intervention, aside from its high cost of hospitalization [17], greatly impair patients' quality of life, preventing participation in daily activities and affecting patients' self-image, particularly that of adolescents and young adults, affecting their psychosocial development and well-being. CS therapy itself can cause numerous side effects [19, 20], some of them severe, such as linear growth retardation and bone demineralization [21]. Children with osteopenia have higher risk of fractures in childhood as well as in adulthood. In addition, other side effects such as striae, acne, and "moon face" present clinical dilemma when administering CS to adolescents. Therefore, the identification of patients susceptible to CS refractoriness (CS resistance or dependence) early during the course of the disease is an important issue in CD therapeutic management.

CS mediates anti-inflammatory responses by first binding to the intracellular glucocorticoid receptor (GR). Activated GR mediates transcriptional regulation of specific target genes when transported to the nucleus of the targeted cells. Consequently, appropriate activation and expression of the *GR (NR3C1)* gene is necessary for anti-inflammatory responses. Stevens et al. [22] have described a haplotype of the *GR* that is associated with dexamethasone resistance in normal individuals. These findings, in addition to the fact that the *GR* gene is located in a region of chromosome 5 previously linked to CD (known as the IBD5 region), suggests that variants in the GR may underlie susceptibility to steroid refractoriness and that *GR* may be an important candidate for determining this phenotype.

The *ABCB1* gene, also called the multidrug resistance gene 1 (*MDR1*), is another key gene. It codes for the P-glycoprotein 170 pump and is expressed in high concentrations on the apical surfaces of superficial columnar epithelial cells of the colon and distal small bowel and functions as an efflux pump, transporting steroids out of the cell, thus reducing their efficacy [22]. The gene is located on chromosome 7q21.1, a region linked to CD [23]. This gene is also involved in the metabolism of xenobiotics [24, 25]. The *MDR1* gene therefore is also potentially a prime candidate gene associated with response to CS.

CS are potent anti-inflammatory agents used to treat various inflammatory, autoimmune and proliferative diseases [26-28] and altered sensitivity to CS has been reported in a variety of medical conditions [29]. For instance, in pediatric asthma [30], an increased heterogeneity in response to CS has been observed [31] wherein about 5 % of patients showing altered response with CS dependency as a major complication [32, 33]. In acute lymphoblastic leukemia (ALL) [34, 35] and rheumatoid arthritis [36] the inter-individual variability in response to CS is a widespread phenomenon and shown to be related to GR and *ABCB1*(*MDR1*) genes [37, 38]. Various malignancies have been shown to have *MDR1* resistant phenotype [39] including leukemia [40], suggesting the role of this gene in inter-individual variability of response to treatment. In asthma, similarly to IBD, several mechanisms have been proposed to account for a failure to respond to CS including a reduced number of GRs, altered affinity of the ligand for GR, reduced ability of the GR to bind to DNA or increased activation of transcription factors, such as AP-1, that compete for DNA binding. The observations that CS dependency is not related to the binding, distribution and clearance of prednisolone supports the notion that CS dependency in asthmatics could be related to defects in steroid receptor sensitivity that is mediated by variation in the GR gene. [41]. The latter is hypothesis is supported by findings by Leung et al (1997) who demonstrated that in asthmatic patients insensitive to CS (or dependent) there were cytokine-induced abnormalities in the DNA binding capability of the GR implying that variability in the GR gene could be related to CS dependency. [42]. Other studies [31, 43] however have pointed that rather than primary structural defects of the GR gene per se, but an increase in pro-inflammatory transcription factors such as AP1 may be related to altered CS response, in particular CS resistance.

The mechanisms of CS resistance in RA are not fully understood, nevertheless existing evidence suggests that this could involve known molecular events related to the mechanisms of CS action. These include alterations in the functional status of GR [44], and perturbations of the cytokine and hormonal milieu [45]. Moreover, significantly elevated T-lymphocyte *MDR1* expression has been shown in patients with RA who

require CS, supporting the importance of *MDR1* gene in response to CS [46]. It is therefore possible that variability in response to CS is a common phenomenon across multiple phenotypes and involved mechanisms could be related to variation in the *ABCB1* and *NR1C3* genes.

Although possible mechanisms underlying the anti-inflammatory effects of CS are relatively well outlined, information on the biological pathways that determine steroid dependence and resistance is limited. Furthermore, there is currently limited information on potential markers that could predict the variability in response to CS. On the basis of the known molecular mechanisms underlying the anti-inflammatory effects of steroids, we have proposed that important proteins, the activities of which are partly under genetic control, could be important determinants of CS response. Modifications in these key proteins due to genetic variation could potentially determine an individual's therapeutic response to corticosteroids. Identifying these stable markers would greatly facilitate the identification of potential subgroups of patients who would be corticosteroid-dependent or resistant and hence benefit from timely alternate treatment strategies.

This study therefore aimed to examine the burden of CS refractoriness in children diagnosed with CD and to examine potential clinical/demographic factors related to response. The second objective was to study the association between DNA variants in the *ABCB1* gene and CS response. The third objective was to investigate the associations between DNA variants in the *NR3C1* gene and CS response. The thesis comprises four chapters. The first describes the knowledge surrounding the inter-individual variation in response to CS in CD patients and other autoimmune inflammatory conditions as well its postulated mechanisms. The second chapter is dedicated to the description of methodology used for examining proposed hypotheses. The third chapter describes the results for the 3 studies that were carried out to investigate the outlined objectives. Finally, a general discussion of acquired results and future perspectives are, outlined in the fourth chapter.

The first article of this thesis characterizes the burden of corticosteroid-refractoriness in paediatric patients and examines potential clinical or demographic predictors related with response to CS. In the second article, associations between variants in the *ABCB1*/*MDR1* gene and CS-dependence are reported and in the third article, those between the *NR3C1*/*GR* gene and CS-dependence are described.

CHAPTER 1

LITERATURE REVIEW AND STUDY RATIONALE

1.1 Crohn's disease (CD)

Crohn's disease (CD) along with ulcerative colitis (UC) comprises inflammatory bowel diseases (IBD) a chronic, relapsing, inflammatory intestinal disorder postulated to result from abnormal host - microbe interactions and involving a complex interaction between genetic and environmental components [47-52]. CD is characterised by transmural inflammation leading to various complications such as fistulae, strictures and the narrowing of inflamed sections of digestive tract. The natural course of CD is characterised by numerous relapses and progression towards more complicated disease [53]. CD can occur at any age but the peak incidence is between the teens and early twenties.

1.1.1 Epidemiology of CD

Burden of pediatric CD is increasing worldwide, particularly in Canada where over the last two decades the highest incidence and prevalence have been reported. According to a province-wide study carried out in Manitoba, the incidence and prevalence of CD among individuals below 20 years of age were $12.7/10^5$ and $56/10^5$ respectively, suggesting that the incidence among children has approached that of adults, which ranges from $13.5/10^5$ to $16.5/10^5$ [1]. Benchimol et al. [54] has reported an increasing trend in CD incidence from 9.5 (in 1994) to 11.4 (in 2005) in Ontario. According to more recent nation-wide study conducted by Bernstein et al. in five Canadian provinces, the average incidence rate of CD for children less than 20 years of age during 1998-2000 was $8.3/10^5$ [3]. This study also revealed a geographic variation with the lowest CD incidence in the western provinces (British Columbia) and highest CD incidence in the eastern provinces (Nova Scotia). Recently, a Quebec based study reported even higher incidence and prevalence of CD [2]. According to this study, the age- and sex-standardized average prevalence for 1993-2002 was $189.7/10^5$ and age- and sex-standardized CD's incidence was $20.2/10^5$ person-years for the 1998-2000 period. The average incidence in those <20 years at diagnosis was $13.9/10^5$.

The female/male ratio among incident cases was 0.74 for the 0-14-year-old group which is in contrast with adult onset of CD (1.77).

Over the past two decades (1983-2003) IBD hospitalization rates remained stable despite of a steady decline in the overall rate of hospitalization in Canada [55]. The percentage of all hospitalizations attributable to IBD has risen as a result. The stability of IBD hospitalization rates reflects a high proportion of readmissions due to chronic and complicated clinical course of the disease. It was shown that hospitalizations due to CD accounted for more than half of direct medical costs in the USA [56]. Kappelman et al. when estimating the direct health care costs of children and adults with CD or UC in the USA [57] reported that the annual health costs attributed to the management of pediatric IBD were higher as compared to adults (9800 $ versus 8000 $).

1.1.2 Clinical features of paediatric CD

CD is characterised by chronic clinical course with many disease flares and increasing occurrence of complications leading to surgeries. Numerous studies have emphasised the particularities of CD in children versus that in adults [58-67]. In comparison to adult patients, children with CD differ in disease phenotype and are more likely to have severe disease course and greater number of complications. More importantly, children are at risk of growth retardation and pubertal delay [68-71] and suffer more from psychological consequences.

1.1.2.1 Disease localization and behaviour

There are suggestions that paediatric CD may be phenotypically different from adult onset CD, implying potential differences in pathogenesis [66]. Disease localization is the most consistent among the reported phenotypic differences. Compared to adults, children, especially those diagnosed in first decade of life, more often tend to have colonic disease presentation [59, 62, 64, 72] and less of ileitis. Children diagnosed at an age prior to 8 years are of special interest, as they tend to have a more isolated colonic localisation of disease [59, 60]. The uncomplicated disease behaviour at diagnosis (80-90 %) can change with

time to stricturing and/or penetrating disease. These rates are much higher in children than in adults (66%). The occurrence of complications such as abscesses, fistulas and strictures often leads to major surgical interventions. In a Scottish [66] study, childhood-onset IBD was characterized by more “pan-enteric” (extensive) CD (L3-L4; 43.2% vs. 3.2%; OR=23.36; 95% CI: 13.45–40.59) than adult-onset CD. According to this study, 24% of children developed stricturing or penetrating complications within 4 years (vs. 9% at diagnosis). A subsequent study in a French [65] paediatric CD cohort reported similar rates, confirming distinctions with adult CD. Clinical management and treatment of CD

The two focuses of CD management are the induction and maintenance of remission. Various treatment strategies are employed to achieve these goals [73].

Treatment of paediatric CD patients is largely, based on experience gained from adult populations. There are however, particular aspects of CD that need distinct management strategies. Since it is widely accepted that CD has an effect on nutritional status and growth in children, the main goals of therapy in paediatric IBD are: to (1) maximize efficacy; (2) maximize adherence; (3) minimize toxicity; (4) maximize quality of life; (5) maintain physical and psychosocial growth; and (6) prevent disease complications [74] .

Depending on presenting symptoms, severity of illness, and individual patient's disease course, treatment can involve multiple medications with varying regimens, dietary changes, and surgery. Historically CD treatment paradigms were changing with the advances in development of pharmacologic agents. Initially a conventional so called “step-up” treatment approach was predominant when patient’s treatment was usually started with 5-Acetyl salicylic acid analogs (ASA-5), and if not successful was followed by corticosteroids, immunosupressors, biologic agents and finally by surgical intervention. Azathioprine and its metabolite 6-mercaptopurine (6-MP) belong to the class of thiopurine immunomodulators. They are prescribed for steroid-refractory patients enabling the tapering of corticosteroids and are useful in the maintenance of remission in moderate to severe CD [75, 76]. These drugs have a slow onset and take about 3 to 6 months to generate

an effect [77]. Methotrexate is also used as an immunosupressor administered in patients with side effects or those failing to respond to azathioprine and 6-MP [78]. One of recognised drawbacks of the "step-up" approach is that patients are often treated with a non-effective agent for prolonged periods without achievement of mucosal healing, thus unnecessarily subjecting them to the potential side effects of these drugs [79]. The presently emerging trend of "top-down" therapy is based on the premise that early use of biological agents can heal mucosa and perhaps change the course of disease [58]. The anti-TNFα is commonly used biological treatment in paediatric CD. The most common is Infliximab, a chimeric monoclonal antibody (IgG1) directed against TNF-α that neutralizes circulating TNF-α and binds to cell-bound TNF-α causing apoptosis. Two other biological agents - adalimumab and certolizumab have also shown efficacy in controlled clinical trials. Infliximab administration however is associated with the development of antibodies to the medication in a large proportion of patients [80, 81].

1.2 Corticosteroids (CS)

CS are a class of steroid hormones that are produced from cholesterol in the cortex of the adrenal gland. Glucocorticoids and mineralcorticoids are two subclasses of these hormones. Glucocorticoids are a class of steroid hormones that binds to the GR receptor. The name "glucocorticoid" derives from their role in the regulation of the metabolism of glucose, their synthesis in the adrenal cortex, and their steroidal structure. In technical terms, "corticosteroid" refers to both glucocorticoids and mineralocorticoids (as both are produced by the adrenal cortex), but is often used as a synonym for glucocorticoid (GC). In this document, the term "corticosteroids" is used interchangeably with "glucocorticoids".

Some common natural glucocorticoid hormones are corticosterone and cortisol. CS exert known immunosuppressive effect and are used widely in the treatment of chronic inflammatory disease such as IBD. They have been used in the treatment of active IBD for several decades and are effective in inducing remission in moderate to severe CD with success rates of about 70% [9, 12]. However, CS are not appropriate for maintenance

therapy because of lack of efficacy [82] and side effects associated with prolonged use. Commonly used systemic CS are available in parenteral hydrocortisone and methylprednisolone formulations and oral prednisone, prednisolone, and budesonide formulations [83]. Hydrocortisone is the active liver metabolite of cortisol. Methylprednisolone, prednisone and prednisolone are the synthetic analogs of cortisol [84]. Budesonide (Entocort^{TM,} Astra Pharmaceuticals) is a corticosteroid with low systemic bioavailability owing to a 90% first-pass liver metabolism.

1.2.1 Anti-inflammatory action of CS

Chronic inflammation is characterised by the increased expression of multiple inflammatory genes regulated by proinflammatory transcription factors, such as nuclear factor-kappa B (*NF-κB)* and activator protein-1 *(AP-1)*. CS mediate their anti-inflammatory action in cells via binding to glucocorticoid receptor (GR) protein – a member of the nuclear receptor subfamily [85]. Subsequently the GR-substrate complex passes into the nucleus where it acts via other genes participating in inflammatory pathways. CS action is dependent on GR-mediated transcriptional regulation of specific target genes as a result of sequence-specific deoxyribonucleic acid (DNA) binding, which in turn inhibits the promoter regions of genes such as *NF-κB* and *AP-1* [26, 86-88] the potent transcription factors for many pro-inflammatory cytokines and adhesion genes (**Figure 1** on page 12). Important to the anti-inflammatory action of CS is the induction of inhibitor kappa B alpha *(IκBα)* which binds to and inhibits *NF-κB* by sequestering it in the cytoplasm [89, 90] and thus regulates the inflammatory process.

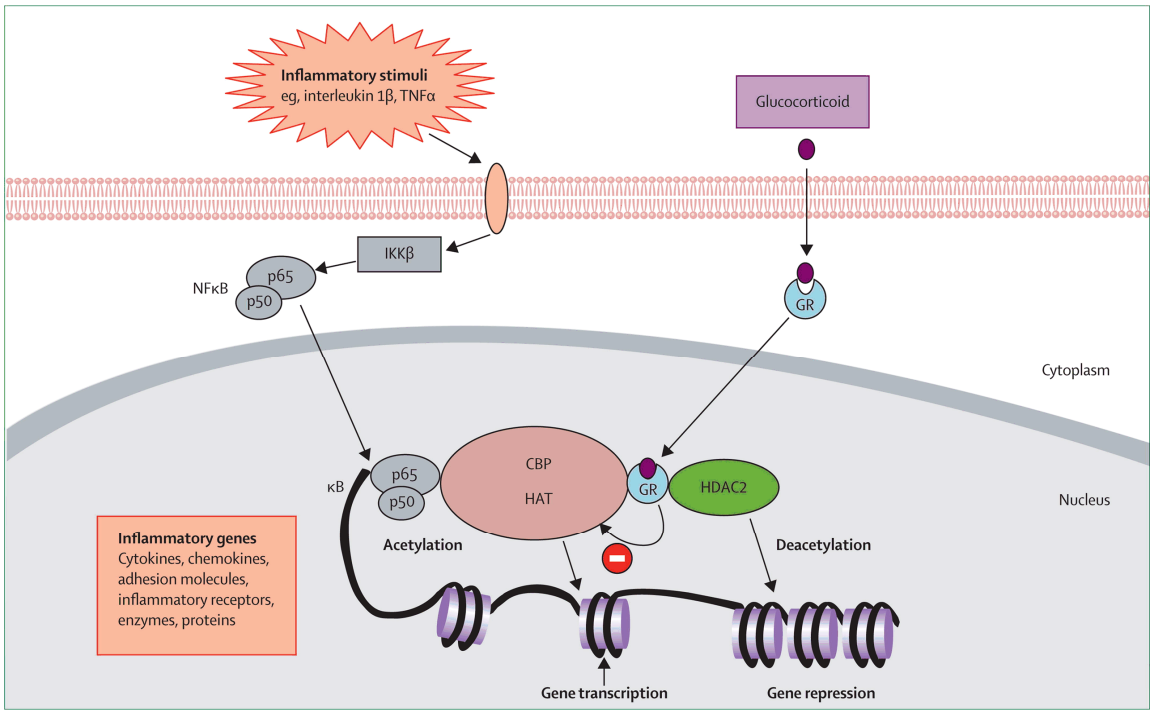


Figure 1. Glucocorticoid suppression of activated inflammatory genes

Legend: Inflammatory stimuli activate inhibitor of nuclear factor κB (NFκB) kinase (IKKβ), which therefore activates NFκB. A dimer of p50 and p65 NFκB proteins translocates to the nucleus and binds to specific κB recognition sites on the promoter regions of inflammatory genes and also to coactivators, such as cyclic AMP response element binding protein (CBP). The co-activators cause acetylation of core histones, activating gene expression of inflammatory proteins. Activated glucocorticoid receptors (GRs) bind to co-activators in the nucleus to inhibit histone acetyltransferase (HAT) activity directly. GRs also recruit histone deacetylase 2 (HDAC2), leading to suppression of the activated inflammatory genes. TNFα=tumour necrosis factor α.

(From Barnes PJ, Adcock IM. "Glucocorticoid resistance in inflammatory diseases". Lancet 2009; 373:1905-1917).

The exact mechanism of action of budesonide in the treatment of CD is not fully understood. However, being a glucocorticosteroid, budesonide has a high local anti-inflammatory effect.

1.2.1.1 Disease activity assessment and CS dosing

Given that treatment decisions are based on disease severity and disease localization [91], it is important to evaluate disease activity in CD at the beginning of treatment. The assessment of therapeutic efficacy is also based on disease activity. Several measures have been proposed to assess disease activity. The Crohn's disease activity index (CDAI) is widely used for the assessment of disease activity in adult patients. This index is based on a 7-day assessment of variables including laboratory values, objective examination and history. After minor modifications CDAI was validated and considered appropriate for use in children [92]. The Pediatric Crohn's Disease Activity Index (PCDAI) is thus a well-validated tool in paediatric patients with CD [93]. The scale is scored 0 - 100 based on subjective criteria (e.g., pain), objective criteria, laboratory findings, and growth parameters. Scores <15, indicate inactive disease; 15-30, mild to moderate disease; and >30, severe disease activity. In comparison to CDAI, the PCDAI index is better able to discriminate between levels of disease activity. Nevertheless, this index incorporates parameters related to growth, which tend to change slowly over relatively short periods of clinical studies, and hence the index is less responsive measure for assessing disease activity in such context. To overcome these limitations, the modified Harvey-Bradshaw index (HBI) [94] was created and has been successfully used to measure disease activity in paediatric patients [76]. This index is less cumbersome, easier to calculate and shown to be highly correlated with CDAI [95] and Physician Global Assessment Index [93].

Usual dose of oral CS is 1 mg per kg of body weight up to 40 mg/day, beyond which additional benefits have not been observed [96]. Budesonide is used in patients with ileal disease. Similar to conventional CS, budesonide is well absorbed from the proximal and distal intestine, relying on rapid hepatic metabolism to reduce systemic impact. Budesonide, administered as 9 mg/day, has been shown to be efficacious for active ileal

and ileocecal Crohn's disease [97-99]. Patients with fulminant inflammatory CD are treated with intravenous corticosteroids, such as methylprednisolone.

1.2.1.2 CS withdrawal schemes

After an achievement of clinical remission, CS cannot be stopped abruptly because of CS withdrawal symptoms. The suppression of Hypothalamic-pituitary-adrenal (HPA) axis occurs early when supraphysiologic doses of CS are used (which is the case in the treatment of CD) and may result in a secondary adrenal insufficiency. Patients undergoing CS dose tapering may experience lethargy, malaise, anorexia, myalgias, headache, and fever. Withdrawal plans are based on a dual goal to complete therapy and at the same time to avoid the potential consequences of adrenal insufficiency. Given that there is insufficient evidence to recommend any particular CS withdrawal regimen [100], various dose tapering schemes are used in clinical practice for prednisone and its analogs. These diverse CS withdrawal schemes however, according to Yang et al. do not appear to influence the long-term outcomes of CS therapy [101]. Traditionally withdrawal schemes begin by incrementally reducing the CS from supraphysiologic to physiologic doses. The physiologic dose is approximately 15-20 mg of hydrocortisone per day or its equivalent. Once patient is on a replacement (physiologic) dose of CS, various strategies may be taken subsequently. To allow the recovery of HPA some authors recommend switching to alternate day therapy [102]. The most common scheme consists of gradual decrease of CS daily dose (by 5 mg per week). Consequently, if at the beginning of therapy an average daily dose is 40 mg, the weaning from CS period takes about 7-8 weeks. For budesonide the dose tapering scheme usually consist of dose decrease by 3 mg/day/month, thus starting from 9 mg/day it takes about 8 weeks to wean of CS.

1.2.1.3 CS sparing medication

CS are rarely administered alone. In order to avoid disease reactivation and to facilitate dose-tapering process, immunomodulators or 5-ASA are introduced. Recently the early use of immunomodulators has become standard of care in IBD and has decreased

corticosteroid dependence. In patients with newly diagnosed moderate to severe CD a multicenter trial of 6-mercaptopurine and prednisone has revealed that an addition of 6-mercaptopurine significantly improves treatment outcome in comparison to the addition of placebo [76].

1.2.2 Inter-individual variation of response to CS in CD patients

Despite successful use of CS in treatment of CD, some patients fail to respond to the standard steroid therapy employed to induce remission and control acute disease flares, thus transforming the status of the disease into corticosteroid-dependent or corticosteroid-resistant [103, 104]. CS dependency in CD is characterized by frequent relapses and a requirement for chronic corticosteroid use. It is important to recognize those patients predisposed to develop an impaired response to CS, since it may allow the timely establishment of alternative or additive treatments, thus avoiding unnecessary exposure to CS side effects. The extent of inter-individual variation of response to CS in adults patients has been well described initially by Munkholm et al. in a population based study carried out in Denmark [13], and subsequently by others [14, 105-109]. The Danish study reported CS resistance in 20 % and CS dependence in 36 % of 109 CD patients (**Table I** on page 16). Similar results were shown by a USA population-based study from Olmstead County, Minnesota [14] carried out on 74 CD patients (28 % CS dependent and 16 % CS resistant). Three other hospital-based retrospective studies in adults carried out in UK, Italy and China reported CS dependence frequencies of 24%, 35 % and 38 % respectively. CS resistance frequencies in these studies were comparable to the Danish and USA studies, with the exception of the Chinese study that reported lower rates (6%).

In paediatric patients, four studies [65, 76, 77, 110] have described rates of CS-dependence and CS-resistance. A population-based study describing the natural history of CD from France included 404 CD patients and reported that 25 % of patients became CS dependent [65]. Another population-based study conducted by Thung et al. in Olmstead County, Minnesota, USA, examined a small number of patients (N=26) and observed CS dependency in 31% of patients [110]. Similarly in a study from a multicenter North

American observational registry, among 109 newly diagnosed children with moderate-severe CD treated with corticosteroids, 31% had become CS dependent [77]. A North American multicenter clinical trial reported the highest frequency (50%) of CS-dependence in the placebo study arm [76]. Barring the later study, rates of CS dependence in paediatric CD were similar to that reported among adults. Rates of CS resistance were by-and-large similar across the paediatric studies [77, 110] and are slightly lower than in adults.

Table I: Studies examining the response to CS therapy in CD patients.

Study		Outcomes, %	
		CS Resistance	CS Dependence
Adult	Munkholm et al., (1994)	20	36
	Faubion et al., (2001)	16	28
	Ho et al., (2006)	20	24
	Papi et al., (2007)	14	35
	Chow et al., (2009)	6	38
	Weiss et al., (2009)	1	21
Children	Thung et al., (2006)	12	31
	Markowitz et al, (2000)	NA	≈ 50
	Markowitz et al, (2006)	17	31
	Vernier-Massouille et al, (2008)	5	25

NA, Not a study's objective.

1.2.2.1 Side effects of prolonged CS therapy

CS can induce side-effects even after short-term treatment of 2-3 weeks [111]. Prolonged use of CS may cause multiple systemic side effects. The toxicity of CS increases with higher doses and prolonged use. Side effects of CS range from reversible short-term effects (moon face, skin striae, weight gain, acne, sleep disturbances, mood swings and psychoses) to serious consequences, such as bone loss, growth retardation and pubertal

delay. Exogenous Cushing's syndrome can occur as a result of prolonged use of CS [112]. Exogenous Cushing's syndrome usually presents with the same signs as spontaneous (endogenous) Cushing's syndrome (**Figure 2** below). However because some patients receive high doses of CS during prolonged period, the onset of the exogenous syndrome may be more striking than in the case of spontaneous Cushing syndrome.

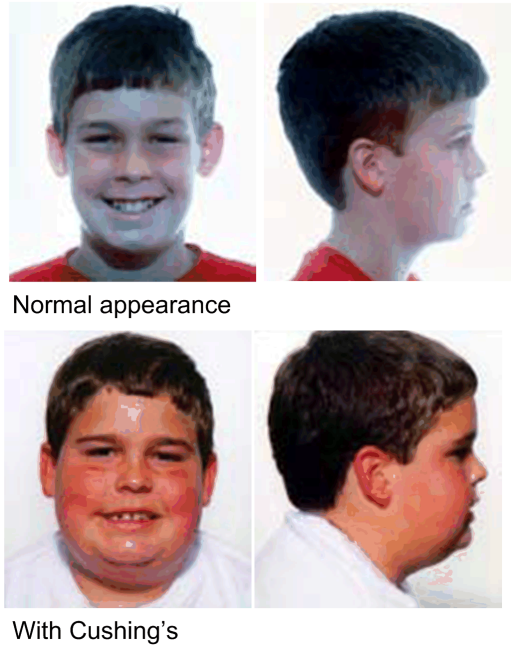


Figure 2. Symptoms of Cushing's syndrome.

The traditional stigmata of Cushing's syndrome include weight gain usually presenting as central obesity (**Figure 3** on page 18 and **Figure 4** on page 19) with redistribution of body fat to truncal and dorsocervical areas and appearance of classical moon face.

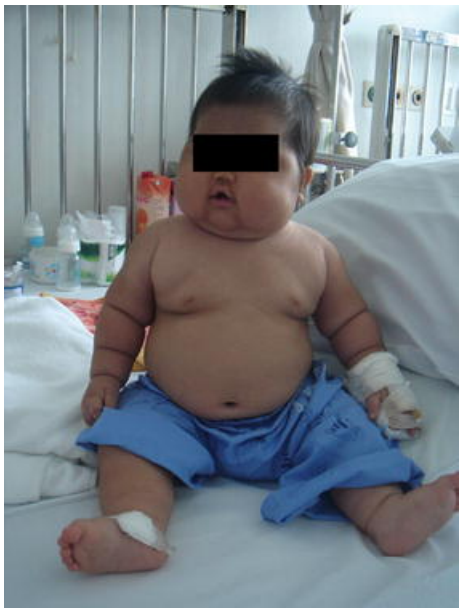
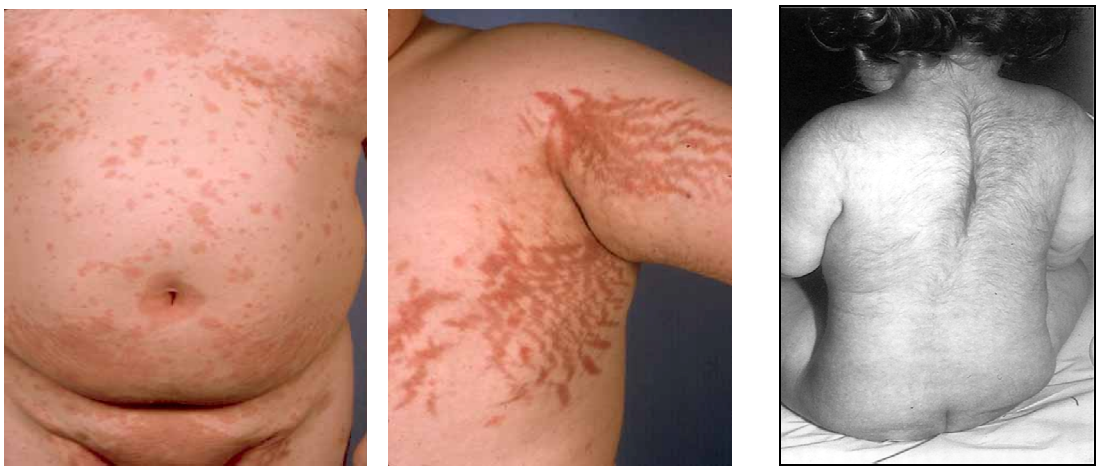


Figure 3. Typical clinical signs of Cushing syndrome including obesity, moon-face and hirsutism.
(From Therdpong Tempark et al, "Exogenous Cushing's syndrome due to topical corticosteroid application: case report and review literature". Endocr., 2010, 38:328–334, with kind permission of Springer Science and Business Media).

Weak muscles, thin skin, striae, easy bruising and hypertrichosis also can be present (**Figure 4** on page 19). Patients became susceptible to poor wound healing and infections [113]. Some manifestations of CS excess appear early in treatment course. For example, psychiatric disturbances, increased appetite and insomnia can occur within hours. In a double blind multicenter clinical trial comparing budesonide to prednisone [114] 55% of patients receiving prednisone experienced one or more side effects associated with CS therapy (p=0.01).



A B C

Figure 4. Side effects of corticosteroids

(A) central obesity; (B) skin striae

(From Edwin K Joe MD**,** "Cushing syndrome secondary to topical glucocorticoids". Dermatology Online Journal 9(4): 16, © 2003 Dermatology Online Journal, with the permission of NYU Langone Medical Center, The Ronald O. Perelman Department of Dermatology).

(C) Hypertrichosis of the back caused by excessive intranasal steroid use.

(From Perry R J et al. "Cushing's syndrome, growth impairment, and occult adrenal suppression associated with intranasal steroids". Arch Dis Child 2002;87:45-48, ©2002 by BMJ Publishing Group Ltd and Royal College of Paediatrics and Child Health).

As mentioned above, among possible side effects of particular concern are the potential for bone loss [21, 115-119] and deleterious effects on linear growth [120, 121]. Osteopenia and osteoporosis are common and severe adverse effects that can lead to increased risk for bone fractures. The relationship between long-term use of CS and risk for bone fractures is well-established [122-124]. In healthy subjects the life-long risk of bone fractures is related to peak bone mass which is achieved in puberty [125]. It is also known that CS have negative impact on calcium homeostasis [126]. Moreover it is suggested that

inhibition of osteoblast function by CS [127, 128] results in decrease of bone formation. The prevalence of osteopenia in paediatric patients is as high as 70% according to some reports [129, 130]. These studies have shown that CS use contributes to decrease of bone density resulting from other factors such as hipoalbuminemia, parenteral nutrition and use of immunomodulators. According to these studies, a cumulative dose of 5g of CS and 12 months exposure was associated with the maximum loss in bone density. Related to losses in bone density, growth impairment and associated pubertal delay [131, 132] are common in paediatric patients with IBD and in particularly with CD. Growth failure is reported to occur in 15 - 40% of children with CD [68]. Although growth failure and significant bone decrease can be present before treatment with corticosteroids [121, 133], exposure to CS can further hinder the achievement of peak bone mass and maintain the growth delay.

Children treated with prednisolone [19, 20] may develop ocular complications. When carrying out ocular examinations of 58 CS-treated paediatric patients with inflammatory bowel disease (IBD) and 58 age-matched controls, Tripathi et al. observed the presence of subcapsular posterior cataract in 20% of cases [19]. An increased intraocular pressure was noted in a case-control study among patients with nephrosis who were receiving CS [134]. Observation that ocular complications can follow CS therapy was further substantiated by findings that steroid cataract and glaucoma occurred in patients on long term (>2 years) treatment with prednisone at doses higher than 15 mg. None of these patients had a history of eye disease prior to CS treatment [135].

CS treatment can also lead to opportunistic infections. Toruner et al. [136] reported strong risks (OR=3.4; 95% CI: 1.8-6.2) of infections associated with CS use in IBD patients. A variety of opportunistic infections, ranging from viral, fungal, bacterial, and mycobacterial organisms were associated with use of CS, according to this study. The severity of infections varied considerably, ranging from mucosal herpes to life-threatening systemic fungal infections. Compared with other immunosuppressant agents CS have also been found to increase the risk of C. difficile infections (RR=3.4; 95% CI: 1.9-6.1) [137]. A meta-analysis [138] that examined the risk associated with CS use has also shown that patents with intestinal diseases who were administered CS were more prone to infections

(RR=1.4; 95% CI: 1.1-1.7). Moreover, CS increase the risk of wound infections by hampering wound healing both by their anti-inflammatory action and by inhibiting collagen synthesis [139].

Regarding central nervous system function, CS have been shown to influence mood, memory and neuronal integrity [140]. A major concern related to CS use has been the resulting psychological health consequences, in particular in children with IBD [141-146]. This is particularly challenging as in spite of recovery from reversible side effects, children's psychological problems persist and contribute to low self esteem. Some studies have suggested that CS may be causally related to depression in children [147, 148]. Psychological side effects from CS therapy can occur early during the course of CS therapy and during CS withdrawal as well. The effects range from minor to moderate mood, behavioural and cognitive effects to acute psychological symptoms. In children with IBD exposed to CS, depressive symptoms were likely to be more prominent than in non exposed patients [149]. In this study subjects receiving CS were more likely to have Children Depression Inventory (CDI) scores > or =12, and those with such scores were on higher doses of CS than were subjects without clinically significant depressive symptoms (both p values < 0.05). The frequency of acute psychological reactions is not negligible with reports that they can occur in about 1.3% of patients receiving 40 mg of oral prednisolone per day [150].

1.2.2.2 Clinical and demographic markers of variability in response to CS

As outlined above, CS treatment although effective in CD, is associated with numerous side effects and shows high variability in response. Identification of immunological, biochemical, and/or clinical parameters that may predict refractoriness to treatment has therefore been an important research priority. **Table II** on page 23 summarizes the findings of the major studies undertaken. In the Munkholm et al. study no correlations between initial symptoms such as diarrhoea, abdominal pain, weight loss, associations with fever, age, sex, duration, extent, and localization of disease, or laboratory values (haemoglobin, white blood cells count, erythrocyte sedimentation rate, serum

albumin) and response to CS treatment were evident [13]. Younger age of diagnosis as a predictor of CS dependence was reported by Franchimont's et al. in a retrospective study carried out in an adult population comparing 20 corticosteroid dependent and 248 non-corticosteroid dependent CD patients [106]. Corticosteroid-dependent patients were younger ($p < 0.05$), more frequently smokers ($p < 0.05$), and suffered more often from colonic ($p < 0.05$) or inflammatory type of CD. Colonic disease localization was negatively associated (HR= 0.3, 95% CI: 0.10-0.80) in a Chinese study [105] and positively (OR=3.2, p=0.009) associated with CS dependency in an Israeli study [109]. Laboratory parameters such as trombocytosis was positively associated with CS dependence (HR=3.0, 95% CI: 1.40-6.40) and only stricturing disease (HR=4.5; 95% CI: 1.80-10.9) was predictive for CS dependence in a Chinese study. An Italian study [108] examining 77 CD patients reported increased C reactive protein (CRP) levels (OR=5.57, 95% CI: 1.20-25.91) during steroid weaning despite clinical remission. This study also noted that penetrating complications (OR=4.20, 95% CI: 1.76-10.04) might predict further steroid requirement in already steroid responsive patients. A North American multicenter paediatric study examined whether clinical or laboratory parameters were associated with CS therapy outcomes [77] and found that growth delay at diagnosis was associated with CS dependence. In this study, amongst both the corticosteroid-dependent and surgical patients, 67% presented with growth impairment, compared with only 18% among those who had a prolonged 1-year response (p=0.001). A paediatric study conducted by Markowitz et al. further confirmed that growth delay at diagnosis could influence the occurrence of CS dependency [77]. In a prospective multicenter study Gelbmann et al. evaluated 30 clinical and 24 laboratory parameters in 239 adult CD patients, but found that prior bowel resection (OR=3.63, 95% CI: 1.79-7.36) and perianal disease (OR=2.28, 95% CI: 1.12-4.66) were associated with CS resistance. In this study, however, none of the studied parameters was associated with CS dependence [151].

Table II: Studies examining potential predictors of response to CS in CD patients.

	Study	Markers examined	Association with CS dependence	Association with CS resistance
Adult population	Munkholm et al., (1994)	C, D, LAB	None	None
	Franchimont et al., (1998)	C, D	Younger age at diagnosis, L2	NA
	Faubion et al., (2001)	C, D	None	None
	Gelbman et al.(2002)	C, D, LAB	None	Prior surgery
	Ho et al., (2006)	C, D, smoking	None	None
	Papi et al., (2007)	C, LAB	Increased CRP, B3	None
	Chow et al., (2009)	C, D, LAB	L2, trombocytosis	B2 B3
	Weiss et al., (2009)	C, D	L2	NA
Children	Thung et al., (2006)	C, D,	None	None
	Markowitz et al, (2000)	Growth	No	NA
	Markowitz et al, (2006)	C, D, LAB, growth	Growth delay	None

C, Clinical variables; D, Demographic variables ; LAB, Laboratory parameters; B2, Stricturing disease; B3, Penetrating disease; CRP, C-reactive protein; L2, Colonic disease; NA, Not applicable.

1.3 Proposed mechanisms for the variability in response to CS therapy

1.3.1 Inter-individual variability of response to CS in autoimmune diseases

CS are used to treat a variety of different immune-mediated diseases [27]. As glucocorticoids they affect a variety of vital functions in the body, such as glucose and fat metabolism, immune response, cell differentiation and response to stress. GC act through the cytoplasmic glucocorticoid receptor (GR) and negatively or positively regulate approximately 20% of genes expressed in leucocytes.

Inter-individual variability in response to CS is encountered in healthy subjects [152-154] as well in those with various medical conditions [45, 155-158] such as rheumatoid arthritis (RA), asthma, leukemia and IBD. Based on clinical and laboratory evidence, subjects can be divided into, “steroid-sensitive” and “steroid-resistant” groups. In this context, it is important to separate the rare familial condition of primary CS resistance from the more common phenomenon of poor response to therapeutic doses of CS. Primary hereditary resistance to glucocorticoids is a rare disorder due to mutations in *NR3C1* gene [159, 160]. In this syndrome, feedback inhibition of the hypothalamo-pituitary-adrenal axis is set at a higher level with slightly elevated plasma adrenocorticotropic hormone (ACTH) and increased circulating cortical concentrations. Generalised cortisol resistance is characterised by hypercortisolism without Cushingoid features. The Cushingoid features are absent because of the lack of receptors in all target organs. Symptoms of resistance to glucocorticoids arise primarily from ACTH-induced adrenocortical overstimulation which results in increased serum concentrations of androgens and mineral-corticoids [161]. Because of the elevated ACTH levels, adrenal androgen levels are also increased, causing - particularly in females - acne, hirsutism and virilisation, male pattern baldness, menstrual irregularity, and infertility. Only a part of resistance to beneficial effects of glucocorticoids in patients with RA and asthma is attributed to generalised (hereditary) form, with the majority of patients experiencing the acquired (local) form due to the high cytokine

production [161]. The local form of glucocorticoid resistance is supposed to be somehow compartmentalised by T-lymphocytes or other forms of inflammatory cells. This assumption is supported by studies of correlation between T-lymphocytes proliferation and drug response in inflammatory conditions such as asthma [162], RA [163] and renal allograft rejection [164].

In CD, Franchimont et al. studied sensitivity to GC by comparing the response to exogenous dexametahsone between quiescent CD patients and healthy controls in blood cells [165]. This study reported a decrease in sensitivity to GC in CD patients compared to healthy controls, suggesting a predominant role for genetic/constitutional predisposition over acquired factors. Nevertheless, no significant difference in sensitivity to GC was observed between CS-dependent and CS non-dependent patients and both subgroups of patients had similar degrees of dexamethasone-mediated cytokine inhibition. The absence of any differences in sensitivity between CS dependent and CS non-dependent patients may be due to: (1) CS dependence not related to sensitivity to CS and (2) improvement of response to CS under the influence of concomitant administration of immunosuppressive drugs.

1.3.2 Mechanisms of inter-individual variability of response to CS in IBD

There is some knowledge of the mechanisms underlying steroid refractoriness in other inflammatory conditions such as asthma and RA (viz. a post-receptor mechanism) [166] and reduced peripheral T-lymphocyte binding affinity [167] as well as an increased expression of β isoform of GR receptor [45, 168]. However, the pathophysiology of steroid refractoriness in CD has not been as well studied. Based on the available evidence, three potential mechanisms have been proposed [156]:

1. Decreased cytoplasmic steroid concentration secondary to increased P-glycoprotein-mediated efflux of steroid from target cells due to over-expression of the *ABCB1* gene;
2. Impaired steroid signalling due to dysfunction at the level of the *NR3C1* gene;

3. Constitutive epithelial activation of proinflammatory mediators, including *NF-κB* and resulting in the inhibition of GR transcriptional activity.

The proposed mechanisms underlying steroid refractoriness are depicted in **Figure 5** below.

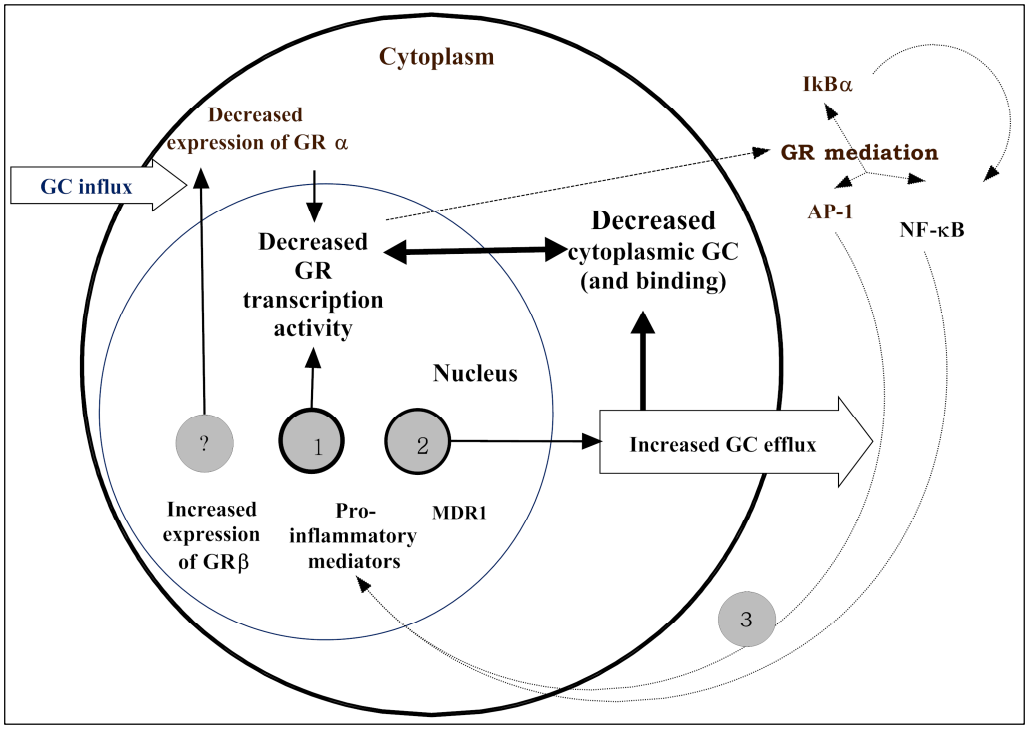


Figure 5. Proposed mechanisms of variation in response to CS

1.4 The *ABCB1* gene

ABCB1 belongs to the family of adenosine triphosphate (ATP) cassette binding transporters and also known as *MDR1*. This gene, located on chromosome 7, spans 200 kb comprising 28 exons and codes a P-glycoprotein-170 efflux pump (P-gp). Many drugs, including therapeutic CS, are substrates of this transporter. Cortisol, dexamethasone and

methylprednisolone have been shown to be the substrates of P-gp. Given the large spectrum of substrates and widespread expression in tissues, *ABCB1*, along with other genes coding for transporters, is thought to play a major role in cell protection from environmental and endogenous harmful substances.

1.4.1 Role of the *ABCB1* gene in modulating response to CS

1.4.1.1 P-glycoprotein's function and substrates

P-gp is a known drug transporter, which eliminates exogenous and endogenous substances from cells. According to currently accepted model, P-gp functions as a transmembrane efflux pump [169], transferring substrates from intracellular to extracellular compartments (**Figure 6** on page 28). It can also remove drug molecules trapped in the cellular membrane. ATP hydrolysis provides energy, allowing the active transport of substrates against steep concentration gradients. The "vacuum cleaner" metaphor is often used to describe this mechanism. A wide spectrum of substrates [24, 170, 171], including a variety of structurally divergent drugs, makes this protein interesting to study in relation to steroid drug metabolism. Several studies have shown that P-gp can bind to and transport different steroids [172-177]. Natural corticosteroids – cortisol, and the CS used in IBD treatments (dexamethasone and methylprednisolone) are shown to be substrates of this protein [173, 176]. More recently, two other steroid drugs, budesonide and prednisone, were also identified as a substrates of the intestinal drug efflux pump [177].

Human P-gp is a complex phosphorylated and glycosylated transmembrane protein that has 1280 amino acids long and is composed of 2 homologous and symmetric sequences, each of which contains 6 transmembrane domains and an ATP-binding motif [178].

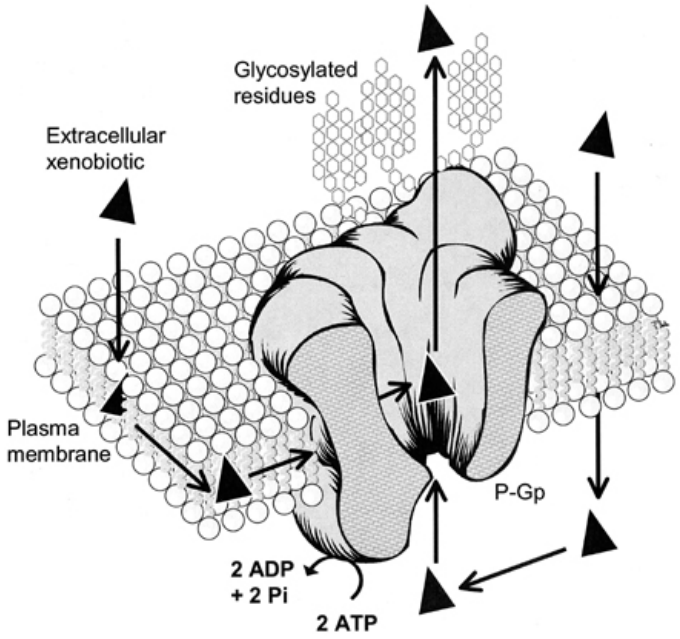


Figure 6. P-glycoprotein (P-gp) function.

Legend: This model shows that P-gp–mediated efflux transport of drug substrates can occur at the level of the plasma membrane or from the intracellular compartment. ATP, Adenosine triphosphate; ADP, adenosine diphosphate; Pi, inorganic phosphate.

(Reprinted by permission from Macmillan Publishers Ltd: From Marzolini C, Paus E, Buclin T, Kim RB. "Polymorphisms in human MDR1 (P-glycoprotein): recent advances and clinical relevance". *Clin Pharmacol Ther* 2004; **75:**13-33. Copyright © 2004, Nature Publishing Group).

1.4.1.2 Expression in tissues

Several studies have found the products of *ABCB1* gene being expressed in many human tissues including the digestive tract. The highest concentrations of P-gp are present in epithelial cells of the colon [179] followed by the ileum, jejunum and stomach [179, 180]. This protein can limit oral drug availability, acting in the small bowel [181] and actively transporting various substrates, including drugs, out of the target cells, thereby reducing their efficacy [182]. Consequently, variability in P-gp expression or function can alter the extent of absorption, distribution in tissues, and excretion of substrate drugs. The

function and the anatomic localization of P-gp suggest that this transporter acts as a protective barrier to keep toxins out of the body by excreting these compounds into bile, urine, and the intestinal lumen.

Variability in drug response is widely observed for several of P-gp substrates. One potential mechanism for this variability is inter-individual differences in P-gp activity that result in altered pharmacokinetics. The number of P-gp transporters on the cell membrane and the level of P-gp transport function are the two most important factors that control the apparent activity of P-gp. Farrel et al. demonstrated that peripheral blood lymphocytes from IBD patients with previous bowel resection due to failed medical therapy had a higher P-gp expression compared with patients with inactive disease [183]. In a follow-up study, the same authors further demonstrated that inhibition of the *MDR1* pump using specific inhibitors could significantly increase intracellular human intestinal epithelial and T-lymphocyte levels of CS [184]. These findings were in line with results from animal studies, which have revealed that mdr1-deficient mice had an increase of dexamethasone levels in the small and large bowel as compared to wild type mice [185-187]. Some other studies have suggested that *ABCB1*/*MDR1* may be a potential target for immunosuppressive therapy in various medical conditions [46, 188-191]. For instance, patients with rheumatoid arthritis who required CS [46] had significantly elevated *MDR1* expression in T-lymphocytes. The relevance of genetic *ABCB1* polymorphisms to altered function of P-gp is also supported by the phenotype of mdr1 knockout mice spontaneously developing colitis [192]. Moreover, the *ABCB1* gene is a very polymorphic site comprising more than 50 coding SNPS 3 insertion/deletion polymorphisms [193]. The consistency of this feature with the very complex structure of P-gp and the wide spectrum of substrates and the results of expression studies allows us to conjecture about the role of genetic variations in response to CS. Therefore, the genetic variations of the *ABCB1* gene in relation to the function of this transporter are of high interest due to its potential to explain the variation in drug response.

1.4.2 Characterization of *ABCB1* genetic variants

The *ABCB1* gene is characterised by multiple variations across the gene. Many synonymous and non-synonymous variations have been described, most of which result in changes to the intracellular protein domain [194] suggesting that they could impact the function of the protein (**Figure 7**, below).

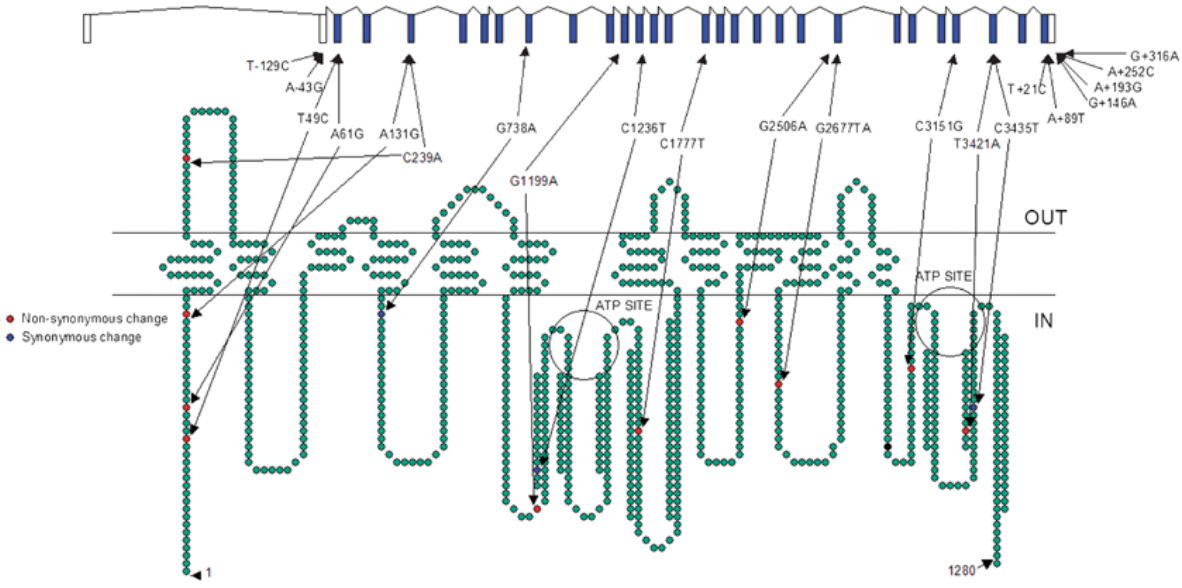


Figure 7. Polymorphisms in *ABCB1* with a minor allele frequency >5%, as confirmed by genotyping in Ensembl, as of January 2006.

(Reprinted with permission from Macmillan Publishers Ltd : from Leschziner GD, Andrew T, Pirmohamed M, Johnson MR. "ABCB1 genotype and PGP expression, function and therapeutic drug response: a critical review and recommendations for future research." *Pharmacogenomics J* 2007; **7:**154-179. Copyright © 2007, Nature Publishing Group).

1.4.2.1 Common variants and haplotypes of ABCB1

The *ABCB1* gene is located in a genomic region with strong linkage disequilibrium (LD). Kroetz et al [195] described the sequence diversity and haplotype structure of this

gene in several populations, including Caucasians. This USA-based study found two common haplotypes that represent 36% of all haplotypes observed. The haplotype *ABCB1**13, containing three SNPs (C1236T, G2677T/A, and C3435T), was most common in the Caucasian population. This study provided evidence that the C3435T site is in tight LD with other regions including G2677T and that multiple haplotypes contain SNP C3435T. The common synonymous variants C1236T and C3435T are evolutionary conserved SNPs whereas SNP G2677T is a tri-allelic non-synonymous SNP.

1.4.2.2 Expression studies in relation to genetic variants of ABCB1

Specific variants in the *ABCB1* gene influence glycoprotein expression. According to the SNP database maintained by the National Center for Biotechnology Information (NCBI), there are more than 50 SNPs in the human *ABCB1* coding region. Among these polymorphisms, two SNPs: SNP C3435T in exon 26 and SNP G2677T/A in exon 21 have been most commonly studied (**Figure 8** on page 32). Both these variants are thought to influence the level, activity and function of P-glycoprotein and thus to influence the transport and uptake of various substrates [196-200]. However, there is a considerable level of controversy about the functional significance of *ABCB1* polymorphisms. Both increased and decreased functions have been associated with in particular the C3435T variation [195]. In one study, Hoffmeyer et al. showed a borderline-significant (p=0.056) association of C3435T polymorphism with expression and function of the protein [196]. In this study, individuals homozygous for the rare allele (TT) of this polymorphism had significantly lower duodenal *MDR-1* expression and the highest digoxin plasma levels. Similarly, in healthy individuals, the rare allele and the rare homozygous genotype of this SNP was significantly correlated with decreased levels of expression of the *MDR1* pump [201, 202]. Consistently, Hiltz et al. have found that healthy individuals homozygous for this polymorphism (CC) have significantly higher *MDR1* expression in natural killer peripheral blood cells and as compared to the TT genotype [203]. More recently, Hitzl et al. demonstrated no association between placental Pgp expression and C3435T in mothers or foetuses, but described a positive association between protein expression and genotype

when wild type homozygous mothers and foetuses were compared with variant homozygous mothers and foetuses [204]. Likewise, allele T was also shown to be associated with better response to anti-depressive drugs in Taiwanese patients [205]. Other studies, however, have failed to replicate these results [199, 206, 207]. Interestingly, Markova et al. when examining *in vitro* the expression and function of *ABCB1* in human peripheral blood mononuclear cells subjected to experimental acute inflammation, reported that CS therapy might be more effective in carriers of the allele C of the C3435T SNP [208]. For the G2677T/A SNP, most of the studies have not showed any influence on protein expression [206, 207, 209, 210], barring one study that observed an association between the GG genotype and reduced mRNA expression [211].

Given these inconsistencies, some studies have suggested that the functional properties of *ABCB1* polymorphisms may reside in the haplotype structure rather than in single SNP. Reasons for the inconsistent results may be related to the fact that the substrate spectrum of *MDR1* is quite wide and may overlap with that of other transporters such as *ABCG2* [212] and *CYP3A4* [213, 214]. Moreover, given the substantial differences in the frequency of the C3435T variant between racial groups, the assessment of its functional significance is further complicated. The frequency of CC genotype of the C3435T SNP ranges from 21% to 42% in Caucasians according to different studies [196, 215-218]. However, recently, Kimshi-Sarfaty et al., have shown that despite an absence in any changes to the amino-acid sequences polymorphisms such as C3435T can influence protein activity and substrate specificity [219].

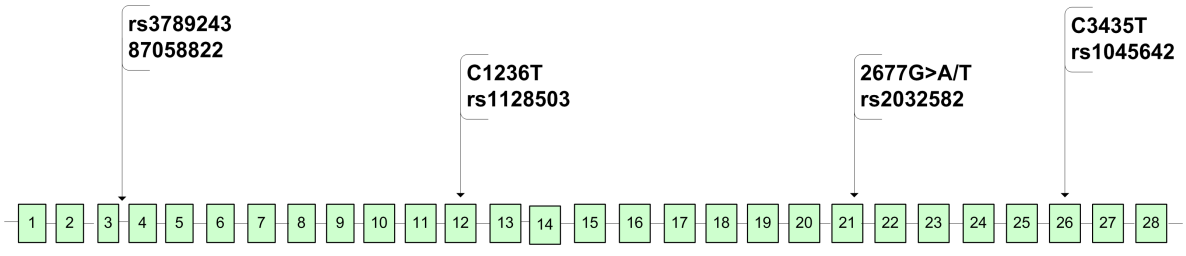


Figure 8. Most commonly studied *ABCB1* gene variants.

Therefore, despite the inconsistent evidence, polymorphisms in the C3435T genotype may potentially play an important role in determining the corticosteroid refractory phenotype.

1.4.3 *ABCB1* gene variants and susceptibility for CS refractoriness

1.4.3.1 Epidemiological studies of CS response in IBD

In spite of the known biological role of the *MDR1* gene in CS metabolism, very few studies have investigated associations between DNA variants in the *MDR1* gene and response to CS. In one study, Potocnik et al. [220] studied whether *ABCB1* gene polymorphisms were associated with corticosteroid refractoriness in a Slovenian population. CD cases with moderately to severely active disease, who failed conventional treatment using 5-ASA, antibiotics, corticosteroids and azathioprine and those patients who developed fistulising disease were considered as corticosteroid refractory. Twenty-four steroid-refractory cases were studied and frequencies of 10 exonic and intronic variants, including one SNP from the promoter region were contrasted with those among healthy blood donors. Significant associations with two intronic SNPs were observed: one in intron 13 (rs2235035) and one in intron 16 (rs1922242) with p-values of 0.014 and 0.024 respectively. These two SNPs were in LD one with another. The frequencies of the rare alleles of these two SNPs were lower among CD-refractory cases in comparison with controls (0.23/0.29 and 0.41/0.47 respectively). A SNP in exon 21 (A893S) (rs2032582) was marginally significantly associated with refractory CD (0.54 versus 0.40, p=0.064). Haplotype analysis using individually associated SNPs did not reveal associations with steroid refractoriness. A common 3-marker haplotype (TTT) comprising SNPs rs2032582, rs1128503 (C1236T exon 12) and rs1045642 (C3435T, exon 26) variants was however associated with a higher risk of refractory CD (OR=3.1, p=0.044).

In a more recent Italian study [221] Palmieri et al. examined the association of two *ABCB1* variants rs2032582 and rs1045642 (examined previously by Potocnik et al. above), with steroid-refractory CD. A responder to CS was defined as a patient who achieved

clinical remission after at least one course of systemic CS and CS-dependent as a patient who continued CS therapy at the end of one year due to relapse after discontinuation of treatment or due to relapse at dose reduction. This study did not detect any associations between either of the two SNPs and CS response in their population. In another study, De Iudicibus et al. examined the C3435T and G2677T variants in 117 Italian IBD patients, but did not find any associations with CS dependence [222]. The T allele of C3435T SNP was associated with significant or complete CS tapering in a cohort studied from Leuven [223]. In the only paediatric association study carried out so far, in an Italian population, the authors [224] did not observe any association between the C3435T SNP and CS response. Various reasons may underlie observed differences between studies carried out in seemingly heterogeneous populations. For one, most of the studies used different classifications of steroid-refractoriness. The significant results described by Potocnik were based on only 24 steroid-refractory cases and potentially susceptible to false-positivity, considering that no adjustments for multiple testing were made. Although Palmieri et al. studied a larger population [221], the phenotype definition was narrower than that in the Potocnik study. Besides, the Italian study examined haplotypes composed only of two markers.

A meta-analysis [225] that included seven studies [220, 225-230] examined associations between the two commonly studied SNPs (C3435T and G2677T/A) in relation CS response in a large sample of IBD patients. This meta-analysis included the Slovenian study [220] but not the two Italian studies [222, 224] that were published subsequently. No associations between the two SNPs and CS response were observed in CD patients. However, both the SNPs appeared to be associated with CS response in UC patients. The meta-analyses however included only adult studies. Given that paediatric CD differs extensively from adult CD, it is however likely that the associations between the *ABCB1* gene and steroid response may differ as well.

1.4.3.2 ABCB1 variants and CS refractoriness in other inflammatory diseases

Refractoriness to the anti-inflammatory action of CS has been observed in many medical conditions, including cancers [231] and immunity-related inflammatory diseases, further supporting the relevance of studying the variations of this gene in relation to CS-refractory IBD. About 30% of patients with acute lymphoblastic lymphoma (ALL) [35] and RA [232] are resistant to CS. In patients with ALL, a worse clinical response to CS was associated with the CC genotype of SNP C3435T (0.62 vs. 0.87; p=0.007) [231]. The C3435T variant has been found to be associated with response to CS in RA [233]. In this study, the probability of remission of RA symptoms after therapy with methotrexate and CS was 2.9 times greater in patients with the 3435TT genotype compared to patients with the genotypes 3435CC and 3435CT. Similarly, in children with heart transplants the TT genotype predicted weaning from CS [234]. Thus, patients with the CC genotype were significantly (p=0.04) more likely to be still on steroids 1 year after transplantation.

1.5 *NR3C1*/*GR* gene and response to CS

NR3C1/*GR* belongs to large family of nuclear receptors and the gene is located at chromosome 5q31-32 corresponding to the IBD-5 susceptibility locus. It extends 157,5 kb and contains 10 exons. The encoded protein has a three-domain structure: an amino-terminal trans-activating domain (TAD), which directs trans-activation of target genes, a DNA-binding domain, interacting with glucocorticoid response elements (GRE) in the DNA and a carboxy-terminal ligand-binding domain, which contains specific steroid and heat shock protein binding sites [235]. The biological role of this gene is well studied. Glucocorticoid receptor (GR) is derived from a single gene, and since its cloning, the prevailing assumption has been that a single receptor protein is responsible for the diverse actions of glucocorticoids. This “one gene-one receptor” paradigm has been challenged by recent studies revealing a large group of functionally distinct GR subtypes that arise from alternative processing of the gene. These receptor isoforms, in turn, are subject to various post-translational modifications that can further modulate their activity. The large variety of

GR isoforms probably explains the large spectrum of physiological functions regulated by *NR3C1*.

1.5.1 GR receptor

The protein encoded by *NR3C1* resides in the cytoplasm until it binds to a substrate, which induces transport into the nucleus. Variations in this gene are known to cause a glucocorticoid resistance (or cortisol resistance). Multiple isoforms of GR: GRα, GRβ and GRγ, arise from alternative splicing, the use of at least three different promoters and from translational events [236-238]. The GRα isoform is the full-length receptor, consisting of 777 amino acids, which binds to a ligand and mediates glucocorticoid action. Two truncated isoforms, GR (742 aa) and GR (676 aa), are unable to bind to a ligand and have been shown to mediate GR activity. The physiological and pathophysiological role of GRβ actually is controversial, as it has been shown to have a dominant-negative effect on GR activity [239] and as well a synergistic effect with GRα via transcriptional repression of cytokine genes *IL5* and *IL13* [240].

A GR-related variable phenotype encompasses a sensitivity and resistance to the suppressive action of GC on the hypothalamic-pituitary-adrenal axis (HPA). Several forms of resistance to GC were described, including familial resistance. Primary glucocorticoid resistance has been described as a rare familial or sporadic syndrome [241] mostly due to inactivating mutations of the GR gene, while several autoimmune-inflammatory diseases, such as rheumatoid arthritis, osteoarthritis, CD, ulcerative colitis and asthma, are often associated with resistance of the inflamed tissues to CS [242-244].

1.5.2 Role of the *NR3C1* gene in modulating response to CS

The anti-inflammatory actions of CS are directly mediated via their contact with the GR in the cell. Therefore, any modifications in the density and function of the GR are likely to mediate resistance to CS. Inside cells, CS bind to an intracellular receptor, so the sensitivity to CS may depend on the number of receptors and their affinity to ligand [245].

Several studies have shown reduced peripheral T-lymphocyte GR binding affinity [167] and abnormalities of GR-AP-1 binding [166]. Flood et al. studied GR receptor density in relation to response to CS in IBD patients, but did not observe a relation between GR density or GR mRNA levels and CS resistance [246].

The identification of several isoforms of the protein coded by *NR3C1* has led to the hypothesis that variations in the gene may influence the function or expression of GR. Particularly, increased expression of GRβ - a truncated splice variant of the normal isoform GRα that does not bind glucocorticoid ligands, and is unable to transactivate glucocorticoid-responsive genes, has been suggested to exert a dominant-negative inhibition of CS action [247]. Observations of increased expression of the GRβ isoform in some CS resistant states lend support of this hypothesis [248-250]. Nevertheless, studies comparing the differences in expression of the GRβ and GRα isoforms in IBD are not conclusive. For example, Honda et al. examined the expression of GRβ isoform in peripheral blood mononuclear cells of IBD patients and showed that the receptor was significantly more frequently expressed in glucocorticoid-resistant UC patients than in glucocorticoid-responsive patients and healthy volunteers [251]. The authors concluded that differential expression of GR isoforms might have a predictive value for response to CS. No differences in GRβ expression in patients with active CD and healthy subjects were however observed. In contrast, Towers et al. reported higher levels of GRβ-mRNA among steroid resistant CD patients in comparison to responsive patients [252]. In a more recent prospective study Hausmann et al. did not observe differences in the expression of GR isoforms in 35 IBD patients compared to healthy controls and CS-naïve patients [253]. Separate findings for CD were not presented. Most of these studies were however based on small sample sizes and using different controls, probably resulting in the discrepant findings.

1.5.3 Variants in *NR3C1/GR* gene

From among the known polymorphisms in the *NR3C1* gene, some such as the ER22/23EK (rs6189/6190), N363S (rs6195), BclI (41423247) and GR-9β are considered

functionally relevant (**Figure 9** below). These polymorphisms have been shown mediate changes in glucocorticoid sensitivity or altered cortisol levels [254-258].

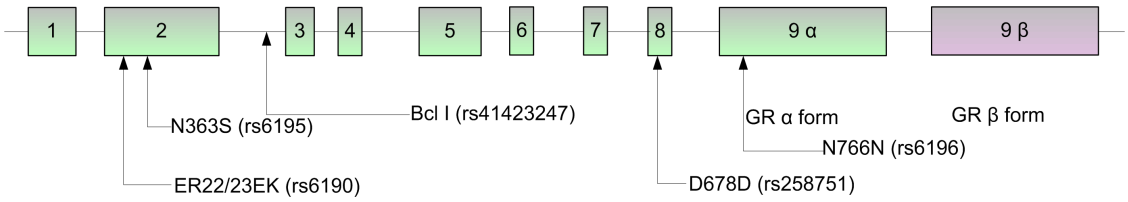


Figure 9. Structure of NR3C1 gene and location of the most commonly studied polymorphisms.

For example, SNPs rs41423247 and rs6195 (A>G) appear to increase sensitivity to dexamethasone [255, 259-261]. On the other hand, SNP, rs6190 (G>A) has been reported to decrease response to dexamethasone, indicating relative resistance to CS [254].

1.5.3.1 NR3C1/GR variants and susceptibility to CS refractory CD

Given the important role of *GR* in mediating the actions of CS and the influence of genetic variants to influence *GR* expression, some studies have examined the association between *GR* gene variants and response to CS in IBD.

Three Italian studies [222, 262, 263] have assessed whether the BclI polymorphism (located in intron 2) was associated with CS response in IBD patients. In one study, De Iudicibus et al. investigated 64 young patients with CD and 100 healthy blood donors [222]. In this retrospective study rare homozygote genotype was more frequent in responders to CS than in CS-dependent patients (OR=0.15, 95% CI: 0.03-0.68). These results were subsequently confirmed by the same author [263] in 84 CD and 72 UC patients. However, Maltese et al. when studying 70 healthy blood donors and 40 CD

patients [262] did not find the associations between this variant and response to CS. A recent Swiss study conducted by Mwinyi et al. analyzed 13 SNPs, mostly from the coding parts of the *NR3C1* gene, in a cohort of 181 (84 CD) IBD patients, but did not observe any association either with single SNPs or haplotypes with response to CS therapy [264]. This study included coding SNPs rs6190 (G>A) (exon 2) and rs6196 (A>G) (exon 9). A separate analysis for CD was however not carried out.

1.5.3.2 NR3C1/GR variants and response to CS in other diseases

Numerous associations between variants in the *NR3C1* gene and the corticosteroid resistance syndrome due to disturbances in the metabolism of endogenous steroids have been reported [265]. There are some reports that certain variants in the exon 9β of the gene (that determines the GRβ isoform) that are associated with a greater GRβ stability may be related to rheumatoid arthritis, [266] but there are no reports on CS response is such patients. Some variants in this gene have been reported to be associated with resistance to CS treatment in asthma [42, 249], leukemia and multiple myeloma [267-269]. More recently, Stevens et al. identified a 3-marker haplotype (GAT) consisting of rs41423247 (BclI), rs33389 and rs33388, associated with low post-dexamethasone cortisol administered in a combined group of healthy individuals and patients with psoriasis, suggesting that markers within the gene could determine population variability in response to exogenously administered steroids [261].

1.6 Summary of the literature and study rationale

CD burden has increased over the past two decades with rising incidence in children leading to major impact on health care costs. CD in children is characterized by a severe, extensive phenotype, the predominance of colonic localization and a greater risk of disease extension in localization and risk of surgery, suggesting the existence of a distinct CD phenotype and of differences in response to drugs.

CS are the mainstay of therapy in CD and are very effective in induction of disease remission. However, one of the drawbacks of this therapy is the occurrence of inter-individual variability in response. Rates of CS resistance and CS dependence in particular are very high in children and the effects of prolonged use can be deleterious in this population because of many side effects. Therefore, it is of high clinical importance to predict patient's atypical treatment response in order to personalise the treatment and to avoid unnecessary exposure to CS.

The search for predictive markers, however, has resulted in inconsistent findings, most acquired from studies in adults. Among putative predictors, younger age at diagnosis, colonic disease localization and penetrating disease behaviour, trombocytosis and CRP levels have been reported to be associated with CS-dependence. The association with younger age at diagnosis may indicate potential differences in mechanisms involved in CS response in children versus adults. In the few paediatric studies that have been carried out so far, only associations with growth retardation at diagnosis and CS dependence appear to be consistent.

The exact mechanism underlying CS response is presently unknown. Not surprisingly, the information on markers that could potentially predict CS response is also limited and inconsistent. Given our understanding of the actions of CS, targeting genes involved therein may help better understand reasons for variability in response. In this context, the *ABCB1/MDR1* and *NR3C1*/*GR* genes are of key interest as they are intimately involved in the CS action pathway and thus can mediate responses to CS treatment.

Despite the known mechanisms of CS action and the important role of the *MDR1* and *GR* genes, evidence for association between variants in these genes and CS response is both limited and inconsistent. Some of the inconsistencies are mostly related to the discrepancy in target coding variants to influence the expression of the relevant protein. This is in particular true for the *MDR1* gene wherein rather than nonsynonymous variants, synonymous coding variants appear to influence protein expression. Concerning the synonymous coding variants, furthermore, evidence appears to be conflicting with studies

showing both increases and decreases in protein expression. These seemingly dual effects limit the interpretability of studies examining the influence of these variants in CS responses in CD, in particular when such studies are carried out in prevalent versus new-onset cases and in adults. In both instances, the larger possibility of potential influence of environmental factors including therapy may alter expression of the relevant protein leading to inconsistent genetic associations. Unfortunately, most previous studies have been carried out in adults and in patients with long-standing disease. Studies to examine the potential influence of the *MDR1* thus need to be carried out in children and within a short time window since disease onset or diagnosis. A further limitation of previous studies has been the use of small number of markers and small sample sizes. In addition, different reference or control groups have been used, potentially leading to either false-positive or false-negative results. Many studies combined both CD and UC populations without separate analysis. Although CD and UC share some pathogenesis mechanisms it is now clear that the genetic predisposition to disease susceptibility varies extensively. Although this does not necessarily presuppose that susceptibility for treatment responses will also vary, given the distinct phenotypes and clinical courses of both these disease, and the differential expression of the *MDR1* and *GR* genes in different parts of the intestines, it would be nonetheless expected. Thus, separately studying the influence of potential predictors/markers in CD that is by-and-large more common in Canadian children (vis-à-vis UC) is required.

In summary, it is quite clear that CS refractoriness is a major clinical challenge in particular in children. Efforts to identify markers to identify children most likely to become refractory are urgently required. The principle aims of our proposed studies were to thus, comprehensively investigate, the hypothesized associations in a large cohort of children.

1.7 Study hypotheses and objectives

It is clear that administration of CS to children with CD although of high clinical benefit, present major challenges. A high proportion of children become either dependent

or are resistant to steroid medication. There is currently very limited information on why some children become refractory to CS while others do not. Some clinical and/or demographic characteristics have been implicated, but evidence is inconsistent. Based on known mechanisms underlying CS metabolism, we have hypothesized that genetic variability in the ability to metabolize steroids may underline susceptibility for inadequate response to CS. Specifically, we have proposed that DNA variations in the *MDR1* and *GR* gene may be important. Based on this hypothesis, the specific objectives of the study were:

- To describe the burden of the variability in response to CS and examine its clinical and/or demographic predictors in paediatric Crohn's disease patients.

- Examine if a variants in *ABCB1*/*MDR1* gene were associated with corticosteroid dependency.

- Examine if the variants in *NR3C1/GR* gene were associated with corticosteroid dependency.

CHAPTER 2

METHODOLOGY

2.1 Study design

Aiming to address our study objectives, we implemented a retrospective cohort study at two Canadian tertiary paediatric gastroenterology centers: Ste. Justine's Hospital (SJH) in Montreal, and the Children's Hospital of Eastern Ontario (CHEO) in Ottawa. The study cohort comprised of CD patients diagnosed prior to age 18 and treated with CS between 1980 and 2008 at SJH and between 2000 and 2008 at CHEO.

In order to establish the study cohort, all patients diagnosed with CD at the two centers for the requisite time periods were identified. The medical charts of these patients were consulted to identify those subjects who were administered steroids during the first year since diagnosis. For these patients, the medical records at each visit were scanned to determine the response of the patient to steroid medication. Published criteria were adapted to classify patients as either steroid resistant or steroid dependent, the two main outcomes of the study. The cohort members were contacted to acquire consent to participate in the study and to provide samples for DNA analysis. Acquired samples were then genotyped for variants in the study genes. The distribution of gene variants according to outcome status was then evaluated to examine associations between study genes and steroid response. Further details on the study population, recruitment and genotyping are presented in the next section.

2.2 Study population, selection of participants

As mentioned above, patients less than 18 years of age at diagnosis were identified and recruited from two Canadian tertiary paediatric gastroenterology centers. In order to define the population for the study (i.e. patients diagnosed with CD), we used multiple sources. One source was a list of patients treated for CD at the Montreal and Ottawa study centers. The gastroenterology clinics at both these centers maintain a comprehensive list of all patients who are treated for IBD at their clinics. The medical files of these patients were scanned to first confirm the diagnosis of CD and identify

those patients who were administered CS during the first year following diagnosis. In order to account for potential subjects not part of the lists and to establish a more comprehensive repository of patients, for the Montreal study center, we consulted the database maintained at the medical archives. The patient record database includes all clinic visits and hospitalization since 1980. Patient diagnoses are classified according to the International Classification of Diseases Ninth revision (ICD9) codes. Diagnosis codes that corresponded to CD (555.0, 555.1 and 555.2) were retrieved. The patient charts corresponding to the retrieved codes were then consulted to confirm the diagnosis of CD. Patients with CD and those who were administered steroids were included for study. Patients' inclusion and exclusion criteria are enumerated below:

1. Inclusion
    - Confirmed diagnosis of Crohn's disease using standard criteria
    - Administration of systemic CS
    - Administration of CS within 1 year since CD diagnosis
2. Exclusion
    - Patient age >18 years at diagnosis
    - Patients that received CS prior to CD diagnosis
    - Presence of serious co-morbidities (such as congenital disorders, neuromuscular degenerative disorders) with several additional medications prescribed

The process of data abstraction was established after preliminary consultations with the study gastroenterologists at the two study centers. At the Ottawa study center, data was abstracted jointly by the study gastroenterologist and the IBD research nurse. For the Montreal study site, the majority of the data was abstracted by the lead author (AK). At this site, important aspects of data abstraction that included deciphering medical synonyms & abbreviations specific to the center were first discussed & clarified by the team. In order to limit inter-observer bias, at the Montreal study center, data for key study variables (disease severity, CS dependence and resistance) was abstracted by

two independent observers (AK and DKA). Discordance if any was resolved by discussion with the study gastroenterologist(s).

2.2.1 Diagnosis of CD

The diagnosis of CD was established according to standard clinical, endoscopic, histopathologic and radiologic criteria [270, 271]. These criteria included: (1) the presence of characteristic small bowel involvement, (2) deep linear ulcers, (3) distinctive cobblestone aspect and discontinuing character of inflammation at endoscopy, or (4) patchy transmucosal inflammation with granulomas in histological samples.

2.2.2 Administration of CS

Patients treated with systemic CS administered within 1 year after diagnosis were considered in order to have a population of patients with limited exposure to concomitant multiple therapeutic agents. Patients who were administered steroids prior to diagnosis with CD were excluded. CS therapy included oral prednisone, intravenous methylprednisolone (administered to patients with very severe, usually fulminant disease) for 1 week and substituted with oral CS subsequently, and budesonide (administered to some patients with pure ileal disease).

CS doses determined empirically by the treating physician were abstracted. The dosage system was standard and corresponded to 1 mg/kg/day, with dosage no higher than 40 mg/day for children whose weight exceeded 40 kg.

All the different dosage decrease regimens were abstracted. The most common consisted of a decrease by 5 mg weekly until 0 was reached. Another recommended regimen was to decrease the dose by 5 mg /week, but when a dose of 20 mg was reached, the current dose would be alternated with one decreased by 5 mg over a week. The following week, both doses would be decreased by 5 mg. For example : if a patient was started on 40 mg - then weaned by 5 mg /week until prednisone reaches 20 mg/day, they would be administered a dose of 15 mg on alternate days each week alternating

with 20 mg. The following week the doses would change to 20 mg and 15 mg; the week after it would be 20 mg and 10 mg. The weeks after the alternate-day dosage would reach zero, the 20 mg dose would be decreased by 5 mg and alternated with zero. (thus 20-15-20-15-20-15-20-10-20-10-20-10-20-5-20-5-20-5-20-5-20-0-20-0-20-0-20-0-20-0-15-0-15-0-15-0-15-0-10-0-10-0-10-0-10-0-5-0-5-0-5-0-5-0). This last regimen was most frequently used at the CHEO study center.

2.3 Study variables

The main study variables are described in **Table III-A** on page 48 and **Table III-B** on page 51. Study outcomes were CS dependence and CS resistance. The main independent variables were the tag-SNPs in *ABCB1* and *NR3C1* genes with the addition of functionally relevant variants. Other co-variates included socio-demographic and clinical factors, some as predictors of the outcome and others as potential confounders.

Table III-A: Main study outcomes and independent variables.

	Name	Description	Related concept	Data source	Scale of measurement	Reference
Outcome (dependent) variables	CS resistance	Absence of response to CS assessed on 30th day after the start of treatment	Steroid refractoriness	Medical charts	Dichotomous: CS-resistant, responders	Munkholm, 1994
	CS dependence	Restarted CS due to relapse after the end of treatment, or inability to wean due to relapse at dose reduction.	Steroid refractoriness	Medical charts	Dichotomous: CS-dependent/ responders	Munkholm, 1994 Faubion, 2001
Independent variables	Tag-SNPs and haplotype in *ABCB1*	Identified using publicly available database as reference	Genetic variation	Blood or saliva (DNA samples) samples)	Dichotomous: Presence or absence of the allele, haplotype Categorical*	Carlson, 2004
	Tag-SNPs and haplotype in *NR3C1*		Genetic variation		Dichotomous: Presence or absence of the allele, haplotype Categorical*	

CS, Corticosteroids; DNA, Deoxyribonucleic acid; Tag-SNPs, Tagging single nucleotide polymorphisms.
*For genotype analysis the categorical variables (0-1-2) were used.

2.3.1 Definition of outcomes

The main study outcome variables were corticosteroid resistance and corticosteroid dependence. The corticosteroid dependent phenotype was assessed based on adaptation of previously recommended criteria [13, 76, 107].

2.3.1.1 CS-dependence

CS-dependence was defined as the reoccurrence of clinical symptoms during the weaning period (1) or clinical relapse occurring during 180 days following the end of treatment, requiring the reintroduction of CS (2) (**Figure 10** on page 50). Comparison groups consisted of responders to CS, i.e. patients that maintained partial or complete remission since the end of corticosteroid therapy.

Patients who had surgeries, who did not meet classification's criteria because of lack of inscription in medical files or who had super-infections such as C. difficile, which could mask symptoms of disease reactivation, were classified as "others".

2.3.1.2 CS-resistance

CS-resistance was defined, based on clinical symptoms, as the absence of response to CS treatment 30 days from the start of therapy. The following clinical symptoms were considered: (1) the number of abnormal stools per day, (2) the presence of mucus, blood or pus in the stool, (3) abdominal pain, (4) fever, weight loss and other extra-intestinal manifestations. Thirty days after the start of CS therapy, patients were classified into three categories:

Complete remission - total regression of clinical symptoms, with 2 bowel movements a day and no pain; no blood, pus or mucus in stool; no fever, weight loss or extra-intestinal manifestations.

Partial remission - regression of symptoms, with ≤ 4 bowel movements a day, less abdominal pain and no fever, weight loss or extra-intestinal manifestations.

Non-response - no improvement within 30 days.

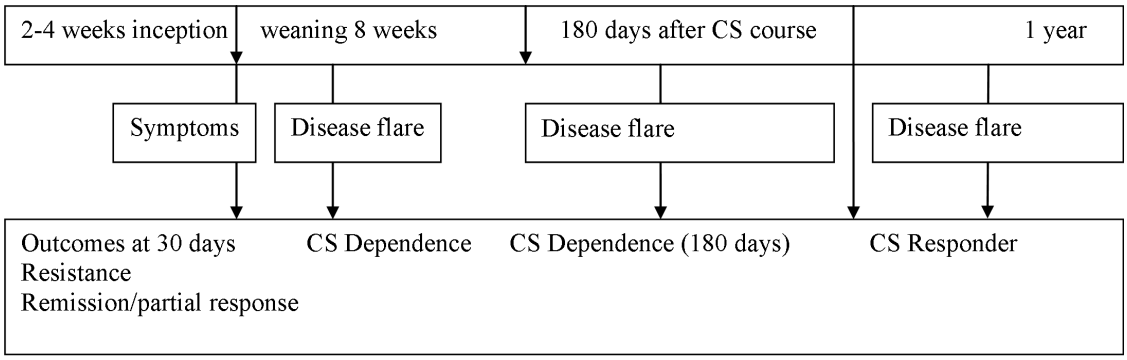


Figure 10. Study outcomes assessment criteria and timeline.

2.3.2 Information on covariates

Socio-demographic and clinical variables were used as potential predictors of the main study outcomes or as potential confounders in genetic association studies. The variables (1) age at diagnosis, (2) gender, (3) disease localization (4) disease behaviour, (5) disease severity, (6) extra-intestinal manifestations, (7) family history of IBD, (8) ancestry and (9) concomitant medication were postulated as clinical and socio-demographic predictors (independent variables) of CS-dependence and CS-resistance. The rationale for their selection, either as predictors or as potential confounders, is provided below.

Table III-B: Main study variables, covariates.

	Name	Description	Related concept	Data source	Scale of measurement	Reference
Covariates	Age at onset of CD*	Patients' age in years	Potential confounder	Medical charts	Categorical:1st versus 2-4 quartile	-
	Gender*	Female /male			Binary (1/0)	-
	Ancestry	European/non-European			Binary (1/0)	
	Disease severity*	HBI			Categorical: Mild-moderate/severe	Markowitz, 2000
	Disease behaviour*	Presence of complications			Binary:B1 versus B2 &B3	Satsangi, 2006
	Concomitant Medication	Given with CS			Categorical: 3 categories	As prescribed
	Disease localization*	Location in gastrointestinal tract			3 categories L1±L4;L2±L4 L3±L4	Satsangi, 2006
	Family history of IBD*	First or second degree relatives with IBD			Binary (1/0)	
	EIM				Binary (1/0)	Su, 2002

*These variables were used as covariates for genetic studies.
B1, Inflammatory disease; B2, Stricturing disease; B3, Penetrating disease; CD, Crohn's disease; EIM, Extraintestinal manifestations, HBI, Harvey Bradshaw Index; L1, Ileal; L2, Colonic; L3, Ileocolon; L4,Upper digestive tract involvement.

2.3.2.1 Age at diagnosis

Age at diagnosis is known to influence the occurrence of CD phenotypes. Although limited, some studies have shown that patients diagnosed at a younger age were more likely to be become steroid refractory [106]. It was thus included as a potential predictor of CS response. Although given the short age range of the study

population (5-18 years), the gene variants under study were unlikely to be differentially distributed within age-groups, based on reports that *GR* polymorphisms may differ according to age, age was considered a covariate in the genetic association studies [272]. For the purpose of the present studies, age at diagnosis was based on the date at which the endoscopy was carried out to confirm the diagnosis of CD.

2.3.2.2 Gender

Gender was considered a potential predictor of CS response. At the same time given observations that gender could be associated with complicated disease course [64, 273], it was also considered to be a potential confounder for other predictors of CS response.

2.3.2.3 Disease localization

Disease localization was classified according to the Montreal classification of Crohn's disease [274]. Disease localisation is an important characteristic of CD. Based on proposed CD classification (**Table IV** on page 53), disease localization was defined as ileal involvement - L1, colonic - L2 or ileocolonic involvement - L3. Upper digestive involvement - L4 was added to each of abovementioned categories, if applicable. Disease localisation was defined according to endoscopic, radiological and/or histological findings abstracted from the medical charts. It was considered as a potential predictor of CS response. Given that many genes are associated with specific disease localizations, it was also considered a potential covariate in the genetic association studies.

Table IV: Montreal classification of Crohn's disease.

Characteristics	
Age at diagnosis	A1 below 16 y A2 between 17 and 40 y A3 above 40 y
Localization	L1 ileal L2 colonic L3 ileo-colonic L4 isolated upper disease*
Behaviour	B1 inflammatory B2 stricturing B3 penetrating p perianal disease modifier†

*L4 is a modifier that can be added to L1–L3 when upper gastrointestinal disease is present.

† '' p'' is added to B1–B3 when concomitant perianal disease is present.

2.3.2.4 Disease behaviour

Disease behaviour was also determined according to the Montreal classification. Based on this classification, behaviour was classified as stricturing disease (B2) if endoscopic, and/or radiological examinations demonstrated the presence of strictures and/or bowel obstruction. It was classified as penetrating (B3) if investigations demonstrated the presence of internal fistulas or abscesses. Patients without structuring or penetrating disease were classified as having inflammatory disease behaviour (B1). The presence of perianal disease was included as a modifier to the three behaviour categories.

2.3.2.5 Disease severity

Disease severity is known to influence the clinical course of CD. It was considered an independent predictor of CS response. Disease severity at the start of

corticosteroid therapy was assessed using a modified Harvey Bradshaw Index (HBI) at SJH and the Physician Global assessment at CHEO. Both these criteria are highly correlated [93]. Other indices such as the Crohn's disease activity index (CDAI) and the Pediatric CDAI (PCDAI) are widely used. These however require prospective data collection that includes maintenance of a 7-day patient diary of subjective symptoms. For retrospective studies, HBI and PGA are thus preferred and the HBI has been shown to be strongly correlated with the CDAI a well. The criteria used to calculate HBI are presented in **Table V**. The information required for estimating the index was abstracted from the patients' medical charts. A binary variable was created by categorizing the scores as <5 and >= 5.

Table V: Harvey Bradshaw Index.

Symptoms	Subscore	Score
1. General well-being		--
Very well	0	
Slightly below par	1	
Poor	2	
Very poor	3	
Terrible	4	
2. Abdominal pain		--
None	0	
Mild	1	
Moderate	2	
Severe	3	
3. Number of liquid stools		--
0	0	
1-2	1	
3-4	2	
5-6	3	
7-8	4	
>8	5	
4. Abdominal mass		--
None	0	
Dubious	1	
Definite	2	
Definite and tender	3	
5. Complications*	1 for each item	--
Total score	Sum of subscores	

* Arthralgia, uveitis, E. *nodosum,* aphthous ulcers, pyoderma gangrenosum, draining fistula, abscess, temperature > 38 °, cutaneous vasculitis

2.3.2.6 Extra-intestinal manifestations

CD is known to present with various extra-intestinal manifestations (EIM). Information on manifestations such as presence of skin, eye, joint, mouth and other symptoms [62, 275] was abstracted (**Table VI**). EIM were considered as independent predictors of CS response. A binary variable corresponding to the absence or presence of any EIM was created.

Table VI: Extra-intestinal manifestations.

Group	Symptoms
Skin	Erythema nodosum
	Pyoderma gangrenosum
Mouth	Cheilitis
	Stomatitis
	Aphtae
Liver	Primary sclerosing cholangitis
	Hepatitis
	Cholelithiasis
Eye	Uveitis
	Episcleritis
	Conjunctivitis
Joints	Arthralgia
	Arthritis
	Ankylosing spondylitis
General	Fever
	Weight loss

2.3.2.7 Family history of IBD

Associations between some CD susceptibility genes and a family history of IBD have been reported. It has been reported, for instance, that the *CARD15* gene is associated with a family history of IBD [276]. We considered having a family history of IBD as a potential predictor of response to CS and included it as a co-variate in the multivariate genetic association analyses as it has been shown to increase the power of association studies [277]. Family history of IBD was defined as the presence (or absence) of family history of IBD (including its both IBD forms: CD and UC) in a first-degree relatives of the patient.

2.3.2.8 Ancestry

Ethnicity is known to be related to susceptibility to diseases, including susceptibility to IBD, and therefore could also influence the response to CS. Genetic population structure is known to influence findings of genetic disease susceptibility association studies [278, 279]. Population structure could have resulted in the noted discrepancies in associations between the *ABCB1* gene and susceptibility to IBD in ethnically similar populations [221, 280]. Such confounding also may have potentially resulted in lack of replication of susceptibility genes and response to CS [221]. Thus, accounting for potential structure may be important. We considered ethnicity as a potential predictor of response to CS. Based on findings of a lack of association between ethnicity and both response to CS and variation in the study genes, ethnicity was not included as a covariate in the genetic association analyses.

Patients were classified into two ancestry categories: European and not European basing on self-reported information about ethnic origin. Self-reported ancestry has been shown to be highly correlated with ancient geographic ancestry [281]. Information on ancestry was acquired by administering a socio-demographic questionnaire (see Appendix 1) to the patient.

2.3.2.9 Concomitant medication

Medication given concurrently (at the time of administration of CS) was considered as concomitant. As immunomodulators are frequently prescribed for the management of CS dependent CD, this definition included medication given only prior to disease relapse. Concomitant medication comprised of ASA-5 analogs or immunosuppressive agents [azathioprine (AZA), 6-mercaptopurine (6-MP), and methotrexate (MTX)] and antibiotics. The following categories were created:

Reference category-Patients who either did not receive any concomitant medication or received only antibiotics.

Category 1- Patients receiving immunomodulators.

Category 2 - Patients receiving ASA-5 analogs.

2.3.3 Genetic information

Patients were concurrently contacted to solicit participation, acquire ethical consent and DNA samples (blood and/or saliva). In order to extract DNA, either peripheral blood or saliva samples were acquired from study participants. Peripheral blood (PB) samples were collected during the patients' regular follow-up visit. Saliva was collected using ORAGENE KITS (DNA Genotek Inc., Ottawa, Canada) if blood samples were not being collected. Instructions for the appropriate collection of the saliva samples were given to each patient. For the subjects for whom a follow-up visit was not scheduled, the ORAGENE kits were mailed to them. Detailed instructions explaining the procedure for collecting saliva samples were sent to patients' (families) and they were contacted by phone to re-enforce the process and offer assistance. By-and-large, the ORAGENE kits are very user-friendly and provide large quantity of high quality DNA. In addition to its effectiveness, and non-invasiveness, this method encourages subjects to consent and is an efficient alternative to collecting DNA from blood samples. Upon receipt, the saliva kits were stored at 4ºC. Subsequently DNA extraction was carried out

according to the extraction methods provided by the manufacturer. For PB samples, DNA extraction was performed using the commercially available PUREGENE DNA isolation kit.

2.3.3.1 The variants selected for genotyping

The main genetic variables of interest were single nucleotide polymorphisms (SNPs) in the *NR3C1* (13 tag-SNPs and 1 functionally relevant SNP) and *ABCB1* (14 tag-SNPs) genes. "Tagging" refers to methods used to select a minimal number of SNPs that retain as much as possible of the genetic variation of the full SNP set. Since many of the SNPs are in LD with others, information acquired from few typed SNPs may serve as proxies for neighbouring SNPs. There are several approaches for selecting tagging variations, but tagging results are highly concordant between different tagging methods, despite the fact that they often involve different sets of tagging SNPs [282]. The tag-SNPs were selected using the LDSELECT program (http://pga.gs.washington.edu), from a publicly available data base of genotyped SNPs of a population of European descent (Seattle SNP public database, which employs an LD-based SNP selection algorithm proposed by Carlson et al. [283]. The regions of 2000 bp from the 5' and 3'ends of the genes were included in the selection process. Based on the Carlson algorithm, using a defined threshold for LD (r^2) and specifying a minimum allele frequency (MAF), SNPs in LD each with other are grouped in subsets called bins. SNPs with the highest LD with others SNPs are selected (tagged) using an iterative algorithm. Accordingly selected tagging SNPs are representative of the un-assayed SNPs. Using this approach, 80% of haplotypes in population could be resolved. The selected tag-SNPs are described in Appendices 2 and 3.

2.3.3.2 Genotyping

Genotyping was done using the GenomeLab Sequenom technology which is based on a newly developed genotyping assay termed iPLEX for use with the Mass ARRAY platform. Genotyping was performed using high-throughput facilities at the

McGill University Genome Quebec Innovation Center in Montreal (http://www.genomequebecplatforms.com/mcgill/home/index.aspx). The multiplexed genotyping assays rely on the molecular weight differences of DNA bases. Analyzing primer composition complementary to the target or by the addition of one or more non-complementary 5' bases to the genotyping primers, mass spectra of interleaved genotyping products can be generated with no ambiguity in allele assignment [284].

2.4 Data management

Clinical and demographic information was abstracted from patients' charts and entered in a structured database. Each patient was provided an alternate ID to maintain confidentiality. The database was constructed such that non-corresponding entries could be easily identified at the time of data entry. Specific algorithms were incorporated in the database that used the raw information to create the appropriate variable definitions. For example, specific algorithms based on the HB index enabled the classification of disease activity based on the raw clinical information required for the definition. Similarly, algorithms were used to classify individuals into CS resistant or dependent. The algorithms were pilot tested to establish their accuracy in a sample of patients (n=40).

The clinical database was linked to a genetic database that kept track of the DNA collection, extraction and analysis process.

2.5 Quality control

Quality control was ensured at each stage of date collection when possible. Clinical data were collected according to data entry forms and entered into the computerised data base. Data entry monitoring was performed regularly in order to identify the problems and errors that occurred, and these were discussed with project team. Data validation mechanisms were implemented in the data base itself, allowing

error detection by various means, such as refusal of incompatible values. The algorithms for study outcome definitions were implemented in the data base in order to diminish the future possibility of errors (**Table VII-A** below and **Table VII-B** below). In addition, to enhance accuracy, information on some key variables such as disease severity and the use of concomitant medication, was abstracted by two independent team members. In situations wherein abstracted clinical information did not enable adequate classification of certain patients, discussions were held with the team gastroenterologists to adopt the best available strategy (such as patient exclusion, use of alternative definitions and use of physician global assessment).

Table VII-A: Algorithms used to assess study short term outcomes.

Symptoms	**Response**				
	Complete	Partial			Absent (CS resistance)
Bowel movement	<=2	3 or 4	<=2	<=2	>4
Pus, mucus	0	0	Or 1	1 not daily	
Blood	0	0	Or 1	1 not daily	NA
Abdominal pain	0	0	Or 1	1 not daily	
Fever	0		0		Or 1
Weight loss	0		0		Or 1
EIM	0		0		Or 1

Table VII-B: Algorithms used to assess study long-term outcomes.

Prolonged response	**CS dependence**	**Other**
No relapse Or relapse later than 180 days from CS end	Relapse date < CS course end	Surgery
	Relapse date < = (CS course end+ 180 days)	Additional infection

In order to ensure the quality and quantity of DNA, genetic material was stored at -80 C and processed for DNA extraction according to standard protocols.

The genotyping process involved a stringent protocol for maintaining the quality of the results acquired. The GenomeLab Sequenom Genotyping System offers multiple advantages including: flexible and efficient assay design (96% success rate), improved call rates (85%) and accuracy (error rate $\leq$ 0.5%) and, significantly reduced cost per genotype. After a reception in the laboratory, samples were re-quantified, the quality of the assays for a particular SNP were validated in a sample of DNA. If the SNP assay performed well in the validation step, then the rest of the samples were genotyped. To assess the reproducibility of the genotyping techniques, 5% of the samples were randomly re-genotyped for all the SNPs. Genotyping results of SNPs showing >95% concordances on the duplicated samples were retained. Those samples that did not show concordance for a particular SNP were excluded from the analysis for that SNP. Samples that did not show concordance for at least 90% of the SNPs were entirely excluded from the analysis. Similarly, SNPs with <90% call rates were excluded from analysis.

Prior to statistical analysis, additional quality control measures were applied. Evaluation of genotyping errors was carried out by testing for HWE using exact test [285] as implemented in the software PLINK [286]. Departures from HWE may indicate genotypic errors in addition to indicating other phenomena such as (1) changing population structure due to non-random mating, (2) mutations and (3) chance. Testing for HWE is commonly used for quality control and is one of the few ways to identify systematic genotyping errors in unrelated individuals [287, 288]. SNPs not meeting HWE equilibrium ($p<0.05$) were excluded. In order to avoid observer bias, genotyping personnel were blind to the phenotype of the patient.

2.6 Study power

In order to estimate the study power required to detect significant associations, the following parameters were considered: the available sample size, the proportion of

patients expected to be CS resistant (and CS dependent); the expected magnitude of the associations (Odds ratios); the frequencies of clinical predictors and allele frequencies of the genes variants in the population; and the expected alpha level of significance. We thus estimated the power of the study based on a fixed sample size.

Based on the preliminary screening of patients diagnosed with CD at the two study centers for the considered time periods (1980-2007 at HSJ and 2000-2008 at CHEO) a maximum of ~750 CD patients were expected to be available. From among these ~60 - 70% (n=450) were expected to have been administered CS during the first year since diagnosis. From among these patients, based on prior experience, ~20 % (n=90) attrition was expected due to either missing clinical data, a lack of participation in the genetic study, inadequate DNA samples, genotyping errors or incomplete call rates. Rates of CS resistance and CS dependence were expected to be respectively ~ 15 (n=54) and 40 % (n=150) in paediatric CD patients' population based on previous literature [76, 110]. An approximate total of 300-360 patients were expected to be available for the analysis of the genetic and clinical associations.

Population frequencies of the study markers ranged from 10 to 50 %, according to publicly accessible databases. According to paediatric studies [59, 66], at the time of diagnosis with CD, 51% of patients have ileo-colonic disease (L3), 36% have pure colonic (L2) disease and 6% have pure ileal (L1) disease. Concomitant upper tract disease involvement is found in approximately 50 % of patients and complicated disease behaviour (stricturing and/or penetrating) is present in about 10-20% of patients. EIM at diagnosis may be present in up to 35 % of patients depending on definitions used [289, 290].

Basing on the above parameters, the power of the study to detect significant associations was determined. Appendices 4 and 5 present these calculations. As shown, the study was adequately powered (power ≥80%) to detect the effects (OR) ranging from 1.8 and higher for the majority of clinical and demographical predictors with frequencies ranging from 25% to 50 % (Appendix 4). For genetic associations, the study was

estimated to have adequate power to detect the ORs of about 1.6 and 1.7 for the markers with minor frequency of 0.50 to 0.30, and to detect the ORs higher than 1.8 and 2.0 for the markers with MAF of 0.30 to 0.10 (Appendix 5). Power estimations were made using the software QUANTO (freely available from http://hydra.usc.edu/gxe).

2.7 Statistical analyses

2.7.1 Statistical methods, article 1

To estimate the frequency of main study outcomes CS resistance and CS dependence a binomial distribution was assumed and 95% confidence intervals (95% CI) were estimated. To examine the role of potential clinical and demographic predictors of CS resistance and CS dependence, univariate and multivariate logistic regression analyses were carried out. Chi square (χ^2) tests were used to estimate the differences in proportions of clinical and demographical variables between CS-resistant and CS-responsive patients in the univariate analysis. In the multivariate analysis, variables such as concomitant medication, year of diagnosis, study center were considered as potential confounders. Considering that CS dependency is not a rare outcome, the odds ratios (OR) were corrected using method described by Zhang et al. [291]. The following formula was used to correct ORs in order to better represent the true relative risk:

$$RR = \frac{OR}{(1 - P_0) + (P_o \times OR)}.$$

P_0 indicates the incidence of the outcome of interest in the non-exposed group; OR, odds ratio; and RR, risk ratio.

Statistical analyses were performed using STATA, version 10.0 (http://www.stata.com/).

2.7.2 Statistical methods, article 2

The aim of this article was to examine the potential contribution of the variations of *ABCB1* gene to CS dependent phenotype. Single-marker association, using various models of inheritance and haplotype analysis using tag-SNPs, was employed to explore possible associations between corticosteroid dependent phenotype and genetic variations in *ABCB1*/*MDR1* gene. For single-marker analysis, logistic regression analysis was implemented to estimate the effect of tag-SNPs on risk of CS dependency. The minor allele of each SNP was considered to be the "risk-conferring allele". Given that mode of inheritance of CS dependent phenotype is unknown and likely to be complex, various models (additive, genotypic, recessive and dominant) were investigated [292]. Odds ratios and 95% confidence intervals were estimated. Power to detect associations may be enhanced using haplotypes as the unit of analysis, as opposed to single alleles. There is strong evidence to indicate that several mutation within a gene can act as a "super-allele" that can have an effect on phenotype [293, 294]. Haplotypes that are not functional can provide a greater power than single marker analyses due to ancestral structure captured in haplotype distribution [295]. The power advantage for haplotype-based methods is expected to be greatest when the marker alleles are not in strong LD with each other, but in strong LD with the causative alleles [296]. Haplotype analysis is therefore appropriate for the tagging SNPs, as based on a priori selection criteria they are not in high LD with each other. Haplotype analysis was carried out using methods described by Barrett et al. [297] and implemented in the HAPLOVIEW software (www.broad.mit.edu/mpg/haploview). Associations were evaluated by comparing the frequency of each haplotype among the cases and controls. Counts for the haplotype association test were obtained by summing the fractional likelihoods of each individual for each haplotype.

Multiple testing is a common problem in genetic association studies leading to increased probability of false positive results given that numerous markers are studied. For single-marker associations, the Bonferroni correction was applied. The Bonferroni adjustment assumes that the hypothesis tests are not correlated, thus it is considered to

be overly conservative in the context of genetic association studies. In case of our study where markers are tag-SNP, this approach is appropriate as tag-SNPs by definition are not supposed to be in high LD, thus a test's independence requirements holds. In order to obtain a measure of significance corrected for multiple testing bias, haplotype association results were permuted (n=1000) using HAPLOVIEW. The permutations approach allows for the independent correction of each p-value. In this approach, after p-values are calculated for the original data set, a pseudo-data set is created by randomly dividing the data into response and non-response groups and the analysis is then repeated to compute p-values using the new data set. This process is repeated multiple times, and the minimum p-value from the original data is compared to the distribution of minimum p-values obtained from the permuted data. The proportion of permuted p-values that are less than the minimum p-value from the original data is the adjusted p-value.

2.7.3 Statistical methods, article 3

The statistical methods utilized for this article were similar to those implemented for article 2 above. Single-marker and haplotype association analyses using tag-SNPs were carried out. General genotype, additive, dominant and recessive models were examined [292] using logistic regression method implemented in STATA software. Haplotype analysis was carried out using on the maximum likelihood based method implemented in HAPLOVIEW [297].

2.7.4 Imputation of un-genotyped markers, articles 2 and 3

The imputation procedure implemented in the MACH software [298] (http://www.sph.umich.edu/csg/abecasis/MACH/) was used to examine potential associations between CS dependence and the un-genotyped SNPs. The term "genotype imputation" refers to the procedure of predicting or imputing SNPs that are not directly assayed in the samples. The genotypes are imputed with uncertainty and a probability

distribution over all three possible genotypes is produced. The use of the "in silico" genotypes helps to boost the number of studied SNPs. This technique was shown to be more powerful than the original analysis of directly genotyped markers in genome wide association studies (GWAS). Another advantage is that the imputation of un-typed variants can help to identify rare causal variants [299]. The HapMap haplotypes are widely used to carry out imputation in samples that have close ancestry to those in the HapMap panels. We used HapMap CEU population panel (Release 22) as the most corresponding to our population's background. SNPs located within1000 bp around gene boundaries (putative transcription start sites) were included. There are several imputation methods based on different algorithms, yet all methods attempt to identify sharing between the underlying haplotypes of the study individuals and the haplotypes in the reference set and use this sharing to impute the missing alleles in study individuals. We used the Markov Chain Haplotyping (MACH) technique implemented in MACH as this method outperforms other available methods [300]. Additionally, in order to validate the results we used the BEAGLE [301] software which uses the hidden Markov model (HMM) algorithm (available at http://www.stat.auckland.ac.nz/~bbrowning/beagle/). In order to access the quality of imputed genotypes at SNPs, MACH uses r^2 which is the ratio of the empirically observed variance of the allele dosage to the expected binomial variance at Hardy–Weinberg equilibrium. We applied the recommended threshold of $r^2 = 0.30$ to filter out the SNPs with better quality of imputation that we have used for subsequent analyses.

CHAPTER 3

RESULTS

ARTICLE 1

ARTICLE 2

ARTICLE 3

The results described in this chapter represent those described in the three articles that are part of this thesis. First article of this thesis describes rates of corticosteroid dependency in our study cohort and explores potential clinical predictors of this condition. The second and third articles examined the associations between variants in two genes *ABCB1* and *NR3C1* and the CS-dependent phenotype. The first author has made an essential contribution at fundamental stages of study design, implementation, data analysis, interpretation and dissemination of results. In addition, she participated in identifying and recruiting patients at the Ste-Justine study site. She has also coordinated the implementation of a data collection tool on a web-based database. Clinical data at Ste-Justine Hospital, used for CD's phenotype definition and study outcomes assessment, was abstracted and input into the database by the first author, who also has reviewed the medical charts. The data extraction from the clinical data base, as well as data management, cleaning and analysis of clinical and genetic data was also done by the first author. The first author has written the manuscripts presented here. Considering the nature of the field, the ultimate successful conduct of the thesis led by the first author, was based on a team effort where the collaborating authors provided guidance and suggestions on the appropriateness of the study design, coordination of its implementation, the conduct of the statistical analyses and interpretation of the results. The results were presented (oral and/or poster presentations) at numerous congresses and conferences[†]

[†] The Ste-Justine Hospital students' conference, 2007, the 75th ACFAS Congress, 7- 11 mai 2007, Université du Québec, Trois-Rivières, the 77th ACFAS Congress, May11-15, 2009 in Ottawa, the Canadian Society of Epidemiology and Biostatistics National Student Conference held on May 23-24, 2009 in Ottawa, the international congress of gastroenterology, Digestive Diseases Week, May 19-24, 2007, Washington, USA, Digestive Diseases Week, May 30-June 4, 2009, Chicago, USA, Digestive Disease Week, May 1-5, 2010, New Orleans, USA, 1st Bruce Kaufman/McGill Symposium on Immunoregulation and Inflammatory Bowel Disease, September 24, 2010, Montreal.

3.1 Article 1:

Immediate and long-term outcomes of corticosteroid therapy in paediatric Crohn's disease patients.

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IMMEDIATE AND LONG-TERM OUTCOMES OF CORTICOSTEROID THERAPY IN PAEDIATRIC CROHN'S DISEASE PATIENTS.

Short title: Corticosteroids in pediatric patients with Crohn's disease.

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3.1.1 Abstract

Background

Although a mainstay of treatment of moderate to severe Crohn's disease (CD), corticosteroids use presents significant challenges because of large inter-individual variability in response. Corticosteroid-dependence is of particular concern in children wherein high rates have been reported.

Aims

We examined the burden of corticosteroid-resistance and dependence in a well-characterized cohort of paediatric CD patients and investigated potential predictors of response.

Methods

Children diagnosed with CD (<18 yr), were recruited from two Canadian pediatric gastroenterology clinics. Immediate and long-term responses to corticosteroid therapy were retrospectively ascertained. Response rates (resistance & dependence) were estimated and potential predictors assessed using logistic regression analysis.

Results

Of the 645 CD patients, 364 (56.2%) received corticosteroids. The frequency of corticosteroid-resistance was (8.0%) (95% CI: 5.0 % - 11%) and 40.9% (95% CI: 39.0% - 46.0%) became dependent. In univariate analysis female gender (OR = 2.49, 95% CI: 1.1-5.5, p = 0.025), disease severity (OR=2.43, 95% CI: 1.10-5.38, p=0.029) and complicated disease (OR = 2.75, 95 % CI: 1.18-6.41, p = 0.019) were associated with resistance. In multivariate analysis lower age at diagnosis (OR = 1.34, 95% CI: 1.03-3.01, p = 0.040), coexisting upper digestive tract involvement (OR=1.35, 95% CI: 1.06-3.07, p =0.031) and concomitant immunomodulators use (OR=0.35, 95% CI: 0.16 - 0.75, p=0.007) were significantly associated with steroid dependency.

Conclusions

Our results demonstrate that steroid dependency is a frequent complication in children with CD. Children with an earlier age at diagnosis and coexisting upper digestive tract involvement could be potentially targeted for steroid-sparing therapy.

Keywords: Crohn's disease, paediatrics, corticosteroids, corticosteroid-dependence, outcomes, predictors

3.1.2 Introduction

Crohn's disease (CD) is a type of inflammatory bowel disease (IBD) characterized by chronic inflammation of the digestive tract, with frequent flares and a progressive clinical course with many complications. During the past decades the incidence and prevalence of CD have increased in most western countries including Canada.[1-5] Of relevance, it has become apparent that the incidence of CD in children and young adults is on the rise, with rates approaching that in adults.[2,5] The latter is of particular concern as CD in children can severely impact physical and psychological wellbeing and can be associated with frequent surgery and growth delay.

Corticosteroids are the mainstay of CD therapy effectively used to induce remission which is an important goal in therapeutic management of CD. However, large inter-individual variability in response to corticosteroid treatment has been noted that presents significant clinical challenges. Some patients do not respond to the initial treatment (resistant) and others require corticosteroids for long periods (dependent). Resistance and dependence to corticosteroids therapy can contribute to inadequate disease control and prolonged drug exposure potentially increasing the risk for steroid associated side-effects.[6-12] Indeed, corticosteroid resistance and dependence are considered markers of treatment failure.[13] Corticosteroid response is of particular importance in children diagnosed with CD as the consequences of failed or long-standing therapy can be severe.[14,15]

Epidemiological studies in adult populations indicate that the rates of resistance to corticosteroids (20%) and dependence (28-38%) are quite high. Some studies focused on paediatric population [16,17] suggest that rates are similarly high and in some instances higher than those among adults. It is unclear what factors contribute to varying responses to corticosteroids in children. Previous studies were mainly retrospective evaluations based on small cohorts of patients and thus were insufficiently powered to investigate actual rates of steroid responsiveness and factors that could influence these rates. In an attempt to further clarify the clinical variation of response to corticosteroids

and to investigate potential markers we studied a large population of well-characterized Canadian children diagnosed with CD and administered corticosteroids for therapy.

3.1.3 Methods

3.1.3.1 Study design, population and data collection

A retrospective cohort study was carried out at two tertiary Canadian gastroenterology clinics: Ste-Justine Hospital (SJH), Montreal and Children's Hospital of Eastern Ontario (CHEO), Ottawa. Patients were children < 18 years of age diagnosed with CD using standard clinical, histological, radiological and endoscopic criteria.[18,19] For the Montreal site, all patients diagnosed between 1980 and 2008 and for the Ottawa site, patients newly diagnosed with CD between 2000 and 2008 were included. The medical records of these children were retrospectively reviewed to identify patients who received corticosteroids for the first time within one year since diagnosis. Clinical details such as disease characteristics (localization and behaviour) at diagnosis, corticosteroids therapy, concomitant medication, extra-intestinal manifestations (EIM), surgeries, family history of IBD and growth retardation (Montreal cohort only) were abstracted. Information on racial and ethnic background was acquired by administering a supplementary questionnaire. Data abstraction at the Montreal site was carried out by 1 investigator (AK) with information on key variables such as disease activity, concomitant medication and steroid dependence validated by a second investigator (DKA). Discrepancies, if any, were resolved by consensus with the site gastroenterologist (CD). For the Ottawa center, data abstraction was carried out by a trained research nurse under the supervision of the gastroenterologist (DRM).

CD was classified according to the World Gastroenterology Organization's Montreal classification.[20] To evaluate response to corticosteroid therapy, we assessed the immediate and long-term outcomes of corticosteroid therapy, based on adaptation of previously reported criteria.[21,22]

Immediate response

Complete remission - total regression of clinical symptoms within 30 days, with 2 bowel movements a day and no pain, no blood, pus or mucus in stool, fever, weight loss or extra-intestinal manifestations.

Partial remission - regression of symptoms within 30 days, with 4 bowel movements a day, less abdominal pain and no fever, weight loss or extra-intestinal manifestations.

Non-response - no improvement within 30 days.

Long-term response

Corticosteroid dependence - reoccurrence of clinical symptoms during the weaning period or clinical relapse occurring during 180 days following the end of treatment, requiring the reintroduction of corticosteroids.

Prolonged response - maintenance of complete or partial remission after treatment had finished.

Other - clinical relapse occurring later than 180 days since discontinuation of corticosteroids and management with alternatives. Also included in this category were patients who had moderate to severe relapse complicated by infections such as cl. difficile, Epstein-Bar virus (EBV)[23] and some patients who had undergone surgery.

Concomitant medication for the purpose of this study was considered if it was introduced during initial corticosteroid therapy before the start of corticosteroid tapering and not if it was given in response to disease relapse.

Disease severity was assessed using a modified Harvey Bradshaw Index (HBI)[24] at the Montreal site and using Physician Global Assessment Index at the Ottawa site. Both these indices have been shown to be strongly correlated [25] enabling the combination of data across the two sites.

The institutional ethical boards of the two study centers approved the study and consent was acquired from the patients.

Statistical analyses

The frequencies (proportions) of corticosteroid response (resistance & dependence) were estimated along with their 95% confidence intervals (95% CI) assuming a binomial distribution. Univariate logistic regression analyses were carried out to examine the influence of potential predictors such as gender, age at diagnosis (categorized as ≤ 10.7 yr and > 10.7 yr based on the distribution in the cohort), family history of IBD (yes/no), EIM (yes/no), use of concomitant medication (categorized according to type of medication), clinical phenotypes of CD (disease localization and behaviour) on response (resistant versus non-resistant and dependent versus non-dependent). In addition, for corticosteroid dependence, a multivariate logistic regression was applied to the data to jointly explore the influence of potential predictors. The latter was not implemented for corticosteroid resistance due to the limited number of subjects. Odds ratios (OR) and respective 95% CI were estimated. Potential temporal and clinic-specific differences in CD management were accounted for by incorporating representative variables in the multivariate logistic model. As for a cohort study, when the frequency of the outcome (corticosteroid response in the present study) is greater than 10%, the estimated odds ratio can overestimate the relative risk, we corrected the ORs' (and corresponding 95% CI) using methods described by Zhang et al.[26] All analyses were carried out using STATA (Stata Statistical Software: Release 10. College Station, TX: StataCorp LP.)

3.1.4 Results

Of the 645 patients diagnosed with CD during the study period, 364 (56.2%) were administered corticosteroids during the first year following diagnosis. The mean age (±SD) at diagnosis was 12.5 (±3.2) (**Table I** on page 90). The majority of patients were male (54.9 %), had inflammatory disease phenotype (84.6%) and ileo-colonic

disease (51.7%) at diagnosis. Most patients (95.6%) were of European ancestry. Corticosteroid treatment included intravenous or oral prednisone with a starting dose of 1 mg/kg per day for 2 to 4 weeks. In patients requiring intravenous treatment, the duration of the corticosteroid treatment was about one week with conversion to oral corticosteroids subsequently. Medication dose was tapered within 8 to 12 weeks using various regimens, but the most frequently used scheme consisted of a decrease of the prednisone dose by 5 mg per week. Budesonide was used in 11.8 % of patients with a starting dose of 9 mg per day and tapered off in decrements of 3 mg over three 4-week time intervals.

The mean (±SD) duration of corticosteroid treatment was 148.2 (±82.4) days. Following treatment initiation, 94 patients (25.8%) (**Figure 1** on page 98) were continuously using corticosteroids for 6 months and 22 patients (6.0%) were on therapy until the end of one year. On the 30th day after the initiation of corticosteroids 29 (8.0%) (95 % CI: 5.0% - 11%) patients did not respond (resistant), 135 (37.0%) (95% CI: 32.0% - 42.0%) patients had achieved partial response and 200 (55.0%) (95% CI: 50.0% - 60.0%) had completely responded (**Figure 2** on page 99).

From among those who initially responded, 148 (44.2%) (95% CI: 39.0% - 50.0%) became dependent (long-term outcome). One patient, initially nonresponsive on the 30th day, later became dependent. From among the patients who initially responded to corticosteroids, 9 (2.7%) had a subsequent course of corticosteroid therapy but did not meet the definition of dependency as they had a clinical relapse occurring later than 180 days following the end of first treatment. Four (1.2%) initially responsive patients had a moderate to severe relapse due to concomitant infections (such as cl. difficile or EBV), 7 (2.1%) had a surgery, 3 (0.9%) had a moderate to severe relapse but were not administered additional corticosteroids and treated with other medications. Overall, 17 (4.7%) patients had surgery at some point during 1 year. Patients non-responsive to corticosteroids were more likely to undergo surgery in comparison to those who initially responded (17.2% versus 3.6%).

The overall frequency of corticosteroid dependence (**Figure 2** on page 99), (including responders and non-responders) was 40.9% (95% CI: 0.39 - 0.46). The frequencies of corticosteroid dependency between the two study centers (Montreal & Ottawa) were comparable (38.4% versus 41.7%, p = 0.58). Similarly, no temporal differences in the rates of corticosteroid dependency were noted (43.9% prior to 2000 and 39.4% in subsequent years, p = 0.411). The frequencies of corticosteroid-dependency were significantly different (p-value = 0.003) between the patients' receiving different types of concomitant medication (none, immunomodulators and ASA-5).

Univariate analysis showed that non-response (resistance) to corticosteroids was more common in females (OR = 2.49, 95% CI: 1.10 - 5.50, p = 0.025) as compared to males (**Table II** on page 92). Similarly, children with complicated disease behaviour (stricturing and/or penetrating) were significantly (OR = 2.75, 95% CI: 1.18 - 6.41, p = 0.019) less likely to respond to corticosteroids. Children who did not respond to therapy were more likely to have surgery (OR = 5.6, 95% CI: 1.82 - 17.2, p = 0.003). Similarly, children with severe disease at the commencement of steroid therapy were more likely to become resistant (OR=2.43, 95% CI: 1.10-5.38, p=0.029). For long-term response (**Table III** on page 94), in univariate analysis, children with coexisting upper digestive tract involvement (L4) (OR = 1.72, 95% CI: 1.05 - 2.83, p = 0.031) and those less than 10.7 years of age at diagnosis (OR = 1.82, 95% CI: 1.10 - 3.03, p = 0.022) were more likely to become corticosteroid-dependent. Concurrent use of immunomodulators was negatively associated with occurrence of corticosteroid-dependency (OR = 0.38, 95% CI: 0.19 - 0.77, p = 0.007). These associations persisted when other potential predictors were adjusted for in the logistic regression model (**Table III** on page 94). Growth retardation was not associated with either steroid resistance or dependence in the Montreal cohort (data not acquired for the Ottawa cohort).

3.1.5 Discussion

In a large cohort of paediatric CD patients administered corticosteroids, we observed that the frequencies of corticosteroid resistance and dependence were 8% and 40.9% respectively. Girls, patients with complications at diagnosis and with severe disease activity were more likely to be resistant to corticosteroids. Children diagnosed with CD at a younger age (≤ 10.7 yr), and having coexisting upper digestive tract involvement were more likely to become corticosteroid dependent. Concomitant use of immunomodulators was associated with significantly lower risks for steroid dependence. Importantly, more than 25% of the patients were continuously using corticosteroids for at least 6 months.

Corticosteroids are an important and effective form of therapy in IBD. Knowing who is best suited for therapy is an important clinical question as known side effects such as moon facies, acne and psychological disturbances can occur even after short term treatment. For example, 55% of patients in the prednisone arm of a randomized controlled trial developed side effects such as moon facies, acne, swollen ankles, easy bruising and insomnia after only 10-weeks of treatment.[27] Other side effects of corticosteroids after more than 12 weeks treatment[28] include striae, cataract, myopathy, susceptibility to infections, and growth retardation. Although the greatest decrease in bone density occurs in the first few months of therapy [29] prolonged corticosteroid therapy may contribute to further decrease.[30] In paediatric patients with CD it is suggested that steroid therapy can explain up to 20% of variation in bone mineral density of the lumbar spine.[8] Thus, minimizing adverse effects and maximizing benefit of corticosteroid therapy is an important clinical challenge.

Our observed frequencies of corticosteroid resistance (8%) are within the range reported in earlier paediatric-CD studies carried out worldwide.[17, 31,32] For example, in a study carried out in France[31] the authors reported corticosteroid resistant rates to be ≈5%, whereas in a study carried out in the Olmsted County in USA, ≈12% of the children were resistant to corticosteroids.[32] Higher rates of resistance (17%) were

reported by Markowitz et al[17] probably due to the use of different criteria (assessment of response after 3 months after therapy initiation) for assessing non-response. Corticosteroid dependence rates in previous paediatric studies [17,31,32] that used criteria similar to that utilized in our study, range from 25% to 31%. These rates are lower than what we observed (40.9%). In two studies[17, 31] the frequency of concomitant use of immunomodulators (61% and 81%) was higher than that in our cohort (37.9%), probably explaining the differences in rates observed. The study by Tung et al[32], was based on a small cohort (n = 26). In the North American multi-centered randomized trial in paediatric CD (6-mercaptopurine versus corticosteroids alone in the placebo arm)[16] noted rates of dependency were expectedly higher (50%). Most of the patients in our cohort were diagnosed prior to the introduction of anti-TNF and thus notably the rate of anti-TNF therapy was low in our patient population (≈6%). In previous adult population- and hospital-based studies [13,21,22,33,34] using similar definitions of response to corticosteroids, rates of resistance and dependency ranged from 16-20% and 20-38% respectively. It is interesting to note that overall slightly higher rates of resistance have been reported in adults than in paediatric studies, with the exception of a Chinese study in adult-CD that reported low rates of non-response (6%).[33] Potential differences in rates of resistance between paediatric and adult-onset CD may be related to the age-dependent expression of the glucocorticoid receptor as shown in a recent experimental study by Kiela et al.[35]

In contrast to rates of resistance, rates of dependency appear to be higher in paediatric-CD in comparison to adult-CD. These differences may not be related to differences in use of concomitant therapy as even among those children who do not receive concomitant immunomodulators, rates of dependence are higher (50%)[16] in comparison with adults who likewise are treated only with corticosteroids (36%)[21]. Other biological mechanisms need to be examined to dissect these potential differences in rates.

As identification of potential predictors of corticosteroid response would be important, we examined whether specific clinical and/or demographic characteristics

could be related to the various outcomes of corticosteroid treatment. Consistent with previous studies [33,36] we observed that female gender and complicated disease were associated with non-response to corticosteroids. Similarly our observations of the association between early age at diagnosis and corticosteroid dependency have been reported earlier.[37] Furthermore, the protective effects of concomitant immunomodulator use on steroid dependency reported previously[16,38] was confirmed in our cohort. Our finding of association between concomitant upper tract involvement and corticosteroid dependence is however novel. Other potential predictors for dependence, such as growth delay [17] and thrombocytosis [33] have been suggested. In a subset of the data, we did not observe any associations between growth retardation and steroid response. Variability in classification of growth impairment/retardation (height <3 percentile in our cohort) between studies may have resulted in varying findings. Taken together, the strongest risk factors for dependency appear to be early-age of diagnosis and coexisting upper digestive disease localization. Further studies are required to examine potential reasons for these associations and to explore whether alternatives to corticosteroids could be considered in these children.

Our study was hospital-based and our observed higher rates of dependence may have resulted from an overall higher clinical severity of the patient population. However, we did not observe any association between the presence of complications at diagnosis and corticosteroid dependence. We were unable to take into account various corticosteroid-tapering regimens when examining responses to therapy. Although, this may have affected our estimation of rates of non-response, as shown previously, rates of dependency are unlikely to be influenced by the choice of weaning scheme implemented.[39,40] It is likely that despite the large cohort, the retrospective nature of the study may have introduced bias in the determination of response predictors. By-and-large we were successfully able to implement reported criteria for defining corticosteroid responses. A small number of patients (n=7) were however assigned to the partial response category, as sufficient information to classify them as complete responders was not available. Such potential misclassification could have influenced the rates for

immediate response but unlikely to have influenced the examination of predictors, given that the information on predictors (at diagnosis) was abstracted prior to knowledge on the responses.

In conclusion, in a cohort of paediatric-onset CD, we observed that corticosteroid dependence is a significant clinical problem, in particular in younger patients, leading to prolonged exposure to corticosteroids. These findings highlight the need for the determination of reliable markers (biologic and/or genetic), that will enable the early identification of patients, susceptible for corticosteroid dependency.

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3.1.6 References

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3.1.7 Tables

Table I: Demographic and clinical characteristics of the CD patients.

Patient's characteristic	All N = 364	Montreal N = 278	Ottawa N = 86
Age at diagnosis, years, Mean	12.5 (±3.2)	12.4 (±3.3)	12.5 (±2.9)
Age at diagnosis, years, by quartiles			
1st Q	10.7	10.7	11.0
2nd Q	12.8	12.8	12.6
3rd Q	14.9	14.9	14.6
Year of diagnosis (%)			
1980-1990	123 (33.8)	123 (44.24)	0
1991-2000	82 (22.5)	73 (26.26)	9 (10.47)
>2000	159 (43.7)	82 (29.50)	77 (89.53)
Gender (%)			
Females	164 (45.1)	132 (47.5)	32 (37.2)
Males	200 (54.9)	146 (52.5)	54 (62.8)
European ancestry (%)			
Yes	348 (95.6)	262 (94.2)	86 (100.0)
No	16 (4.4)	16 (5.8)	0
Duration of corticosteroid therapy (days), mean (±SD)	148.2 (±82.15)	145.58	153.39(±63.96)
Disease localization (%)*			
L1±L4	70 (19.2)	40 (14.4)	30 (34.9)
L2±L4	106 (29.1)	73 (26.3)	33 (38.4)
L3±L4	188 (51.7)	165 (59.3)	23 (26.7)
Co-existing L4 (%)			
Yes	101 (27.8)	68 (24.5)	33 (38.4)
No	263 (72.2)	210 (75.5)	53 (61.6)

Table I. (Continued)

Patient's characteristic	All N = 364	Montreal N = 278	Ottawa N = 86
Disease behaviour (%)*			
B1±p	308 (84.6)	245 (88.1)	63 (73.3)
B2±p	32 (8.8)	21 (7.6)	11 (12.8)
B3±p	24 (6.6)	12 (4.3)	12 (13.9)
EIM (%)†			
Yes	88 (24.2)	70 (25.2)	18 (20.9)
No	276 (75.8)	208 (74.8)	68 (79.1)
Family history of IBD‡ (%)			
Yes	43 (11.8)	32 (11.5)	11 (12.8)
No	321 (88.2)	246 (88.5)	75 (87.2)
Concomitant medication (%)			
None	56 (15.4)	33 (11.9)	23 (26.8)
Immunomodulators	138 (37.9)	93 (33.4)	45 (52.3)
ASA	170 (46.7)	152 (54.7)	18 (20.9)
Use of anti-TNF§ (%)			
Yes	21 (5.8)	14 (5.0)	7 (8.1)
No	343 (94.2)	264 (95.0)	79 (91.9)
Surgery (%)			
No	347 (95.3)	265 (95.3)	82 (95.3)
Yes	17 (4.7)	13 (4.7)	4 (4.7)
Disease severity††			
Mild-moderate	198 (54.4)	154 (55.4)	44 (51.2)
Severe	166 (45.60)	124 (44.6)	42 (48.8)
Growth retardation‡‡ (%)			
Yes	-	72 (25.9)	-
No	-	206 (74.1)	-

Table I (Continued)

*Disease localization and behaviour classified according to World Gastroenterology Organisation's Montreal classification.

† Extra intestinal manifestations.

‡ IBD in first-degree relatives.

§ Anti-TNF administered in response to corticosteroid dependency, resistance, side effects or failure of immunomodulators.

†† HBI for Montreal and Physician General assessment for Ottawa patients.

‡‡ Data available for Montreal cohort only.

L1 - Ileal

L2 - Colonic

L3 - Ileo-colonic

L4 - Upper digestive tract

B1 - Inflammatory disease

B2 - Stricturing disease

B3 - Penetrating disease

p - Perianal disease

Table II: Univariate analysis of short term response to corticosteroids in CD patients.

Patient's characteristics	N = 364			
	Non response N = 29	Response N = 335	OR (CI 95%)	P value
Age at diagnosis, (%)				
1st Q	4 (13.8)	87 (26.0)	0.46 (0.15 - 1.35)	0.156
2nd 3rd and 4th Q	25 (86.2)	248 (74.0)		
Year of diagnosis (%)				
<2000	10 (34.5)	113 (33.7)		
>2000	19 (65.5)	222 (66.3)	0.96 (0.44 - 2.1)	0.935
Study centre (%)				
Montreal	24 (82.8)	254 (75.8)	1.53 (0.56 - 4.14)	0.402
Ottawa	5 (17.2)	81 (24.2)		
Gender (%)				
Females	19 (65.0)	145 (43.3)	2.49 (1.10 - 5.50)	0.025
Males	10 (35.0)	190 (56.7)		
European ancestry (%)				
Yes	29 (100.0)	319 (95.2)	-	
No	-	16 (4.8)	-	0.229
Duration of corticosteroid therapy (days), mean (±SD)	109.8 (15.8)	151.6 (4.5)		0.004
Disease localization (%)				
L1±L4	3 (10.3)	67 (20.0)	Reference	
L2±L4	11 (37.9)	95 (28.4)	2.58 (0.69 - 9.62)	0.157
L3±L4	15 (51.7)	173 (51.6)	1.94 (0.54 - 6.90)	0.308
Coexisting L4 (%)				
Yes	5 (17.2)	96 (28.7)	0.52 (0.19 - 1.40)	0.195
No	24 (82.8)	239 (71.3)		

Table II. (Continued)

Patient's characteristics	N = 364			
	Non response N = 29	Response N = 335	OR (CI 95%)	P value
Disease behaviour (%)				
Complicated	9 (31.0)	47 (14.0)	2.75 (1.18 - 6.41)	0.019
Inflammatory	20 (69.0)	288 (86.0)		
Family history of IBD* (%)				
Yes	4 (13.8)	39 (11.6)	1.20 (0.40 - 3.7)	0.731
No	25 (86.2)	296 (88.4)		
EIM† (%)				
Yes	9 (31.0)	79 (23.6)	0.80 (0.32 - 2.04)	0.648
No	20 (69.0)	256 (76.4)		
Concomitant medication (%)				
None	6 (20.7)	50 (14.9)	Reference	
Immunomodulators	16 (55.2)	122 (36.4)	1.09 (0.40 - 2.9)	0.861
ASA	7 (24.1)	163 (48.7)	0.36 (0.11 - 1.11)	0.076
Surgery (%)				
Yes	5 (17.2)	12 (3.6)	5.6 (1.82 - 17.23)	0.003
No	24 (82.8)	323 (96.4)		
Disease severity (%)				
Mild-moderate	10 (34.5)	188 (56.1)		
Severe	19 (65.5)	147 (43.9)	2.43 (1.10 - 5.38)	0.029
Growth retardation‡ (%)				
Yes	4 (16.7)	68 (26.8)	0.55 (0.21 - 1.86)	0.286
No	20 (83.3)	186 (73.2)		

* IBD in first-degree relatives.

† Extra intestinal manifestations.

‡ Data available for Montreal cohort only.

Table III: Univariate and multivariate analyses of long term response to corticosteroids in CD patients.

Patient's characteristics N = 313	Univariate analysis			Multivariate analysis	
	Dependence N = 149	Prolonged N = 164	OR (CI 95%)	Adjusted OR	P value
Age at diagnosis (%)					
1st Q	48 (32.2)	34 (20.7)	1.82 (1.10 - 3.03) ‡	1.34* (1.03 – 3.01)	0.040
2nd 3rd and 4th Q	101 (67.8)	130 (79.3)			
Year of diagnosis (%)					
<2000	54 (36.2)	52 (31.7)			
>2000	95 (63.8)	112 (68.3)	0.82 (0.51 - 1.30)	1.44 (0.80 - 2.60)	0.227
Study centre (%)					
Montreal	116 (77.8)	122 (74.4)	1.21 (0.72 - 2.04)	1.13 (0.58 - 2.21)	0.579
Ottawa	33 (22.15)	42 (25.6)			
Gender (%)					
Females	70 (47.0)	69 (42.1)	1.22 (0.78 - 1.90)	1.36 (0.83 - 2.21)	0.217
Males	79 (53.0)	95(57.9)			

Table III (Continued)

Patient's characteristics N = 313	Univariate analysis			Multivariate analysis	
	Dependence	Prolonged			
	N = 149	N = 164	OR (CI 95%)	Adjusted OR	P value
European ancestry (%)					
Yes	143 (96.0)	156 (95.1)	1.22 (0.41 - 3.60)	1.34 (0.43- 4.21)	0.611
No	6 (4.0)	8 (4.9)			
Disease behaviour (%)					
Complicated	18 (12.1)	20 (12.2)	0.99 (0.50 - 1.95)	1.10 (0.51 - 2.38)	0.806
Inflammatory	131 (87.9)	144 (87.8)			
Disease localization (%)					
L1±L4	24 (16.1)	36 (21.9)	Reference	Reference	
L2±L4	44 (29.5)	48 (29.3)	1.37 (0.71 - 2.65)	1.46 (0.72 - 2.95)	0.295
L3±L4	81 (54.4)	80 (48.8)	1.52 (0.83 - 2.77)	1.63 (0.84 - 3.16)	0.150
Coexisting L4 (%)					
Yes	51 (34.2)	38 (23.2)	1.72 (1.05 - 2.83)**	1.35* (1.06 - 3.07)	0.031
No	98 (65.8)	126 (76.8)			

Table III (Continued)

Patient's characteristics N = 313	Univariate analysis			Multivariate analysis	
	Dependence	Prolonged			
	N = 149	N = 164	OR (CI 95%)	Adjusted OR	P value
Family history of IBD (%)					
Yes	16 (10.7)	21 (12.8)	0.82 (0.41 - 1.64)	0.94 (0.45 - 1.96)	0.877
No	133 (89.3)	143 (87.2)			
EIM (%)[†]					
Yes	39 (26.2)	38 (23.2)	1.17 (0.70 - 1.97)	1.21 (0.70 - 2.09)	0.489
No	110 (73.8)	126 (76.8)			
Concomitant medication (%)					
None	27 (18.1)	19 (11.6)	Reference	Reference	
Immunomodulators	41 (27.5)	75 (45.7)	0.38 (0.19 - 0.77) [††]	0.35 (0.16 - 0.75)	0.007
ASA	81 (54.4)	70 (42.7)	0.81 (0.42-1.59)	0.94 (0.45 - 1.96)	0.871
Disease severity (%)					
Mild- moderate	78 (52.4)	95 (57.9)			
Severe	71(47.6)	69 (42.1)	1.25 (0.80 - 1.96)	1.45 (0.89 – 2.35)	0.136

Table III (Continued)

Patient's characteristics N = 313	Univariate analysis			Multivariate analysis	
	Dependence	Prolonged			
	N = 149	N = 164	OR (CI 95%)	Adjusted OR	P value
Growth retardation (%)					
Yes	34 (22.8)	28 (17.1)	1.39 (0.78 - 2.49)	-	-
No	115 (77.2)	136 (82.9)			

*Bias corrected estimates[26]

† Extra intestinal manifestations.

‡ p-value = 0.022.

** p-value = 0.031.

†† p-value = 0.007

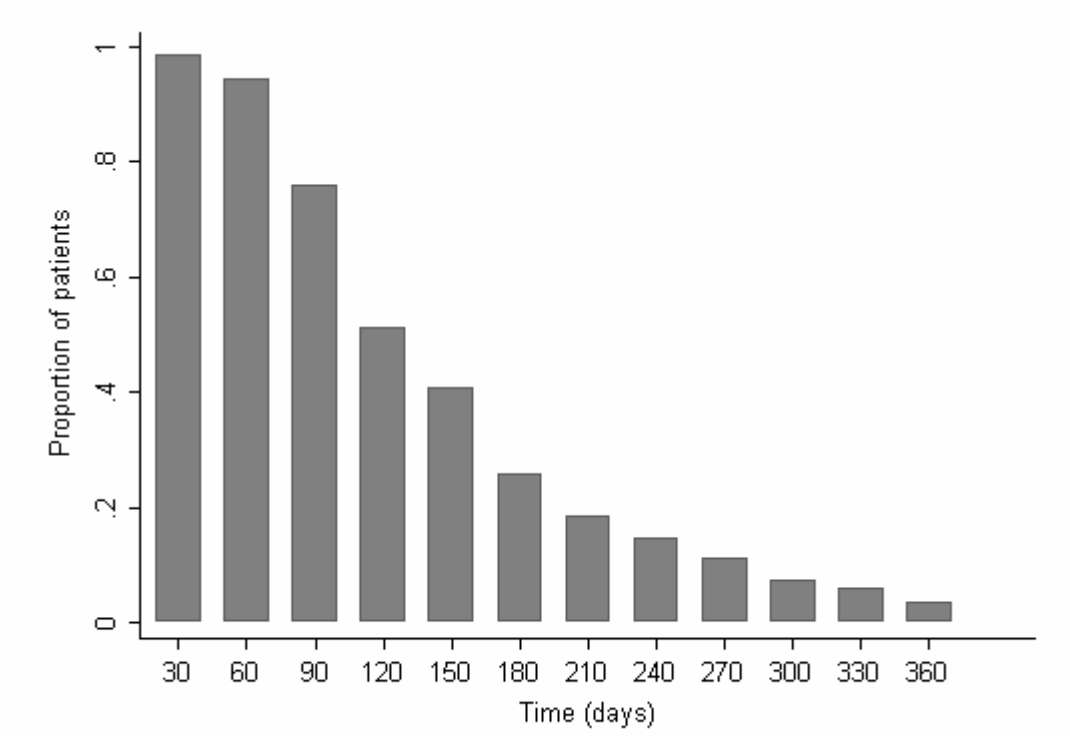

Figure 1. Proportion of patients on continuous corticosteroid therapy during the year following initiation.

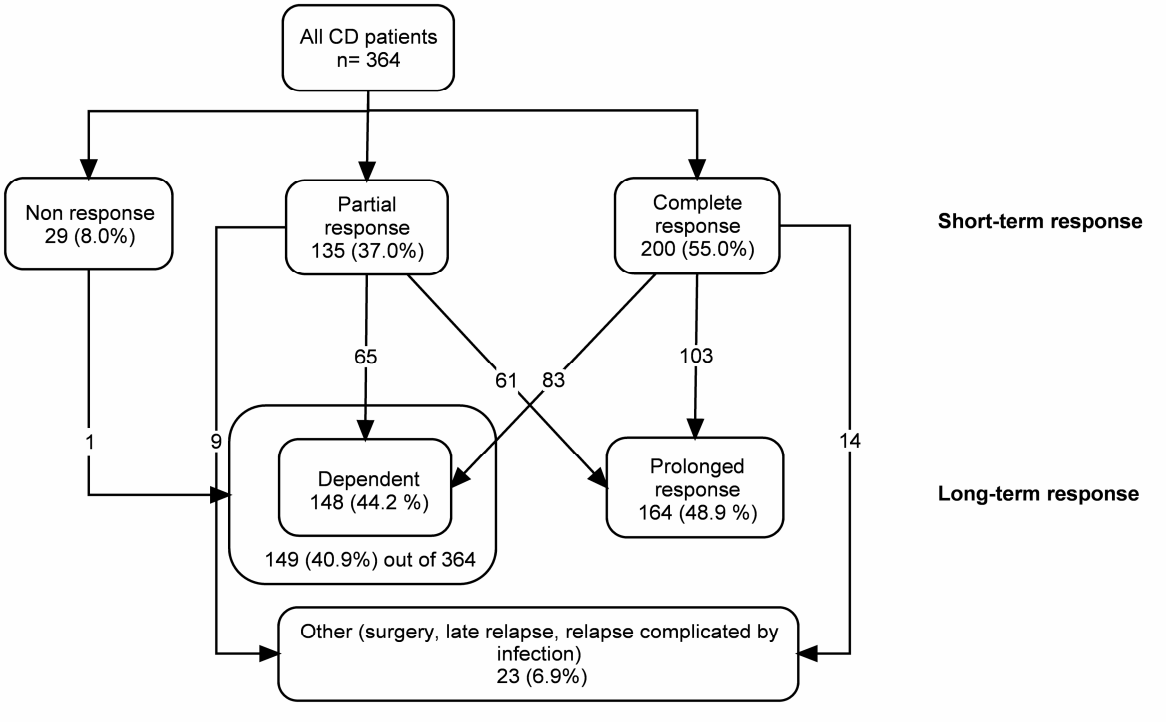

Figure 2. Short- and long-term responses in CD patients after the first course of corticosteroids

3.2 Article 2:

Associations between variants in the *ABCB1 (MDR1)* gene and corticosteroid dependence in children with Crohn's disease.

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ASSOCIATIONS BETWEEN VARIANTS IN THE ABCB1 (MDR1) GENE AND CORTICOSTEROID DEPENDENCE IN CHILDREN WITH CROHN'S DISEASE.

Short title: ABCB1 gene and corticosteroid dependency in pediatric Crohn's disease

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3.2.1 Abstract

Background

Corticosteroids (CS) effectively induce remission in patients with moderate to severe Crohn's disease (CD). However, CS dependence in children is a significant clinical problem associated with numerous side-effects. Identification of molecular markers of CS dependence is of paramount importance. The *ABCB1* gene codes for P-glycoprotein a transporter involved in the metabolism of CS. We examined whether DNA variation in the *ABCB1* gene was associated with CS dependency in children with CD.

Methods

A retrospective study was carried out in two Canadian tertiary paediatric gastroenterology centers. Clinical information was abstracted from medical charts of CD patients (N=260) diagnosed with CD prior to age 18 and administered a first course of CS during the one year since diagnosis. Patients were classified as CS dependent if they relapsed during drug tapering or after the end of therapy. DNA was extracted from blood or saliva. Thirteen tagging single nucleotide polymorphisms (tag-SNPs) and a synonymous variation (C3435T) in *ABCB1* gene were genotyped. Allelic, genotype and haplotype associations were examined using logistic regression and HAPLOVIEW.

Results

Tag-SNP rs2032583 was statistically significantly associated with CS dependency. The rare C allele of this SNP (OR=0.56, 95% CI: 0.34-0.95, p=0.029) and heterozygous genotype TC (OR=0.52, 95% CI: 0.28-0.95, p=0.035) conferred protection from CS dependency. A 3-marker haplotype was significantly associated with CS dependence (multiple comparison corrected p-value=0.004).

Conclusion

Our results suggest that the *ABCB1* gene may be associated with CS dependence in paediatric CD patients.

Keywords: Pharmacogenetics; steroids in IBD; Gene/Drug response; *ABCB1*; Crohn's disease; paediatric

3.2.2 Introduction

Inter-individual variability in response to corticosteroid (CS) therapy is widespread in patients with various diseases treated with this medication.[1-4] In moderate and severe Crohn's disease (CD) management, CS are the mainstay of therapy. Although effective in inducing remission in the majority of patients, some of patients experience a flare of disease during dose tapering or shortly after an initial response and require reintroduction of CS (dependent). Patients treated with CS for long duration experience numerous side effects many of which are serious and irreversible.[5-10] The consequences of these side effects are even more significant in children potentially affecting their psychological & physical wellbeing and development. We and others have shown that CS dependence is a frequent outcome (ranging from 30 to 40%)[11-13] in children with CD, presenting a serious clinical dilemma. Some studies have attempted to identify clinical and demographical markers of CS dependence, but results have been inconsistent. Researching such markers is nonetheless paramount as they would potentially enable the early identification of patients most susceptible for becoming CS dependent. Susceptible children could then benefit from alternative therapeutic strategies.

The steroid metabolic pathway is under genetic control. An individual's ability to metabolize steroids is intimately related to the kind of genetic variation he or she harbours in key proteins within the pathway. We have recently observed that variation in the glucocorticoid receptor gene (*NR3C1*) was associated with steroid dependence in children with CD.[14] Another candidate gene of interest is the *MDR1 (ABCB1)* gene that codes for P-glycoprotein (Pg) a trans-membrane transporter. The gene is involved in the metabolism of various xenobiotics (including steroids) and has been the subject of study for susceptibility for CD. Most studies focused on two single nucleotide polymorphisms (SNPs) in the gene, a synonymous SNP C3435T (rs1045642) and a non-synonymous triallelic variation SNP G2677T/A. Some studies have found associations between these variants and CD [15-17], however others have not.[18-26] Using a gene-wide approach in

Canadian children, we did not observe overall associations with the gene and CD, although some evidence for associations with colonic disease were evident.[26] None of the genome-wide association studies (GWAS) have reported associations between the gene and CD [27-30], as well. Although intimately associated with steroid metabolism, previous studies on whether variations in *MDR1* gene are associated with steroid response have however been unclear.[16,20,24,31] Much of the inconsistency is probably related to the examination of few markers in small sample sizes and differing definitions of steroid response. In order to further clarify the role of the *MDR1* gene vis-à-vis steroid dependency in children we comprehensively examined variation across the gene in a large cohort of Canadian children who were administered steroids for CD.

3.2.3 Methods

3.2.3.1 Study design and population

A retrospective cohort study was carried out, including patients recruited from two tertiary paediatric gastroenterology clinics in Canada (Montreal and Ottawa). This cohort have been recently described.[11] Briefly, patients diagnosed with CD according to established criteria[32,33] prior to age 18 and treated with an initial course of CS, were identified and their follow-up information covering the period of 1 year was abstracted from the medical charts. CS treatment consisted of prednisone (1 mg/kg/day) for 2–4 weeks and subsequent dose tapering by 5 mg/week or budesonide (9 mg) for 1 month with subsequent dose tapering by 3 mg/month. Previously reported criteria[4,34] were adapted to define response to corticosteroid therapy. Patients were classified as CS dependent if after an initial response they experienced clinical relapses during drug tapering or after the end of therapy resulting in reintroduction of CS. Patients that maintained partial or total remission after the end of corticosteroid therapy were classified as responders to CS. Information on clinical and socio-demographic parameters such as disease localization & behaviour at diagnosis, disease activity at steroid initiation, family history of inflammatory bowel disease (IBD) and ethnicity was also acquired. Disease localization and behaviour were classified according to Montreal classification.[35] A total of 364 CD patients diagnosed between 1980 and 2008 and administered a first course of CS were identified. Of these 313 (86%) patients could be classified according to the definition set for CS dependency or CS responsiveness. The mean (± SD) age at diagnosis was 12.3 (± 3.2) years. The majority of patients were male (54.0 %), of Caucasian ancestry (96.9%) (**Table I** on page 121) and had ileocolonic disease (54.2 %) and inflammatory behaviour (88.8%). Blood and/or saliva samples were available for 260 patients (83.1%).

The institutional ethical boards of the two study centers approved the study and informed consent was acquired from the patients.

3.2.3.2 Selection of SNPs and genotyping

Blood or saliva was obtained as a source of DNA. We used an established approach[36] to select tagging single nucleotide polymorphisms (tag-SNPs) for genotyping. Tag-SNPs across the *ABCB1* gene were identified from publicly available databases (http://pga.gs.washington.edu). A linkage disequilibrium (LD) threshold of r2 $\geq$ 0.8 and minor allele frequency of >10% was utilized to select the tag-SNPs. In addition to 13 selected tag-SNPs, SNP C3435T, a synonymous SNP known to influence *MDR1* expression was included. Genotyping was carried out at the McGill University & Genome Quebec Innovation Center using the GenomeLab Sequenom technology which is based on a newly developed genotyping assay termed iPLEX for use with the Mass ARRAY platform. Personnel performing genotyping were unaware of the phenotype of interest.

3.2.3.3 Statistical analyses

Data were checked for missing genotypes and Hardy-Weinberg Equilibrium (HWE). Single marker associations between selected SNPs and CS dependence were examined using logistic regression analysis (STATA). Associations were examined under additive, recessive and dominant models after adjusting for potential confounding variables such as disease localization and behaviour, disease severity, age at diagnosis and gender. Odds ratios (ORs) along with 95% confidence intervals (95% CI) were estimated. LD between tag-SNPs was visualized and haplotype analysis carried out using HAPLOVIEW.[37] Ungenotyped SNPs were imputed using the Hapmap release 22, CEU population as reference based on procedures implemented in the MACH software.[38] Association p-values were corrected for multiple testing using Bonferroni correction (for single marker analysis) and permutation methods (for haplotype associations, implemented in HAPLOVIEW).

3.2.3.4 Study power estimation

Study power was determined using QUANTO software (http://hydra.usc.edu/gxe.). Based on the expected sample size and assuming a range of allele frequencies (0.10 to 0.50), an alpha level of=0.05, and a case-control ratio of ~1.0 to 1.1, the range of risks (OR) that could be detected with adequate power (≥ 80%) was estimated.

3.2.4 Results

The overall genotyping rate for the 14 SNPs (**Table II** on page 122, **Figure 1** on page 133) was 99%. All SNPs were in HWE. Of these 14 SNPs, a minor allele (C) of SNP rs2032583 showed a statistically significant association with CS dependence (**Table III** on page 123) when an additive model was fit (OR=0.56; 95% CI: 0.34 - 0.95, p= 0.029). Individuals heterozygous (TC) for the SNP were less likely to become CS dependent (OR=0.52, 95%CI: 0.28-0.95, p=0.035). Associations with this SNP were also evident under a dominant model (OR=0.51; 95% CI: 0.20-0.92, p = 0.026). There were suggestions for associations with SNP rs3789243 under an additive model (OR =0.71, 95%CI: 0.49-1.02, p = 0.07) and with rare homozygous genotype (OR=0.51, 95%CI: 0.24-1.06, p=0.07), but these were borderline non-significant. Haplotype analysis was carried out to examine associations between SNPs that showed evidence for association in the single SNP analysis (rs2032583 and rs3789243) and SNP rs1045642 that is known to alter MDR1 expression. The 3 marker combination resulted in 6 haplotypes (**Table IV** on page 130) of which haplotype C-T-C was significantly associated with CS dependency (p = 0.0014) and this association remained significant after correction for multiple testing (p = 0.004).

On imputation, among 111 SNPs that met the quality requirements (r^2 >0.3), 24 SNPs in addition to one genotyped SNP (rs2032583) were statistically significantly (p < 0.05) associated with CS dependence at the allelic level (**Table V** on page 131). These significant associations did not withstand adjustment for multiple testing. Haplotype analysis of non-genotyped SNPs showed that six haplotypes belonging to nine blocks (defined according to the "four gamete rule" implemented in HAPLOVIEW) were statistically significantly associated with CS dependence. None of these however maintained significance after corrections for multiple comparisons (data not shown).

Assuming the frequency of CS dependence about 40 % and expecting a cohort size of ~260 our study had > 80% power to detect risks > 1.6 for the markers with minor allele frequencies of ~0.50. For the alleles frequencies between 0.30 and 0.40 power was

sufficient to detect the effects > 1.7, for allele's frequencies of 0.20 – > 1.8, and for alleles frequencies less than 0.20 the study was powered to detect effects > 2.0.

3.2.5 Discussion

We carried out a comprehensive gene-wide study to examine associations between the *MDR1* gene and CS dependence in children with CD. Of the 14 markers studied, associations with 1 marker were evident whereas associations with another marker were borderline non-significant. Haplotype analysis suggested multi-marker variation in the gene may be associated with CS dependence. Analysis of non-genotyped SNPs revealed associations but these did not persist after accounting for multiple comparisons.

The Pg system is an efflux pump that protects cells from foreign molecules including CS [39] by moving them from the intracellular to the extracellular compartments.[40] It is well known that the *ABCB1* gene is involved in the response to various drugs administered for several medical conditions.[41,42] The gene is highly expressed in various tissues exposed to foreign substances, such as the kidneys, liver and intestines[43-45], highlighting its protective role against harmful influences. In the gastrointestinal tract the gene is variably expressed, with the highest expression observed in the colon.[45] Of interest, Farrel et al.[46] demonstrated that the expression of P-glycoprotein was higher in peripheral lymphocites of CD and UC patients not responding to medication suggesting that genetic variation may underlie the variability in observed responses to steroid treatment. Indeed three polymorphisms in the gene, C3435T (rs1045642), T1236C (rs1128503) and triallelic SNP G2677T/A, have been shown to affect the expression of the P-glycoprotein.[47]

Epidemiological evidence however for association between *MDR1* variants and steroid response in CD have been inconsistent. Two Italian studies[20,24] did not find associations between the C3435T and G2677T/A variants and response to CS in CD. A Slovenian study[16] examined 10 SNPs in the gene and also did not observe associations

with any SNP including the C3435T SNP. However associations between haplotypes comprised of two intronic SNPs (rs2235035 and rs1922242) were found with refractory CD (defined as the failure to respond to corticosteroids and immunomodulators and subsequent treatment with anti-TNF medication). Although interesting, these findings, however, were based on only 24 patients. A study on North Indian ulcerative colitis patients[31] did not find associations between *MDR1* SNPs and CS dependence, however some associations with partial response to CS were evident.

Similar to previous reports, we did not observe associations between SNPs rs1045642 and rs1128503 and CS dependence in our study in single marker analysis. However, haplotype analysis suggested that a haplotype encompassing one previously studied SNP (rs1045642) was associated with CS dependence. Both decreased and increased P-glycoprotein function has been shown to be associated with this polymorphism.[48-53] Moreover, rs1045642 has been shown to affect serum cortisol level[54] in healthy women. It is intriguing that intronic SNP (rs3789243) that was a part of the haplotype significantly associated with CS dependence in our study, was previously shown by Ho et al.[22] to be associated with susceptibility to UC and as a part of haplotypes, with differences in response to CS in Chinese epilepsy patients.[55] It is also of interest that another SNP (rs2032583), that was a part of the haplotype significantly associated with CS dependence in our study, has been shown to influence remission in depressive patients treated with antidepressants.[56,57] Taken together these observations support the notion[47] that haplotypes, rather than individual SNPs, were likely to affect the function of P-glycoprotein and thus likely to modify response to steroids. Our results however should be interpreted with caution considering the modest sample size. Noted associations (both single SNP and haplotype) although withstanding corrections for multiple comparisons, could nonetheless be false positive. Similarly lack of associations noted for many SNPs may be the result of low power of the study to detect associations <1.5. Studies to replicate the findings in larger cohorts are required.

In our recent study[14] examining the role of *NR3C1* genetic variations in CS dependence we have shown statistically significant haplotype associations with CS

dependence considering both genotyped and imputed SNPs. Altogether these findings suggest that both genes (*ABCB1* and *NR3C1*) are implicated and CS dependence appears to be complex phenotype. In the context of a lack of reliable clinical markers that could predict the response to CS in IBD our findings are of interest and need to be confirmed in larger patients' cohorts. Further studies to explore functional mechanisms and potential interactions with other candidate genes such as the *GR* gene will be also needed.

In conclusion, the *ABCB1* gene appears to be relevant to CS dependency in paediatric CD.

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3.2.7 Tables

Table I: Demographic and clinical characteristics of studied patients.

Clinical characteristics	CS dependent patients N=127	CS responsive patients N=133
Age at diagnosis, mean (SD)	11.6 (±3.5)	13.0 (±2.8)
Gender, n (%)		
Females	60 (47.2)	59 (44.4)
Males	67 (52.8)	74 (55.6)
Ethnicity, Caucasians, n (%)	124 (97.6)	128 (96.2)
Intestinal localization [a], n (%)		
L1±L4	16 (12.6)	28 (21.1)
L2±L4	38 (29.9)	37 (27.8)
L3±L4	73 (57.5)	68 (51.1)
Disease behaviour [a], n (%)		
Inflammatory disease B1±p	113 (89.0)	118 (88.7)
B2±p and B3±p	14 (11.0)	15 (11.3)
Disease severity, n (%)		
Mild-to-moderate	68 (53.5)	77 (57.9)
Moderate-severe	59 (46.5)	56 (42.1)

CD, Crohn's disease; CS, corticosteroids;L1±L4, Ileum with or without upper digestive tract involvement; L2±L4, Colon with or without upper digestive tract involvement; L3±L4,Ileocolon with or without upper digestive tract involvement;B1±p, Inflammatory disease with or without perianal involvement; B2±p, Stricturing disease with or without perianal involvement; B3±p, Penetrating disease with or without perianal involvement.

[a] Defined according to Montreal classification.

Table II: Characteristics of the SNPs in the ABCB1 gene selected for study.

db SNP id	Genomic location	Position, Bp	Alleles	Type	MAF
rs1128503	Exon 12	86824252	C/T	CS;T1236C	0.41
rs1202186	Intron	86857909	A/G	NC	0.32
rs1045642	Exon 26	86976581	T/C	CS;C3435T	0.50
rs2032583	Intron 21	86998497	C/T	NC	0.14
rs10248420	Intron 20	87002922	A/G	NC	0.18
rs2235046	Intron 16	87012002	G/A	NC	0.43
rs2091766	Intron 15	87012440	C/T	NC	0.39
rs2235035	Intron 13	87017022	C/T	NC	0.33
rs6950978	Intron 5	87038403	A/T	NC	0.42
rs10264990	Intron 5	87040551	T/C	NC	0.35
rs17327442	Intron 5	87050926	T/A	NC	0.16
rs1202184	Intron 5	87051837	A/G	NC	0.48
rs17327624	Intron 4	87054753	G/T	NC	0.21
rs3789243	Intron 3	87058822	C/T	NC	0.49

Bp, Base pairs; CS, Coding - Synonymous; NC, Non-Coding; MAF, Minor Allele Frequency.

Table III: Associations between ABCB1 gene SNPs and CS dependence in paediatric CD patients.

db SNP id	CS dependent N (%)	CS responsive N (%)	Odds ratio (95% CI)	P value	Adjusted Odds ratio (95% CI)	P value [a]
rs1128503						
C	143 (57.2)	157 (60.4)				
T	107 (42.8)	103 (39.6)	1.14 (0.80-1.62)	0.465	1.17 (0.81-1.68)	0.412
CC	40 (32.0)	48 (36.9)				
CT	63 (50.4)	61 (46.9)	1.24 (0.72-2.14)	0.443	1.33 (0.76-2.35)	0.310
TT	22 (17.6)	21 (16.2)	1.26 (0.60-2.61)	0.539	1.28 (0.60-2.73)	0.519
Dom			1.24 (0.74-2.09)	0.409	1.32 (0.78-2.26)	0.303
Rec			1.11 (0.58-2.13)	0.760	1.07 (0.55-2.11)	0.834
HWE				1.00		
rs1202186						
A	170 (67.7)	170 (63.9)				
G	81 (32.3)	96 (36.1)	0.86 (0.57-1.20)	0.335	0.82 (0.55-1.20)	0.299
AA	57 (45.24)	53 (39.9)				
AG	57 (45.24)	64 (48.1)	0.83 (0.49-1.38)	0.475	0.85 (0.49-1.44)	0.551
GG	12 (9.52)	16 (12.0)	0.69 (0.30-1.61)	0.398	0.63 (0.26-1.51)	0.305
Dom			0.80 (0.48-1.35)	0.381	0.81 (0.48-1.34)	0.409
Rec			0.77 (0.32-1.82)	0.516	0.69 (0.31-1.57)	0.380
HWE				1.00		

Table III (Continued)

db SNP id	CS dependent N (%)	CS responsive N (%)	Odds ratio (95% CI)	P value	Adjusted Odds ratio (95% CI)	P value[a]
rs1045642						
T	124 (48.8)	135 (54.4)				
C	130 (51.2)	113 (45.6)	1.07 (0.77-1.50)	0.668	1.01 (0.77-1.53)	0.647
TT	32 (25.2)	36 (27.0)				
TC	60 (47.2)	63 (47.4)	1.10 (0.60-1.93)	0.820	1.12 (0.60-2.08)	0.716
CC	35 (27.6)	34 (25.6)	1.16 (0.60-2.26)	0.668	1.18 (0.59-2.35)	0.645
Dom			1.10 (0.61-1.99)	0.732	1.14 (0.64-2.03)	0.654
Rec			1.11 (0.62-1.99)	0.716	1.09 (0.62-1.92)	0.759
HWE				0.387		
rs2032583						
T	223 (89.2)	217 (82.2)				
C	27 (10.8)	47 (17.8)	0.56 (0.34-0.94)	0.027	0.56 (0.33-0.94)	0.029
TT	100 (80.0)	89 (67.4)				
TC	23 (18.4)	39 (29.6)	0.52 (0.29-0.94)	0.032	0.52 (0.28-0.95)	0.035
CC	2 (1.6)	4 (3.0)	0.44 (0.80-2.49)	0.357	0.43(0.07-2.56)	0.354
Dom			0.52 (0.28-0.95)	0.022	0.51 (0.29-0.92)	0.026
Rec			0.52 (0.05-3.71)	0.448	0.51 (0.09-2.99)	0.455
HWE				0.799		

Table III (Continued)

db SNP id	CS dependent N (%)	CS responsive N (%)	Odds ratio (95% CI)	P value	Adjusted Odds ratio (95% CI)	P value[a]
rs10248420						
A	204 (84.3)	183 (78.9)				
G	38 (15.7)	49 (21.2)	0.70 (0.44-1.12)	0.135	0.71 (0.44-1.14)	0.158
AA	86 (71.1)	73 (62.9)				
AG	32 (26.4)	37 (31.9)	0.73 (0.42-1.29)	0.285	0.75 (0.42-1.34)	0.337
GG	3 (2.5)	6 (5.2)	0.42 (0.10-1.76)	0.237	0.41 (0.09-1.78)	0.235
Dom			0.69 (0.39-1.23)	0.182	0.70 (0.40-1.23)	0.220
Rec			0.47 (0.07-2.25)	0.229	0.45 (0.10-1.92)	0.281
HWE				0.665		
rs2235046						
G	144 (56.7)	154 (57.9)				
A	110 (43.3)	112 (42.1)	1.05 (0.74-1.49)	0.779	1.01(0.75- 1.55)	0.695
GG	39 (30.7)	45 (33.8)				
GA	66 (52.0)	64 (48.1)	1.19 (069-2.10)	0.535	1.32 (0.75-2.33)	0.335
AA	22 (17.3)	24 (18.1)	1.06 (0.48-2.17)	0.879	1.07 (0.51-2.27)	0.849
Dom			1.15 (0.66-2.01)	0.590	1.25 (0.73-2.15)	0.411
Rec			0.87 (0.44-1.72)	0.664	0.90 (0.47-1.74)	0.765
HWE				0.800		

Table III (Continued)

db SNP id	CS dependent N (%)	CS responsive N (%)	Odds ratio (95% CI)	P value	Adjusted Odds ratio (95% CI)	P value[a]
rs2091766						
C	150 (59.1)	164 (62.1)				
T	104 (40.9)	100 (37.9)	1.13 (0.80-1.60)	0.482	1.10(0.76-1.56)	0.631
CC	44 (34.7)	53 (40.2)				
CT	62 (48.8)	58 (43.9)	1.28 (0.75-2.20)	0.356	1.38(0.80-2.39)	0.245
TT	21 (16.5)	21 (15.9)	1.20 (0.58-2.49)	0.615	1.04(0.49-2.22)	0.917
Dom			1.26 (0.74-2.16)	0.360	1.29 (.77-2.16)	0.334
Rec			1.05 (0.51-2.14)	0.891	0.87(0.43-1.76)	0.705
HWE				0.696		
rs2235035						
C	166 (65.4)	178 (67.4)				
T	88 (34.6)	86 (32.6)	1.10 (0.77-1.58)	0.582	1.01(0.72-1.53)	0.781
CC	54 (42.5)	63 (47.4)				
CT	58 (45.7)	54 (40.6)	1.25(0.75-2.11)	0.394	1.33 (0.78-2.27)	0.298
TT	15 (11.8)	16 (12.0)	1.10 (0.50-2.42)	0.825	0.88 (0.38-2.06)	0.771
Dom			1.22 (0.72-2.04)	0.432	1.22 (0.74-2.02)	0.437
Rec			0.98 (0.43-2.23)	0.957	0.77 (0.34-1.72)	0.522
HWE				0.580		

Table III (Continued)

db SNP id	CS dependent N (%)	CS responsive N (%)	Odds ratio (95% CI)	P value	Adjusted Odds ratio (95% CI)	P value[a]
rs6950978						
A	145 (57.1)	156 (58.6)				
T	109 (42.9)	110 (41.4)	1.06 (0.76-1.46)	0.738	1.00(0.74-1.45)	0.825
AA	45 (35.4)	52 (39.1)				
AT	55 (43.3)	52 (39.1)	1.22 (0.70-2.12)	0.475	1.24 (0.70-2.19)	0.454
TT	27 (21.3)	29 (21.8)	1.1 (0.56-2.08)	0.828	1.03 (.52-2.04)	0.929
Dom			1.19 (0.71-1.94)	0.541	1.17 (0.69-1.96)	0.562
Rec			0.97 (0.51-1.83)	0.915	0.92 (0.50-1.69)	0.781
HWE				0.015		
rs10264990						
T	158 (62.2)	180 (67.7)				
C	96 (37.8)	86 (32.3)	1.26 (0.88-1.79)	0.204	1.24(0.86-1.78)	0.250
TT	51 (40.2)	62 (46.6)				
TC	56 (44.1)	56 (42.1)	1.22 (0.72-2.05)	0.465	1.23 (0.72-2.09)	0.454
CC	20 (15.7)	15 (11.3)	1.62 (0.75-3.48)	0.216	1.54 (0.70-3.39)	0.280
Dom			1.30 (0.77-2.19)	0.294	1.29 (0.78-2.14)	0.317
Rec			1.47 (0.72-3.02)	0.291	1.39 (0.66-2.91)	0.381
HWE				0.414		

Table III (Continued)

db SNP id	CS dependent N (%)	CS responsive N (%)	Odds ratio (95% CI)	P value	Adjusted Odds ratio (95% CI)	P value[a]
rs17327442						
T	216 (85.0)	220 (82.7)				
A	38 (15.0)	46 (17.3)	0.84 (0.53-1.34)	0.472	0.80 (0.49-1.29)	0.362
TT	91 (71.6)	92 (69.2)				
TA	34 (26.8)	36 (27.1)	0.95(0.55-1.66)	0.869	1.0 (0.57-1.79)	0.976
AA	2 (1.6)	5 (3.7)	0.40 (0.08-2.14)	0.287	0.23 (0.04-1.3)	0.102
Dom			0.88 (0.50-1.56)	0.661	0.88 (0.51-1.53)	0.654
Rec			0.41 (0.08-2.15)	0.291	0.23 (.034-1.34)	0.101
HWE				0.822		
rs1202184						
A	126 (49.6)	144 (54.1)				
G	128 (50.4)	122 (45.9)	0.95 (0.61-1.48)	0.811	1.26 (0.88-0.81)	0.20
AA	32 (25.2)	39 (29.3)				
AG	62 (48.8)	66 (49.6)	1.14 (0.64-2.05)	0.649	1.29 (0.71-2.36)	0.401
GG	33 (26.0)	28 (21.1)	1.44 (0.72-2.85)	0.302	1.59 (0.78-3.27)	0.203
Dom			1.23(0.71-2.13)	0.456	1.38 (0.78-2.44)	0.268
Rec			1.32 (0.74-2.30)	0.349	1.35 (0.74-2.44)	0.330
HWE				0.805		

Table III (Continued)

db SNP id	CS dependent N (%)	CS responsive N (%)	Odds ratio (95% CI)	P value	Adjusted Odds ratio (95% CI)	P value[a]
rs17327624						
G	205 (80.7)	207 (77.8)				
T	49 (19.3)	59 (22.2)	0.84 (0.55-1.28)	0.422	0.80 (0.52-1.24)	0.328
GG	83 (65.4)	81 (60.9)				
GT	39 (30.7)	45 (33.8)	0.85 (0.50-1.43)	0.533	0.87 (0.51-1.49)	0.609
TT	5 (3.9)	7 (5.3)	0.70 (0.21-2.29)	0.552	0.53 (.15-1.83)	0.314
Dom			0.83 (0.50-1.37)	0.457	0.82 (0.49-1.37)	0.446
Rec			0.74 (0.23-2.39)	0.612	0.55 (0.16-1.90)	0.345
HWE				0.710		
rs3789243						
T	133 (52.4)	122 (45.9)				
C	121 (47.6)	144 (54.1)	0.77 (0.54-1.08)	0.138	0.71 (0.49-1.02)	0.070
TT	35 (27.6)	27 (20.3)				
TC	63 (49.6)	68 (51.1)	0.71 (0.39-1.31)	0.279	0.70 (0.38-1.32)	0.275
CC	29 (22.8)	38 (28.6)	0.59 (0.29-1.18)	0.136	0.51 (0.24-1.06)	0.070
Dom			0.66 (0.38-1.17)	0.091	0.64 (0.35-1.16)	0.138
Rec			0.74 (0.41-1.34)	0.291	0.65 (0.36-1.17)	0.147
HWE				1.00		

HWE, Hardy-Weinberg Equilibrium; CI, Confidence interval; Dom, Dominant; Rec, Recessive.

[a] Adjusted for demographic and clinical variables.

Table IV: Haplotype associations between variations in ABCB1 gene and CS dependence.

SNPs			Frequencies			
rs1045642	rs2032583	rs3789243	Overall Freq	Cases/ Controls	Chi2	P value
T	T	C	0.396	0.392, 0.400	0.035	0.8525
C	T	T	0.279	0.286, 0.273	0.098	0.754
C	C	T	0.129	0.095, 0.161	5.04	**0.0248**
T	T	T	0.102	0.096, 0.107	0.183	0.6685
C	T	C	0.078	0.117, 0.041	10.251	**0.0014**[a]
C	C	C	0.016	0.014, 0.017	0.052	0.8199

[a] Empiric P value = 0.004

Table V: Single marker associations between imputed SNPs in ABCB1 gene and CS dependence.

SNP id	Type of SNP	Imputation Quality, r^2	Frequencies, Case, Control	Chi^2	P value
rs6979885	Intr	0.91	0.339, 0.263	3.52	0.061
rs2235067	Intr	0.99	0.886, 0.820	4.524	0.033
rs11979702	Intr	0.99	0.886, 0.820	4.524	0.033
rs10280101	Intr	0.99	0.886, 0.820	4.524	0.033
rs10225473	Intr	0.99	0.886, 0.820	4.524	0.033
rs7787082	Intr	0.93	0.886, 0.820	4.524	0.033
rs2032583	Intr	0.99	0.886, 0.820	4.524	0.033
rs4148739	Intr	0.99	0.886, 0.820	4.524	0.033
rs11983225	Intr	0.99	0.886, 0.820	4.524	0.033
rs11760837	Intr	0.99	0.886, 0.820	4.524	0.033
rs10274587	Intr	0.99	0.886, 0.820	4.524	0.033
rs2235040	Intr	0.99	0.886, 0.823	4.069	0.044
rs12720067	Intr	0.99	0.886, 0.823	4.069	0.044
rs10268314	Intr	0.99	0.886, 0.823	4.069	0.044
rs10276603	Intr	0.93	0.886, 0.823	4.069	0.044
rs11772987	Intr	0.98	0.886, 0.823	4.069	0.044

Table V (Continued)

SNP id	Type of SNP	Imputation Quality, r^2	Frequencies, Case, Control	Chi^2	P value
rs10244266	Intr	0.94	0.886, 0.823	4.069	0.044
rs1882479	Intr	0.98.	0.886, 0.823	4.069	0.044
rs2188525	Intr	0.90	0.063, 0.026	4.134	0.042
rs2214103	Intr	0.90	0.063, 0.026	4.134	0.042
rs2235074	Intr	0.90	0.063, 0.026	4.134	0.042
rs2888599	Intr	0.90	0.063, 0.026	4.134	0.042
rs3213619	5` UTR	0.83	0.063, 0.026	4.134	0.042
rs10486996	Intr	0.74	0.063, 0.026	4.134	0.042
rs12539395	Intr	0.57	0.063, 0.030	3.198	0.074
rs7790722	Intr	0.62	0.067, 0.030	3.856	0.05
rs17149864	Intr	0.56	0.063, 0.030	3.198	0.074
rs2188528	Intr	0.72	0.063, 0.026	4.134	0.042

Intr, Intronic; 5` UTR, Five Prime Untranslated Region.

Only statistically significantly ($p< 0.1$) associated SNPs are presented.

Genotyped marker highlighted in bold.

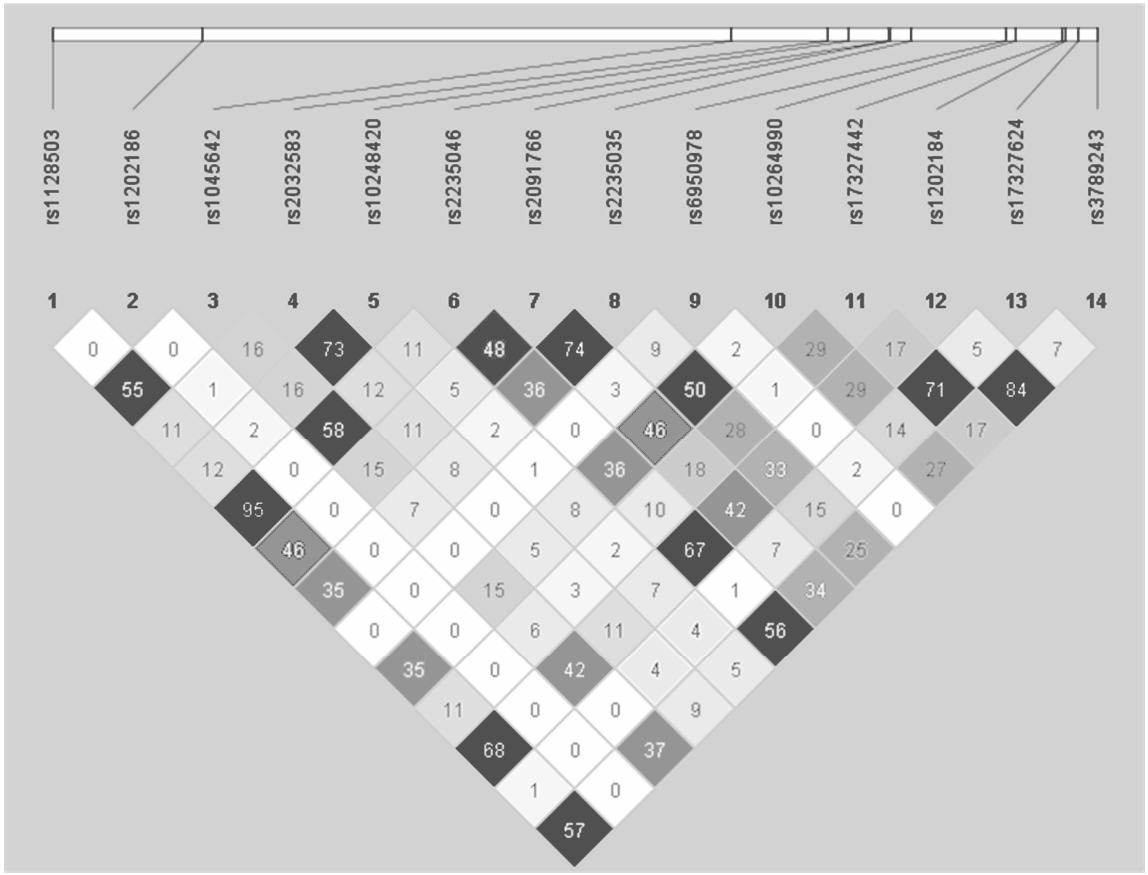

Figure 1. LD (r2 x 100) between genotyped SNPS in ABCB1 gene.

3.3 Article 3:

Variation in the glucocorticoid receptor gene *(NR3C1)* may be associated with corticosteroid dependency in children with Crohn's disease

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VARIATION IN THE GLUCOCORTICOID RECEPTOR GENE (NR3C1) MAY BE ASSOCIATED WITH CORTICOSTEROID DEPENDENCY IN CHILDREN WITH CROHN'S DISEASE

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3.3.1 Abstract

Objectives

In pediatric-onset of Crohn's disease (CD) corticosteroids (CS) dependency (~40%) is a significant clinical problem associated with numerous side-effects. Given the known effects of the glucocorticoid receptor (*NR3C1*) gene in CS metabolism, we investigated whether variation in the gene was associated with CS-dependency.

Methods

A retrospective cohort study was carried out including CD patients diagnosed before 18 years and treated with a first course of CS in two Canadian tertiary pediatric gastroenterology clinics. DNA was obtained from blood or saliva. Tagging single nucleotide polymorphisms (Tag-SNPs) and functionally important SNPs were genotyped. Ungenotyped SNPs were imputed using the HAPMAP CEU reference panel. Allelic, genotype and haplotype associations between the *GR* SNPs and CS-dependency were examined.

Results

A total of 255 CS-dependent and CS-responsive CD patients were studied. Of the 12 SNPs examined, 3 SNPs rs10482682 (OR=1.43, 95% CI:0.99-2.08, *P*=0.047)**,** rs6196 (OR=0.55, 95% CI:0.31-0.95, *P*=0.024), and rs2963155 (OR=0.64, 95% CI:0.42-0.98, *P*=0.039), showed associations under an additive model whereas rs4912911 (OR=0.37, 95% CI:0.13-1.00, *P*=0.03) and rs2963156 (OR=0.32, 95% CI:0.07-1.12, *P*=0.047) showed significant associations under a recessive model. Haplotype analysis of 5 associated markers revealed significant associations between two haplotypes and CS-dependence (*P*-values 0.002 and 0.004). On imputation, a further 19 SNPs were associated with CS-dependency. Two multi-marker haplotypes (*P*-values=0.001 each) including genotyped and imputed SNPs conferred susceptibility for CS-dependency.

Conclusion

Our results suggest that variations in the *GR*/*NR3C1* gene are associated with CS-dependency in paediatric-onset CD. Studies to replicate these findings and identify the potentially relevant variants are required.

Keywords: GR-receptor; *NR3C1*; pharmacogenetics; corticosteroids; paediatric; Crohn's disease

3.3.2 Introduction

Crohn's disease (CD) is a chronic inflammatory bowel disease (IBD) characterized by a progressive course and various complications. The incidence of CD is on the rise among younger individuals particularly in Canada [1-5]. Corticosteroids (CS) are the mainstay of therapy used to induce remission in patients with moderate to severe CD. However, inter-individual variability in the response to these agents is frequently observed. About 30 % to 40% of initially responsive patient's experience disease flares during drug tapering or after drug discontinuation (become CS dependent) [6-11]. Most of these patients require either surgery or reintroduction of steroids. Long-term administration of CS is frequently associated with harmful side-effects in children [12-14]. Determination of predictors that will enable identification of paediatric-CD patients most likely to become CS dependent is thus paramount. Demographic (younger age at diagnosis) and/or clinical [6-9, 11, 15-17] markers (colonic localization, coexisting upper tract disease localization, and growth impairment) [17] have been examined but associations have not been consistently replicated. Given their stability genetic markers are likely to complement clinical and/or demographic predictors. Few studies have however investigated such markers [18, 19].

A potential genetic marker is the glucocorticoid receptor (*NR3C1*) gene, spanning ≈150 kb and located on chromosome 5. This gene is intimately involved in the metabolism of natural substrates - steroid hormones and exogenous steroids, its action being mediated via its regulation of glucocorticoid-responsive genes [20]. DNA variations in the gene are known to be associated with inter-individual variation in steroid metabolism. Although *NR3C1* variation has been shown to influence steroid response in some conditions, it is not known whether such variation can determine response to CS in paediatric-onset CD. We investigated the latter in a well-characterized cohort of Canadian children diagnosed with CD and who were administered CS for treatment.

3.3.3 Material and Methods

3.3.3.1 Study design and patients

A retrospective cohort study was carried out, including patients recruited from two tertiary paediatric gastroenterology clinics in Canada (Montreal and Ottawa). Details on this cohort have been recently reported [21]. In brief, patients diagnosed with CD according to established criteria [22, 23] prior to age 18 and treated with an initial course of CS, were identified and their follow-up information covering the period of 1 year was abstracted from the medical charts. CS treatment consisted of prednisone (1 mg/kg/day) for 2–4 weeks and subsequent dose tapering by 5 mg/week. Some patients (11.8%) were receiving budesonide (9 mg) for 1 month with subsequent dose tapering by 3 mg/month. Previously reported criteria were adapted to define response to corticosteroid therapy [7, 10]. Patients were classified as steroid dependent if after an initial response, clinical relapse either during drug tapering or shortly after the end of treatment was experienced resulting in reintroduction of CS. Patients that maintained partial or total remission since the end of corticosteroid therapy were classified as responders to CS. Information on clinical and socio-demographic parameters such as disease localization and behaviour (at diagnosis), disease activity at steroid initiation, family history of inflammatory bowel disease (IBD), ethnicity etc. was also acquired.

The institutional ethical boards of the two study centers approved the study and consent was acquired from the patients.

3.3.3.2 Selection of SNPs and genotyping

Blood or saliva was obtained as a source of DNA. Fourteen tag-SNPs (single nucleotide polymorphisms) across the *NR3C1* gene were identified using the methods described by Carlson et al [24]. A linkage disequilibrium (LD) threshold of $r^2 \geq 0.8$ and minor allele frequency of >10% was utilized to select the tag- SNPs. In addition to the tag-SNPs, three SNPs (rs6190, rs6195, rs258751) were added to set of markers based on

their functional/clinical significance. Genotyping was carried out at McGill University's Genomics Innovation Center using high throughput genotyping technology. Personnel performing genotyping were unaware of the phenotype of interest.

3.3.3.3 Statistical analyses

Genotyped SNPs

Each genetic marker was tested for Hardy-Weinberg equilibrium in the cohort. Single marker associations between the SNPs and CS-dependency were initially examined using univariate logistic regression analysis. Subsequently multivariate logistic regression adjusting for demographic and clinical variables (gender, age at diagnosis, disease localization and behavior, disease severity, family history of IBD) was carried out. Various models of inheritance including additive, dominant and recessive were fit. Odds ratios (OR) and respective 95% confidence intervals (95% CI) were estimated. The STATA statistical package (Stata Statistical Software: Release 10. College Station, TX: StataCorp) was used to carry out these analyses. Association between specific haplotypes comprising the selected markers were investigated using HAPLOVIEW (v.4.2 http://www.broad.mit.edu/mpg/haploview/) [25].

Imputation

Ungenotyped SNPs in and around the NR3C1 gene were imputed using the HapMap reference panel (Release 22). SNPs with MAF $\geq$ 0.01 and genotyping frequency > 0.95 in the 60 CEU founders were utilized. Imputation analysis was carried out using procedures implemented in MACH (http://www.sph.umich.edu/csg/abecasis/mach/) [26] and validated using BEAGLE [27]. Only SNPs with r^2 values > 0.3 (indicator of imputation quality) were considered for further association analysis. Single marker and haplotype analysis was carried out as described above for the genotyped SNPs.

Adjustment for multiple comparisons

P values were corrected using Bonferroni method for single marker analyses and permutation (n=10000) for haplotype analyses (implemented in HAPLOVIEW).

3.3.4 Results

A total of 364 CD patients diagnosed between 1980 and 2008 and administered a first course of CS were identified. Of these 313 (86%) patients could be classified according to the definition set for CS dependency or CS responsiveness. Blood and/or saliva samples were available for 255 patients (81.5%). The mean age (±SD) at diagnosis was 12.4 (±3.2) years (**Table I** on page 151). Most patients were male (53.7%) and Caucasian (96.9%). Most patients had ileo-colonic disease (54.5%) and inflammatory behaviour (89 %) at diagnosis. A total of 125 (49 %) patients became CS dependent.

From 14 tag-SNPs we were able to successfully genotype 10 SNPs. One of functionally significant SNP (rs258751) was not in HWE, so our final set of analyzed markers included 12 SNPs (**Table II** on page 152). The overall genotyping rate in individuals and markers was high (97.0 % and 96.8 % respectively). Single SNP analysis showed that of the 12 markers, three markers (rs10482682, rs6196 and rs2963155) were associated at the allelic level (unadjusted for multiple comparisons, P values of 0.05; 0.02 and 0.036 respectively) with corticosteroid dependency (**Table III** on page 153; **Figure 1** on page 162). For two other markers (rs2963156 and rs4912911) associations were evident when a recessive model was fit. Adjusting for demographic and clinical variables did not change the effect of the allelic associations with CS-dependency for the majority of SNPs (excepting rs4912911). For this SNP allelic association became significant (P value 0.039) after adjustment for demographic and clinical variables (**Table IV** on page 157). Haplotype analysis based on these 5 significantly associated SNPs showed that two haplotypes (AAACA and GGGCG) were significantly associated with the corticosteroid-dependent phenotype (**Table V** on page 158). Haplotype AAACA was risk conferring whereas haplotype GGGCG was protective. These associations remained significant (respective empirical *P* values 0.006 and 0.01) after permutation testing (10000).

Imputation results acquired using MACH were retained given the similarity with those obtained using BEAGLE. Imputation of ungenotyped SNPs revealed that from

among the 74 SNPs that passed the quality control criteria (r^2 >0.3), in addition to the 3 genotyped SNPs, 19 SNPs were statistically significantly associated with CS dependent phenotype at the allelic level (**Table VI** on page 159). Five of these markers remained significantly associated with CS dependence after adjusting for multiple testing. The LD pattern of genotyped and imputed SNPs revealed the presence of 6 haplotype blocks (based on implementing the "four gamete rule" algorithm in HAPLOVIEW). Haplotype association analysis showed that one haplotype in each of two blocks (Block 1 and 2) (**Figure 2** on page 163) were associated with CS dependency and remained statistically significantly associated after accounting for multiple comparisons (respective permuted *P* values 0.03 and 0.02) (**Table VII-A** on page 160 and **Table VII-B** on page 161).

3.3.5 Discussion

Using tag-SNP approach, we examined whether variants in the *NR3C1* gene were associated with the development of CS dependency in paediatric patients with CD. In single-marker analysis, we observed that 5 SNPs were potentially associated with CS dependency. Haplotype analysis of associated SNPs provided evidence for associations as well. Analysis of imputed and genotyped SNPs showed potential associations with haplotypes within 2 distinct LD blocks in the gene.

CS dependency is a serious clinical challenge during the management of pediatric-onset CD. We [21] and others have shown that ≈40% of children administered steroids can become CS dependent. CS dependency is known to be associated with serious side-effects and investigating markers to enable identification of susceptible children is an important priority. However many studies that searched for clinical or socio-demographical markers have not produced consistent results. Based on the intimate relationship between the *NR3C1* gene and steroid metabolism and the large inter-individual variability in the *NR3C1* gene, we hypothesized that DNA variation in the gene may predispose children to susceptibility for steroid dependency. Our findings suggest that indeed the *NR3C1* gene may be an important predictor of CS dependency in children diagnosed with CD. To our knowledge, this is the first study that has

comprehensively explored the role of genetic variations across the *NR3C1* gene with respect to corticosteroid-dependence in either children or adults diagnosed with CD.

The GR protein is expressed in the cytoplasm until it binds to a ligand, which induces transport into the nucleus. There are several isoforms of GR: GRα, GRβ and GRγ, arising from alternative splicing and translational events [28, 29]. The alpha form of GR is a ligand-activated transcription factor that modulates the expression of glucocorticoid-responsive genes. The beta form of GR is not transcriptionnally active but is able to inhibit the effect of glucocorticoid activated GRα and therefore may be physiologically and pathophysiologically relevant to tissue sensitivity to glucocorticoids. Various lines of evidence indicate that variation in the *NR3C1* gene can influence sensitivity to glucocorticoids in normal and diseased conditions and modify response to CS [30-33]. Two non-synonymous coding polymorphisms (rs6195, rs6190) and an intronic SNP rs41423247) in the gene have been most investigated. For example, the BclI (rs41423247), ER22/23EK (rs6189/6190), and N363S (rs6195) polymorphisms have been shown to be associated with a variation in sensitivity to exogenous glucocorticoids [31, 33] in patients with rheumatoid arthritis and in healthy individuals. Although we did not detect significant associations with SNPs rs6195 and rs6190 [probably due to low power, given the lower frequency (≈2%)], there were suggestions that they could be potentially associated with CS dependency (ORs of 1.65 and 2.4 respectively). We did not genotype SNP rs41423247, however, it is in high LD (D` 0.95) with the coding synonymous SNP rs6196 [34], which was significantly associated with CS dependency in our study. The rs6196 polymorphism resides in exon 9α which codes for transcriptionally active form of GR [35, 36]. Another SNP found to be associated with CS dependency in our study (rs33389) was previously shown to be associated with sensitivity to CS by Stevens et al. [37].

With regards to IBD, De Iudicibus et al. [18], reported that the homozygous rare genotype of the BclI polymorphism was significantly more frequent in responders to corticosteroids than in CS-dependent patients. It is interesting to note that in our study rare homozygotes for SNP rs6196 that is in high LD with the BclI polymorphism, were

absent among children with CS dependency and that carriage of the minor allele was protective (OR=0.55, 95% CI=0.13-0.95), indirectly supporting the findings of De Iudicibus et al. [18]. In another study, [38] the authors found a higher frequency of the BclI polymorphism in CD patients versus healthy controls, but stratification based on steroid response was not carried out. Other studies have reported an increased expression of GRβ isoform in CS resistant ulcerative colitis (UC) patients [39, 40], but did not studied associations with CS dependence.

The *NR3C1* gene is located in a genomic region characterized by high LD and the identification of causal variants is therefore hampered by numerous mutually associated SNPs. Our results suggest that *NR3C1* haplotypes are likely to play role in CS dependent phenotype. Two haplotypes comprising 5 markers that were individually nominally associated with CS dependence were associated with CS dependence even after adjustment for multiple comparisons, suggesting that multi-marker variation in the gene were responsible for the observed associations. At least two SNPs that were associated with CS dependency in our study (one encompassed in the associated haplotypes) have been previously shown to influence various glucocorticoid-mediated phenotypes [34, 37, 41], further supporting the role of the gene in determining CS response in CD. A more comprehensive analysis that examined imputed and genotyped SNPs further revealed that variation across two distinct haplotype blocks could be potentially associated with CS dependency.

In conclusion, our findings suggest that variations across the *NR3C1* gene play role in response to CS in paediatric CD. These associations need to be investigated further in larger cohorts. Furthermore, functional studies to assess the implications of these associations for therapeutic modulation of steroid administration need to be carried out.

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3.3.6 References

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3.3.7 Tables

Table I: Demographic and clinical characteristics of the CD patients.

Clinical characteristics	CS dependent patients N=125	CS responsive patients N=130
Age at diagnosis, mean (SD)	11.6 (±3.5)	13.0 (±2.8)
Gender, n (%)		
Females	60 (48.0)	58 (44.6)
Males	65 (52.0)	72 (55.4)
Ethnicity, Caucasians, n (%)	122 (97.6)	125 (96.2)
Intestinal location, n (%)		
L1±L4	16 (12.8)	27 (20.8)
L2±L4	37 (29.6)	36 (27.7)
L3±L4	72 (57.6)	67 (51.5)
Disease behaviour a, n (%)		
Inflammatory disease B1±p	111 (88.8)	116 (89.2)
B2±p and B3±p	14 (11.2)	14 (10.8)
Disease severity, n (%)		
Mild-to-moderate	68 (54.4)	75 (57.7)
Moderate-severe	57 (45.6)	55 (42.3)

CD, Crohn's disease; CS, corticosteroids; L1±L4, Ileum with or without upper digestive tract involvement; L2±L4, Colon with or without upper digestive tract involvement;L3±L4, Ileocolon with or without upper digestive tract involvement; B1±p, Inflammatory disease with or without perianal involvement; B2±p, Stricturing disease with or without perianal involvement; B3±p, Penetrating disease with or without perianal involvement.

a Defined according to Montreal classification.

Table II: Characteristics of studied markers in NR3C1 gene.

SNP id	Location in the gene	Position, Bp	Alleles	MAF	Type
rs6196	Exon 9	142641683	A/G	0.14	CS
rs10482682	Intron 5	142659590	G/A	0.40	NC
rs860457	Intron 4	142668516	T/C	0.29	NC
rs4912905	Intron 2	142710569	G/C	0.22	NC
rs2963155	Intron 2	142736197	A/G	0.22	NC
rs2963156	Intron 2	142738689	C/T	0.24	NC
rs6195	Exon 2	142759510	A/G	0.026	CNS
rs6190	Exon 2	142760530	G/A	0.025	CNS
rs10482616	Intron 1	142761760	G/A	0.14	NC
rs7701443	Intron 1	142772843	A/G	0.44	NC
rs4244032	Intron 1	142774918	A/G	0.22	NC
rs4912911	Intron 1	142787225	A/G	0.33	NC

CS, Coding synonymous; CNS, Coding non-synonymous; Bp, Base pairs; MAF, Minor allele frequency; NC, Non-coding; SNP, Single nucleotide polymorphism.

Table III: Allelic and genotypic associations between NR3C1 gene variants and response to CS in children with CD.

SNP id	CS dependent n (%)	CS responsive n (%)	Odds ratios (95% CI)	*P* values
rs6196				
A	223 (89.9)	216 (83.1)		
G	25 (10.1)	44 (16.9)	0.55 (0.31 - 0.95)	0.024[a]
AA	99 (79.8)	89 (68.5)	ref	
AG	25 (20.2)	38 (29.2)	0.59 (0.33 - 1.06)	0.076
GG	0	3 (2.3)	-	-
Dominant			0.55 (0.29 - 1.01)	0.039[a]
Recessive			-	-
rs10482682				
G	140 (56.0)	168 (64.6)		
A	110 (44.0)	92 (35.4)	1.43 (0.99 - 2.08)	0.047 [a]
GG	36 (28.8)	59 (45.4)	ref	
GA	68 (54.4)	50 (38.5)	2.23 (1.28 - 3.87)	0.004[a]
AA	21 (16.8)	21 (16.1)	1.64 (0.79 - 3.41)	0.187
Dominant			2.05 (1.18 - 3.57)	0.006 [a]
Recessive			1.05 (0.51 - 2.14)	0.890
rs860457				
T	170 (68.5)	188 (72.9)		
C	78 (31.5)	70 (27.1)	1.23 (0.82 - 1.84)	0.285
TT	55 (44.3)	65 (50.4)	ref	
TC	60 (48.4)	58 (45.0)	1.22 (0.73 - 2.03)	0.439
CC	9 (7.3)	6 (4.6)	1.77 (0.59 - 5.29)	0.305
Dominant			1.27(0.75 - 2.15)	0.337
Recessive			1.60 (0.49 - 5.65)	0.380

Table III (Continued)

SNP id	CS dependent n (%)	CS responsive n (%)	Odds ratios (95% CI)	P values
rs4912905				
G	192 (78.7)	201 (77.9)		
C	52 (21.3)	57 (22.1)	0.95 (0.61 - 1.49)	0.832
GG	75(61.5)	75 (58.2)	ref	
GC	42 (34.4)	51 (39.5)	0.82 (0.49 - 1.38)	0.463
CC	5 (4.1)	3 (2.3)	1.67 (0.38 - 7.22)	0.495
Dominant			0.87 (0.51 - 1.49)	0.590
Recessive			1.79 (0.34 - 11.78)	0.424
rs2963155				
A	206 (82.4)	194 (74.6)		
G	44 (17.6)	66 (25.4)	0.64 (0.42 - 0.98)	0.039 [a]
AA	85 (68.0)	74 (56.9)	ref	
AG	36 (28.8)	46 (35.4)	1.47 (0.86 - 2.51)	0.161
GG	4 (3.2)	10 (7.7)	0.51 (0.15 - 1.76)	0.288
Dominant			0.62 (0.36 - 1.07)	0.068
Recessive			0.40 (0.09 - 1.43)	0.115
rs2963156				
C	193 (77.2)	196 (75.4)		
T	57 (22.8)	64 (24.6)	0.90 (0.59 - 1.39)	0.630
CC	72 (57.6)	78 (60.0)	ref	
CT	49 (39.2)	40 (30.8)	1.33 (0.78 - 2.25)	0.292
TT	4 (3.2)	12 (9.2)	0.36 (0.11 - 1.17)	0.090
Dominant			1.10 (0.65 - 1.87)	0.697
Recessive			0.32 (0.07 - 1.12)	0.047 [a]

Table III (Continued)

SNP id	CS dependent n (%)	CS responsive n (%)	Odds ratios (95% CI)	P values
rs6195				
A	180 (96.8)	198 (98.0)		
G	6 (3.2)	4 (2.0)	1.65 (0.38 - 8.07)	0.439
AA	87 (93.5)	97 (96.0)	ref	
AG	6 (6.5)	4 (4.0)	1.67 (0.46 - 6.12)	0.437
GG	0	0	-	-
Dominant			1.67 (0.46 - 6.12)	0.433
Recessive			-	-
rs6190				
G	241 (96.4)	256 (98.5)		
A	9 (3.6)	4 (1.5)	2.39 (0.65 - 10.74)	0.139
GG	116 (92.8)	126 (96.9)	ref	
GA	9 (7.2)	4 (3.1)	2.44 (0.73 - 8.15)	0.146
AA	0	0	-	-
Dominant			-	-
Recessive			-	-
rs10482616				
G	217 (86.8)	222 (85.4)		
A	33 (13.2)	38 (14.6)	0.89 (0.52 - 1.51)	0.644
GG	94 (75.2)	95 (73.1)	ref	
GA	29 (23.2)	32 (24.6)	0.92 (0.51 - 1.63)	0.766
AA	2 (1.6)	3 (2.3)	0.67 (0.11 - 4.12)	0.669
Dominant			0.89 (0.49 - 1.63)	0.698
Recessive			0.69 (0.06 - 6.12)	0.683

Table III (Continued)

SNP id	CS dependent n (%)	CS responsive n (%)	Odds ratios (95% CI)	P values
rs7701443				
A	137 (54.8)	148 (56.9)		
G	113 (45.2)	112 (43.1)	0.92 (0.76 - 1.57)	0.629
AA	43 (34.4)	41 (31.5)		
AG	51 (40.8)	66 (50.8)	0.74 (0.42 - 1.30)	0.287
GG	31 (24.8)	23 (17.7)	1.28 (0.64 - 2.56)	0.475
Dominant			0.88 (0.50 - 1.53)	0.627
Recessive			1.53 (0.80 - 2.96)	0.164
rs4244032				
A	185 (77.1)	185 (79.1)		
G	55 (22.9)	49 (20.9)	1.12 (0.71 - 1.78)	0.603
AA	70 (58.3)	74 (63.3)	ref	
AG	45 (37.5)	37 (31.6)	1.28 (0.75 - 2.21)	0.365
GG	5 (4.2)	6 (5.1)	0.88 (0.26 - 3.02)	0.840
Dominant			1.22 (0.70 - 2.14)	0.438
Recessive			0.80 (0.19 - 3.27)	0.725
rs4912911				
A	168 (70.0)	151 (62.9)		
G	72 (30.0)	89 (37.1)	0.73 (0.49 - 1.08)	0.100
AA	55 (45.8)	48 (40.0)	ref	
AG	58 (48.4)	55 (45.8)	0.92 (0.54 - 1.57)	0.761
GG	7 (5.8)	17 (14.2)	0.36 (0.14 - 0.94)	0.037 [a]
Dominant			0.79 (0.46 - 1.36)	0.361
Recessive			0.37 (0.13 - 1.00)	0.031 [a]

CS, corticosteroid; SNP, Single nucleotide polymorphism.

[-] Counts were not sufficient to permit calculation.

[a] *P* values < 0.05 unadjusted for multiple comparisons.

Table IV: Allelic associations between tag-SNPs in NR3C1 gene and response to CS before and after adjustment for patient's demographic and clinical characteristics.

SNP id	OR (95% CI)	P value	Adjusted [a] OR (95% CI)	Adjusted [a] P value
rs6196	0.55 (0.31 - 0.95)	0.024	0.52 (0.29 - 0.91)	0.021
rs10482682	1.43 (0.99 - 2.08)	0.047	1.37 (0.96 - 1.97)	0.091
rs860457	1.23 (0.82 - 1.84)	0.285	1.20 (0.79 - 1.83)	0.390
rs4912905	0.95 (0.61 - 1.49)	0.832	0.98 (0.61 - 1.55)	0.918
rs2963155	0.64 (0.42 - 0.98)	0.039	0.62 (0.40 - 0.96)	0.033
rs2963156	0.90 (0.59 - 1.39)	0.630	0.88 (0.58 - 1.34)	0.559
rs6195	1.65 (0.38 - 8.07)	0.439	1.93 (0.50 -7.36)	0.336
rs6190	2.39 (0.65 -10.74)	0.139	2.41 (0.71 - 8.20)	0.160
rs10482616	0.89 (0.52 - 1.51)	0.644	0.86 (0.52 - 1.45)	0.577
rs7701443	0.92 (0.76 - 1.57)	0.629	1.12 (0.79 - 1.59)	0.514
rs4244032	1.12 (0.71 - 1.78)	0.603	1.12 (0.72 - 1.75)	0.614
rs4912911	0.73 (0.49 - 1.08)	0.100	0.65 (0.43 - 0.99)	0.039

[a] Adjusted for age at diagnosis, gender, disease localization, disease behaviour,family history of IBD & disease severity in multivariate models.

Statistically significant (<0.05) P values are highlighted in bold.

Table V: Associations between haplotypes in GR NR3C1 gene and response to CS in paediatric CD patients.

SNPs					Frequency			
rs6196	rs10482682	rs2963155	rs2963156	rs4912911	Overall	CS dependent	CS responsive	*P*-value
A	G	A	C	A	0.253	0.240	0.265	0.515
A	A	A	T	A	0.180	0.176	0.184	0.818
A	A	A	C	A	0.152	0.202	0.105	**0.002**[a]
A	G	A	C	G	0.136	0.150	0.122	0.347
G	G	G	C	G	0.101	0.062	0.139	**0.004**[b]
A	A	A	T	G	0.057	0.052	0.062	0.616
A	G	G	C	A	0.048	0.044	0.051	0.685
A	G	G	C	G	0.031	0.024	0.039	0.324
G	G	G	C	A	0.029	0.036	0.022	0.343

CS, corticosteroid.

[a] Empiric *P* value = 0.006.

[b] Empiric *P* value = 0.01.

Table VI: Allelic associations between imputed SNPs and CS dependence in children with CD.

SNP id	Association Allele	Imputation Quality, r2	Frequencies, Case, Control	Chi square	P value
rs10482689	T	0.740	0.212, 0.108	10.379	0.001[a]
rs10482682	A	1.00	0.440, 0.354	3.955	0.048
rs17287758	A	0.628	0.208, 0.108	9.696	0.002[a]
rs6196	A	0.997	0.896, 0.831	4.58	0.032
rs10482642	C	0.813	0.204, 0.108	9.030	0.003[a]
rs10515521	A	0.830	0.192, 0.104	7.896	0.005[a]
rs17339831	C	0.845	0.192, 0.104	7.896	0.005[a]
rs2963155	A	1.00	0.824, 0.746	4.566	0.033
rs11740792	G	0.856	0.192, 0.108	7.144	0.007
rs10482633	G	0.867	0.184, 0.108	5.983	0.014
rs4128428	C	0.875	0.184, 0.108	5.983	0.014
rs1438732	G	0.913	0.900, 0.838	4.23	0.040
rs10515522	T	0.937	0.900, 0.838	4.23	0.040
rs9324918	T	0.891	0.900, 0.838	4.23	0.040
rs4912903	T	0.697	0.440, 0.354	3.955	0.050
rs17287745	G	0.754	0.440, 0.354	3.955	0.050
rs33389	C	0.925	0.900, 0.842	3.765	0.050
rs2918419	T	0.922	0.900, 0.842	3.765	0.050
rs2918418	G	0.867	0.900, 0.842	3.765	0.050
rs2963151	T	0.929	0.900, 0.842	3.765	0.050
rs2963154	T	0.880	0.900, 0.842	3.765	0.050
rs2918415	A	0.942	0.900, 0.842	3.765	0.050

[a] Empiric *P* values.

Only significantly ($P \leq 0.05$) associated markers are shown.

Genotyped markers are highlighted in bold.

Table VII-A: Association between haplotypes in the NR3C1 gene with response to CS in paediatric CD using genotyped and imputed SNPs data, block 1.

SNPs													Frequencies			
rs17209216	s865482	rs853184	rs4912903	rs244465	rs174048	rs258763	rs17287745	rs258748	rs258747	rs17287758	rs17209237	rs6196	Overall Freq.	Freq. C/Ctr.	Chi2	*P* value
G	C	C	C	C	T	T	A	C	A	G	A	A	0.404	0.384/0.423	0.81	0.369
G	C	C	T	C	T	A	G	C	G	A	A	A	0.155	0.208/0.104	10.56	0.001[a]
A	C	C	T	C	T	A	G	C	G	G	G	A	0.143	0.140/0.146	0.04	0.843
G	A	T	C	C	C	A	A	G	G	G	A	G	0.131	0.104/0.158	3.22	0.073
G	C	C	T	C	T	A	G	C	G	G	G	A	0.094	0.088/0.100	0.21	0.643
G	C	C	C	T	T	T	A	C	A	G	A	A	0.065	0.072/0.058	0.43	0.511

C, Cases; Ctr., Controls.

[a] Empiric *P* value = 0.03.

Genotyped marker highlighted in bold.

Table VII-B: Association between haplotypes in the NR3C1 gene with response to CS in paediatric CD using genotyped and imputed SNPs data, block 2.

SNPs							Frequencies			
rs258750	rs17209251	rs190488	rs11750172	rs17209258	rs258813	rs10482689	Overall Freq	Freq C/Ctr.	Chi2	P value
A	A	T	C	A	G	C	0.467	0.456/0.477	0.22	0.636
A	G	T	G	G	G	C	0.237	0.228/0.246	0.23	0.630
G	A	G	C	A	A	T	0.157	0.208/0.108	9.69	0.0018 [a]
G	A	G	C	A	A	C	0.131	0.104/0.158	3.22	0.073

C, Cases; Ctr., Controls.

[a] Empiric *P* value = 0.025.

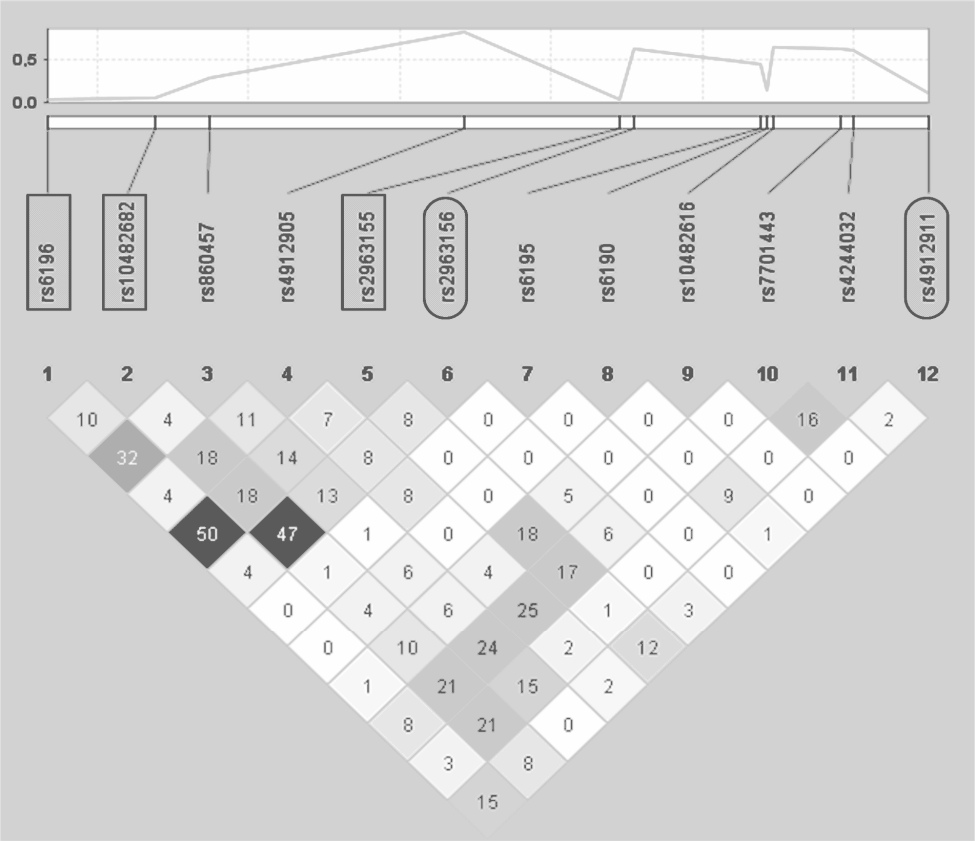

Figure 1. LD (r2 x 100) between genotyped Tag-SNPS in NR3C1 gene.

Legend: The line at top of the figure represents association between the SNPs and CS dependence (P-values). The markers significantly associated ($P<0.05$) with CS dependence under an additive model are boxed. Under a recessive model they are in rounded boxes.

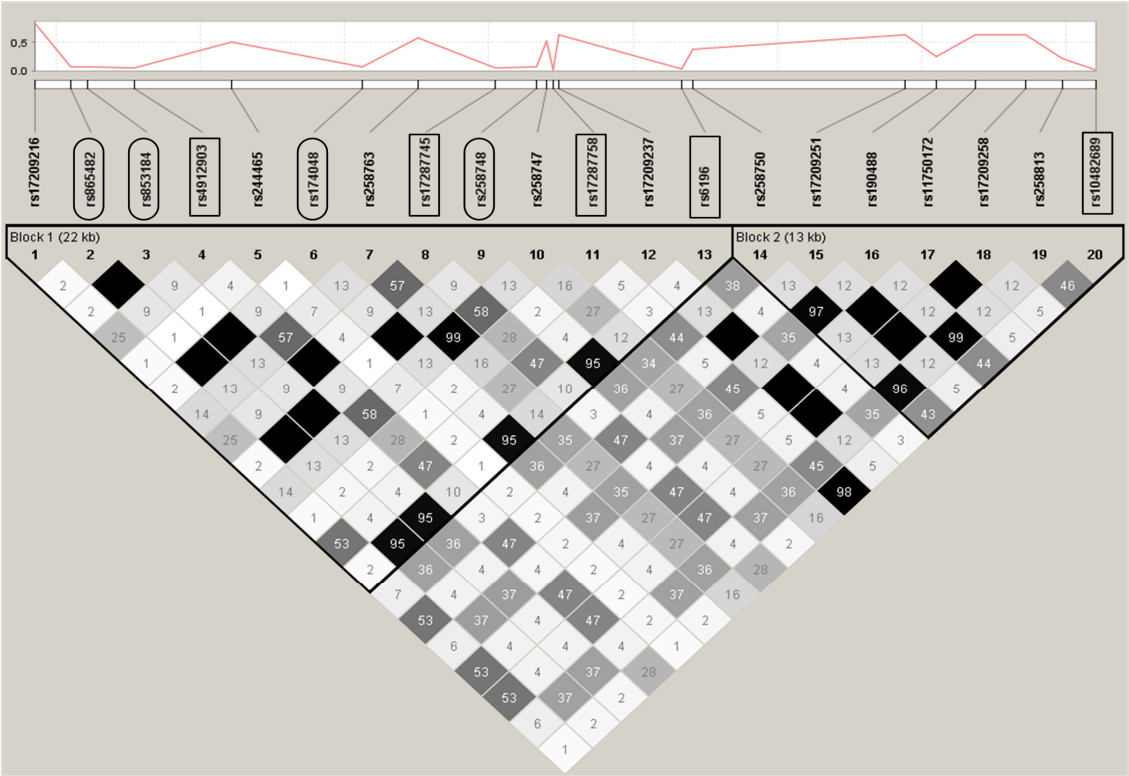

Figure 2. LD between genotyped and imputed SNPs, and haplotype blocks.

Legend: The line on the top designates P values of single marker association analysis for genotyped and imputed SNPs. The markers significantly (P value <0.05) associated with CS dependence are in boxes and in rounded boxes are the markers marginally significantly (P<0.10) associated with CS dependence. rs6196 is a genotyped SNP.

CHAPTER 4

DISCUSSION OF THE RESULTS

4.1 Summary of the results

This study confirms that CS dependence is very common among children diagnosed with CD. The high rates of ~40% suggest that approximately 1 in 2 children treated with CS are likely to become dependent. These high rates are particularly challenging and make the search for the identification of clinical and molecular predictors that much more urgent. Concerning clinical and demographic markers, our study has shown that children diagnosed with CD at a very young age (<10.7) are more likely to become CS dependent. Similarly, those children who had co-existing upper digestive tract disease were also more likely to be susceptible. Findings related to a younger age at diagnosis and susceptibility for CS dependence have been reported previously. Associations with upper digestive tract disease, however, are novel. Concerning genetic markers, we found some evidence that the *MDR1* gene was associated with CS dependency. Associations appear to be confined to 1-2 markers within the gene, although haplotype combinations with SNP C3435T (Exon 26), which is known to alter *MDR1* expression, seem important as well. As for the *GR* gene, multiple markers individually and at the haplotype level were found to be related to CS dependence.

The rate of CS resistance that we report (8%) is consistent with the majority of previous paediatric studies but is substantially lower than those in adult studies. This difference in rates between paediatric and adult patients may be related to the age-dependent expression of GR receptor [302]. Moreover, a lesser extent of the previous exposure to various environmental influences, including CS, in a paediatric IBD population could possibly explain the difference, given that a sensitizing effect of CS on the development of the sub-population of T cells, which contributes to CS resistance, has been suggested [303]. We also observed a higher incidence of CS resistance in girls than in boys (11, 6% vs. 5%). This finding may reflect more severe disease in girls in

accordance with USA based study [304]. However in our study disease severity was not different between boys and girls. Another explanation could be related to differences in hormonal background between girls and boys or to use of contraceptives. Although interesting these differences were based on small number of patients and need to be further examined in larger samples.

The rates of CS dependence that we report are comparable to rates reported in some paediatric studies and are slightly higher than those reported in adult patients. This difference could be attributed to the differential expression of studied genes in children and adults. Indeed, the association of younger age at diagnosis and upper digestive tract involvement reported by this study suggests that the CS dependent phenotype may be heterogeneous. It is of interest that an experimental animal study [302] suggested that the loss and gain of glucocorticoid responsiveness in the proximal and distal small intestine, respectively, were related to age- and segment-dependent expression of GR. It could thus be speculated that variations in the *GR* gene may be related to CS dependence via its influence on GR expression in different parts of the intestinal tract.

LD among some tag-SNPs (rs1202184 and rs37789243) exceeds the selected threshold of 0.80 in our population as it shown in **Figure 1** (page 133). Higher LD in our population than in the HAPMAP data could be explained by composition of our population that comprise CD patients as opposed to the healthy individuals in the HAPMAP. With respect to associations between the *ABCB1* gene and CS dependence, from among the 14 SNPs investigated we observed associations only with SNP rs2032583 (Intron 21). In our study, he minor allele (C) of rs2032583 was inversely associated with CS dependence (OR=0.56; 95% CI: 0.34 - 0.95, p=0.029). The comparison of our findings with other similar studies in the paediatric population is not possible as none of the earlier studies examined associations between this SNP (or a proxy) and CS dependence in CD. However, consistent with our findings in CD, in German [305] and USA based [306] studies, the C allele of SNP rs2032583 has been shown to be associated with a higher probability of remission from depression after 4 weeks in carriers treated with antidepressants. Nevertheless, these comparisons should

be interpreted with caution because of the different Pgp substrates (CS versus anti-depressants) and the different phenotypes involved. It is also notable that in our earlier study [307] the same SNP (rs2032583) was associated with colonic CD. It is possible that the same variant contributes to the CS dependent phenotype and colonic disease. However, no association was found between colonic disease and CS dependence in our present study. It is therefore possible that, the link between the *MDR1* gene and colonic CD may be independent from that between the *MDR1* gene and CS response and that different pathways may be implicated in these associations.

In addition to the single SNP association, we found a haplotype, comprising the functionally relevant SNP C3435T (rs1045642) in exon 26, to be associated with CS dependence. Gene-wide haplotype analysis carried out as well revealed that haplotypes in one of blocks were associated with CS-dependence, but did not withstand permutation test. The association appeared to be driven by C3435T and rs2032583 variants, so further analysis strategy focused on theses SNPs with addition of marginally significant one (rs3789243) in single marker analysis. The association of haplotype CTC, comprising two intronic (rs3789243 and rs2032583) in intron 2 and intron 21 and one coding synonymous (rs1045642) variant, was significant after correction for multiple testing. In our study, the C allele of rs1045642 was part of the haplotype that was more frequent in CS-dependent patients than in responders to CS. This finding supports the hypothesis that a decrease in intracellular concentration of the drug due to the over-expression of Pgp may be involved in CS dependence, given that the C allele has been shown to be associated with increased activity of the Pgp pump in expression studies [203, 204]. Our results nonetheless differ from some previous association studies that have explored *ABCB1* variants and CS response. For example, in contrast to our study, Potocnik et al. [220] observed positive associations between a haplotype encompassing the T allele of rs1045642, and refractory CD. This study was however based on a small sample, used a comparison group different from our study and had a broader definition for refractory CD (it included all patients not responding to various drugs used to treat IBD). In the Belgium [223] study, observations of positive associations between the TT

genotype and complete tapering of CS are however consistent with those in our study. As also noted by the authors of this study, observed associations of the T allele with CS response may be related to the T allele being associated with more extensive and severe disease [227]. In our cohort however, disease severity was not correlated with CS dependence, indicating that associations between the SNP and CS dependence may be independent of disease severity. Two other SNPs (rs3789243 and rs2032583) comprising the haplotype associated with CS dependence in our study have also been shown to influence response to treatment in other medical conditions. Particularly, the C allele of rs3789243 (Intron 2) was shown to be more frequent in responders to antiepileptic drugs [308], and the C allele of rs2032583 (Intron 21) was shown to be more frequent in patients responding to antidepressants [305, 306], implying that associations between variants in the *MDR1* gene may not be specific to CD. This is not surprising given that the *MDR1* gene has multiple substrate specificity and influences the metabolism of a variety of drugs besides steroids.

With regard to known haplotype comprising SNPs at the positions 1236, 2677 and 3435, we were not able to explore its association with CS dependence, because marker 2677 could not be typed due to technological limitations. The haplotype comprising two other markers of interest (1236 and 3435) was not significantly associated with outcome of interest.

One of known SNPs in *NR3C1* gene (rs41423247), shown by Mill et al.[309] to be in high LD (D` 0.95) with the coding synonymous SNP rs6196 that was significantly associated with CS dependency in our study. Although there appears to be high D` between these two markers, their allele frequencies are quite disparate (50% versus 14%) and hence r2 is likely to be quite low. Therefore, given potentially low LD, the rs41423247 SNP may require specific investigation. We observed associations between multiple SNPs across the *NR3C1* gene (both single marker and haplotype) and CS dependency implicating the gene in CS response. The haplotype associations noted were of particular interest. Both, gene-wide and 5-marker haplotype associations comprising SNPs significant in single marker analysis, withstood adjustment for multiple

comparisons. A 6-marker haplotype AACGAC (defined using solid spine of LD), that included the coding-synonymous variant rs6196 (Exon 9) in gene-wide analysis was positively associated with CS-dependence (p-value 0.0013). A 5-marker haplotype AAACA that also comprised allele A of rs6196, was positively associated with CS dependence. These observations are consistent with the inverse associations between the G-allele of SNP rs6196 and CS dependence noted in the single-SNP analysis in our study. Our findings, however, differ from the only other study that examined associations between the rs6196 variant and CS response in IBD. In a Swiss study [264], in 181 IBD patients, no associations were noted either at the single SNP or haplotype level with SNP rs6196. The Swiss study was however potentially underpowered to detect associations, did not analyse CD separately and used different definitions for steroid response, making comparisons between studies difficult.

Recently it has become clear that multiple isoforms of the GR protein are generated endogenously as a result of alternative RNA splicing and alternative translation initiation [245]. In addition, each isoform is subject to a variety of post-translational modifications. Consequently, the potential existence of numerous receptor variants, each having differential characteristics in expression, localization, transcriptional activity, and degradation, contributes substantially to unique biological responses. In our study, the haplotype associated with CS-dependency contained rs6196, a coding-synonymous SNP located in exon 9α, encoding the transcriptionally active form of a GR receptor, consequently supporting the role of α isoform in variability to drug response. Since GRα can also undergo a variety of other post-translational modifications [310], leading to other isomers such as the β isoforms, the variants coding for this isoform may also be related to CS dependency. It was suggested previously that the variability of GC sensitivity could depend on the ratio of GR alternative splicing and therefore on imbalance in α and β isoforms. However, as our study did not include variations in exon 9β encoding for the β isomer we were unable to examine its associations with CS response. Certainly, additional studies will be required to further differentiate associations between the various GR isomers and CS response.

Our examination of both the *MDR1* and *GR* genes, by-and-large, suggested that synonymous coding variations were likely to be associated with CS response. This is in line with recent evidence indicating that synonymous sites are not neutral or non-functional [219, 311]. Incidentally, synonymous variations have been shown to affect gene function by altering the stability, splicing or localisation of m-RNA [312-314], thus affecting the expression and activity of the coded protein.

4.2 Alternate pathways mediating variation in response to CS

The mechanism of CS refractoriness is likely complex and unlikely to be associated with a single pathway. Although CS refractoriness may be a primary phenomenon related to abnormalities in the proteins in the upstream pathway of steroid metabolism, it is also likely that the anti-inflammatory capacity of CS is overwhelmed by inflammation-driven excessive synthesis and the activity of intracellular transcription factors that may reduce the affinity of GR for its intracellular substrate (CS). For instance, an excessive constitutive activation of the pro-inflammatory molecule NF-κB, and *IL-10* cannot be ruled out. It has been recently shown that [315] in CD patients who are resistant to CS, there is an increase in activity and level of NF-κB in the epithelial cells of the intestinal mucosa and reporter gene assays indicate that these higher levels inhibit the activity of GRα preventing its transcriptional activity. Similarly, associations between the *IL-10* gene and CS response have been reported [316]. It is quite plausible that an excess of inflammatory cytokines accumulated locally during relapses cannot be efficiently down-regulated in response to CS because of an insufficient up-regulation of *IL-10* synthesis in IBD patient in particular in carriers of the low *IL-10* producer genotype. In addition to *IL-10*, variation in the *IL-2* and *CYP3AA* genes may also be of interest. Lee et al. [303] have reported an increased proliferation of CD4+ T cells expressing the interleukin receptor (IL-2) after exposure to dexamethasone in adult patients with UC. A higher prevalence of these cells was observed in subjects with a history of CS resistance. A higher resistance of lymphocytes to steroids has also been

reported in healthy subjects [317] with suggestions that up to 30% of the healthy population would fail to respond to CS therapy for severe inflammatory conditions [317, 318] as a consequence. As for the *CYP3A4* gene, it is known to catalyze the 6-hydroxylation of a number of steroids, including dexamethasone and prednisone [213, 214], implying that variations in this gene may also underlie susceptibility for CS dependence. It is therefore clear that CS-dependency is apparently a multi-factorial phenotype involving perhaps the contribution of several genes and unknown environmental influences. These potential influences need to be further explored.

4.3 Strengths and limitations of the study

The studies undertaken had some strengths and limitations as well. A major strength in comparison to previous studies was that that our studies were based on a relatively large cohort of well-characterized CD patients. Another strength was the inclusion of only CS-naïve patients so that the possible influence of previous CS exposure could be limited. A comprehensive investigation of the target genes was carried out by: (1) selecting tag-SNPs that provided adequate coverage of variation across the genes and (2) using imputation methods to allow us to infer the associations with other markers possibly not captured by the SNP-tagging approach. This approach ensured that any associations between the target genes and CS response would not be missed.

The studies also had some limitations. They were based on a cohort for whom data was acquired retrospectively. They were thus susceptible to the different biases inherent in such designs. The potential for such biases, the methods utilized to reduce them and their impact on the study findings are summarized below.

In the context of retrospective cohort studies, selection bias may arise when the selected study population is not representative of the target population, limiting the generalisation of findings [319]. For our studies, we have used two sources to identify patients diagnosed with CD: a patient list maintained at the gastroenterology clinics and

the medical archives' databases at the main study hospital, which is one of the largest paediatric hospitals in Canada. From within this cohort, the study cohort (patients with moderate-severe disease who were administered steroids during the first year after diagnosis) was established. This comprehensive approach would have limited the potential for selection bias, if any. It is nonetheless possible that many patients with moderate-severe CD who visited the other paediatric IBD center in Montreal (Montreal Children's Hospital) and who were administered steroids, may differ in some ways from our study cohort. Similarly, a minority of patients with mild CD are also at times administered steroids. Such patients are more likely to be treated by their family physicians and hence would not have been eligible for selection in our cohort. Therefore, the potential for selection bias remains but is likely to be limited.

Another potential for selection bias in a cohort study may be due to the rates of participation and/or follow-up that differ according to the exposure or the outcome under study. Among the ~ (n=450) patients that were eligible for selection, participation and follow-up, in our clinical and/or genetic studies, 86% were finally included. Thus complete data was available for 82% to 86% of patients. These participation rates can be considered high (and therefore acceptable) and unlikely to have resulted in major selection bias, if any. Among those patients who were eligible for inclusion it is nonetheless possible that patients who responded to CS versus those who became CS dependent had different propensities to attrition or to provide the biomaterial. However, the main reason for the missing samples of biomaterial was the impossibility to retrace patients' addresses with only few refusals to provide samples. It has to be noted however that the follow-up period in our study was quite short (1 year since diagnosis) and that the majority of patients continued to visit the hospitals for this time period, ensuring that follow-up was near-complete for all included patients. We also believe that the distribution of alleles was unlikely to be different in patients who either did not participate or did not have complete clinical data or did not provide DNA samples, as a result ensuring that selection bias was also likely to be limited. Nevertheless, in order to verify potential bias, we compared main characteristics of patients (such as age, sex,

clinical characteristics at diagnosis) that were lost to follow-up (i.e. patients who did not have complete clinical information for the follow-up duration) with those with complete follow-up. Similarly, in order to identify the occurrence of analogous bias due to the refusal to provide samples of biomaterial, patients' baseline characteristics and the response to CS were compared between those who contributed DNA material and those who did not. No meaningful differences were evident, suggesting that selection bias was limited. We also believe that possibility of survival bias is very limited given that firstly, mortality during 1-2 years since diagnosis in early onset of CD is quite low and secondly none of the examined genes are known to be associated with survival.

We have considered a possibility of the presence of information bias resulting from potential genotyping errors. Aiming to decrease the possibility of such bias and the extent of random errors, which may lead to reduced power to detect true associations, the genotyping process involved a stringent protocol for maintaining the quality of the acquired results. Each of the SNPs was tested for genotyping reproducibility in 5 % of samples. We observed >98% concordance in results in the duplicate samples. The samples of biological material of all subjects were handled in a similar way adhering to stringent protocols during DNA extraction process, so possible contamination, which may lead to low quality of DNA, is likely to be equally distributed among the comparison groups. Moreover, laboratory personnel that performed genotyping were unaware of patients' outcome status. Consequently, if genotyping errors due to the "human factor" have occurred, they should be equally distributed among comparison groups as well. Most of the tested SNPs, barring 1, were in HWE. Although this test may have limited power to detect genotyping errors in conjunction with the quality control methods implemented, we believe that information bias with regards to genotyping was likely to be limited. In any case, if present, it would be expected, on average, to be non-differential and have resulted in an underestimation of the true associations.

With respect to majority of clinical and demographic predictors of CS dependence, information bias was unlikely to occur, since characteristics such as age at

diagnosis, gender, CD phenotypes and other clinical characteristics were well-documented in the medical charts and were not susceptible to misinterpretation. Nevertheless, some misclassification in the assignment of patients to the studied outcomes was possible. This is because the assessment of study outcomes was based on inscriptions in medical charts. The description of some symptoms required in particular for classifying individual short term outcomes (response and partial response to CS) was not complete. For these patients, classification was based on the physician's assessment of the patients' profile. If misclassification was present, it may have resulted in lower/higher estimates of the true prevalence rates. It was however unlikely to influence comparisons between groups as it was unlikely to be related to the socio-demographic, clinical variables and genotypes of the patient. With respect to long-term study outcomes (CS-dependence and CS response) the possibility of such misclassification is minimal because of the nature of the definitions used. The definition of CS-dependency was based on the occurrence of clinical relapse of disease, which was in turn well-documented in the medical charts by the treating physicians. In doubtful instances, the outcome was validated after consulting with the treating physician. Moreover, inter- rater reliability assessed for long-term outcomes on the subset of patients was satisfactory. Nevertheless, the potential for misclassification remains, but was likely to be non-differential as well and may have lead to the underestimation of the true effects.

Confounding refers to the mixing of the estimated effect with other risk factors [319]. .In our study, some patients' characteristics were considered as potential confounders given that they could be associated with response to CS and the studied genes. These include variables such as concomitant medication, age at diagnosis and disease localization or behaviour. In order to assess the true effects of the genetic variants of interest on the occurrence of CS dependence, we have applied an adjustment in analysis using the multivariate models. The anti-TNF medication was introduced in Quebec in about 2000 and a little earlier in Ontario. This medication however according to recent practice was used only in cases of non-response or dependence to CS, thus after the study outcomes were developed. Therefore, the possibility of confounding by

these therapeutic agents is not likely. The same procedure, control in analysis, was applied to the assessment of the effects of potential clinical and demographic markers of a response to CS, based on the knowledge that many of them may be associated each with another and with CS resistance or/and CS dependence.

Confounding by ethnicity, also called population stratification, is an inherent limitation of genetic association studies, occurring when both the frequencies of genetic variations and the proportions of outcome differ in the ethnic subgroups of the study population. In the context of our study, ethnicity could have potentially confounded genetic associations if the gene frequencies varied according to ethnicity and CS response was different between ethnic groups. The majority of the studied cohort was Caucasian (>90%). An analysis excluding non-Caucasians did not alter the findings, indicating that ethnicity was unlikely to have played a major role.

Although by-and-large our studies were adequately powered to examine genetic markers for CS dependence, power was limited for comprehensively examining associations with CS resistance. Also, the power to detect associations between some low frequency functional SNPs such as rs6195 and rs6190 in *NR3C1* gene and CS dependence was potentially limited as well. Furthermore, our study did not have adequate power to investigate gene-gene interactions, that could potentially influence CS response.

4.4 Clinical and public health implications

Advances in pharmacogenetics and genomics can improve the overall results of patients' care and thus contribute to the improvement of health services. The prevention of disease outcomes based on the risk stratification approach has long been proposed [320]. It has been maintained, that the clarification of the role of genes and environment could lead to new combined approaches towards prevention based on risk stratification. Personalized medicine and personalized genomics approaches have recently emerged in

clinical medicine [321], promising the improvement of efficiency of treatment strategies by adjusting drug choices according to genetic patient's profile.

In the context of our study, the identification of molecular markers could permit the timely introduction of appropriate alternate therapies to patients potentially susceptible to develop CS-dependence. Ultimately, such alternate strategies can result in the improvement of the quality of life of patients and would decrease the burden imposed on health services due to disease flares and complications requiring hospitalization. The observed associations between the *NR3C1* and *ABCB1* genes and the CS dependent phenotype among paediatric patients, if confirmed, could potentially aid clinicians in pursuing individualized treatments and researchers in pursuing the search for mechanisms underlying variability in response to CS.

The clinical relevance of potential markers of drug response largely depends on the frequency of a variant in patients' population. The characteristic of a good clinical marker depends on its prevalence in a population of interest, its specificity and sensitivity. The proportion of children' under 11 years of age in our population is about 25%, and those with upper digestive disease is about 26%. These two potential markers for CS dependency (young age at diagnosis and upper digestive disease) would not represent all CS-dependent patients. Moreover, the two mentioned potential markers do not appear to explain all variations related to the CS-dependent phenotype, highlighting the importance of studying the role of genetic variations, including those in other putative genes. Given that the overall frequencies of markers associated with CS dependency in our study were from 10 to 50% and the frequencies of associated haplotypes range from 10 to 15.5 %, it is unlikely that the results of this study could be translated into clinical practice presently. Future exploration would be necessary in order to delineate the simultaneous contribution of the two studied genes, the role of other putative genes and other potentially clinical predictors. Nonetheless, our findings do provide clues to possible pathogenesis of CS dependence that could be investigated using functional studies.

Although pharmacogenetics is a promising field that already contributes to a better understanding of some of the underlying mechanisms of action of drugs used in IBD, until now the only discovery translated into daily practice is the relation between the thiopurine S-methyltransferase (TPMT) gene polymorphisms and the hematological toxicity of thiopurine treatment. In reality, the majority of currently established genetic polymorphisms that influence drug therapy are mono-genic traits, such as those found in TPMT. As CS dependence is very likely to be a complex phenotype, on a cautious note, the translation of findings of genetic associations into clinical practice may be more complicated and require extensive study.

These findings are of relevance to other inflammatory and auto-immune medical conditions where CS are commonly used. The magnitude of the problem related to altered response to CS depends on prevalence of given disease and the extent of inter-individual variability of response to CS. Childhood ALL is the most common malignancy in children below age of 15 years [322] with an annual incidence in Europe and the United States of 3.5 cases per 100,000 children 0 to 14.9 years of age [323]. Notably, in ALL CS are widely used as a part of induction therapy according to current protocols. About 10-30% of ALL patients do not respond well to CS therapy [324]. In childhood asthma despite the relatively small fraction (5%) of CS dependent/resistant patients, this problem presents significant clinical challenge due to the difficulties of disease management and is associated with 2-fold increase of health costs [325, 326].

Given that other inflammatory and autoimmune conditions share common pathways related to CS action and CS metabolism, the results of this thesis could help future investigations.

4.5 Future directions

Our findings of associations between the variations in *NR3C1* and *ABCB1* genes and CS dependency have to be replicated in larger prospective cohorts of paediatric or adult CD patients. Furthermore, expression studies examining the relationship between

CS dependent phenotype and haplotypes in two studied genes would be of relevance. The role of the GR isoform α and β should also be further explored. Regarding the clinical predictors of CS dependency, the mechanisms underlying the association with younger age at diagnosis need exploration as well. The studies of the interaction between the two investigated genes and the CS dependent phenotype would be of interest. Another focus of interest could be the exploration of the underlying mechanisms that differentiate between the resistance and dependence to CS, as well as the mechanisms involved in secondary resistance and dependence to CS. In our study, the patients were not previously exposed to CS, precluding the examination of secondary CS dependence. As some patients change from a CS resistant (with an increased dose of CS) to a CS dependent phenotype, studying the mechanisms underlying these changing phenotypes would be of interest as well.

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APPENDICES

Appendix 1

Short questionnaire, medical history and ancestry

QUESTIONNAIRE

_____

Date d'achèvement du questionnaire : _____(J/M/A)

A. ID DU SUJET: _____

Sexe: Garçon _____ Fille _____

Diagnostic : _____ Crohn _____ Colite ulcéreuse _____ Indéterminée
_____Contrôle

Pour les questions suivantes, veuillez cocher la case si nécessaire (NR = ne souhaite pas ou ne connaît pas la réponse).

	Quel est le rang de l'individu dans la famille? (ordre de naissance) (mettez 1 s'il est né le premier, 2 s'il est né le second etc.)	_____	NR ☐
	Combien de frères et/ou soeurs a votre enfant?	_____	NR ☐
	Taille actuelle de l'individu (pieds/pouce)	___ pieds ___ pouce	NR ☐
	Poids actuel de l'individu (kg/grammes)	___kg ___gm	NR ☐

Pour chaque frère/sœur, veuillez indiquer l'âge et le sexe (M pour garçon, F pour fille) :

Frère/sœur 1 Prénom : _____ Age : _____ Sexe : _____

Frère/sœur 2 Prénom : _____ Age : _____ Sexe : _____

Frère/sœur 3 Prénom : _____ Age : _____ Sexe : _____

Frère/sœur 4 Prénom : _____ Age : _____ Sexe : _____

Frère/sœur 5 Prénom : _____ Age : _____ Sexe : _____

(Vous pouvez ajouter ci-dessous d'autres frères/sœurs si nécessaire)

B. HISTOIRE MÉDICALE ANTÉRIEURE

Les questions suivantes portent sur les maladies affectant ou ayant affecté un membre de la famille. Lorsque nécessaire, veuillez cocher les cases correspondantes. (NA = ne s'applique pas, NR = ne souhaite pas répondre ou ne connaît pas la réponse)

Maladie de Crohn	**Colite ulcéreuse**
Père ____Oui ___ Non ___ NR ___ NA	Père ____Oui ___ Non ___ NR ___ NA
Age au moment du diagnostic: _____ ans	Age au moment du diagnostic: _____ ans
Mère ____Oui ___ Non ___ NR ___ NA	Mère ____Oui ___ Non ___ NR ___ NA
Age au moment du diagnostic: _____ ans	Age au moment du diagnostic: _____ ans
Grand-père ____Oui ___ Non ___ NR ___ NA	Grand-père ____Oui ___ Non ___ NR ___ NA
Age au moment du diagnostic: _____ ans	Age au moment du diagnostic: _____ ans
Grand-mère ____Oui ___ Non ___ NR ___ NA	Grand-mère ____Oui ___ Non ___ NR ___ NA
Age au moment du diagnostic: _____ ans	Age au moment du diagnostic: _____ ans
Oncle(s): maternel(s) ou paternel(s)	Oncle(s): maternel(s) ou paternel(s)
1. ____Oui ___ Non ___ NR ___ NA	1. ____Oui ___ Non ___ NR ___

Age au moment du diagnostic: _____ ans 2. ____Oui ___ Non ___ NR ___ NA Age au moment du diagnostic: _____ ans 3. ____Oui ___ Non ___ NR ___ NA Age au moment du diagnostic: _____ ans Tante(s):maternel(s) ou paternel(s) 1. ____Oui ___ Non ___ NR ___ NA Age au moment du diagnostic: _____ ans 2. ____Oui ___ Non ___ NR ___ NA Age au moment du diagnostic: _____ ans 3. ____Oui ___ Non ___ NR ___ NA Age au moment du diagnostic: _____ ans Premier(s) cousin(s) : maternel(s) ou paternel(s) 1. ____Oui ___ Non ___ NR ___ NA Age au moment du diagnostic: _____ ans 2. ____Oui ___ Non ___ NR ___ NA Age au moment du diagnostic: _____ ans 3. ____Oui ___ Non ___ NR ___ NA Age au moment du diagnostic: _____ ans	NA Age au moment du diagnostic: _____ ans 2. ____Oui ___ Non ___ NR ___ NA Age au moment du diagnostic: _____ ans 3. ____Oui ___ Non ___ NR ___ NA Age au moment du diagnostic: _____ ans Tante(s): maternel(s) ou paternel(s) 1. ____Oui ___ Non ___ NR ___ NA Age au moment du diagnostic: _____ ans 2. ____Oui ___ Non ___ NR ___ NA Age au moment du diagnostic: _____ ans 3. ____Oui ___ Non ___ NR ___ NA Age au moment du diagnostic: _____ ans Premier(s) cousin(s) : maternel(s) ou paternel(s) 1. ____Oui ___ Non ___ NR ___ NA Age au moment du diagnostic:

Frère(s)/sœur(s) 1. ____Oui ___ Non ___ NR ___ NA Age au moment du diagnostic: _____ ans 2. ____Oui ___ Non ___ NR ___ NA Age au moment du diagnostic: _____ ans 3. ____Oui ___ Non ___ NR ___ NA Age au moment du diagnostic: _____ ans	_____ ans 2. ____Oui ___ Non ___ NR ___ NA Age au moment du diagnostic: _____ ans 3. ____Oui ___ Non ___ NR ___ NA Age au moment du diagnostic: _____ ans Frère(s)/sœur(s) 1. ____Oui ___ Non ___ NR ___ NA Age au moment du diagnostic: _____ ans 2. ____Oui ___ Non ___ NR ___ NA Age au moment du diagnostic: _____ ans 3. ____Oui ___ Non ___ NR ___ NA Age au moment du diagnostic: _____ ans

C. Les questions suivantes portent sur les maladies affectant ou ayant affecté certains membres de la famille. Lorsque nécessaire, veuillez cocher les cases correspondantes. (NA = ne s'applique pas, NR = ne souhaite pas répondre ou ne connaît pas la réponse)

Asthme	Oui ☐ Non ☐ NR ☐	Quel(s) membre (s):_____
Arthrite rhumatoïde	Oui ☐ Non ☐ NR ☐	Quel(s) membre (s):_____
Scléroses multiples	Oui ☐ Non ☐ NR ☐	Quel(s) membre (s):_____
Diabète	Oui ☐ Non ☐ NR ☐	Quel(s) membre (s):_____
Lupus Erythémateux aigu	Oui ☐ Non ☐ NR ☐	Quel(s) membre (s):_____
Colon irritable	Oui ☐ Non ☐ NR ☐	Quel(s) membre (s):_____
Infection Hélicobacter Pylori	Oui ☐ Non ☐ NR ☐	Quel(s) membre (s):_____
Autre(s) (spécifiez):	Oui ☐ Non ☐ NR ☐	Quel(s) membre (s):_____

D. GROUPE ETHNIQUE

Auquel des groupes suivants appartenez-vous ? (il s'agit ici de la provenance du sujet-cas ou sujet témoin). Si plus d'un groupe, sélectionnez les groupes pertinents.

Blanc	☐
Noir, Africain Américain	☐
Amérindien ou natif d'Alaska	☐
Asiatique Indien	☐
Japonais	☐
Natif d'Hawaii	☐
Chinois	☐
Coréen	☐
Guamanien ou Chamorro	☐
Philippin	☐
Vietnamien	☐
Samoan	☐
Autre Asiatique (préciser): _____	☐
Autre insulaire du Pacifique (préciser): _____	☐
Autre (préciser) : _____	☐
NR	☐

Ce questionnaire est maintenant terminé, nous vous remercions pour le temps que vous y avez consacré.

Appendix 2

List of selected tag-SNPs in *ABCB1/MDR1* gene

Gene Name: ABCB1

Gene ID: 5243

Chromosome 7: 86970884 - 87180500 (-)

Genes in this region: ABCB1 RUNDC3B

Allele Frequency Cutoff (%): 10, monomorphic sites included

R2 Threshold for Clusters: 0.8

Minimal Genotype Coverage (%) of Snps to Be TagSnps: 85

Minimal Genotype Coverage (%) of Snps to Be Clustered: 80

Data Merging: common samples with combined variations

Population: AFD_EUR_PANEL, Submitter: PERLEGEN

Bin	Total Number of Sites	Average Minor Allele Frequency	Tag SNPs	Other SNPs
1	14	47 %	rs2235046 rs10276036	rs1202167 rs1202168 rs1202170 rs2235013 rs2520464 rs3789244 rs4148738 rs6949448 rs6961665 rs6969155 rs10808072 rs12539098
2	9	13 %	rs1882479 rs2032583	

Bin	Total Number of Sites	Average Minor Allele Frequency	Tag SNPs	Other SNPs
			rs2235067 rs4148739 rs4148740 rs10276603 rs10280101 rs11983225 rs12720067	
3	8	28 %	rs1202171 rs1202172 rs1202179 rs1202181 rs1202182 rs1202185 rs1202186 rs1989830	
4	6	31 %	rs2091766	rs956825 rs1922240 rs4148735 rs4148736 rs4148737
5	3	21 %	rs6950978 rs10256836 rs10259849	
6	2	38 %	rs1045642	

Bin	Total Number of Sites	Average Minor Allele Frequency	Tag SNPs	Other SNPs
			rs2235048	
7	2	15 %	rs4148733 rs17327442	
8	2	21 %	rs7787082 rs10248420	
9	1	48 %	rs1128503	
10	1	48 %	rs1202184	
11	1	33 %	rs2235035	
12	1	46 %	rs3789243	
13	1	29 %	rs10264990	
14	1	25 %	rs17327624	

Legend:

Variation Color code

splice-site

coding-nonsynonymous

coding-synonymous

coding

mrna-utr

SNPs that were included in panel to genotype are underlined

Appendix 3

List of selected tag-SNPs in *NR3C1/GR* gene

Gene Name: NR3C1

Gene ID: 2908

Chromosome 5: 142637689 - 142795270 (-)

Total chromosome span: 142635689 - 142797270

Allele Frequency Cutoff (%): 10, monomorphic sites included

R2 Threshold for Clusters: 0.8

Minimal Genotype Coverage (%) of Snps to Be TagSnps: 85

Minimal Genotype Coverage (%) of Snps to Be Clustered: 80

Data Merging: common samples with combined variations

Population: AFD_EUR_PANEL, Submitter: PERLEGEN

Bin	Total Number of Sites	Average Minor Allele Frequency	Tag SNPs	Other SNPs
1	12	21 %	rs2963156 rs4986593 rs9324916 rs10482634 rs10482655 rs11750172 rs17209237 rs17209251 rs17209258 rs17339455 rs17399352	rs11745958
2	11	18 %	rs6196 rs33389 rs258748	

Bin	Total Number of Sites	Average Minor Allele Frequency	Tag SNPs	Other SNPs
			rs1438732 rs2918415 rs2918418 rs2918419 rs2963151 rs2963154 rs9324918 rs10515522	
3	10	34 %	rs190488 rs258750 rs258813 rs852977 rs852982 rs860457 rs1866388 rs2918416 rs2918417 rs10052957	
4	8	16 %	rs4128428 rs10482633 rs10482642 rs10482689 rs10515521	

Bin	Total Number of Sites	Average Minor Allele Frequency	Tag SNPs	Other SNPs
			rs11740792 rs17287758 rs17339831	
5	7	45 %	rs33383 rs33388 rs258747 rs852980 rs4634384 rs6877893 rs10041520	
6	5	27 %	rs4912911	rs4912910 rs12655166 rs12054797 rs17100289
7	3	14 %	rs10482616 rs10482672 rs17100236	
8	2	20 %	rs4244032 rs13182800	
9	1	30 %	rs2963155	
10	1	45 %	rs4607376	
11	1	23 %	rs4912905	

Bin	Total Number of Sites	Average Minor Allele Frequency	Tag SNPs	Other SNPs
12	1	29 %	rs7701443	
13	1	32 %	rs9324924	
14	1	37 %	rs10482682	

Legend:

Variation Color code

splice-site

coding-nonsynonymous

coding-synonymous

coding

mrna-utr

SNPs that were included in panel to genotype are underlined

Appendix 4

Study power estimation for article 1

Model # 1
Outcome: Disease
Design: Unmatched case-control (1:1)
Hypothesis: Environment only
Sample size: 180 cases, 1 control(s) per case are required
Significance: 0.050000, 2-sided
Binary environmental factor
Prevalence: 0.1000
Disease model Summary parameters
P0 0.000100 *kP 0.000100
RE: 1.0000 (*indicates calculated value)

Parameter	Null	Full	Reduced
Environment	bE=0	bE	----

Power		
RE	Environment	kP
1.0000	0.0500	0.000100
1.1000	0.0588	0.000101
1.2000	0.0837	0.000102
1.3000	0.1232	0.000103
1.4000	0.1756	0.000104
1.5000	0.2388	0.000105
1.6000	0.3101	0.000106
1.7000	0.3862	0.000107

1.8000	0.4639	0.000108
1.9000	0.5400	0.000109
2.0000	0.6121	0.000110
2.1000	0.6782	0.000111
2.2000	0.7372	0.000112
2.3000	0.7885	0.000113
2.4000	0.8321	0.000114
2.5000	0.8684	0.000115

Model # 2
Outcome: Disease
Design: Unmatched case-control (1:1)
Hypothesis: Environment only
Sample size: 180 cases, 1 control(s) per case are required
Significance: 0.050000, 2-sided
Binary environmental factor
Prevalence: 0.1500
Disease model Summary parameters
P0 0.000100 *kP 0.000100
RE: 1.0000 (*indicates calculated value)

Parameter	Null	Full	Reduced
Environment	bE=0	bE	----

Power

RE	Environment	kP
1.0000	0.0500	0.000100
1.1000	0.0624	0.000101
1.2000	0.0976	0.000103
1.3000	0.1532	0.000104
1.4000	0.2260	0.000106
1.5000	0.3118	0.000107
1.6000	0.4048	0.000109
1.7000	0.4992	0.000110
1.8000	0.5896	0.000112
1.9000	0.6722	0.000113
2.0000	0.7442	0.000115
2.1000	0.8048	0.000116
2.2000	0.8540	0.000118
2.3000	0.8928	0.000119
2.4000	0.9226	0.000121
2.5000	0.9450	0.000122

Model # 3
Outcome: Disease
Design: Unmatched case-control (1:1)
Hypothesis: Environment only
Sample size: 180 cases, 1 control(s) per case are required
Significance: 0.050000, 2-sided
Binary environmental factor
Prevalence: 0.2000
Disease model Summary parameters

P0 0.000100 *kP 0.000100

RE: 1.0000 (*indicates calculated value)

Parameter	Null	Full	Reduced
Environment	bE=0	bE	----

Power		
RE	Environment	kP
1.0000	0.0500	0.000100
1.1000	0.0655	0.000102
1.2000	0.1095	0.000104
1.3000	0.1784	0.000106
1.4000	0.2675	0.000108
1.5000	0.3696	0.000110
1.6000	0.4762	0.000112
1.7000	0.5795	0.000114
1.8000	0.6731	0.000116
1.9000	0.7535	0.000118
2.0000	0.8191	0.000120
2.1000	0.8706	0.000122
2.2000	0.9095	0.000124
2.3000	0.9379	0.000126
2.4000	0.9582	0.000128
2.5000	0.9723	0.000130

Model # 4

Outcome: Disease

Design: Unmatched case-control (1:1)

Hypothesis: Environment only

Sample size: 180 cases, 1 control(s) per case are required

Significance: 0.050000, 2-sided

Binary environmental factor

Prevalence: 0.2500

Disease model Summary parameters

P0 0.000100 *kP 0.000100

RE: 1.0000 (*indicates calculated value)

Parameter	Null	Full	Reduced
Environment	bE=0	bE	----

	Power		
RE	Environment	kP	
1.0000	0.0500	0.000100	
1.1000	0.0682	0.000102	
1.2000	0.1193	0.000105	
1.3000	0.1990	0.000107	
1.4000	0.3003	0.000110	
1.5000	0.4137	0.000112	
1.6000	0.5282	0.000115	

1.7000	0.6349	0.000117
1.8000	0.7274	0.000120
1.9000	0.8030	0.000122
2.0000	0.8618	0.000125
2.1000	0.9056	0.000127
2.2000	0.9369	0.000130
2.3000	0.9587	0.000132
2.4000	0.9735	0.000135
2.5000	0.9832	0.000137

Model # 5

Outcome: Disease

Design: Unmatched case-control (1:1)

Hypothesis: Environment only

Sample size: 180 cases, 1 control(s) per case are required

Significance: 0.050000, 2-sided

Binary environmental factor

Prevalence: 0.3000

Disease model Summary parameters

P0 0.000100 *kP 0.000100

RE: 1.0000 (*indicates calculated value)

Parameter	Null	Full	Reduced
Environment	bE=0	bE	----

	Power	
RE	Environment	kP
1.0000	0.0500	0.000100
1.1000	0.0703	0.000103
1.2000	0.1271	0.000106
1.3000	0.2150	0.000109
1.4000	0.3251	0.000112
1.5000	0.4457	0.000115
1.6000	0.5645	0.000118
1.7000	0.6716	0.000121
1.8000	0.7616	0.000124
1.9000	0.8326	0.000127
2.0000	0.8859	0.000130
2.1000	0.9243	0.000133
2.2000	0.9508	0.000136
2.3000	0.9687	0.000139
2.4000	0.9804	0.000142
2.5000	0.9878	0.000145

Model # 6

Outcome: Disease

Design: Unmatched case-control (1:1)

Hypothesis: Environment only

Sample size: 180 cases, 1 control(s) per case are required

Significance: 0.050000, 2-sided

Binary environmental factor

Prevalence: 0.3500

Disease model Summary parameters

P0 0.000100 *kP 0.000100

RE: 1.0000 (*indicates calculated value)

Parameter	Null	Full	Reduced
Environment	bE=0	bE	----

Power		
RE	Environment	kP
1.0000	0.0500	0.000100
1.1000	0.0719	0.000103
1.2000	0.1329	0.000107
1.3000	0.2265	0.000110
1.4000	0.3425	0.000114
1.5000	0.4673	0.000117
1.6000	0.5877	0.000121
1.7000	0.6942	0.000124
1.8000	0.7815	0.000128
1.9000	0.8490	0.000131
2.0000	0.8986	0.000135
2.1000	0.9336	0.000138
2.2000	0.9574	0.000142
2.3000	0.9731	0.000145
2.4000	0.9833	0.000149

2.5000	0.9897	0.000152

Model # 7

Outcome: Disease

Design: Unmatched case-control (1:1)

Hypothesis: Environment only

Sample size: 180 cases, 1 control(s) per case are required

Significance: 0.050000, 2-sided

Binary environmental factor

Prevalence: 0.4000

Disease model Summary parameters

P0 0.000100 *kP 0.000100

RE: 1.0000 (*indicates calculated value)

Parameter	Null	Full	Reduced
Environment	bE=0	bE	----

	Power	
RE	Environment	kP
1.0000	0.0500	0.000100
1.1000	0.0730	0.000104
1.2000	0.1368	0.000108
1.3000	0.2339	0.000112
1.4000	0.3530	0.000116

1.5000	0.4796	0.000120
1.6000	0.6002	0.000124
1.7000	0.7054	0.000128
1.8000	0.7907	0.000132
1.9000	0.8560	0.000136
2.0000	0.9036	0.000140
2.1000	0.9369	0.000144
2.2000	0.9594	0.000148
2.3000	0.9743	0.000152
2.4000	0.9840	0.000156
2.5000	0.9901	0.000160

Legend:

K_p	Overall disease risk in the general population
P_0	*Baseline disease risk*: disease risk in unexposed (E=0 or Z=0) genetically normal (G=0, H=0) subjects
R_e	*Environmental relative risk (or odds ratio)*: Risk relative to P_0 when E=1 or Z=1, in genetically normal (G=0) subjects
R_g	*Genetic relative risk (or odds ratio)*: Risk relative to P_0 when G=1, in environmentally unexposed (E=0 or Z=0) subjects

Appendix 5

Study power estimation for articles 2 and 3

Model # 1

Outcome: Disease

Design: Unmatched case-control (1:1)

Hypothesis: Gene only

Sample size: 150 cases, 1 control(s) per case are required

Significance: 0.050000, 2-sided

Gene

Mode of inheritance: Log-additive

Allele frequency: 0.1000 to 0.5000 by 0.0500

Disease model Summary parameters

P0 0.400000 *kP 0.400000

RG: 1.0000 (*indicates calculated value)

Parameter	Null	Full	Reduced
Gene	bG=0	bG	----

		Power	
Frequency	RG	Gene	kP
0.100000	1.0000	0.0500	0.400000
	1.1000	0.0643	0.404619
	1.2000	0.1033	0.408898
	1.3000	0.1625	0.412869
	1.4000	0.2369	0.416561
	1.5000	0.3209	0.420000
	1.6000	0.4085	0.423209
	1.7000	0.4947	0.426208
	1.8000	0.5755	0.429017
	1.9000	0.6484	0.431653
	2.0000	0.7123	0.434130
	2.1000	0.7668	0.436462

	2.2000	0.8124	0.438661
	2.3000	0.8501	0.440738
	2.4000	0.8807	0.442703
	2.5000	0.9054	0.444565
0.150000	1.0000	0.0500	0.400000
	1.1000	0.0702	0.406931
	1.2000	0.1259	0.413354
	1.3000	0.2102	0.419313
	1.4000	0.3138	0.424849
	1.5000	0.4259	0.430000
	1.6000	0.5357	0.434800
	1.7000	0.6357	0.439281
	1.8000	0.7213	0.443471
	1.9000	0.7913	0.447395
	2.0000	0.8463	0.451078
	2.1000	0.8884	0.454539
	2.2000	0.9198	0.457798
	2.3000	0.9428	0.460872
	2.4000	0.9595	0.463774
	2.5000	0.9714	0.466520
0.200000	1.0000	0.0500	0.400000
	1.1000	0.0754	0.409244
	1.2000	0.1457	0.417814
	1.3000	0.2512	0.425763
	1.4000	0.3776	0.433142
	1.5000	0.5083	0.440000
	1.6000	0.6288	0.446383
	1.7000	0.7306	0.452333
	1.8000	0.8108	0.457887
	1.9000	0.8707	0.463082
	2.0000	0.9134	0.467948
	2.1000	0.9430	0.472514
	2.2000	0.9629	0.476807

	2.3000	0.9761	0.480848
	2.4000	0.9847	0.484659
	2.5000	0.9902	0.488258
0.250000	1.0000	0.0500	0.400000
	1.1000	0.0798	0.411560
	1.2000	0.1624	0.422279
	1.3000	0.2853	0.432218
	1.4000	0.4289	0.441439
	1.5000	0.5712	0.450000
	1.6000	0.6954	0.457957
	1.7000	0.7936	0.465363
	1.8000	0.8655	0.472267
	1.9000	0.9150	0.478712
	2.0000	0.9476	0.484740
	2.1000	0.9683	0.490387
	2.2000	0.9811	0.495686
	2.3000	0.9888	0.500667
	2.4000	0.9934	0.505356
	2.5000	0.9962	0.509778
0.300000	1.0000	0.0500	0.400000
	1.1000	0.0834	0.413877
	1.2000	0.1760	0.426748
	1.3000	0.3127	0.438680
	1.4000	0.4688	0.449741
	1.5000	0.6179	0.460000
	1.6000	0.7421	0.469523
	1.7000	0.8350	0.478373
	1.8000	0.8990	0.486610
	1.9000	0.9403	0.494287
	2.0000	0.9656	0.501455
	2.1000	0.9806	0.508157
	2.2000	0.9892	0.514436
	2.3000	0.9941	0.520328

	2.4000	0.9968	0.525866
	2.5000	0.9982	0.531081
0.350000	1.0000	0.0500	0.400000
	1.1000	0.0862	0.416196
	1.2000	0.1866	0.431222
	1.3000	0.3336	0.445148
	1.4000	0.4984	0.458048
	1.5000	0.6513	0.470000
	1.6000	0.7740	0.481080
	1.7000	0.8619	0.491362
	1.8000	0.9196	0.500916
	1.9000	0.9549	0.509806
	2.0000	0.9754	0.518091
	2.1000	0.9869	0.525825
	2.2000	0.9931	0.533058
	2.3000	0.9964	0.539833
	2.4000	0.9982	0.546190
	2.5000	0.9991	0.552165
0.400000	1.0000	0.0500	0.400000
	1.1000	0.0882	0.418516
	1.2000	0.1941	0.435701
	1.3000	0.3483	0.451622
	1.4000	0.5188	0.466360
	1.5000	0.6737	0.480000
	1.6000	0.7947	0.492629
	1.7000	0.8787	0.504330
	1.8000	0.9319	0.515185
	1.9000	0.9633	0.525269
	2.0000	0.9808	0.534649
	2.1000	0.9902	0.543391
	2.2000	0.9951	0.551551
	2.3000	0.9976	0.559180
	2.4000	0.9988	0.566327

	2.5000	0.9994	0.573032
0.450000	1.0000	0.0500	0.400000
	1.1000	0.0894	0.420838
	1.2000	0.1985	0.440184
	1.3000	0.3569	0.458102
	1.4000	0.5306	0.474676
	1.5000	0.6865	0.490000
	1.6000	0.8064	0.504169
	1.7000	0.8880	0.517277
	1.8000	0.9386	0.529418
	1.9000	0.9678	0.540675
	2.0000	0.9836	0.551130
	2.1000	0.9919	0.560854
	2.2000	0.9960	0.569914
	2.3000	0.9981	0.578370
	2.4000	0.9991	0.586277
	2.5000	0.9996	0.593681
0.500000	1.0000	0.0500	0.400000
	1.1000	0.0898	0.423162
	1.2000	0.1999	0.444671
	1.3000	0.3595	0.464588
	1.4000	0.5343	0.482998
	1.5000	0.6907	0.500000
	1.6000	0.8103	0.515700
	1.7000	0.8913	0.530204
	1.8000	0.9411	0.543613
	1.9000	0.9694	0.556026
	2.0000	0.9847	0.567532
	2.1000	0.9925	0.578215
	2.2000	0.9964	0.588149
	2.3000	0.9983	0.597403
	2.4000	0.9992	0.606039
	2.5000	0.9996	0.614113

Legend:

K_p	Overall disease risk in the general population
P_0	*Baseline disease risk*: disease risk in unexposed (E=0 or Z=0) genetically normal (G=0, H=0) subjects
R_e	*Environmental relative risk (or odds ratio)*: Risk relative to P_0 when E=1 or Z=1, in genetically normal (G=0) subjects
R_g	*Genetic relative risk (or odds ratio)*: Risk relative to P_0 when G=1, in environmentally unexposed (E=0 or Z=0) subjects
R_{ge}	*Interaction effect (Relative-risk ratio, or Odds-ratio ratio)*: Risk relative to to P_0 when G=1 and E=1 (or G=1 and Z=1), divided by the product $R_e \times R_g$
${}_eR$	*Marginal environmental relative risk (or odds ratio)*: Risk relative to P_0 when E=1 or Z=1, irrespective of genetic status
${}_gR$	*Marginal genetic relative risk (or odds ratio)*: Risk relative to P_0 when G=1, irrespective of environmental exposure status