UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE BIOLOGIA ANIMAL

PHARMACOGENETICS AND NUTRIGENETICS IN CROHN'S DISEASE

PAULA SOUSA ALVES FERREIRA

DOUTORAMENTO EM BIOLOGIA
(GENÉTICA)

2014

UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE BIOLOGIA ANIMAL

PHARMACOGENETICS AND NUTRIGENETICS IN CROHN'S DISEASE

PAULA SOUSA ALVES FERREIRA

Tese orientada pelo Professor Doutor Rui Miguel Brito e pela Professora Doutora Maria Manuela Coelho, especialmente elaborada para a obtenção do grau de doutor em Biologia (Genética)

2014

A presente tese foi elaborada com o apoio financeiro da Fundação para a Ciência e Tecnologia pela atribuição da Bolsa de doutoramento com a referência SFRH/BD/64172/2009.

Este estudo foi apoiado por uma bolsa do Grupo de Estudos da Doença Inflamatória Intestinal (2009-2012).

A dissertação deve ser citada como:
Ferreira, P., 2014. Pharmacogenetics and Nutrigenetics in Crohn's Disease. Ph.D. Thesis. Faculty of Sciences. University of Lisbon. Portugal.

The present thesis was conducted with the financial support of the Fundação para a Ciência e Tecnologia through the attribution of a PhD grant with the reference SFRH/BD/64172/2009.

This study was supported by a grant from the Grupo de Estudos da Doença Inflamatória Intestinal (2009-2012).

The dissertation should be cited as:
Ferreira, P., 2014. Pharmacogenetics and Nutrigenetics in Crohn's Disease. Ph.D. Thesis. Faculty of Sciences. University of Lisbon. Portugal.

NOTA PRÉVIA

De acordo com o previsto no nº 1 do artigo 45º do Regulamento de Estudos Pós-Graduados da Universidade de Lisboa, publicado no Diário da República, 2.ª série, n.º 65, de 30 de março de 2012, a candidata esclarece que na elaboração da presente tese foram utilizados integralmente artigos científicos já publicados (3) ou submetidos para publicação (1) em revistas indexadas de circulação internacional, os quais integram os Capítulos II e III da presente tese. Tendo os referidos trabalhos sido realizados em colaboração, a candidata esclarece que participou integralmente no planeamento, obtenção dos dados, análise e discussão dos resultados, bem como na redação de todos os artigos científicos.

A tese, por ser uma compilação de publicações internacionais, está redigida em Inglês. Esclarece-se ainda que a formatação dos vários artigos que integram a presente dissertação obedece às regras das revistas em que foram publicados ou submetidos para publicação. Por este motivo, não foi possível adotar um critério uniforme ao longo dos vários capítulos.

Lisboa, dezembro 2014
Paula Sousa Alves Ferreira

PRELIMINARY NOTE

According to Article 45 nr.1 of the Post-graduate Studies Regulation (Diário da República, 2ªsérie, nº65, 30 Março 2012) this dissertation includes papers published (3) or submitted for publication (1) in international scientific journals, which are comprised in Chapters II and III. The candidate, as co-author, states that was involved in the scientific planning, sampling design, data collection, statistical analysis and writing of all manuscripts.

This dissertation, being composed of a series of international publications, is written in English and obeys the formatting rules of the journals were the articles were published or submitted for publishing. For this reason, it wasn't possible to adopt a uniform criterion along the different chapters.

Lisbon, December 2014
Paula Sousa Alves Ferreira

“A ambição da ciência não é abrir a porta do saber infinito,
mas pôr um limite ao erro infinito”
Bertolt Brecht

AGRADECIMENTOS

Em 2006, estava eu no meu último ano de licenciatura a iniciar o último semestre que consistia num trabalho de investigação, quando tive contacto com as diferentes oportunidades de estágio para escolher. De entre todas, houve uma que mais me chamou a atenção e que ia de encontro aos meus interesses e objetivos e, portanto, decidi arriscar e questionar a possibilidade de preencher a vaga de estágio. Mão do destino ou fortuito do acaso, o facto é que volvidos 8 anos chega ao fim um longo percurso construído por um conjunto de projetos que conduziram aos resultados finais compilados aqui nesta tese de doutoramento.

Foi um caminho trabalhoso, com alguns obstáculos, mas que permitiu o teste das minhas capacidades e vontade de vencer. Permitiu também o crescimento pessoal e profissional, a evolução do conhecimento, a presença em congressos internacionais, a escrita de artigos científicos publicados em revistas internacionais e nacionais de renome, a experiência da docência e o conhecimento de pessoas especiais que tornaram este caminho menos sinuoso e mais prazeroso, às quais na generalidade agradeço. Particularizando, gostaria de agradecer:

- Ao Professor Doutor Miguel Brito, que no ano de 2006 me recebeu de braços abertos e me permitiu integrar o seu grupo de investigação na ESTeSL, sem nenhuma referência sobre a minha pessoa ou trabalho. Foi um voto de confiança que espero ter correspondido ao longo de todos estes anos, cumprindo todos os objetivos que me foram sendo propostos e levando a bom porto este enorme projeto que foi o projeto Crohn. Muitos foram os momentos pessoais e profissionais partilhados que permitiram enriquecer toda esta experiência de aprendizagem e convívio.

- À Professora Doutora Maria Manuela Coelho da Faculdade de Ciências da Universidade de Lisboa, pela gentileza de me ter recebido como sua aluna de doutoramento e por ter facilitado a minha ligação à Faculdade de Ciências. Saliento todas as suas palavras de incentivo aquando das apresentações periódicas do meu trabalho, decorrentes da cadeira de Seminário integrado no plano de estudos de formação avançada, e todo o seu cuidado no apoio à finalização da tese de doutoramento.

- À Professora Doutora Marília Cravo, médica gastroenterologista responsável por todo o projeto das Doenças Inflamatórias Intestinais, pela coordenação entre os diferentes hospitais incluídos no estudo e o nosso laboratório de investigação, pelo acompanhamento de todo o trabalho e pela perseverança no cumprimento de todos os objetivos propostos que possibilitaram a escrita de artigos científicos e presença em congressos internacionais.

- À Professora Doutora Isabel Marques Carreira e a todas as pessoas do Laboratório de Citogenética e Genómica da Faculdade de Medicina da Universidade de Coimbra que dirige, pela possibilidade e ótimo acolhimento durante o período em que pude desenvolver um pequeno estágio neste

laboratório, permitindo novos conhecimentos em temáticas complementares ao meu trabalho de investigação.

- À Fundação para a Ciência e Tecnologia pela atribuição de uma bolsa de doutoramento que permitiu o desenvolvimento de um trabalho de investigação e consequente tese de doutoramento, conduzindo à realização de um objetivo pessoal e profissional.

- À Professora Doutora Catarina Sousa Guerreiro, com a qual trabalhei de perto para o cumprimento de alguns dos objetivos de trabalho propostos, nomeadamente os relacionados com toda a componente nutricional deste projeto, assim como na escrita de artigos científicos publicados em revistas internacionais e nacionais.

- A todos os colegas do Laboratório de Genética Humana da ESTeSL que me receberam muito bem e tornaram esta jornada de trabalho mais fácil e apoiada, nomeadamente Ana Moleirinho, Carina Ladeira, João Lourenço, Joana Malta Vacas e Susana Gonçalves. Um agradecimento especial para aqueles que se tornaram mais próximos, garantiram a sanidade mental e bons momentos de diversão, e que de certeza permanecerão para a vida, Alice Melão, Bruno Carmona, Carla Mota e Ana Oliveira.

- A todos os amigos de uma vida inteira que me acompanham em todos os momentos desde que me lembro de ser alguém, e que permitem que exista um equilíbrio salutar entre os compromissos profissionais e a vida pessoal.

- À minha família, pelo apoio estrutural e a possibilidade da realização de todos os meus objetivos pessoais e profissionais.

- Ao João, que me acompanha desde antes do início desta etapa, e que permitiu que todos os impossíveis se tornassem possíveis e que todos os obstáculos parecessem fáceis de ultrapassar. Hoje, já meu marido, é o lado otimista da vida, o meu equilíbrio para conseguir realizar todos os meus objetivos e o cúmplice no meu projeto de vida.

RESUMO

A doença de Crohn, de etiologia desconhecida, caracteriza-se por uma resposta inflamatória crónica que envolve todo o trato gastrointestinal, uma elevada heterogeneidade clínica e um forte envolvimento de fatores genéticos. Atualmente, ainda não existe nenhuma cura disponível e é sabido que os doentes de Crohn, ao longo da sua vida, vão alternando entre períodos de doença ativa e períodos de remissão. Trata-se de uma doença que ocorre principalmente entre os 20-30 anos e apresenta uma maior incidência em países industrializados, o que parece sugerir a importância de fatores ambientais na sua patogénese, onde a dieta tem sido apontada como um fator possível.

A doença de Crohn encontra-se vulgarmente associada a má nutrição e perda de peso resultantes da redução da ingestão de alimentos, hábitos alimentares incorretos, desenvolvimento do processo inflamatório característico desta doença e efeitos secundários derivados das múltiplas terapias aplicadas no tratamento da doença. Vários estudos têm sido realizados com o intuito de identificar quais os alimentos responsáveis pelo agravamento dos sintomas observados na doença de Crohn. Os principais macronutrientes prejudiciais a este tipo de doentes são os açúcares e as gorduras, principalmente as saturadas, polinsaturadas e trans. Vulgarmente, são também observadas deficiências em micronutrientes como o ferro, magnésio, zinco, cálcio e vitaminas A, D, K, B6, ácido fólico, cobalamina e ácido ascórbico. Como principais recomendações, os doentes de Crohn devem ter uma dieta rica em ácidos gordos, principalmente ómega 3, fruta, vegetais e proteínas, nomeadamente carnes magras e ovos. A importância da definição de hábitos alimentares específicos a cada doente reside nas premissas que os nutrientes conseguem influenciar direta ou indiretamente a expressão de genes e os efeitos da dieta no desenvolvimento da doença dependem do perfil de suscetibilidade genética individual. Desta forma, é possível a identificação e caracterização de polimorfismos genéticos responsáveis pela alteração do metabolismo dos nutrientes, permitindo deste modo a personalização da dieta a cada doente de Crohn com base no seu perfil genético.

Atualmente, a doença de Crohn define-se como uma doença crónica resultante da interação entre fatores clínicos, genéticos e ambientais, apresentando na Europa uma taxa de mortalidade de aproximadamente 40%. Em conjunto com a Colite Ulcerosa, constitui o grupo das Doenças Inflamatórias Intestinais. A Colite Ulcerosa é também uma doença inflamatória crónica caracterizada por um fenótipo específico, predisposição genética, fatores ambientais e resposta imunitária não controlada ao microbioma intestinal.

Com a utilização de estudos de associações genéticas foi possível a identificação de genes de suscetibilidade ao desenvolvimento da doença de Crohn. Um dos primeiros e mais importantes genes de suscetibilidade associados à doença de Crohn é o gene do núcleo de oligomerização de domínio 2 (*NOD2*), já anteriormente descrito como um gene significativamente associado ao risco de desenvolvimento da doença de Crohn. Outros genes foram posteriormente identificados e encontram-se relacionados com o processo inflamatório, nomeadamente citocinas pró-inflamatórias como interferão gama (IFNγ), fator de necrose tumoral alfa (TNFα), interleucina 1, 6, 12 e 23 e citocinas anti-inflamatórias como o antagonista do recetor da interleucina 1. Foram também identificados diferentes genes apoptóticos como o Fas, ligando do Fas e caspase 9, em consequência da persistente não resposta à apoptose usualmente observada em diferentes tipos de células nos vários

locais de inflamação intestinal, que é característica da doença de Crohn. Recentemente, grande atenção tem sido dada à investigação de genes autofágicos, nomeadamente os genes *ATG16L1* e *IRGM*, e ao gene de resistência a drogas (*MDR1*), dada a sua associação à patogénese da doença de Crohn.

Atualmente, a terapia aplicada aos doentes de Crohn envolve o tratamento com aminosalicilatos, corticosteroides, imunossupressores e terapia biológica. As recomendações para a terapêutica a aplicar aos doentes de Crohn aconselham o início do tratamento com mesalamina e corticosteroides, seguindo-se a azatioprina e, finalmente, as terapias biológicas com anticorpos monoclonais anti-TNFα em pacientes cuja terapia convencional anteriormente aplicada não tenha sido eficaz.

O tratamento desta doença é complexo devido à severidade dos parâmetros clínicos e à variedade de respostas do doente a cada terapia, e deve por isso contabilizar fatores clínicos como a localização da doença, o comportamento da doença e a agressividade da doença, mas também a conjugação com fatores genéticos que englobem polimorfismos em genes chave envolvidos na resposta inflamatória, na apoptose, na autofagia e no metabolismo e transporte de fármacos.

O grande desafio existente resulta da dificuldade em prever o desenvolvimento da doença ao longo dos anos, o que dificulta a escolha da terapêutica a aplicar e o controlo dos sintomas.

Com o intuito de responder a esta questão, desenvolvemos um conjunto de estudos aqui compilados nesta dissertação que tiveram como objetivo principal identificar fatores clínicos e genéticos preditivos da resposta à terapêutica numa população de doentes de Crohn provenientes de diferentes hospitais do centro de Portugal. Para tal, procedemos à recolha dos dados clínicos dos pacientes, à análise genética através das técnicas de PCR/RFLP e PCR em tempo real de polimorfismos relevantes envolvidos no processo inflamatório, apoptose, autofagia e transporte de drogas, ao estudo dos seus hábitos alimentares e, por fim, à recolha dos dados de resposta à terapêutica aplicada a cada indivíduo. Como objectivos específicos pretendemos: a) estudar as associações entre os genes *MDR1*, *IL23R, ATG16L1*, *Fas*, *FasL*, *Casp9* e os parâmetros clínicos como idade de diagnóstico, localização da doença, comportamento da doença, agressividade da doença, fístulas e manifestações extraintestinais; b) estudar a associação entre os polimorfismos no gene *MDR1* e a resposta à terapia com aminosalicilatos, corticosteroides e imunossupressores; c) estudar a associação entre os polimorfismos nos genes apoptóticos *Fas*, *FasL* e *Casp9* e a resposta à terapêutica biológica; d) estudar a associação entre os polimorfismos no gene *IL23R* e a resposta à terapêutica com corticosteroides, imunossupressores e terapia biológica; e) avaliação global da importância da farmacogenética no tratamento da doença de Crohn e, finalmente, a importância da nutrigenética no tratamento da doença de Crohn.

Estes fatores vão permitir a identificação de parâmetros específicos de forma a que o clínico possa proceder a uma estratificação dos seus pacientes, aplicando uma terapia mais personalizada de acordo com o perfil genético de cada indivíduo.

Como principais resultados referem-se a identificação de polimorfismos genéticos em citocinas como TNFα, LTα, IL1 e IL6 como associados à severidade e desenvolvimento da doença. O consumo elevado de açúcares, lípidos e gorduras saturadas, monoinsaturadas e polinsaturadas foi identificado como fator de risco para uma elevada atividade da doença. Fatores clínicos preditivos da

resposta à terapêutica como a idade, a realização de cirurgia e o envolvimento perianal e fatores genéticos preditivos da resposta à terapêutica como polimorfismos genéticos nos genes *Casp9* e *MDR1* foram também identificados.

Durante a próxima década são esperados desenvolvimentos no conhecimento da terapêutica vulgarmente aplicada para o tratamento da doença de Crohn, o aparecimento de novos alvos terapêuticos e um maior conhecimento dos fatores genéticos que influenciam a resposta à terapia.

O futuro do tratamento da doença de Crohn reside no investimento em estudos nas componentes de farmacogenética e nutrigenética, como os aqui descritos, uma vez que surgem como uma mais valia para a rotina médica diária. Este tipo de estudos vão permitir uma aplicação da terapêutica e dieta nutricional direcionadas a cada paciente, de acordo com um conjunto de recomendações identificadas que possibilitam uma terapia mais personalizada, com uma maior taxa de sucesso e redução de efeitos secundários, permitindo assim um maior controlo da doença na rotina diária dos doentes de Crohn.

Termos-chave: Doença de Crohn, Doenças Inflamatórias Intestinais, Farmacogenética, Nutrigenética, Medicina personalizada.

ABSTRACT

Crohn's disease, a pathology of unknown origin, is characterized by a chronic inflammatory response that involves the entire gastrointestinal tract, a high heterogeneity in phenotype and a strong genetic component. It's a disease with a greater incidence in industrialized countries, what suggests the importance of environmental factors in its pathogenesis, where diet patterns have been pointed out as possible cause.

The treatment of this disease is very complex due to the severity of the clinical parameters and the variety of response to the existing therapies. This emphasizes the importance of the conjugation with genetic factors such as polymorphisms in key genes presented in inflammatory pathways, apoptosis, autophagy and metabolism and drug transportation.

The main existing challenge results from the difficulty in predicting the disease development along the years, which turns the choice of appropriate therapeutic and control of symptoms problematic.

With the purpose of answering to these questions, we have developed several studies, here compiled in this dissertation, that have the main goal of identifying clinical and genetic predictors of response to the normally used therapies that allowed the physicians to stratified their patients in order to apply a more personalized therapeutic based on individuals genetic profile.

As main results emerges the identification of genetic polymorphisms in cytokine genes such as TNFα, LTα, IL1 e IL6 as associated with disease aggressiveness and development. The high consumption of glicids, lipids, saturated, monounsaturated and polyunsaturated fats appears as risk factors to greater disease aggressiveness. Clinical predictors such as patient's age, surgery and perianal involvement and genetic predictors like *Casp9* and *MDR1* gene polymorphisms were associated with response to therapy.

The future for the treatment of Crohn's disease resides in the investment in pharmacogenetics and nutrigenetics studies, such as the studies described here, since they emerge as a benefit to the routinely clinical practice that contributes to a therapeutic and nutritional therapy personalized to each patient and, therefore, a better quality of life.

Keywords: Crohn's Disease, Inflammatory Bowel Disease, Pharmacogenetics, Nutrigenetics, Personalized medicine.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

5'ASA – 5'-aminosalicylic acid.

ADA – Adalimumab.

AZA – Azathioprine.

ATG16L1 - Autophagy-related protein 16-1.

Casp9 - Caspase 9.

CI – Confidence interval.

CD – Crohn's disease.

CDAI – Crohn's disease activity index.

CPZ – Certolizumab pegol.

CS – Corticosteroids.

DME – Drug-metabolizing enzymes.

ECCO - European Crohn's and Colitis Organisation.

EIM – Extraintestinal manifestations.

FasL - Fas Ligand.

FFQ - Food Frequency Questionnaire.

GI – Gastrointestinal tract.

GWA – Genome-wide association studies.

HBI - Harvey-Bradshaw index.

HLA – Human leukocyte antigen.

IBD – Inflammatory bowel disease.

IBD5 – Inflammatory bowel disease 5.

IFNγ – Interferon gamma.

IFX – Infliximab.

IL1 – Interleukin 1.

IL1β – Interleukin 1 beta.

IL1RN – Interleukin 1 receptor antagonist.

IL6 – Interleukin 6.

IL12 – Interleukin 12.

IL17 – Interleukin 17.

IL18 – Interleukin 18.

IL21 – Interleukin 21.

IL23 – Interleukin 23.

IL23R – Interleukin 23 receptor.

IRGM - Immunity-related GTPase family M protein.

ITLN1 – Intelectin 1.

JAK2 - Janus kinase 2.

LTα - Lymphotoxin alpha.

MDR1 – Multidrug resistance gene.

MTX – Methotrexate.

NOD2 – Nucleotide-binding oligomerization domain containg 2.

NSAIDs - Nonsteroidal antiinflammatory drugs.

NTZ – Natalizumab.

ω-3 fatty acids – Omega-3 fatty acids.

ω-6 fatty acids – Omega-6 fatty acids.

OR – Odds ratio.

PCR – Polimerase chain reaction.

PPARγ - Peroxisome proliferator-activated receptor gamma.

RFLP – Restriction fragment length polymorphism.

SNP - Single nucleotide polymorphisms.

TNFα – Tumor necrosis factor alpha.

TLR – Toll like receptor.

UC - Ulcerative colitis.

Vitamin B9 - Folic acid.

Vitamin B12 – Cobalamin.

Vitamin C - L-ascorbic acid.

VNTR - Variable number tandem repeat.

CHAPTER I - INTRODUCTION

I.1 CROHN'S DISEASE

Crohn's disease (CD) is a chronic inflammatory bowel disease (IBD) of unknown origin that is characterized by an uncontrolled inflammatory response in any part of the gastrointestinal tract (GI) that is thought to be related to alterations in the gut microbiome in genetically predisposed individuals (Cottone & Criscuoli 2011). Until now, there is no cure available and patients go through periods of active disease, that may lead to progressive bowel damage and complications as fistulas, abscesses and strictures, followed by periods of relapses and remission (Panaccione *et al*. 2012).

It can vary from mild to severe and the major symptoms embrace abdominal pain, diarrhea, GI bleeding, nausea, weight loss, fever and fatigue. Crohn's disease can also affect other parts of the body like joints, skin, liver and eyes, which are referred as extraintestinal manifestations (EIM).

The diagnosis is currently based on a combination of clinical presentation, endoscopic appearance, surgical findings and radiologic, histologic and serologic criteria (Van Assche *et al.* 2010).

Important assessment tools as a CD activity index (CDAI) and Harvey-Bradshaw index (HBI) exist for CD symptoms evaluation (Papay *et al.* 2013).

The prevalence of CD in Europe varies from 1.522 to 21312 cases per 100 000 individuals (Burisch *et al. 2013).* The incidence of CD is considered variable for different regions and groups of population and has been increasing in recent years. It is commonly higher in developed countries, mainly in North America and Western Europe and more predominant in urban than rural areas and northern than southern areas (Magro *et al*. 2012) (Burisch *et al. 2013).* A study by (Shivananda *et al*. 1996) reported that in Portugal between 1991 and 1993 it was estimated a CD incidence of 2.4 per 100 000 subjects.

It can be developed at any age, but it mainly occurs between ages 20-30 years (Magro *et al.* 2012*).*

For European CD patients, mortality is up to 40% when compared with the general population (Colombel *et al.* 2014*)*

To date, it is known that is a lifelong disease that results from the interaction between clinical, environmental and genetic factors (Van Assche *et al.* 2010).

I.1.1 CLINICAL AND ENVIRONMENTAL FACTORS

Crohn's disease is classified as a heterogeneous disease once it presents a variety of phenotypes in terms of age of onset, disease location and disease behaviour (Van Assche *et al.* 2010) (Louis *et al.* 2011). Due to this heterogeneity, a classification system named Montreal classification was developed based on different phenotypes (Table 1).

Table I.1. Montreal classification of Crohn's Disease. (Adapted from Silverber *et al*., 2005)

Age at diagnosis (A)	A1- ≤ 16 years
	A2– 17-40 years
	A3- > 40 years
Location (L)	L1- Terminal ileum
	L2- Colon
	L3- Ileocolon
	L4- Upper gastrointestinal
Behaviour (B)	B1- Inflammatory/ nonstricturing and nonpenetrating
	B2- Stricturing
	B3- Penetrating

It is also possible to complement disease phenotype analysis based on Montreal classification with disease modifiers of location, namely location on upper gastrointestinal that can be divided into L1+L4, L2+L4 and L3+L4, and disease modifiers on behaviour with perianal disease that can assume B1p, B2p and B3p (Gisbert *et al.* 2008).

Several studies have demonstrated that disease location is the clinical feature most associated with the disease course, with the terminal ileum location strongly associated with a greater risk of stricture and internal penetrating behaviour and with the risk of surgery (Louis *et al.* 2010).

Environmental factors such as smoking habits, diet and drugs are pointed as important parameters in Crohn's disease development, but there aren't enough studies to confirm this for use routinely in clinical practice. It is hypothesised that smoking habits and the use of nonsteroidal antiinflammatory drugs (NSAIDs) lead to a more permeable mucosa of the intestine and that the introduction of antibiotics or occurrence of gastrointestinal infections causes the alteration of bacteria that normally lives in the colon (Parkes *et al.* 2014).

The most studied environmental factor is smoking and has been reported that not only increases the risk to CD, but also aggravates the course of disease (Parkes *et al.* 2014).

I.1.2 IMMUNOLOGY AND GUT MICROBIOME

During intestinal inflammation in CD it has been observed that occurs an improper host immune response to intestinal flora (Allez & Lémann 2009).

Several studies have been suggesting that the pathogenesis of CD is mostly related to genetic, environmental and immunological factors (Chen *et al.* 2014) (Huebner *et al.* 2009). In fact, it has been shown that CD is a Th1-mediated disease characterized by an excessive Th1-cell activity (Hendrickson *et al.* 2002).

It has been suggested that increased intestinal permeability may play a role in CD pathogenesis (Ardizzone & Bianchi 2002).

Regarding pathogenic traits, CD patients present in greater number bacteria with proinflammatory properties like *Escherichia coli* in opposition to a more reduced number of *Faecalibacterium prausnitzii*, which have antiinflammatory properties (Colombel *et al.* 2014).

Recently, the study of underlying inflammatory processes and the appearance of new biological therapies directly targeted to proinflammatory mediators has gained major attention due to its important contribution to the future of CD management (D'Haens *et al.* 2014) (Stappenbeck *et al.* 2014).

Although continuously investigation is on progress on these thematic, the underlying pathogenesis of CD remains unclear.

I.2 GENETIC POLYMORPHISMS

The importance of genetics in CD pathogenesis was demonstrated by strong familial aggregation, twin studies and established genetic associations (Rioux *et al.* 2007). In fact, the data shown in several studies in twins strongly supports the importance of the genetic component in this disease, once it was demonstrated a significant increase in the concordance of CD in monozygotic twins when compared to the existing in dizygotic twins (Peña & Crusius 1998).

The common use of genome-wide associations (GWA) led to the comprehension of the molecular pathways that were determining in CD. These studies allowed the identification of specific polymorphisms responsible for individual's susceptibility to disease and those that can be used as therapeutic targets in the development of more effective and safer treatments for CD patients (Budarf *et al.* 2009) (Cho & Weavet 2007). Identified genes conferring disease susceptibility seem to differ from those responsible for clinical phenotype determination, such as extent and severity of disease, and its response to medical therapy (Ardizzone & Bianchi 2002).

In the recent years, much progress has been achieved and the discovering and confirmation of 163 IBD susceptibility genetic regions by the end of 2012 is a proof of that. The nucleotide-binding oligomerization domain containing 2 gene (*NOD2*) on chromosome 16q12, involved in innate immunity, was the first identified CD risk gene and continues to be the most proven CD susceptibility gene so far, with three variants most usually associated with CD (R702W, G908R and L100fs) (Kabi *et al.* 2012). Up to one third of CD patients carry one of these three allelic variants against 10-15% of the normal population (Cummings & Rubin 2006).

The contribution of CD genetics has gain great importance in changing clinical practice once it may predict the disease phenotype such as disease location, disease behaviour and the occurrence of EIM and the decision of treatment strategies to adopt to each individual (Figure1).

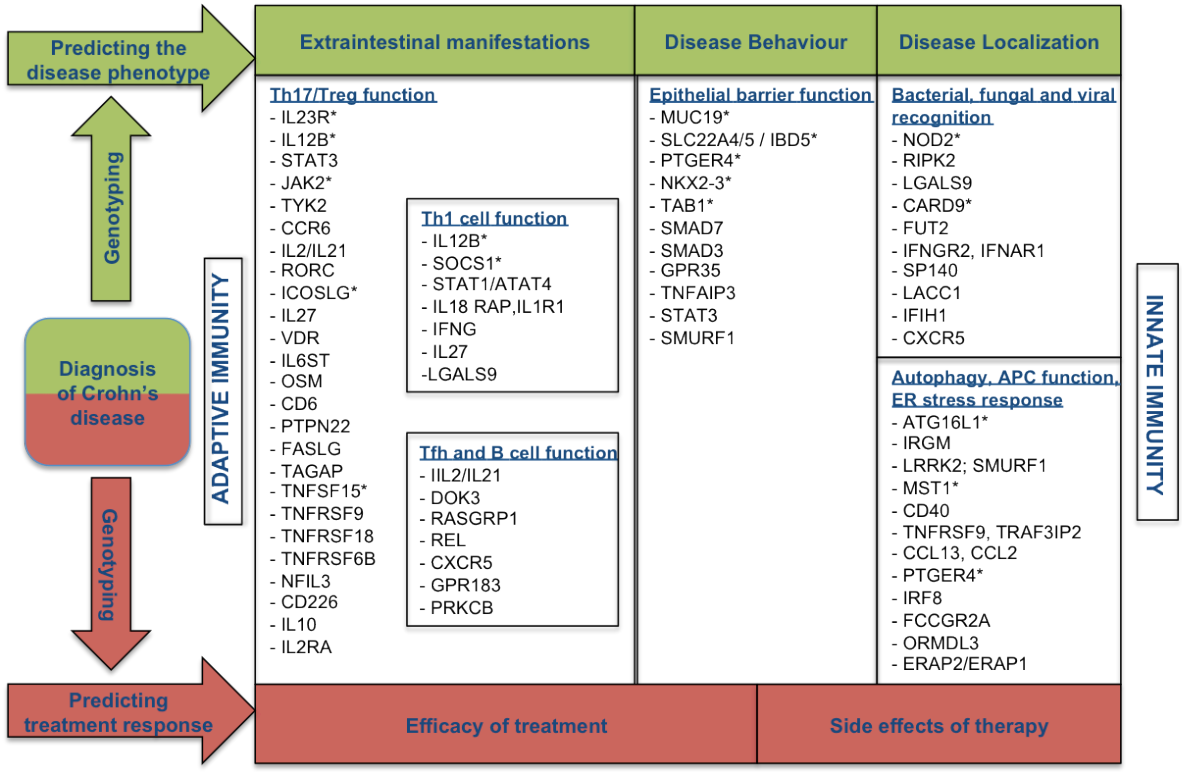

Figure I.1. Most important susceptibility genes to Crohn's disease (CD) and its association with disease phenotype and response to therapy. (Adapted from Brand 2013)

The most relevant association was described for *NOD2* as the most important genetic predictor for CD ileal disease, ileal stenoses, fistula and surgery (Xia *et al.* 2005). Another gene, yet with a weaker association than *NOD2*, significantly associated with ileocolonic disease involvement and stenosing disease behaviour was janus kinase 2 gene (*JAK2*), an important element of the signal transduction pathway of several cytokines like interleukin 12 (IL12) and interleukin 23 (IL23) that are involved in CD pathogenesis (Xia *et al.* 2005).

Greater importance has been paid to the existing variability in efficacy of the therapeutic applied to CD patients. It has been shown that this variability is influenced by disease severity, environmental factors and genetics factors. It was predicted that 20-95% in variability of drug effects in CD patients is due to genetic polymorphisms, with the main gene candidates those encoding for drug receptors, metabolizing enzymes, transporters and disease susceptibility genes (Pierik *et al.* 2006).

I.2.1 CYTOKINE GENES

One of the most important inflammatory mediators in CD are cytokines and its described genetic polymorphisms are very well studied, since it's though to influence inflammatory response and consequently disease susceptibility and/or development (Ardizzone & Bianchi 2002).

The uncontrolled inflammatory response results from an imbalance between proinflammatory cytokines such as interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), interleukin 1 (IL1), interleukin 6 (IL6), IL12 and antiinflammatory cytokines like interleukin 1 receptor antagonist (IL1RN) (Guidi *et al.* 2011). Some single nucleotide polymorphisms (SNP) have been already identified as associated to CD, particularly *IL1*β +3953 C/T and -511C/T, *TNF*α -857 C/T and -308 G/A, lymphotoxin alpha (*LT*α) +252 A/G, *IL6* -174 G/C and *IL1RN* variable number tandem repeat of 86 base pairs (VNTR) (Waterer & Wunderink 2003).

Another known CD gene risk is interleukin 23 receptor (*IL23R*) on chromosome 1p31 (Duraes *et al.* 2013) (Brand 2013). It was reported a strong protective effect of the Arg381Gln allele of the *IL23R* against CD developing, contrarily to variants of *IL23* gene that conferred increased risk (Budarf *et al.* 2009) (Cho & Weavet 2007). The demonstrated genetic association and the proinflammatory role of IL23 turn this pathway into a therapeutic target for CD (Cho & Weavet 2007).

I.2.2 APOPTOSIS GENES

Much have been speculated about the importance of apoptosis in CD, but recent studies suggested that Fas-mediated apoptosis influences CD pathogenesis by inducing gut inflammation, either by increased apoptosis of intestinal epithelium or decreased apoptosis of lamina propria lymphocytes (Xia *et al.* 2005).

The interaction between Fas and its ligand, Fas Ligand (FasL), activates the extrinsic pathway of apoptosis that leads to the activation of caspase 8 and the initiation of all of the apoptotic process, including the intrinsic apoptotic pathway (Xia *et al.* 2005).

One of the most described polymorphism is the *FasL* -843 C/T, in the promoter and near the local of ligation to the CAAT activator protein, with the CC genotype associated with a three times greater capacity of ligation to the CAAT protein and, consequently, a three times higher expression of FasL (Nagata 1994).

The Caspase 9 (Casp9) is an apoptosis related protein that forms an apoptosome, after ligation to cytochrome C and Apaf-1, which activates caspases 3, 6 and 7. Although its association with the apoptotic process, its functionality remains unclear (Krammer *et al.* 1994). It has been described that *Casp9* +93C/T polymorphism influenced response to biologic therapy in luminal CD cohort (Hlavatay *et al.* 2005).

In Crohn's disease it has been observed that different cells are unresponsive to apoptosis, which may represent an underlying genetic defect in lymphocyte and monocyte functioning and justified its persistence in sites of intestinal inflammation (Souza *et al.* 2005).

I.2.3 AUTOPHAGY GENES

Autophagy is the homeostatic process, through which cytosol or intracellular organelles are sequestered by autophagosomes to be delivered to lysosomes and consequently degraded (Deretic 2006). This biologic process is involved in protein degradation, antigen processing, regulation of cell signalling and several other pathways essential to the initiation and regulation of inflammatory response, which suggests that autophagy is likely to have an important role in CD pathogenesis (Rioux *et al.* 2007).

The GWA studies showed the importance of *ATG16L1* and *IRGM,* autophagic genes, as related to CD pathogenesis. The *ATG16L1* gene is part of the autophagosome pathway and has been associated with the processing of intracellular bacteria (Cho 2008). The *ATG16L1* Thr300Ala variant is directly associated to CD pathogenesis (Budarf *et al.* 2009).

It is important to refer a significant association between the *ATG16L1* gene and the *NOD2* gene, which is known to be a CD risk gene (Márquez *et al.* 2009).

In GOIA Study II (Duraes *et al.* 2013), it was reported that in the Portuguese population a genetic profile involving SNPs in three autophagy-related genes, *ATG16L1*, *IRGM* and intelectin 1 (*ITLN1*), helps to predict Crohn's ileal or ileacolonic disease, involvement of the upper digestive tract, response to steroids and to biologic therapy.

I.3 NUTRITION

For the past decades an emerging growing in the incidence and prevalence of IBD has been observed, which strongly suggests the importance of environmental factors as triggers for this disease (Hou *et al.* 2014). One of these suggested triggers is dietary patterns, based on the spreading of "western" diet high in fat and protein and low in fruits and vegetables and through its influence in intestinal inflammation, namely by altering gut microbiome and affecting gastrointestinal permeability (Hou *et al.* 2014).

Crohn's disease is commonly associated with malnutrition and weight loss that results from reduction of food intake, inappropriate dietary patterns, inflammatory process and side effects of multiple therapies applied in CD treatment. Nowadays, this pattern has become less common due to advances in treatment options, greater knowledge of how diet can influence disease course and an increased number of patients attaining clinical remission (Hwang *et al.* 2012).

Along the years, several studies have been trying to establish a link between diet and CD development, once it is very important that CD patients identify and avoid foods that worsen symptoms (Fergunson 2013).

Distinct associations between fatty acids, higher fruit intake and protein with the development of CD have been described, suggesting that they play a protective role against flares (Hou *et al.* 2014).

Various dietary components have been proposed to increase the risk of developing or exacerbating symptoms of CD. The main macronutrients pointed as prejudicial for CD patients are sugars and fats,

particularly saturated, polyunsaturated and trans (Hou *et al.* 2014). General recommendations also suggest reducing high-fiber foods and limit consumption of dairy foods during flares (Fergunson 2013).

It is commonly observed in CD patients micronutrients deficiencies such as iron, magnesium, zinc, calcium and vitamins A, D, K, B6, B9 (folic acid), B12 (cobalamin) and C (L-ascorbic acid). Those micronutrients deficiencies lead to important EIM like anemia, bone disease, hypercoagulability, wound healing and colorectal cancer risk (Hwang *et al.* 2012) (Gassul 2003).

Studies have demonstrated that CD patients have ω-3 fatty acids deficiencies. These fatty acids are known to be involved in immunomodulatory mechanisms in IBD, such as altering proinflammatory eicosanoid synthesis, cell membrane fluidity, cell signal transduction, intraluminal bacterial content and expression of inflammatory genes such as *TNFα*, *IL1* and *IL6* (MacLean *et al.* 2005). Because of this, it's usually recommended supplemental ω-3 fatty acids as beneficial in treating or preventing relapses in CD (MacLean *et al.* 2005).

In Europe it has been considered the use of exclusive enteral nutrition with elemental, semi-elemental and defined formula diets for CD as first line therapy for induction of remission, due to its results in mucosal healing, prolong clinical remission and highly favourable safety profile, although its use over long periods of time it's still uncertain (Hou *et al.* 2014) (Hirai *et al.* 2013).

Although all these facts, the identification and use of guidelines based on diet for IBD patients is still growing and data on how altering diets can influence disease susceptibility and development are limited.

I.4 THERAPEUTIC

Several pharmacological therapies aimed at controlling intestinal inflammation have been developed along the years.

The treatment goals in CD are not just concerning control of symptoms and GI inflammation, but mainly preventing bowel damage, reducing long-term disability and maintaining patients quality of life (Papay *et al.* 2013).

The treatment of active CD is very complex and approximately 20% of patients do not respond to conventional therapy, namely corticosteroids (CS) and immunosuppressors like azathioprine (AZA) and methotrexate (MTX) (Orlando *et al.* 2005). The development of new therapies led to the use of chimeric monoclonal antibodies that specifically blockage and neutralise the human tumour necrosis factor-a (TNF-a), an important proinflammatory cytokine in bowel mucosal inflammation (Mascheretti *et al.* 2004).

I.4.1. THERAPIES FOR CROHN'S DISEASE

Several therapies are used as CD treatment, namely:

Aminosalycilates

The application of aminosalycilates, mainly 5-aminosalicylic acid (5'ASA), has long been used as first-line treatment in CD. However, its use as induction and maintenance therapy still remains conflicting, once it has been shown that its relative inefficacy may be due to the fact that it only addresses mucosal disease and may not have activity in deeper layers of the bowel (Williams *et al.* 2011) (Herrlinger & Jewell 2006).

Corticosteroids

Corticosteroids offer a more efficacious and rapid relief of symptoms in the majority of IBD patients (Panaccione *et al.* 2012). Although most patients initially respond to corticosteroids, after 1 year, approximately 25% become steroid-dependent (Gisbert *et al.* 2009).

Immunosuppressors

The decision to begin immunosuppressive therapy should rely on the fact that most patients should present a chronic active disease course, corticosteroid dependence and resistance and recurring flares of CD (Gisbert *et al.* 2008). It's also referred for development of complicated disease course, presence of perianal fistulising disease and EIM (Wenger *et al.* 2012). Early introduction of immunosuppressive treatment in mild CD patients should be prevented at all cost because of overtreatment, unnecessary side effects and therapeutic toxicity (Cosnes *et al.* 2013).

Biologic therapy

Monoclonal antibodies that bind to TNFα have revolutionary the management of moderate to severe CD that is refractory to conventional therapy (Reenaers 2010). It is known that TNFα affects the epithelial barrier, induces apoptosis of the villous epithelial cells and secretes chemokines from the intestinal epithelial cells (Cottone & Criscuoli 2011).

The ideal biologic agent for CD treatment should target a specific event of the inflammatory pathway, induce and maintain sustained remission, be well tolerated and do not induce any immunogenicity (Reenaers 2010).

It is recommended that biologic therapy should be use as first-line therapy in patients with complicated disease or bowel damage and poor prognostic factors and/or severe disease (Peyrin-Biroulet *et al.* 2013). Despite all the successfully treatments with biologic therapy it is important to note that one-third of patients will not respond to this therapy, although this percentage may decrease if patients begin this type of treatment as early as possible (Panaccione & Ghosh 2010).

One of the mostly used monoclonal antibodies is Infliximab (IFX), which has proven to be highly effective agent in induction and maintenance in patients with refractory luminal and fistulising CD (Cottone & Criscuoli 2011). Therapeutic development led to the arrival of Adalimumab (ADA), subcutaneously administered, that is effective for the induction and maintenance of therapy in patients with moderate to severe CD (Orlando *et al.* 2012) and Certolizumab pegol (CPZ) that has been shown to be effective as induction therapy in patients with moderate to severe CD, offering a rapid treatment response and symptom relief (Schreiber 2011).

Combined therapy

The evidence suggests that the early use of biologic therapy, in combination with immunosuppressors, culminated in the achievement of a more rapid remission than with conventional progression of treatment, with a longer time to relapse, decreased need for treatment with corticosteroids, faster reduction in clinical symptoms, decreased inflammatory markers and improved mucosal healing (Cottone & Criscuoli 2011) (Colombel 2012).

Nevertheless, it remains unclear the optimal use of combined therapy, mainly because of doubts about immunogenicity, efficacy and safety (Panaccione & Ghosh 2010).

The application of the described therapies is commonly referred as "step-up" therapy, a model normally described as a pyramid scheme, with the milder and less toxic therapies at the base of the pyramid and the more efficacious and powerful therapies at the top like shown in Figure 2 (Pithadia & Jain 2011).

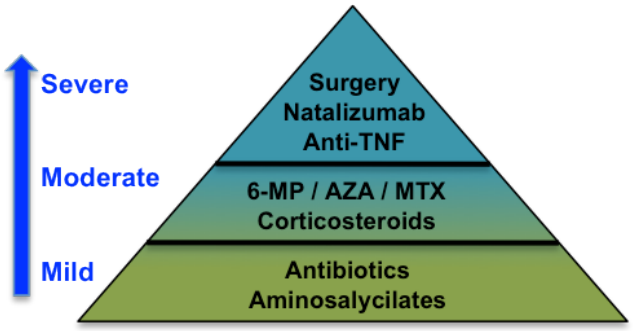

Figure I.2. Conventional "step-up" treatment strategy for Crohn's disease patients. (Adapted from Hutfless *et al.* 2014)

Conventional treatment strategies have focused on induction of a clinical remission using a step-wise approach to medical therapy with 5-aminosalicylates, corticosteroids and immunosuppressors, but in recent years clinical trials of earlier use of immune-modifying or biologic therapies (or combinations of them) have been associated with more rapid remission and improved short- and longer-term outcomes (Rubin *et al.* 2012).

Several studies have been demonstrating that an early introduction of intensive therapy in patients with disabling and/or severe CD of immunosuppressors and/or biologic therapies generates an increased probability of mucosal healing and early continuous remission without steroids (Van Assche *et al.* 2010). It's assumed that when at least two clinical predictors are present in one patient, early treatment with azathioprine and/or biologics should be considered (Van Assche *et al.* 2010).

The traditional treatment paradigm includes a "step-up" approach of corticosteroids and immunosuppressors, with or without biologic agents as severity progresses or patients fail to respond. Whereas this approach may be effective in the near term, it may not prevent overall disease progression (Tamboli *et al.* 2011). Within 10 years of diagnosis, more than half of CD patients still require surgical resection and within 20 years, approximately 50%–70% of CD patients develop a stricturing or penetrating intestinal complication, and the cumulative risk of hospitalization rises to nearly 80%. The risk of hospitalization is greater within the first year after diagnosis of CD (32%–83%

of patients), with the annual incidence of hospitalizations remaining steady at 20% over the next 5 years. “Top-down” therapy, with the earlier introduction of biologic agents such as antitumor necrosis factor alpha (anti-TNF-α) antibodies, has demonstrated high rates of remission and mucosal healing (Tamboli *et al.* 2011).

Recent studies of ‘top-down’ versus ‘step-up’ therapy for CD have shown conflicting results. A prospective randomized comparison between a step-up regimen with corticosteroids and a top-down strategy starting with infliximab showed favorable results for the top-down approach after six months, but significance was lost after 12 months (Kruis *et al.* 2013). A five-year prospective observational study concluded that indiscriminate use of biological therapy (‘top-down’ strategy) is not appropriate for moderate to severe CD. Indeed, the recent European Crohn's and Colitis Organisation (ECCO) management guidelines point out that for selected patients with mild CD, one option is to start no active treatment.

Long-term efficacy, safety and cost are main concerns of risk/benefit assessment of different treatment strategies. In step-up approach, infections associated with corticosteroids and lymphomas caused by azathioprine are commonly seen adverse events. In top-down approach, serious infections, lymphoma and malignancies are side effects of anti-TNF agents and immunosuppressives (Chen *et al.* 2014).

Nevertheless, the prediction of which approach should be used in each patient depends on a combination of clinical parameters, namely disease aggressiveness, serologic markers and individual genetic profile, emphasizing the importance of more pharmacogenetic studies in the near future.

I.5 OTHER INFLAMMATORY BOWEL DISEASES

In addition to Crohn’s disease, Ulcerative colitis (UC) is the other chronic inflammatory disorder that completes the known Inflammatory Bowel Diseases group, which is characterised by specific phenotype, genetic predisposition, environmental factors and uncontrolled immune response to the gut microbiome (Duricova *et al.* 2014).

The prevalence of UC in Europe varies from 2.422 to 2.946 cases per 100 000 individuals, whereas the incidence for the total European population suggests that may be up to 2.1 million persons (Burish *et al.* 2013). Although considered as “Western” diseases, it is now known that IBD incidence and prevalence are rapidly increasing in areas like India, Japan, China and Middle East (Lee *et al.* 2011) (Norgard *et al.* 2014).

Ulcerative Colitis specifically presents continuous mucosal inflammation of the colon, rectal bleeding, diarrhea and abdominal pain, showing a relapsing and remitting disease course (Louis *et al.* 2010). The disease classification is mainly based in disease location divided in proctitis (E1), left-sided UC (distal UC) (E2) and extensive UC (pancolitis) (E3) (Louis *et al.* 2011). Affects equally both sexes and has been shown that first-degree relatives of UC patients have a 10-15 fold risk of developing the disease (Dignass *et al.* 2012).

The environmental factor most studied in UC is smoking. In fact, it has been already shown and it is well established that occurs a protective effect of smoking against the development of UC (Parkes *et al.* 2014).

The dietary guidelines available for UC patients are very similar to the ones for CD patients. It is also a disease commonly associated with malnutrition and weight loss resulting from nutritional deficiencies, inflammatory process; reduction of food intake; inappropriate dietary patterns and side effects of therapy (Hwang *et al.* 2012). Recommendations emphasized the use of fatty acids, higher fruit intake and protein, and alert against the excessive use of sugars and fats, particularly saturated, polyunsaturated and trans as well as suggest reducing high-fiber foods and limit consumption of dairy foods during flares (Brown *et al.* 2011). It is commonly observed in UC patients micronutrients deficiencies such as vitamin B12, folic acid and especially iron, since iron-deficient anemia due to blood loss occurs in up to 80% of these patients (Brown *et al.* 2011).

Regarding UC genetics, GWA studies have led to the identification of several susceptibility genes, particularly the *HLA* region, which is the most strongly associated with UC, but also the *IL23R* gene, the *DLG5* gene, the *JAK/STAT* pathway, the *MDR1* gene and *TLR* gene (Dignass *et al.* 2012) (Brant 2013).

Medical treatment comprises mainly 5-aminosalicylates in both induction and maintenance of clinical and endoscopic remission, corticosteroids and immunosuppressors, including cyclosporine that is an option for severe acute UC if intravenous corticosteroids fail to induce remission (Williams *et al.* 2011) (Rutgeerts *et al.* 2005) (Lichtenstein & Rutgeerts 2010) (Pierik *et al.* 2006). It is believed that TNFα promotes the inflammatory response in UC patients so it is important to include TNF inhibitors, like the approved Infliximab and Adalimumab, that emerges like an alternative to the conventional treatment with aminosalicylates, corticosteroids and immunosuppressors when patients do not respond (Thorlund *et al.* 2014).

Similar to the strategies adopted in the treatment of CD, there is also a “step-up” approach in UC treatment normally described in a pyramid scheme with the milder and less toxic therapies at the base of the pyramid and the more efficacious and powerful therapies at the top, like shown in Figure 3 (D’Haens *et al.* 2014).

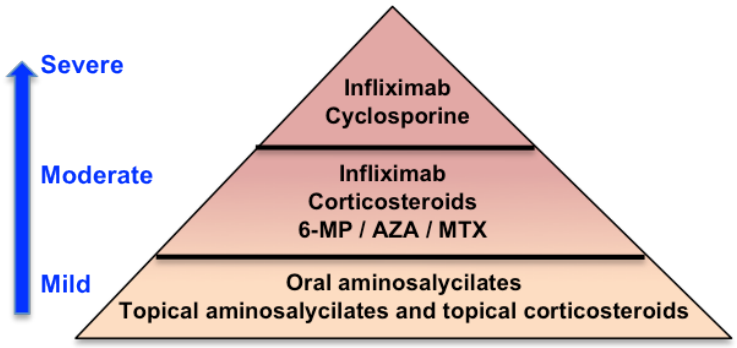

Figure I.3. Conventional “step-up” treatment strategy for Ulcerative colitis patients. (Adapted from Hutfless *et al.* 2014).

Inflammatory bowel diseases present a highly variable clinical course, which complicates diagnosis prediction and consequently, the determination of appropriate therapeutic strategies. A major obstacle to the introduction of personalized medicine in IBD patients is the lack of applicable clinical, genetic, environmental and therapeutical predictors to guide treatment at early diagnosis (Reenaers *et al.* 2012).

I.6 AIMS AND THESIS STRUCTURE

Crohn's disease is a multifactorial disease of unknown origin, characterized by clinical and genetic heterogeneity that has been studied for several years in the pursuit of a better understanding of its pathogenesis, clinical management and development of more efficacious and safer therapies for its treatment.

One of the immediate difficulties is the precise characterization of CD patients due to the great variability in clinical traits and response to therapeutic. Because of this, and with the purpose of facilitate diagnosis and specially improve therapeutic response, we have developed a study based in the clinical, genetic and therapeutical component of the disease.

To achieve our goal we have design a study based in the analysis by Real Time PCR of genetic polymorphisms in cytokines, apoptosis and autophagy genes that allow us to stipulate phenotype-genotype relations and its association with response to therapy conventionally used to treat CD. With this in mind, the primary purpose is the establishment of guidelines that leads to the application of individualized therapeutic. As main aims we propose:

1. Study of the association between polymorphisms in the *MDR1, IL23R, ATG16L1, Fas, Fas Ligand* e *Casp9* genes and the phenotype parameters such as age at diagnosis, disease location, disease behaviour, disease aggressiveness, fistulas and extraintestinal manifestations;
2. Study of the association between polymorphisms in the *MDR1* gene and the response to aminosalycilates, corticosteroids and immunosuppressive therapy;
3. Study of the association between polymorphisms in the *Fas, Fas Ligand* e *Casp9* apoptotic genes and the response to biologic therapy;
4. Study of the association between polymorphisms in the *IL23R* gene and the response to corticosteroids, immunosuppressive and biologic therapy;
5. Global evaluation of the importance of pharmacogenetics in the management of IBD;
6. Understanding the relevance of nutrigenetics in IBD management.

The present dissertation summarizes all the results obtained in the past years, where we have been developing several studies, namely a study entitled "Doença de Crohn: fatores genéticos e nutricionais", with a scholarship funding by Sociedade Portuguesa de Gastroenterologia (2006), that allowed us to obtain consistent results of the analysis of polymorphisms in cytokines genes as *IL1*, *TNFα* e *IL6* and the influence of the association between the genetic factors and the intake of fats and fatty acids in the disease aggressiveness and the "Estudo de farmacogenética nas Doenças

Inflamatórias Intestinais: Doença de Crohn e Colite Ulcerosa" study, with a scholarship funding by Grupo de Estudos das Doenças Inflamatórias Intestinais (2009-2012), that permitted the identification of associations between genotype-phenotype relations and response to therapeutic normally used in IBD and the associations between polymorphisms in inflammatory, apoptosis and autophagy genes and response to therapy used to treat IBD.

For a better understanding and compilation of all of the results and knowledge obtain along the several studies, this dissertation is organized in five chapters:

- In chapter I, Introduction, it's presented a global view thought to approach all of the important themes for the understanding of the work in question and proposed aims;
- In chapter II, Nutrigenetics, it's presented one article that approaches the interaction of fat intake with polymorphisms in *Caspase9*, *Fas Ligand* and *PPARgamma* apoptotic genes in modulating Crohn's disease activity in a population of 99 individuals with Crohn's disease and 116 control individuals;
- In chapter III, Pharmacogenetics, it's presented three articles where the first one is the study of clinical and genetic factors that may be used as predictors of response for several therapies in Crohn's disease in 242 CD patients from several participating hospitals from Central Portugal, the second one is the study of *IL23R* polymorphisms that may influence phenotype and response to therapy in Ulcerative colitis in 174 patients from several participating hospitals from Central Portugal, and the last one reviews the importance of genetics and susceptibility genes in IBD and its use as predictors to individualized therapy;
- In chapter IV, Discussion, it's summarized all of the results achieved in the different studies supported by previous published results from other authors and it's enlighten all of the main assumptions to remember. This analysis is divided into the discussion of clinical and genetic associations, nutrition effects on Crohn's disease patients and, finally, phenotye-genotype relations in association with response to therapy;
- Finally, in chapter V, Final Remarks and Future Perspectives, based on a synopsis of the aims and importance of the work made, it's suggested future study goals and thematic in order to pursuit a better understanding of IBD, specially CD, towards the use of pharmacogenetics and nutrigenetics as routinely tools for the management of Crohn's disease.

I.7 REFERENCES

Allez M, Lémann M (2009) New directions and new strategies in inflammatory bowel diseases. Gastroenterol Clin Biol. **33**(Suppl 3): S121-122.

Ardizzone S, Bianchi PG (2002) Inflammatory bowel disease: new insights into pathogenesis and treatment. J Intern Med. **252**(6): 475-496.

Brand S (2013) Moving the genetics of inflammatory bowel disease from bench to bedside: first steps towards personalized medicine. Gut **62**(11): 1531-1533.

Brant SR (2013) Promises, delivery, and challenges of inflammatory bowel disease risk gene discovery. Clin Gastroenterol Hepatol **11**(1): 22-26.

Brown AC, Rampertab SD and Mullin GE (2011) Existing dietary guidelines for Crohn's disease and ulcerative colitis. Expert Rev. Gastroenterol. Hepatol. **5**(3): 411-425.

Budarf ML, Labbé C, *et al.* (2009) GWA studies: rewriting the story of IBD. Trends Genet **25**(3): 137-146.

Burisch J, Jess T, *et al.* (2013) The burden of inflammatory bowel disease in Europe. J Crohns Colitis. **7**(4): 322-337.

Chen Q, Yian L, Wan J (2014) Select a suitable treatment strategy for Crohn's disease: step-up or top-down. EXCLI Journal **13**: 111-122.

Cho JH and Weaver CT (2007) The genetics of inflammatory bowel disease. Gastroenterology **133**(4): 1327-1339.

Cho JH (2008) Inflammatory bowel disease: Genetic and epidemiologic considerations. World J Gastroenterol. **14**(3): 338–347.

Colombel JF (2012) When Should Combination Therapy for Patients with Crohn's Disease Be Discontinued? Gastroenterology & Hepatology **8**(4): 259-262.

Colombel JF (2014) Decade in review-IBD: IBD-genes, bacteria and new therapeutic strategies. Nat Rev Gastroenterol Hepatol **11**(11): 652-654.

Cosnes J, Bourrier A *et al.* (2013) Early administration of azathioprine vs conventional management of Crohn's Disease: a randomized controlled trial. Gastroenterology **145**(4): 758-765.

Cottone M and Criscuoli V (2011) Infliximab to treat Crohn's disease: an update. Clin Exp Gastroenterol **4**: 227-238.

Cummings SA, Rubin D (2006) The complexity and challenges of genetic counseling and testing for inflammatory bowel disease. J Genet Couns **15**(6): 465-476.

Deretic V (2006). Autophagy as an immune defense mechanism. Curr Opin Immunol. **18**(4): 375-382.
D'Haens GR, Sartor RB *et al.* (2014) Future directions in inflammatory bowel disease management. J Crohns Colitis. **8**(8): 726-734.

Dignass A, Eliakim R *et al.* (2012) Second European evidence-based consensus on the diagnosis and management of ulcerative colitis part 1: definitions and diagnosis. J Crohns Colitis **6**(10): 965-990.

Duraes C, Machado JC *et al.* (2013) Phenotype-genotype profiles in Crohn's disease predicted by genetic markers in autophagy-related genes (GOIA study II). Inflamm Bowel Dis **19**(2): 230-239.

Duricova D, Burish J *et al.* (2014) Age-related differences in presentation and course of inflammatory bowel disease: an update on the population-based literature. J Crohns Colitis. **8**(11): 1351–1361.

Ferguson LR (2013). Nutrigenomics and Nutrigenetics in Functional Foods and Personalized Nutrition, CRC Press.

Gassull MA (2003) Nutrition and inflammatory bowel disease: its relation to pathophysiology, outcome and therapy. Dig Dis **21**(3): 220-227.

Gisbert JP, Nino P *et al.* (2008) Comparative effectiveness of azathioprine in Crohn's disease and ulcerative colitis: prospective, long-term, follow-up study of 394 patients. Aliment Pharmacol Ther **28**(2): 228-238.

Gisbert JP, Linares PM *et al.* (2009) Meta-analysis: the efficacy of azathioprine and mercaptopurine in ulcerative colitis. Aliment Pharmacol Ther **30**(2): 126-137.

Guidi L, Pugliese D, Armuzzi A (2011) Update on the management of inflammatory bowel disease: specific role of adalimumab. Clin Exp Gastroenterol. **4**: 163-172.

Hendrickson BA, Gokhale R *et al.* (2002) Clinical aspects and pathophysiology of inflammatory bowel disease. Clin Microbiol Rev **15**(1): 79-94.

Herrlinger KR, Jewell DP (2006) Review article: interactions between genotype and response to therapy in inflammatory bowel diseases. Aliment Pharmacol Ther. **24**(10): 1403-1412.

Hirai F, Ishihara H *et al.* (2013) Effectiveness of concomitant enteral nutrition therapy and infliximab for maintenance treatment of Crohn's disease in adults. Dig Dis Sci. **58**(5): 1329-1334.

Hlavatay T, Pierik M *et al.* (2005) Polymorphisms in apoptosis genes predict response to infliximab therapy in luminal and fistulizing Crohn's disease. Aliment Pharmacol Ther **22**(7): 613-626.

Hou JK, Lee D *et al.* (2014) Diet and inflammatory bowel disease: review of patient-targeted recommendations. Clin Gastroenterol Hepatol **12**(10): 1592-1600.

Huebner C, Browning BL *et al.* (2009) Genetic analysis of MDR1 and inflammatory bowel disease reveals protective effect of heterozygous variants for ulcerative colitis. Inflamm Bowel Dis. **15**(12): 1784-1793.

Hutfless S, Almashat S, Berger Z *et al.* (2014) Pharmacologic therapies for the management of Crohn's disease: Comparative effectiveness. Comparative Effectiveness Reviews. **131**: 1-26.

Hwang C, Ross V *et al.* (2012) Micronutrient deficiencies in inflammatory bowel disease: from A to zinc. Inflamm Bowel Dis **18**(10): 1961-1981.

Kabi A, Nickerson K *et al.* (2012) Digesting the genetics of inflammatory bowel disease: insights from studies of autophagy risk genes. Inflamm Bowel Dis. **18**(4): 782-792.

Krammer PH, Dhein J *et al.* (1994) The role of APO-1-mediated apoptosis in the immune system. Immunol Rev. **142**: 175-191.

Kruis W, Katalinic A *et al.* (2013) Predictive factors for an uncomplicated long-term course of Crohn's disease: a retrospective analysis. J Crohns Colitis **7**(7): e263-270.

Lee JC, Lyons PA *et al.* (2011) Gene expression profiling of CD8+ T cells predicts prognosis in patients with Crohn disease and ulcerative colitis. J Clin Invest **121**(10): 4170-4179.

Lichtenstein GR and Rutgeerts P (2010) Importance of mucosal healing in ulcerative colitis. Inflamm Bowel Dis **16**(2): 338-346.

Louis E, Belaiche J, Reenaers C (2010) Do clinical factors help to predict disease course in inflammatory bowel disease? World J Gastroenterol. **16**(21): 2600-2603.

Louis E, Van Kemseke C, Reenaers C (2011) Necessity of phenotypic classification of inflammatory bowel disease. Best Pract Res Clin Gastroenterol. **25**(Suppl 1): S2-7.

MacLean CH, Mojica WA *et al.* (2005) Systematic review of the effects of n-3 fatty acids in inflammatory bowel disease. Am J Clin Nutr **82**(3): 611-619.

Magro F, Cravo M, Lago P *et al.* (2012) Portuguese consensus on the best practice for the management of inflammatory bowel disease: IBD ahead 2010 meeting results. GE Jornal Português de Gastrenterologia **19**(4): 190-198.

Márquez A, Núñez C *et al.* (2009) Role of ATG16L1 Thr300Ala polymorphism in inflammatory bowel disease: a Study in the Spanish population and a meta-analysis. Inflamm Bowel Dis. **15**(11): 1697-1704.

Mascheretti S, Croucher JP, Schreiber S. (2004) Pharmacogenetics of inflammatory bowel disease. Best Practice & Research Clinical Gastroenterology **18**(3): 597–609.

Nagata S (1994) Fas and Fas ligand: a death factor and its receptor. Adv Immunol **57**: 129-144.

Norgård BM, Nielsen J *et al.* (2014) The incidence of ulcerative colitis (1995-2011) and Crohn's disease (1995-2012) - Based on nationwide Danish registry data. J Crohns Colitis **8**(10): 1274-1280.

Orlando A, Colombo E *et al.* (2005) Infliximab in the treatment of Crohn's disease: Predictors of response in an Italian multicentric open study. Digestive and Liver Disease **37**: 577–583.

Orlando A, Renna S *et al.* (2012) Adalimumab in steroid-dependent Crohn's disease patients: prognostic factors for clinical benefit. Inflamm Bowel Dis **18**(5): 826-831.

Panaccione R, Ghosh S (2010) Optimal use of biologics in the management of Crohn's disease. Therap Adv Gastroenterol. **3**(3): 179-189.

Panaccione R, Hibi T *et al.* (2012) Implementing changes in clinical practice to improve the management of Crohn's disease. J Crohns Colitis **6 Suppl 2**: S235-242.

Papay P, Ignjatovic A *et al.* (2013) Optimising monitoring in the management of Crohn's disease: a physician's perspective. J Crohns Colitis. **7**(8): 653-669.

Parkes GC, Whelan K *et al.* (2014) Smoking in inflammatory bowel disease: impact on disease course and insights into the aetiology of its effect. J Crohns Colitis **8**(8): 717-725.

Peña AS, Crusius JB (1998) Genetics of inflammatory bowel disease: implications for the future. World J Surg. **22**(4): 390-393.

Peyrin-Biroulet L, Florino G *et al.* (2013) First-line therapy in adult Crohn's disease: who should receive anti-TNF agents? Nat Rev Gastroenterol Hepatol. **10**(6): 345-351.

Pierik M, Rutgeerts P *et al.* (2006) Pharmacogenetics in inflammatory bowel disease. World J Gastroenterol. **12**(23): 3657-3667.

Pithadia AB, Jain S (2011) Treatment of inflammatory bowel disease (IBD). Pharmacological Reports **63**: 629-642.

Reenaers C (2010) Current directions of biologic therapies in inflammatory bowel disease. Therap Adv Gastroenterol. **3**(2): 99–106.

Reenaers C, Belaiche J, Louis E (2012) Impact of medical therapies on inflammatory bowel disease complication rate. World J Gastroenterol. **18**(29): 3823-3827.

Rioux JD, Xavier RJ *et al.* (2007) Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. Nat Genet. **39**(5): 596-604.

Rubin DT, Uluscu O, Sederman R (2012) Response to biologic therapy in Crohn's disease is improved with early treatment: an analysis of health claims data. Inflamm Bowel Dis. **18**(12): 2225-2231.

Rutgeerts P, Sandborn WJ *et al.* (2005) Infliximab for induction and maintenance therapy for ulcerative colitis. N Engl J Med **353**(23): 2462-2476.

Schreiber S (2011) Certolizumab pegol for the treatment of Crohn's disease. Therap Adv Gastroenterol **4**(6): 375-389.

Shivananda S, Lennard-Jones J *et al.* (1996) Incidence of inflammatory bowel disease across Europe: is there a difference between north and south? Results of the European Collaborative Study on Inflammatory Bowel Disease (EC-IBD). Gut **39**(5): 690-697.

Souza HS, Tortori CJ *et al.* (2005) Apoptosis in the intestinal mucosa of patients with inflammatory bowel disease: evidence of altered expression of FasL and perforin cytotoxic pathways. Int J Colorectal Dis. **20**(3): 277-286.

Stappenbeck TS, Rioux JD *et al.* (2011) Crohn disease: a current perspective on genetics, autophagy and immunity. Autophagy **7**(4): 355-374.

Tamboli CP, Doman DB *et al.* (2011) Current and future role of biomarkers in Crohn's disease risk assessment and treatment. Clin Exp Gastroenterol **4**: 127-140.

Thorlund K, Druyts E *et al.* (2014) Adalimumab versus infliximab for the treatment of moderate to severe ulcerative colitis in adult patients naïve to anti-TNF therapy: an indirect treatment comparison meta-analysis. J Crohns Colitis **8**(7): 571-581.

Van Assche G, Dignass A *et al.* (2010) The second European evidence-based Consensus on the diagnosis and management of Crohn's disease: Definitions and diagnosis. J Crohns Colitis **4**(1): 7-27.

Waterer GW and Wunderink RG (2003) Science review: Genetic variability in the systemic inflammatory response. Crit Care **7**(4): 308-314.

Wenger S, Nikolaus S *et al.* (2012) Predictors for subsequent need for immunosuppressive therapy in early Crohn's disease. J Crohns Colitis **6**(1): 21-28.

Williams C, Panaccione R et al. (2011) Optimizing clinical use of mesalazine (5-aminosalicylic acid) in inflammatory bowel disease. Therap Adv Gastroenterol. **4**(4): 237-248.

Xia B, Yu YH *et al.* (2005) Association of Fas-670 gene polymorphism with inflammatory bowel disease in Chinese patients. World J Gastroenterol **11**(3): 415-417.

CHAPTER II – NUTRIGENETICS

II.1 PAPER 1

Ferreira P., Cravo M., Sousa Guerreiro C., Tavares L., Moura-Santos P. and Brito M. (2010). Fat intake interacts with polymorphisms of Caspase9, FasLigand and PPARgamma apoptotic genes in modulating Crohn's disease activity. *Clinical Nutrition*. **29**:819-823.

Clinical Nutrition 29 (2010) 819–823

Contents lists available at ScienceDirect

Clinical Nutrition

journal homepage: http://www.elsevier.com/locate/clnu

Original Article

Fat intake interacts with polymorphisms of Caspase9, FasLigand and PPARgamma apoptotic genes in modulating Crohn's disease activity

Paula Ferreira [a], Marília Cravo [b], Catarina Sousa Guerreiro [a,c], Lourdes Tavares [d], Paula Moura Santos [d], Miguel Brito [a,*]

[a] Escola Superior de Tecnologia da Saúde de Lisboa, Lisboa, Portugal
[b] Serviço de Gastrenterologia do Instituto Português de Oncologia Francisco Gentil, EPE, Lisboa, Portugal
[c] Unidade de Nutrição e Metabolismo do Instituto de Medicina Molecular da Universidade de Lisboa, Lisboa, Portugal
[d] Serviço de Gastrenterologia do Hospital de Santa Maria, EPE, Lisboa, Portugal

ARTICLE INFO

Article history:
Received 30 March 2010
Received in revised form
24 May 2010
Accepted 14 June 2010

Keywords:
Crohn's disease
Caspase 9
Fas Ligand protein
PPAR gamma
Polymorphisms
Fat intake

SUMMARY

Background & aims: Crohn's disease (CD) is a multifactorial disease where resistance to apoptosis is one major defect. Also, dietary fat intake has been shown to modulate disease activity. We aimed to explore the interaction between four single nucleotide polymorphisms (SNPs) in apoptotic genes and dietary fat intake in modulating disease activity in CD patients.

Methods: Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP) techniques were used to analyze Caspase9+93C/T, FasLigand-843C/T, Peroxisome Proliferator-Activated Receptor gamma+161C/T and Peroxisome Proliferator-Activated Receptor gamma Pro12Ala SNPs in 99 patients with CD and 116 healthy controls. Interactions between SNPs and fat intake in modulating disease activity were analyzed using regression analysis.

Results: None of the polymorphisms analyzed influenced disease susceptibility and/or activity, but a high intake of total, saturated and monounsaturated fats and a higher ratio of n-6/n-3 polyunsaturated fatty acids(PUFA), was associated with a more active phenotype ($p < 0.05$). We observed that the detrimental effect of a high intake of total and trans fat was more marked in wild type carriers of the Caspase9+93C/T polymorphism [O.R(95%CI) 4.64(1.27–16.89) and O.R(95%CI) 4.84(1.34–17.50)]. In the Peroxisome Proliferator-Activated Receptor gamma Pro12Ala SNP, we also observed that a high intake of saturated and monounsaturated fat was associated to a more active disease in wild type carriers [OR(95%CI) 4.21 (1.33–13.26) and 4.37(1.52–12.51)]. Finally, a high intake of n-6 PUFA was associated with a more active disease in wild type carriers for the FasLigand-843C/T polymorphism [O.R(95%CI) 5.15(1.07–24.74)].

Conclusions: To our knowledge, this is the first study to disclose a synergism between fat intake and SNPs in apoptotic genes in modulating disease activity in CD patients.

1. Introduction

Crohn's disease (CD) is a chronic inflammatory disease characterized by a transmural and discontinuous inflammation of the intestinal wall. Although the precise etiology of this disease is unknown, most authors believe that the on-going inflammation results from the interaction between genetic and environmental factors, namely bacterial flora and luminal nutrients.[1,2] Among the most probably involved pathogenetic mechanisms is the imbalance between proinflammatory and anti-inflammatory cytokines as well as the resistance of the inflammatory cells to apoptotic stimuli.[3,4] Both of these abnormalities are believed to contribute to perpetuate the inflammation in CD.

On the other hand, some studies have focused on the nutritional manipulation of inflammation, namely through the administration of n-3 fatty acids.[5] As opposed to n-3 PUFA, and contrary to what was formerly expected, monounsaturated acids may have a detrimental effect,[6] whereas a recent study showed that medium-chain triglycerides (MCT) may exhibit an anti-inflammatory effect in an experimental model of colitis.[7] In this regard, Gassull *et al.*, believe that the pro or anti-inflammatory effect of fat depends more on a certain fatty acid profile than on a single fatty acid.[6] The exact mechanism whereby

* Corresponding author at. Escola Superior de Tecnologia da Saúde de Lisboa, Av. D. João II lote 4.69.01, 1990-096 Lisboa, Portugal. Tel.: +351 218980400; fax: +351 218980460.
E-mail address: miguel.brito@estesl.ipl.pt (M. Brito).

0261-5614/$ – see front matter © 2010 Elsevier Ltd and European Society for Clinical Nutrition and Metabolism. All rights reserved.
doi:10.1016/j.clnu.2010.06.008

these agents interfere with inflammation is still not fully understood, but probably relates to its ability of reducing the secretion of proinflammatory cytokines as well as to its capacity of inducing apoptosis, as has been shown in previous studies.[8]

However, the beneficial or deleterious effect of the fatty acids is not consistently observed in all studies performed. One reason for these discrepancies could be ascribed to polymorphisms of cytokine genes.[9] Thus, Grimble *et al.*[10] have shown that in a group of healthy individuals, different genotypes for *TNFa* were not only associated with different levels of *TNFa* production, but also with different responses to the anti-inflammatory effects of fish oil. Consistent with this hypothesis, in a previous study performed by our group in this same population and aimed at examining the interaction between genetic polymorphisms of pro and anti-inflammatory cytokines and fat intake, we observed that different types of fat did interact with cytokine genotype, modulating disease activity.[11]

Similarly, in the present study we explored the interaction between fat intake and four single nucleotide polymorphisms in apoptotic genes namely Caspase-9, FasLigand and Peroxisome Proliferator-Activated Receptor gamma in modifying both susceptibility for CD as well as disease activity.

2. Materials and methods

2.1. Subjects

Ninety nine consecutive outpatients with a confirmed diagnosis of CD, who were coming for a routine visit to the outpatient clinic of the two hospitals involved in the study, during the period between September 2004 and November 2007, were asked to participate in the study. Demographic and clinical characteristics of study population have been described previously.[11] The diagnosis was based on previously defined criteria[12] and disease activity was assessed according to Harvey and Bradshaw Index.[13] None of the patients was on steroids at the time of the study and none was hospitalized. Only patients with mild to moderate disease were included. The reason for that was because we intended to analyze the association between nutritional intake during the last year and pattern of disease activity during this same period. Thus, it would be a major bias if we included patients hospitalized for severe disease. Patients with severe active disease (HBI>7) at the time of patient inclusion or with need of systemic steroids were excluded ($n = 7$). One hundred and sixteen healthy blood donors from the Instituto Português de Oncologia Francisco Gentil S.A (IPOFG), with no previous history of inflammatory bowel disease (IBD), were recruited as a control group for genotyping.

2.2. Laboratory methods

Blood samples from CD patients were collected from the institutions previously mentioned and the DNA extraction was performed with phenol/chloroform extraction,[14] while for controls, the DNA extraction was made from blood samples with methodology described in Generation Capture Card Kit – DNA Purification/DNA Elution (Gentra Systems, Inc., Minneapolis).

Polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) techniques were used to analyze gene polymorphisms in the *CASP9*, *FASLG* and *PPARγ* genes in 99 patients with CD and 116 controls.

PCR reaction mixture included 10× buffer ([Taq buffer +$(NH_4)_2SO_2$]), 25 mM $MgCl_2$, 2 mM dNTPs, 6 μM forward/reverse primer, 5 U/μl Taq DNA polymerase and H_2O until the final volume of 20 μl. Primer sequences of the polymorphisms in study: CASP9+93C/T (F: 5′- GGAAGAGCTGCAGGTGGAC -3′/R: 5′- ATGGCAT GGAATCGCTTTAG -3′), FASLG-843C/T (F:5′- TGGGCAAACAATGAA AATGA -3′/R: 5′- TCATCTCTTCCCCACACACA -3′), PPARγ+161C/T (F: 5′- TGAATGTGAAGCCCATTGAA -3′/R: 5′- TGGAAGAAGGGAAATG TTGG -3′) and PPARγPro12Ala (F: 5′- ACTCTGGGAGATTCTCCT ATTGGC -3′/R: 5′- CGATAGCAACGAGCTAAGCA -3′).

RFLP reaction mixture included 10x appropriate reaction buffer (New England Biolabs (NEB)), 0.08 U/μL NruI for CASP9+93C/T, 0.17 U/μL BsrdI for FASLG-843C/T, 0.04 U/μL PmlI for PPARγ+161C/T and 0.09 U/μL HaeIII for PPARγPro12Ala (New England Biolabs (NEB)), 100 μg/ml BSA for BsrdI *and* PmlI enzymes and H_2O until the final volume of 30 μl. The reaction took place for 2 h at 37 °C, except for BsrdI that digests at 65 °C.

After digestion, the product was run in a 4% gel electrophoresis and, according to the restriction profile, the genotypes for each subject were recorded.

2.3. Nutritional intake evaluation

For quantification of the nutrients intake we used a Food Frequency Questionnaire,[15] validated for the Portuguese population. Participants were asked to recall their habits in the year before the interview. Colored photographs of most food items, showing 3 different portions sizes, as well as measuring cups and spoons were used to facilitate quantification of intake. Type and quantity of food intake was then analyzed in a modified database Food Processor *software*, version 7 (Esha Research, Inc, Salem, USA, 2000) including some Portuguese food items, which allow the quantification of different macro and micronutrients. Nutrient values were calculated from foods and supplements.

2.4. Statistical analysis

Differences in genotype frequencies, Fisher's exact tests and Hardy–Weinberg tests were calculated by GENEPOP (version 3.4). Statistical analysis was performed using SPSS version 16.0 for Windows (SPSS Inc., Chicago, EUA 2007). Data were expressed as mean ± standard deviation, as number of subjects and (percentage) or as Odds ratio (OR) and 95% confidence interval (CI). Bivariate analyses were conducted using Student's *t*-test or Mann–Whitney test for continuous variables and chi-square test for categorical ones. Multiple logistic regressions were used to study the association between variables and disease activity.

Statistical significance was established for $p < 0.05$.

Subjects were classified as homozygous for the variant if they carried two mutated alleles, heterozygous if they carried only one mutated allele, and finally homozygous for the wild type when they had no mutant alleles. When analyzing the interaction between genetic and nutritional variables, the median value for each nutrient was considered.

2.5. Ethical issues

The study was approved by the Ethics and Scientific Committees of Instituto Português de Oncologia Francisco Gentil S.A (IPOFG) and Hospital Santa Maria. All subjects gave their informed and written consent before entering the study.

3. Results

Clinical features and demographics of study population are described in Table 1. Ninety nine consecutive outpatients with a confirmed diagnosis of CD (60F:39M, mean age 40.4 ± 14.6 years) had a stable body weight during the 3 months preceding the study. According to the Harvey and Bradshaw Index (HBI), 36/99 (36.4%) of patients had moderately active disease with an HBI≥ 4 whereas

Table 1
Clinical features of study population.[a]

Clinical features	Cases % (*N*)	Controls % (*N*)
Age (years)[b]	40.4 ± 14.6	59.7 ± 11.8
Gender		
Male	39.4 (39)	44.0 (51)
Female	60.6 (60)	56.0 (65)
Smoking habits	14.2 (30)	48.1 (102)
Years of disease[b]	11.2 ± 8.9	–
Disease location		
Ileum	29.3 (29)	–
Colon	17.2 (17)	–
Ileum + Colon	53.5 (53)	–
Disease phenotype		
Inflammatory	27.3 (27)	–
Penetrating	34.3 (34)	–
Stricturing	38.4 (38)	–
Harvey–Bradshaw Index		
≤3	64.3 (63)	–
≥4	35.7 (36)	–
Previous surgery	49.5 (49)	
Current and past medication		
Previous corticotherapy	80.8 (80)	–
Metothrexate	6.2 (6)	–
Azathioprine	78.4 (76)	–
Infliximab	1.0 (1)	–

[a] *N* = 99 patients with Crohn's disease and 116 healthy controls.
[b] $\bar{x} \pm$ SD.

the remaining 63 (63.6%) had inactive disease. These activity indices refer to at least three visits during the 1-year period. Although controls were slightly older as compared to CD patients (49.7 ± 11.8 vs 40.4 ± 14.6; $p < 0.01$) gender distribution was similar in both groups (65F:51M vs 60F:39M).

For the genetic analysis of the population we studied the genotypic frequencies (Table 2). Except for the PPARγ+161C/T in controls, all polymorphisms were in Hardy–Weinberg equilibrium. When examining whether any of the polymorphisms studied had any influence in increasing the risk of developing Crohn's disease, we didn't observe any significant differences in odds ratio. The same applies to SNPs analyses and disease activity (Table 3), thereby suggesting that none of these SNPs alone, or in conjunction (data not shown) predispose to a more active phenotype. Also, no significant association was observed between SNPs analyzed and disease location, phenotype, age of disease onset or other characteristics of disease (Data not shown). Table 4 shows the association

Table 2
Genotypic frequencies. Odd ratio for disease susceptibility.[a]

Polymorphism	Genotypic frequencies case/control % (*N*)	O.R (95% C.I)[b]
CASP9 + 93 C/T		
CC	52.5 (52)/66.7 (76)	1
CT	43.4 (43)/31.6 (37)	1.64 (0.80–3.36) (Ns)
TT	4.0 (4)/1.8 (3)	1.03 (0.17–6.38) (Ns)
FASLG-843 C/T		
CC	35.4 (35)/28.1 (33)	1
CT	42.4 (42)/55.3 (64)	0.60 (0.27–1.29) (Ns)
TT	22.2 (22)/16.7 (19)	1.02 (0.38–2.73) (Ns)
PPARγPro12Ala		
CC	82.2 (74)/81.6 (95)	1
GC	16.7 (15)/16.7 (19)	1.04 (0.04–29.56) (Ns)
GG	1.1 (1)/1.8 (2)	0.96 (0.38–2.44) (Ns)
PPARγ+161C/T		
TT	93.8 (90)/100 (116)	–
CT	6.3 (6)/0	–
CC	0/0	

Ns, not significant.
[a] N = 99 patients in the case group and *N* = 116 in the control group.
[b] OR (Multiple Logistic Regression) was adjusted for age and gender.

Table 3
Influence of polymorphisms genotype on CD activity.[a]

Polymorphism	Inactive disease % (*N*)	Active disease % (*N*)	*p* value[b]	O.R (95% C.I)[c]
CASP9+93 C/T				
CC	34.7 (34)	18.4 (18)	Ns	1
CT	26.5 (26)	16.3 (16)		1.13 (0.48–2.65)
TT	3.1 (3)	1.0 (1)		0.68 (0.06–6.87)
FASLG-843 C/T				
CC	21.4 (21)	13.3 (13)	Ns	1
CT	26.5 (26)	16.3 (16)		1.03 (0.40–2.63)
TT	16.3 (16)	6.1 (6)		0.65 (0.20–2.13)
PPARγPro12Ala				
CC	53.9 (48)	29.2 (26)	Ns	1
GC	10.1 (9)	5.6 (5)		0 (0)
GG	1.1 (1)	0 (0)		1.19 (0.35–4.09)
PPARγ+161C/T				
TT	60.0 (57)	33.7 (32)	Ns	1
CT	3.2 (3)	3.2 (3)		2.33 (0.41–13.13)
CC	–	–		–

Ns, not significant.
[a] High activity was defined if Harvey-Bradshaw Index≥4.
[b] *P* values are from chi-square test.
[c] OR (Multiple Logistic Regression) was adjusted for age and gender.

between different types of fat intake and disease activity. We observed that a higher intake of total, saturated and monounsaturated fat was associated with a higher risk for active disease [O.R (95% C.I) 2.56 (1.08–6.03); O.R (95% C.I) 3.56 (1.46–8.65); O.R (95% C.I) 3.32 (1.38–7.95), respectively]. Also, patients with a higher n-6/n-3 PUFA intake had a significantly higher disease activity [O.R (95% C.I) 2.30 (1.02–5.30)]. Regarding the percentage of energy coming from dietary fat we have a mean value of 32.89%, with

Table 4
Influence of fat intake[a] on CD activity.[b]

	Inactive disease %(*N*)	Active disease % (*N*)	*p* value[c]	OR (95% CI)[d]
Total fat				
High	42.9 (27)	65.7 (23)	<0.01	2.56 (1.08–6.03)
Low	57.1 (36)	34.3 (12)		1
Saturated fat				
High	41.3 (26)	71.4 (25)	<0.01	3.56 (1.46–8.65)
Low	58.7 (37)	28.6 (10)		1
Monounsaturated fat				
High	39.7 (25)	68.6 (24)	<0.01	3.32 (1.38–7.95)
Low	60.3 (38)	31.4 (11)		1
Polyunsaturated fat				
High	46 (29)	57.1 (20)	Ns	1.56 (0.68–3.60)
Low	54 (34)	42.9 (15)		1
Trans fat				
High	44.4 (28)	60 (21)	Ns	1.86 (0.81–4.34)
Low	55.6 (35)	40 (14)		1
n-3 PUFA				
High	47.6 (30)	48.6 (17)	Ns	1.04 (0.45–2.38)
Low	52.4 (33)	51.4 (18)		1
n-6 PUFA				
High	44.4 (28)	60 (21)	Ns	1.88 (0.81–4.34)
Low	55.6 (35)	40 (14)		1
n-6/n-3 ratio				
High	47.6 (30)	57.1 (20)	0.04	2.30 (1.02–5.30)
Low	52.4 (33)	42.9 (15)		1

The cut-off points were for total fat = 76.7 g, saturated fat = 24.7 g, monounsaturated fat = 33.3 g, polyunsaturated fat = 11.5 g, trans fat = 0.7 g, and n-3 PUFA = 1.2 g; n-6 PUFA = 7.6 g; n-6/n-3 = 7.3.
Ns, not significant.
[a] Low or high intake refers to values above or under nutrient median intake.
[b] High activity was defined if Harvey-Bradshaw Index≥4.
[c] P values are from chi-square test.
[d] OR was determined using the values above or under nutrient median intake. OR (Multiple Logistic Regression) was adjusted for age and gender.

a range between 17.41 and 52.78, and we have observed a mean value of 32.81% for patients with inactive Crohn's disease and 33.27% for patients with a more aggressive disease, although these differences are not statistically significant ($p = 0.722$).

Interactions between dietary fat and polymorphisms of apoptotic genes on disease risk are shown in Table 5. Only significant associations are displayed. To increase statistical power in diet–gene association, risk was assessed combining the heterozygous and variant homozygous against the reference category of homozygous for the more frequent allele. In respect to total fat which *per se*, was already associated with a more active phenotype, we observed that the proinflammatory effect of a high total fat intake was more prominent in wild type carriers of the CASP9+93C/T polymorphism [O.R (95% CI) 4.64 (1.27–16.89)]. The same applies to a high trans fat intake [O.R (95% CI) 4.84 (1.34–17.50)]. In respect to the intake of saturated and monounsaturated fat, the magnitude of being associated with a more active phenotype was similar for both wild type as well as polymorphic allele carriers (Table 5). In regard to the PPARγPro12Ala SNP, we also observed that a high intake of saturated and monounsaturated fat was associated to a more active disease, but in wild type carriers only [OR (95%IC) 4.21 (1.33–13.26) and 4.37 (1.52–12.51), respectively]. Finally, a high intake of n-6 PUFA was associated with a more active phenotype, again in wild type carriers only for the FASLG-843C/T polymorphism [O.R (95% CI) 5.15 (1.07–24.74)].

4. Discussion

Previous studies have shown that in CD one of the basic pathogenic defects is resistance to apoptosis, namely of T-cells, which certainly contributes to perpetuate inflammation in the intestinal mucosa. It is also clear from previous studies that SNPs in various genes, namely apoptotic ones, may explain not only the heterogeneous phenotypes but also the different responses to similar treatments. Thus, Hlavaty and colleagues[16] recently demonstrated that polymorphisms in *FASLG/FAS* system and *CASP9* influence the response to Infliximab in luminal and fistulizing Crohn's disease.

Table 5
Influence of the interaction between dietary[a] and apoptotic polymorphisms in CD activity.[b]

	Polymorphism	
	Wild type	With variant
Total fat/CASP9+93C/T		
High	4.64 (1.27–16.89)	2.87 (0.79–10.52)
Low	1.0	2.23 (0.57–8.64)
Saturated fat/CASP9+93C/T		
High	6.14 (1.52–24.86)	5.14 (1.21–21.75)
Low	1.0	2.03 (0.47–8.70)
Monounsaturated fat/CASP9+93C/T		
High	3.48 (0.98–12.29)	3.78 (1.03–13.92)
Low	1.0	1.27 (0.32–5.07)
Trans fat/CASP9+93C/T		
High	4.84 (1.34–17.50)	2.27 (0.60–8.49)
Low	1.0	3.04 (0.80–11.54)
Saturated fat/PPARγ Pro12Ala		
High	4.21 (1.33–13.26)	2.57 (0.35–18.87)
Low	1.0	2.41 (0.45–12.89)
Monounsaturated Fat/PPARγ Pro12Ala		
High	4.37 (1.52–12.51)	1.66 (0.26–10.47)
Low	1.0	3.04 (0.54–17.14)
n-6 PUFA/FASLG-843C/T		
High	5.15 (1.07–24.74)	2.35 (0.54–10.11)
Low	1.0	2.26 (0.53–9.69)

Analyzed by multiple logistic regression. OR, adjusted for age and gender was determined using the values above or under nutrient median intake. The cut-off points were for total fat = 76.7 g, saturated fat = 24.7 g, monounsaturated fat = 33.3 g, trans fat = 0.7 g and n-6 PUFA = 7.6 g. Combined genotype CC +93 *CASP9*, CC Pro12Ala *PPARγ*, CC -843 *FASLG* and low intake was the reference category. All tests interactions showed $p > 0.05$.

[a] Low or high intake refers to values above or under nutrient median intake.

[b] High activity was defined if Harvey-Bradshaw Index≥4.

These observations were also the rationale to explore the associations between these same SNPs and dietary fat intake. A number of previous experimental and human studies have shown that dietary fat has the capacity of influencing cellular kinetics by interfering with crucial processes such as apoptosis induction, cell proliferation and cell differentiation. Thus, in an experimental study using cell lines, Llor *et al.*[8] showed that supplementation with fish oil and olive oil resulted in an induction of apoptosis which could be an explanation for the putative beneficial effect of these fatty acids in the treatment of CD.

Therefore, in the present study we examined whether there was any interaction between SNPs of apoptotic genes and dietary fat intake in modulating disease activity in CD patients.

When analyzing the effect of fat intake *per se* in modulating disease activity, we observed that a high intake of total, saturated and monounsaturated fat was associated with more active disease which is consistent with previous findings of Pischon *et al.*[17], where the results suggest that the combination of both n-6 and n-3 types of fatty acids is associated with the lowest levels of inflammation. In contrast, SNPs of apoptotic genes did not show any significant association with increased susceptibility to develop CD or to exhibit a more active phenotype.

Caspase-9 (1p36) is an apoptosis-related cystein protease, which upon binding with cytochrome *c* and Apaf-1 forms an apoptosome complex and activates the executive caspases 3, 6 and 7.[18] Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis.[18] To our knowledge the functionality of this polymorphism is not yet known. The fact that, in the present study we observed that a high intake of total and trans fat was associated with a more active phenotype, mainly in wild type carriers, leads us to hypothesize that wild type carriers might exhibit more resistance to apoptosis and, therefore there would be a synergism between two potentially harmful factors. This is also consistent with the observations by Hlavaty *et al.*[16] who observed that homozygotes for the polymorphic allele had a better response to Infliximab. However, this might not be straightforward as a high intake of saturated and monounsaturated fat, which by themselves are already associated to a more active phenotype, have comparable effect both in wild type as well as in polymorphic allele carriers.

PPARγ (3p25) encodes a member of the peroxisome proliferator-activated receptor (*PPAR*) subfamily of nuclear receptors.[19] Rare *PPARγ* polymorphisms were found to be associated with human Crohn's disease.[19] *PPARγ* inhibits NFκB activity, which forms part of a central signaling pathway that stimulates the transcription of multiple genes that encode proinflammatory molecules, and diets enriched with n-3 fatty acids are powerful *PPARγ* activators, which may be a plausible explanation for the anti-inflammatory effects of these fatty acids.[20] A common structural polymorphism in the *PPARγ* gene, exon 1 (CCA-GCA, producing a Pro→Ala substitution at codon 12), has been described.[20] This amino acid is located in the *PPARγ* domain that enhances ligand-independent activation. The Pro→Ala change may cause a conformational change in the protein,[21] and thus patients wild type carriers of this polymorphism would have less inhibition of NFκB pathway and therefore would exhibit a more aggressive and active phenotype. Thus, our results are consistent with the observations that a synergism between a high intake of saturated and monounsaturated fat exists, thereby leading to a more active disease.

In regard to FAS receptor (*FAS*, *CD95*) and FAS ligand (*FASLG*, *CD95LG*) these are complementary members of a particular

apoptotic pathway which play a major role in immune regulation.[18] Recent studies suggest that *FAS*-mediated apoptosis is involved in the pathogenesis of IBD.[22,23] *FASLG* (1q23) is a key apoptosis inducing ligand of the *TNF* family of death factors.[18] The FASLG-843C/T polymorphism is located in the promoter region in a binding site for the CAAT enhancer protein.[24] A recent study reported that carriers of the C allele of this polymorphism have a threefold increased binding capacity to the CAAT enhancer protein and subsequently a threefold higher expression of *FASLG* that leads to an increase of apoptosis of the active cells which are expressing *FAS*.[16,24] Thus, wild type carriers would be more susceptible to apoptosis and, theoretically, exhibit a less severe phenotype. We observed that a high intake of n-6 PUFA, which is known to promote inflammation, was more deleterious in wild type carriers, as opposed to what could be expected. The reasons for these inconsistencies are not clear but it is worth recalling that interactions between nutrients and genes may be extremely complex. Furthermore, there are at least two main pathways of apoptosis. The extrinsic pathway induced by so-called death ligands, i.e. *FAS* dependent, and the intrinsic one. We may hypothesize that n-6 PUFA influence cellular apoptosis by interfering in the intrinsic pathway which does not involve the *FASLG*. On the other hand, Grimble *et al.*[10] when examining in a group of healthy individuals the effect of a fish oil emulsion on cytokine production, observed that this suppressive effect was maximal when the basal production of cytokines was also maximal. Theoretically, the latter group would correspond to the most aggressive phenotype and therefore a beneficial effect could be harder to obtain, as opposed to what was observed. Therefore, a number of different hypotheses may have arisen to explain the results observed.

To our knowledge this is the first study examining the interaction between dietary fat intake and polymorphisms of apoptotic genes. Both are known to affect the rate of apoptosis in inflammatory cells which is known to be a major defect in the pathogenesis of CD. However, this is an evolving field and the long accepted concept that saturated fat had a deleterious effect on chronic inflammation, is now called into question, since a recent experimental study showed that MCT oil, which is a saturated lipid, might prevent a form of colitis in an animal model.[7] Thus, future studies examining the roles that these several types of dietary fat might exert in the treatment of CD according to specific genotypes, will be most welcome.

Conflict of interest

Potential competing interests: None of the authors had any financial or personal conflicts of interest related to this manuscript.

Statement of authorship

Specific author contributions: Ferreira P.: study design, data collection and analysis, polymorphism analysis and writing of the manuscript; Cravo M.: study design, patient inclusion from Instituto Português de Oncologia and clinical data collection, data analysis and writing of the manuscript; Guerreiro C.S.: nutritional evaluation of patients, data collection, data analysis and manuscript preparation; Tavares L and Moura Santos P.: patient inclusion from Hospital Santa Maria and clinical data collection and Brito M.: polymorphism analysis and manuscript preparation.

Acknowledgment

Financial support: This study was partly funded by a grant from Sociedade Portuguesa de Gastrenterologia.

References

1. Ahmad T, Armuzzi A, Bunce M, Mulcahy-Hawes K, Marshall SE, Orchard TR, et al. The molecular classification of the clinical manifestations of Crohn's disease. *Gastroenterology* 2002;**122**:854–66.
2. Satsangi J, Morecroft J, Shah NB, Nimmo E. Genetics of inflammatory bowel disease: scientific and clinical implications. *Best Pract Res Clin Gastroenterol* 2003;**17**(1):3–18.
3. Louis E, Satsangi J, Roussomoustakaki M, Parkes M, Fanning G, Welsh K, et al. Cytokine gene polymorphisms in inflammatory bowel disease. *Gut* 1996;**39**:705–10.
4. Souza HS, Tortori CJ, Castelo-Branco MT, Carvalho AT, Margallo VS, Delgado CF, et al. Apoptosis in the intestinal mucosa of patients with inflammatory bowel disease: evidence of altered expression of FasL and perforin cytotoxic pathways. *Int J Colorectal Dis* 2005;**20**:277–86.
5. Teitelbaum JE, Walker WA. Review: the role of omega 3 fatty acids in intestinal inflammation. *J Nutr Biochem* 2001;**12**:21–32.
6. Gassull MA, Fernández-Bañares F, Cabré E, Papo M, Giaffer MH, Sánchez-Lombraña JL, et al. Fat composition may be a clue to explain the primary therapeutic effect of enteral nutrition in Crohn's disease: results of a double blind randomized multicentre European trial. *Gut* 2002;**51**:164–8.
7. Mañé J, Pedrosa E, Lorén V, Ojanguren I, Fluvià L, Cabré E, et al. Partial replacement of dietary (n-6) fatty acids with medium-chain triglycerides decreases the incidence of spontaneous colitis in interleukin-10-deficient mice. *J Nutr* 2009;**139**(3):603–10.
8. Llor X, Pons E, Roca A, Alvarez M, Mañé J, Fernández-Bañares F, et al. The effects of fish oil, olive oil, oleic acid and linoleic acid on colorectal neoplastic processes. *Clin Nutr* 2003;**22**(1):71–9.
9. Sadeghi S, Wallace FA, Calder PC. Dietary lipids modify the cytokine response to bacterial lipopolysaccharide in mice. *Immunology* 1999;**96**:404–10.
10. Grimble R, Howell W, O'Reilly G, Turner S, Markovic O, Hirrell S, et al. The ability of fish oil to suppress tumour necrosis factor x production by peripheral blood monuclear cells in healthy men is associated with polymorphisms in genes that influence tumour necrosis factor α production. *Am J Clin Nutr* 2002;**76**:454–9.
11. Guerreiro CS, Ferreira P, Tavares L, Santos PM, Neves M, Brito M, et al. Fatty acids, IL-6 and TNF-α polymorphisms: an example of nutrigenetics in Crohn's disease. *Am J Gastroenterol* 2009;**104**(9):2241–9.
12. Lennard-Jones JE. Classification of inflammatory bowel disease. *Scand J Gastroenterol* 1989;**24**(170):2–6.
13. Harvey RF, Bradshaw JM. A simple index of Crohn's-disease activity. *Lancet* 1980;**1**(8167):514.
14. Sambrook J, Maniatis T, Fritsch EF. *Molecular cloning: a laboratory manual*. 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; 1989.
15. Lopes C.M. Reprodutibilidade e validação do questionário semiquantitativo de frequência alimentar. In: alimentação e Enfarte agudo do miocárdio: estudo de caso-controlo de base comunitária. Tese de Doutoramento Porto 2000;pp. 78–115.
16. Hlavaty T, Pierik M, Henckaerts L, Ferrante M, Joossens S, van Schuerbeek N, et al. Polymorphisms in apoptosis genes predict response to infliximab therapy in luminal and fistulizing Crohn's disease. *Aliment Pharmacol Ther* 2005;**22**:613–26.
17. Pischon T, Hankinson SE, Hotamisligil GS, Rifai N, Willett WC, Rimm EB, et al. Habitual dietary intake of n-3 and n-6 fatty acids in relation to inflammatory markers among US men and women. *Circulation* 2003;**108**:155–60.
18. Srivastava R. *Apoptosis, cell signaling, and human diseases – molecular mechanisms*, vol. 2. Totowa, New Jersey: Humana Press Inc.; 2007.
19. Fajas L, Auboeuf D, Raspé E, Schoonjans K, Lefebvre AM, Saladin R, et al. The organization, promoter analysis, and expression of the Human PPARg Gene. *J Biol Chem* 1997;**272**(30):18779–89.
20. Debril MB, Renaud JP, Fajas L, Auwerx J. The pleiotropic functions of peroxisome proliferator-activated receptor γ. *J Mol Med* 2001;**79**:30–47.
21. Gong Z, Xie D, Deng Z, Bostick RM, Muga SJ, Hurley TG, et al. The PPARg Pro12Ala polymorphism and risk for incident sporadic colorectal adenomas. *Carcinogenesis* 2005;**26**(3):579–85.
22. Xia B, Yu YH, Guo QS, Li XY, Jiang L, Li J. Association of Fas-670 gene polymorphism with inflammatory bowel disease in Chinese patients. *World J Gastroenterol* 2005;**11**(3):415–7.
23. Bu P, Keshavarzian A, Stone DD, Liu J, Le PT, Fisher S, et al. Apoptosis: one of the mechanisms that maintains unresponsiveness of the intestinal mucosal immune system. *J Immunol* 2001;**166**:6399–403.
24. Wu J, Metz C, Xu X, Abe R, Gibson AW, Edberg JC, et al. A novel polymorphic CAAT/enhancer-binding protein β element in the *FasL* gene promoter alters FasLigand expression: a candidate background gene in African American systemic Lupus Erythematosus patients. *J Immunol* 2003;**170**:132–8.

CHAPTER III – PHARMACOGENETICS

III.1 PAPER 2

Cravo M., Ferreira P., Sousa P., Moura-Santos P., Velho S., Tavares L., Ramos Deus J., Ministro P., Pereira da Silva J., Correia L., Velosa J., Maio R. and Brito M. (2014). Clinical and genetic factors predicting response to therapy in patients with Crohn's disease. *United European Gastroenterology Journal.* **2**(1):47-56.

III.2 PAPER 3

Cravo M., Ferreira P., Sousa P., Moura-Santos P., Velho S., Tavares L., Ramos Deus J., Ministro P., Peixe P., Correia L., Velosa J., Maio R. and Brito M. (2014). Il23R polymophisms influence phenotype and response to therapy in patients with Ulcerative colitis. *European Journal of Gastroenterology & Hepatology.* **26**(1):26-32.

III.3 PAPER 4

Ferreira P., Cravo M. and Brito M. (2014). Is it personalized therapy around the corner for IBD patients? A review and a perspective from a Portuguese population. Submitted

UNITED EUROPEAN GASTROENTEROLOGY ueg journal

Original Article

United European Gastroenterology Journal
2014, Vol. 2(1) 47–56

DOI: 10.1177/2050640613519626
ueg.sagepub.com
SAGE

Clinical and genetic factors predicting response to therapy in patients with Crohn's disease

Marilia Cravo[1,2], Paula Ferreira[3], Patricia Sousa[4], Paula Moura-Santos[2,4], Sonia Velho[1], Lurdes Tavares[4], João Ramos Deus[5], Paula Ministro[6], João Pereira da Silva[7], Luis Correia[2,4], Jose Velosa[2,4], Rui Maio[1] and Miguel Brito[3]

Abstract

Aim: To identify clinical and/or genetic predictors of response to several therapies in Crohn's disease (CD) patients.

Methods: We included 242 patients with CD (133 females) aged (mean ± standard deviation) 39 ± 12 years and a disease duration of 12 ± 8 years. The single-nucleotide polymorphisms (SNPs) studied were *ABCB1* C3435T and G2677T/A, *IL23R* G1142A, C2370A, and G9T, *CASP9* C93T, *Fas* G670A and LgC844T, and *ATG16L1* A898G. Genotyping was performed with real-time PCR with Taqman probes.

Results: Older patients responded better to 5-aminosalicylic acid (5-ASA) and to azathioprine (OR 1.07, $p = 0.003$ and OR 1.03, $p = 0.01$, respectively) while younger ones responded better to biologicals (OR 0.95, $p = 0.06$). Previous surgery negatively influenced response to 5-ASA compounds (OR 0.25, $p = 0.05$), but favoured response to azathioprine (OR 2.1, $p = 0.04$). In respect to genetic predictors, we observed that heterozygotes for *ATGL16L1* SNP had a significantly higher chance of responding to corticosteroids (OR 2.51, $p = 0.04$), while homozygotes for *Casp9* C93T SNP had a lower chance of responding both to corticosteroids and to azathioprine (OR 0.23, $p = 0.03$ and OR 0.08, $p = 0.02$,). TT carriers of *ABCB1* C3435T SNP had a higher chance of responding to azathioprine (OR 2.38, $p = 0.01$), while carriers of *ABCB1* G2677T/A SNP, as well as responding better to azathioprine (OR 1.89, $p = 0.07$), had a lower chance of responding to biologicals (OR 0.31, $p = 0.07$), which became significant after adjusting for gender (OR 0.75, $p = 0.005$).

Conclusions: In the present study, we were able to identify a number of clinical and genetic predictors of response to several therapies which may become of potential utility in clinical practice. These are preliminary results that need to be replicated in future pharmacogenomic studies.

Keywords

Crohn's disease, clinical genetic predictors, response to therapy

Received: 6 August 2013; accepted: 14 December 2013

Introduction

Crohn's disease (CD) is a chronic and relapsing condition, with the majority of patients experiencing a progressive and disabling course over time.[1,2] Management of disease is complex and should take into account several factors such as disease location, activity, and behaviour.[3] Additional disease aspects that make management difficult are the lack of correlation between severity of clinical symptoms and severity of intestinal inflammation and the difficulty in predicting a patient's clinical course and the individual response to a given treatment.

According to ECCO guidelines, 5-aminosalicylic acid (5-ASA) compounds could be used to treat patients with mild-to-moderate ileocecal disease, whereas the use of azathioprine/6-mercaptopurine and/or anti-tumour necrosis factor (TNF) therapy is

[1]Hospital Beatriz Angelo, Loures, Portugal
[2]Faculdade de Medicina da Universidade de Lisboa, Lisboa, Portugal
[3]Escola Superior de Tecnologias da Saude, Lisbon, Portugal
[4]Hospital Santa Maria, Lisboa, Portugal
[5]Hospital Fernando da Fonseca, Amadora, Portugal
[6]Hospital S Teotónio, Viseu, Portugal
[7]Instituto Portugués de Oncologia, Lisboa, Portugal

Corresponding author:
Marilia Cravo, Hospital Beatriz Angelo, Av Carlos Teixeira n3, Loures, 2674–514, Portugal.
Email: marilia.cravo@sapo.pt

recommended for patients with moderate to severe disease who relapse after responding to steroids.[3]

In the past years, numerous efforts have been made with the aim of identifying early predictors of aggressive disease[4–6] because, intuitively, patients presenting indicators of bad outcome at diagnosis would be the ideal candidates for early and more aggressive therapy in order to avoid long-term structural damage. However, patients who present with disease bad prognostic factors are not necessarily those who will respond better or worse to certain therapies. Although tailored therapy in CD is still far from reality, reliable prediction of response to treatment, combined with clinical indicators of aggressive disease, could allow a better selection of candidates for treatment, allowing the choise of not only the most effective therapies but also the less toxic ones. It is certainly important to remember that, according to previous studies,[7,8] 30% of CD patients will have a mild disease course and will never need corticosteroids.

Previous studies have mainly focused on clinical predictors of response to biologicals. Short duration of disease is probably the single most important factor of response to anti-TNF agents and perhaps to azathioprine as well.[9–11] Some found better responses to biologicals in nonsmoking patients with recent onset and colonic disease, while others could not confirm these observations.[12–15] A high value of basal C-reactive protein also seems to be a predictive factor of response to biologicals.[16]

As well as clinical and laboratory predictors, genetics could also influence patients' individual response to a drug.[17,18] So far, pharmacogenomic research in inflammatory bowel disease has witnessed only modest success and sometimes conflicting results, mainly because response to treatment in this disease is very heterogeneous as it is influenced by many factors, such as disease duration, behaviour, and severity, which certainly interact with genetic variables and modulate response to treatment.[19–21] While some authors reported that carriers of *ABCB1* single-nucleotide polymorphisms (SNPs) present a worse response to azathioprine or corticosteroids, others could not confirm these observations.[22–25] *ABCB1* codes for the ATP-dependent membrane efflux transporter P-glycoprotein 1, which is expressed in various cells including in those in the gastrointestinal tract, which is responsible for resistance to a number of structurally and functionally unrelated drugs. *IL23R* has been recently implicated in the pathogenesis of CD. Although previous studies found that SNPs in this gene could increase susceptibility to develop CD,[26] few studies explored the association between these SNPs and phenotype or response to therapy in patients with inflammatory bowel disease.[18,27,28] *Caspase9*, *Fas*, and *fas ligand* encode for proteins involved in apoptosis, which has been shown to be defective in CD. Hlavaty et al.[29,30] observed that carriers of SNPs of genes involved in apoptosis, responded less frequently to biologicals. Finally, *ATG16L1* is involved in autophagy, a key pathway for innate immunity and important for maintaining the epithelial barrier. Several drugs already used in the treatment of CD might exert at least part of their effect through the regulation of autophagy.[31] A recently published study found an association between SNPs in this gene and response to corticosteroids, azathioprine, and biologicals.[32]

The primary aim of the present study was to identify clinical and/or genetic factors that, alone or in combination, could predict response of CD patients to several therapies. Our secondary end point was to distinguish between those patients who will be in remission with azathioprine only from those who will need escalation to anti-TNF agents.

Materials and methods

This was a multicentre study with participating hospitals from Central Portugal. Informed consent was obtained from all patients entering the study, which was approved by Scientific and Ethical committees of the several participating hospitals.

Patients with the diagnosis of CD[33] were classified according to the Montreal classification[34] based on age at diagnosis (A), location (L), and behaviour (B). Disease modifiers were also considered: L4 when the upper digestive tract was involved and P for perianal involvement. No families with CD were included in the present series. Phenotypic characteristics retrospectively collected from charts included demographic data, age of disease onset, disease extension, and behaviour, time of follow up, smoking habits, presence of extraintestinal manifestations, and previous therapies including surgery. All phenotypic data were collected in a blinded fashion to the results of the genotypic data.

Patients were selected to enter the study if a definitive classification in terms of response to a specific drug could be clearly obtained after reviewing the chart and interviewing the patient at the moment of entering the study. Patients were considered responders if they presented long-term sustained remission, defined as a Harvey Bradshaw Index (HBI) lower than 4, lasting at least 1 year after a certain therapy was started, not needing steroids, surgery, or escalation of therapy to biologicals for those taking azathioprine or switch to another biological (infliximab vs. adalimumab) for those on biologicals. Patients requiring the addition or switching to other therapies, corticosteroids or surgery before 1 year were considered nonresponders. If a clear distinction between these two scenarios was not

possible for a specific drug, the patient was not considered either as a responder or as a nonresponder at least for this drug. Because our secondary end point was to distinguish between patients who responded to azathioprine from those who came to require biologicals, if a patient had a clinical remission for less than 1 year with azathioprine but came to require biologicals, he or she was considered a nonresponder to azathioprine.

Decision to switch therapy was made by the treating physician. HBI was calculated after a new therapy was started until the drug was discontinued or until end of follow up. Optimization of dose and/or interval of administration in patients on biologicals, was considered as therapy optimization and not as nonresponse. Biological parameters such as C-reactive protein levels or endoscopic response were not used to classify patients as responders or nonresponders because identification of these data on the precise moment on which a specific therapy was started was not clear in a large number of patients.

For steroids, only short-term response was considered. Steroid dependence was defined as recurrent flare up on withdrawal of glucocorticoids or as the need for glucocorticoids treatment twice within 6 months. Patients were considered refractory to steroids when no remission was obtained with a dose of 1 mg/kg during a period of at least 4 weeks.

DNA extraction and genotyping

Blood samples were taken from all study participants, and genomic DNA was isolated from peripheral blood using a DNA blood mini kit from Quiagen (Hiden, Germany) according to the manufacturer's guidelines. A total of 10 SNPs were studied: *ABCB1* C3435T (rs1045642) and G2677T/A (rs2032582), *IL23R* G1142A (rs11209026), C2370A (rs10889677), G43045A (rs 1004819) and G9T (rs1884444), *CASP9* C93T (rs4645983), *Fas* G670A (rs1800682), *Faslg* C844T (rs763110), and *ATG16L1* A898G (rs2241880). All genotypes were determined using real-time PCR with TaqMan Pre-Designed SNP Genotyping Assays (Applied Biosystems, USA), except for *Fas* G670A and *FasLg* C844TT, which were determined using Custom TaqMan SNP genotyping Assays probes (Applied Biosystems). To perform the genotype analysis, the target fragments were amplified in 20-µl reaction mixture containing 10 µl TaqMan Universal PCR Master Mix, 1 µl primers, 5 µl Milli-Q water, and 4 µl DNA. Real-time PCR was conducted using a iCycler iQ® Multicolor Real-Time PCR Detection System (BIO-RAD) with the following thermal cycling program: 10 min at 95°C and 50 cycles of 15 s at 92°C and 1 min at 60°C.

Statistical analysis

Statistical analysis was performed using SPSS version 14.0 (SPSS, Chicago, IL, USA) and SNPassoc 1.6 package in R software. Data were expressed as mean ± standard deviation, number of subjects (%), or odds ratio (OR) with 95% confidence interval (CI). Primary analyses were performed using chi-squared test and univariate logistic regression. Multivariate logistic regression was performed to adjust for potential confounding variables. Receiver operating characteristic (ROC) curves were plotted for multivariate models, and sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and area under the curve (AUC) were calculated. Whenever the AUC value was under 0.65, the model performance was considered unacceptably low and data are not shown. Association between outcome and SNP was analysed with SNPassoc library in R and SPSS 19 (IBM SPSS statistics). Different inheritance models (dominant, recessive, log-add, and overdominant) were considered and were presented as eligible. Haplotype analysis was performed for *ABCB1* C3435T and *ABCB1* G2677T/A. To adjust for multiple testing, a Bonferroni correction was applied. Statistical significance was established for $p < 0.05$. Differences in genotypic and allelic frequencies and Hardy–Weinberg tests were performed using GENEPOP Web version 4.0.10 program. To obtain the exact p-value of the Hardy–Weinberg equilibrium, the Markov Chain method (Guo and Thompson, 1992) with a dememorization number of 1000, 100 batches, and 1000 iterations per batch was used. The p-value returned by this method is calculated as the sum of the probabilities of all tables and its standard error. Genotypic frequencies are under Hardy–Weinberg equilibrium when $p > 0.001$

Results

A total of 242 CD patients were eligible to enter the study; mean follow-up period was 2.5 years. There were no patients treated according to the top-down strategy. Because the criteria used to consider patients as responders or nonresponders required sustained clinical remission for 1 year or more, there were only four patients who were considered responders simultaneously to azathioprine and biologicals. The demographic, clinical, and response to therapy data are shown on Table 1.

Allelic and genotypic frequencies

Allelic and genotypic frequencies for all the studied SNPs are shown in Table 2. The genotypic frequencies of all SNPs did not deviate significantly from those

United European Gastroenterology Journal 2(1)

Table 1. Demographic and clinical data from patients included in the study

Characteristic	Study population ($n=242$)
Gender	
Male	109 (45)
Female	133 (55)
Age (years)	39 ± 12.8
Duration of disease (years)	11.8 ± 8.4
Duration of follow up (years)	2.5 ± 3.2
Age of disease onset	
A1	28 (12)
A2	183 (75)
A3	31 (13)
Location of disease	
L1	54 (22)
L2	48 (20)
L3	140 (58)
L4	20 (8)
Behaviour of disease	
B1	110 (45)
B2	93 (39)
B3	39 (16)
Perianal involvement	90 (39)
Previous surgery	
Yes	64 (26)
No	178 (74)
Perianal procedures	38 (16)
Smoking habits	
Yes	66 (27)
No	141 (58)
Unknown	35 (14)
Extraintestinal manifestations	
Yes	79 (33)
No	163 (67)
5-Aminosalicylic acid	
Yes	29 (48/12)
No	31 (52)
Corticosteroids	
Yes	107 (74/44)
No	37 (26)[a]
Azathioprine	
Yes	90 (56/41)
No	70 (44)
Biologicals	
Yes	81 (81/33)
No	19 (19)

Values are *n* (%), mean ± standard deviation, or % responders to drug/% responders of total.

[a]Response to corticosteroids refers to short-term (1 month) response. Of these 37 patients, seven were refractory to corticosteroids and 30 were corticodependent.

Table 2. Genotypic frequencies, allelic frequencies, and Hardy-Weinberg equilibrium

Polymorphism	Genotypes		Alleles	Hardy-Weinberg equilibrium
ABCB1 C3435T				
CC ($n=71$)	0.298			
CT ($n=100$)	0.420	C	0.508	
TT ($n=67$)	0.282	T	0.492	$p=0.0147$
ABCB1 G2677T/A				
GG ($n=66$)	0.282			
GT + GA ($n=119$)	0.517	G	0.527	
TT + AA + TA ($n=53$)	0.202	T/A	0.473	$p=1.0000$
IL23R G1142A				
GG ($n=193$)	0.801			
GA ($n=47$)	0.195	G	0.898	
AA ($n=1$)	0.400	A	0.102	$p=0.4826$
IL23R C2370A				
CC ($n=93$)	0.389			
CA ($n=114$)	0.477	C	0.628	
AA ($n=32$)	0.134	A	0.372	$p=0.8902$
IL23R G9T				
TT ($n=58$)	0.244			
TG ($n=119$)	0.500	T	0.494	
GG ($n=61$)	0.256	G	0.506	$p=1.0000$
IL23R C/T				
CC ($n=83$)	0.353			
CT ($n=123$)	0.523	C	0.615	
TT ($n=29$)	0.123	T	0.385	$p=0.1353$
Casp9 C93T				
CC ($n=131$)	0.567			
CT ($n=86$)	0.372	C	0.753	
TT ($n=29$)	0.610	T	0.247	$p=1.0000$
Fas G670A				
GG ($n=44$)	0.191			
GA ($n=120$)	0.522	G	0.452	
AA ($n=66$)	0.287	A	0.548	$p=0.5047$
Fas LgC844T				
CC ($n=73$)	0.319			
CT ($n=104$)	0.454	C	0.546	
TT ($n=52$)	0.227	T	0.454	$p=0.2317$
ATG16L1 A898G				
AA ($n=43$)	0.297			
AG ($n=100$)	0.437	A	0.406	
GG ($n=86$)	0.266	G	0.594	$p=0.1750$

There was no deviation from Hardy-Weinberg equilibrium.

expected under Hardy–Weinberg equilibrium ($p > 0.001$).

Associations between genetic polymorphisms and phenotypic characteristics

We observed that carriers for *IL23R* G9T and *IL23R* C2370A SNPs had less frequently upper GI involvement as compared to wild-type carriers (OR 0.4, 95% CI 0.2–0.82, $p = 0.008$, and OR 0.25, 95% CI 0.06–0.86, $p = 0.03$, respectively). Also, TT carriers for *FasLg* C844T SNP exhibited more often an inflammatory behaviour (B1) (OR 0.38, 95% CI 0.18–0.82, $p = 0.014$). No other significant associations were observed between the remaining SNPs and disease characteristics.

Associations between clinical characteristics, SNPs, and response to therapy

An analysis of associations between clinical variables and response to several drugs according to univariate analysis was performed. Only significant or near significant associations are displayed in Table 3.

We observed that older patients and those with a longer duration of disease responded better to 5-ASA compounds (OR 1.07, $p = 0.003$ and OR 1.16, $p = 0.002$, respectively). On multivariate analysis, after adjusting for age and previous surgery, disease duration remained statistically significant (OR 1.21, $p = 0.005$); however, age was no longer statistically significant. The same trend was observed in respect to response to azathioprine and age (OR 1.03, $p = 0.01$), which means that there is a 3% increase in the chance of responding to azathioprine for each additional year of life. In respect to biologicals, an opposite trend was observed with a higher chance of response in younger patients ($p = 0.06$) and with more recent disease onset ($p = 0.008$). However, on multivariate analysis, both age and duration of disease lost significance.

Previous surgery negatively influenced response to 5-ASA compounds (OR 0.25, $p = 0.05$), while the opposite effect was observed in regard to azathioprine (OR 2.1, $p = 0.04$) (Table 3). In respect to perianal involvement, we observed that these patients did significantly worse on corticosteroids (OR 0.32, $p = 0.006$) (Table 3). No significant associations were found between disease behaviour, disease location including L4 involvement, smoking habits, presence of extraintestinal manifestations, and response to various therapies. Stricturing and penetrating phenotypes (B2 and B3, respectively) were more often associated with surgical therapy in contrast with inflammatory phenotype (B1) (OR 18.3, 95% CI 7.5–54.8, $p < 0.0001$).

Interestingly, previous therapies seemed to influence response to therapy in various ways (Table 3). Thus, previous 5-ASA users responded better to biologicals, although not reaching statistical significance (OR 2.86, 95% CI 0.90–8.94, $p = 0.06$). Previous corticosteroid therapy negatively influenced response to 5-ASA (OR 0.05, 95% CI 0.003–0.333, $p = 0.008$). Finally, previous use of biologicals decreased the chance of responding

Table 3. Association between clinical phenotypic characteristics and response to therapy according to univariate analysis

Characteristic	5-ASA ($n = 60$)	Corticosteroids ($n = 144$)	Azathioprine ($n = 160$)	Biologicals ($n = 100$)
Responders/nonresponders	29/31	107/37	90/70	81/19
Age	1.07 (1.02–1.14) $p = 0.003$	–	1.03 (1.006–1.06) $p = 0.01$	0.95 (0.90–1.00) $p = 0.06$
Duration of disease	1.16 (1.07–1.31) $p = 0.002$	–	–	0.91 (0.85–0.98) $p = 0.008$
Previous surgery	0.25 (0.05–0.96) $p = 0.05$	–	2.1 (1.04–4.49) $p = 0.04$	–
Perianal disease	–	0.32 (0.14–0.72) $p = 0.006$	–	–
Previous 5-ASA	–	–	–	2.86 (0.90–8.94) $p = 0.06$
Previous steroids	0.05 (0.03–0.33) $p = 0.008$	–	–	–
Previous biologicals	–	0.26 (0.11–0.60) $p = 0.002$	0.05 (0.02–0.13) $p = 1.26 \times 10^{-9}$	–

Values are OR (95% CI). OR < 1.00, less responsive; OR > 1.00, better response. Table only displays significant or near significant results.
5-ASA, 5-Aminosalicylic acid.

Table 4. Association between genetic polymorphism and response to therapy according to univariate analysis

Polymorphism and genotype	5-ASA ($n=60$)	Corticosteroids ($n=144$)	Azathioprine ($n=160$)	Biologicals ($n=100$)
Responders/nonresponders	29/31	107/37	90/70	81/19
IL23R G9T: GT	0.29 (0.08–1.03) $p=0.06$	–	–	3.06 (0.91–10.4) $p=0.06$
Casp9 C93T: TT	–	0.23 (0.36–0.88) $p=0.03$	0.08 (0.004–0.51) $p=0.02$	–
ABCB1 C3435T: TT	–	–	2.38 (1.13–5.02) $p=0.01$	–
ABCB1 G2677T/A: TT vs. G carrier	–	–	1.89 (0.94–3.81) $p=0.07$	0.31 (0.08–1.07) $p=0.07$

Values are OR (95% CI). OR < 1.00, less responsive; OR > 1.00, better response. Table only displays significant or near significant results.
5-ASA, 5-Aminosalicylic acid.

both to corticosteroids (OR 0.26, $p=0.002$) and to azathioprine (OR 0.05, $p=1.26\times10^{-9}$).

The associations between genetic polymorphisms and response to therapy on univariate analysis are shown on Table 4. We observed that heterozygotes for the polymorphic allele of *IL23R* G9T had a lower chance of responding to 5-ASA compounds (OR 0.29, $p=0.06$) but a higher one of responding to biologicals (OR 3.06, $p=0.06$).

Homozygotes for *Casp9* C93T SNP had a significantly lower chance of responding both to corticosteroids and to azathioprine (OR 0.23, $p=0.03$ and OR 0.08, $p=0.02$, respectively; Table 4). After adjusting for previous response to biologicals, gender, and *ATGL16L1*, the probability of responding to corticosteroids was even further decreased (OR 0.14, 95% CI 0.03–0.71 $p=0.014$). A ROC curve was plotted for the previous model: 71.1% sensibility, 71.9% specificity, 54.9% PPV, 11.5% NPV, and an AUC of 0.778 was obtained (Figure 1).

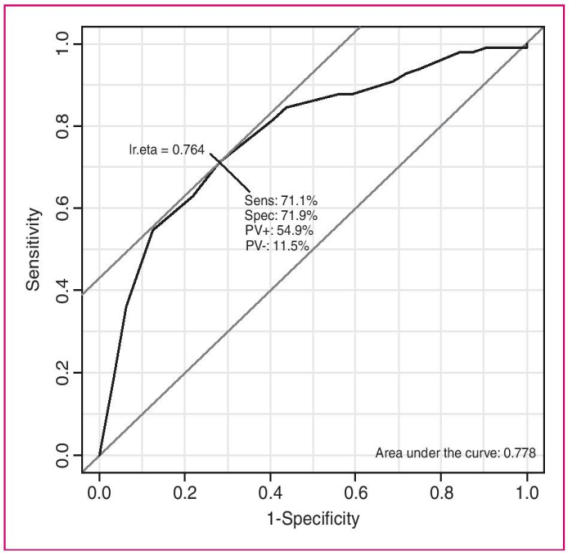

Figure 1. Receiver operating characteristic curve for model assessment of the *Casp9* C93T SNP
Multivariate logistic regression model: dependent variable, therapy response to corticosteroids; independent variables, *Casp9* C93T SNP, gender, previous therapy with infliximab, and ATGL16L1.

In respect to the *ABCB1* C3435T SNP, we observed that TT carriers had a significantly higher chance of responding to azathioprine (OR 2.38, $p=0.01$; Table 4). After adjusting for gender distribution, we observed that this association remained significant (OR 2.4, 95% CI 1.13–5.03 $p=0.019$) and became even more significant after further adjustment for age and smoking habits (OR 3.22, 95% CI 1.13–5.03 $p=0.005$). Model assessment with a ROC curve generated a 60,5% sensibility, 73% specificity, 39.5% PPV, 27% NPV, and an AUC of 0.687 (Figure 2).

The same was observed for the polymorphic allele carriers of the other SNP of this gene (*ABCB1* G2677T/A), although not reaching statistical significance (OR 1.89, $p=0.07$). Carriers of both haplotypes had a higher chance of responding to azathioprine (OR 1.53, 95% CI 0.94–2.49, $p=0.08$). The *ABCB1* G2677T/A SNP, as well as responding better to azathioprine, had a significantly lower chance of responding to biologicals (OR 0.31, $p=0.07$), which became significant after adjusting for gender (OR 0.75, 95% CI 0.24–0.63, $p=0.005$). Adjusting for duration of disease also increased the strength of this association (OR 0.23, 95% CI 0.07–0.70, $p=0.01$). When considering the ROC curve for the former model, 70.1% sensibility, 63.2% specificity, 65.7% PPV, 11.5% NPV, and an AUC of 0.714 was obtained (Figure 3).

We also examined whether there was any interaction between clinical and genetic predictors of response. As shown in Table 5, we observed that carriers for both *ABCB1* SNPs responded better to azathioprine only if

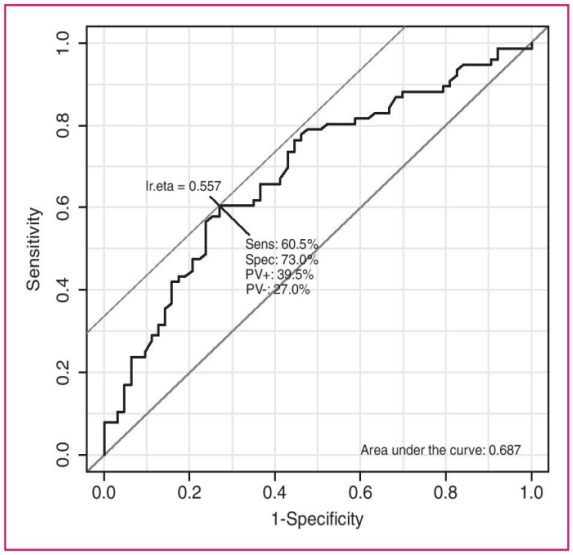

Figure 2. Receiver operating characteristic curve for model assessment of the *ABCB1* C3435T SNP
Multivariate logistic regression model: dependent variable, therapy response to azathioprine; independent variables, *ABCB1* C3435T SNP, gender, smoking habits, and age.

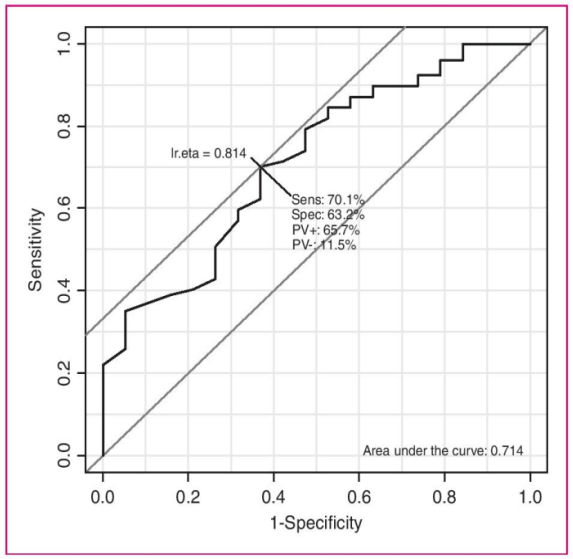

Figure 3. Receiver operating characteristic curve for model assessment of the *ABCB1* G2677T/A SNP
Multivariate logistic regression model: dependent variable, therapy response to biologics; independent variables, *ABCB1* G2677T/A SNP, gender, and disease duration.

they had an ileocolonic involvement as compared to isolated ileal or colonic disease.

Discussion

CD is a heterogeneous disease both in terms of clinical manifestations as well as in terms of response to therapy. Great effort has been dedicated to develop treatment algorithms with the aim of choosing the most effective treatment with less adverse effects and risks.[35–39] In this sense, a number of previous studies tried to identify clinical,[12–16,40] serological,[41,42] or genetic[17–25] predictors of response to several therapies available; however, so far, the results have not been very consistent across studies. One of the reasons for these discrepancies might result from what is considered response. In the present study, we decided to use long-term response (more than 1 year) because we believe that this concept is clinically more relevant. Thus, a patient who responds to azathioprine or biologicals at 2–3 months but later relapses with the need of corticosteroids or escalation of therapy was considered a nonresponder to this specific therapy.

In regard to predictors of response, we used both clinical and genetic markers. The latter may be more attractive because they do not change over time. However, in clinical practice they are of little value until their utility can be clearly demonstrated and validated in other studies. In contrast, clinical or phenotypic variables are more readily used in clinical practice.

In the present study, 25% of patients (60/242) were treated with 5-ASA: 29 of these responded to this therapy whereas the remaining 31 did not and required further escalation of therapy. Although overall this means a low number of patients (29/242, 12%), we think that it is certainly important to identify those patients who do very well on less aggressive and less toxic therapies. According to the last ECCO consensus,[3] no treatment may be an option in patients with mild disease, because a systematic review of clinical trials[43] showed that 18% of patients (95% CI 14–24%) of patients entered remission with placebo alone. In line with these findings, it is plausible that these patients could be treated with 5-ASA compounds only.

Previous surgery was a negative predictor of response in regard to 5-ASA (OR 0.25), but previously operated patients had a twice-higher chance of responding to azathioprine (OR 2.1). Clinically, we are often faced with the patient who does not respond to medical therapy, needs surgery, and post operatively

Table 5. Interaction between ABCB1 haplotypes (C3435T and G2677T/A) and disease location

Haplotype	Ileal disease (L1)	Colonic disease (L2)	Ileocolonic disease (L3)
CG	1.00	1.00	1.00
TG	0.57 (0.11–3.10)	0.28 (0.02–3.63)	3.51 (1.02–11.99)*
CT	1.45 (0.17–12.07)	0.00	5.9 (1.28–22.19)*
TT	2.05 (0.62–6.43)	1.18 (0.40–3.52)	2.0 (0.98–4.09)

*$p = 0.01$.

has a mild course, further raising the question of whether, once the diseased segment is resected, he can be maintained on 5-ASA therapy or whether immunosuppression should be started. Our data strongly supports the latter.

In regard to corticosteroids we observed that previous therapy with biologicals was a negative predictor of response to both corticosteroids (OR 0.26) and to azathioprine (OR 0.05). These are important observations from a clinical point of view because patients who are on biologicals and relapse are often treated with corticosteroids.[3] According to our results, this is not contraindicated but they are less likely to respond. Also, patients who are doing well on combination therapy and in whom we want to de-escalate therapy, the immunosuppressor (azathioprine) should preferentially be stopped.

Genetic polymorphisms included in the present study were chosen according to previous studies, which had shown some type of association with response to therapy. Although some previously reported associations could not be confirmed in the present study,[23,29,30] we found some interesting associations in the sense that genotypes identified patients who were simultaneously less likely to respond to certain therapies but more prone to respond to others. Thus, individuals who are heterozygotic for *IL23R* G9T SNP were less likely to respond to 5-ASA (OR 0.29) but 3-times more likely to respond to biologicals (OR 3.06). TT carriers of *Casp9* C93T SNP had a significant reduction in the probability of responding both to corticosteroids and azathioprine (OR 0.23 and 0.08, respectively), while TT carriers for both *ABCB1* C3435T and *ABCB1* G2677T/A showed a higher chance of responding to azathioprine. Concomitantly, TT carriers for *ABCB1* G2677T/A had a 25% reduction in the probability of responding to biologicals, which might be relevant in clinical decisions. ROC curves were plotted to test the performance of each of these models. This analysis allowed us to assess the performance of each of these models including SNPs for the several genetic associations found in multivariate analysis and response to corticosteroids, azathioprine, and biologicals. AUC values close or higher than 0.70 are considered reasonable classifiers, further reinforcing the reliability of the associations found between SNPs tested and response to several therapies.

This study had some limitations that need to be addressed. A major drawback relies on the fact that response to drugs was evaluated retrospectively from data recorded in the charts. However, in our opinion, after having the perspective of long-term evolution and whether the patient came to require therapy escalation after a transitory response which lasted less than 1 year, evaluating the response to a specific drug is clinically more relevant than evaluating short-term responses. Another limitation refers to the number of patients included in the study, which may be considered low for a pharmacogenomic study. Therefore, the data obtained in the present study can only be considered preliminary until prospectively validated in an independent cohort of CD patients.

In conclusion, in the present study we were able to identify a number of clinical predictors of response to several therapies available to treat CD patients, that might be of help in clinical practice, not only to select patients for more potent therapies such as immunosuppressors or biologicals, but also to less toxic ones such as 5-ASA compounds. In regard to genetic predictors, associations found are certainly promising but can only, at best, be considered hypothesis generating until these results are confirmed in larger populations.

Funding

This research was supported by Grupo de Estudo da Doença Inflamatória Intestinal (2009–2012).

Conflict of interest

The authors declare that there is no conflict of interest.

References

1. Cosnes J, Cattan S, Blain A, et al. Long-term evolution of disease behavior of Crohn's disease. *Inflamm Bowel Dis* 2002; 8: 244–250.
2. Thia KT, Sandborn WJ, Harmsen WS, et al. Risk factors associated with progression to intestinal complications of Crohn's disease in a population-based cohort. *Gastroenterology* 2010; 139: 1147–1155.
3. Dignass A, Van Assche G, Lindsay JO, et al. The second European evidence-based consensus on the diagnosis and management of Crohn's disease: current management. *J Crohns Colitis* 2010; 4: 28–62.
4. Beaugerie L, Seksik P, Nion-Larmurier I, et al. Predictors of Crohn's disease. *Gastroenterology* 2006; 130: 650–656.
5. Loly C, Belaiche J and Louis E. Predictors of severe Crohn's disease. *Scand J Gastroenterol* 2008; 43: 948–954.
6. Seksik P, Loftus EV, Beaugerie L, et al. Validation of predictors of 5-year disabling CD in a population-based cohort from Olmsted County, Minnesota, 1983–1996. *Gastroenterology* 2007; 132: A–17.
7. Froslie KF, Janhsen J, Moum BA, et al. Mucosal healing in inflammatory bowel disease: results from a Norwegian population-based cohort. *Gastroenterology* 2007; 133: 412–422.
8. Bokemeyer B, atalinic A, Klugmann T, et al. Predictive factors for a mild course of Crohn's colitis. *J Crohn's Colitis* 2009; 3: S82.
9. Colombel J-F, Sandborn WJ, Rutgeerts P, et al. Adalimumab for maintenance of clinical response and remission in patients with Crohn's disease: the CHARM trial. *Gastroenterology* 2007; 132: 52–65.

10. Schreiber S, Khaliq-Kareemi M, Lawrence IC, et al. Maintenance therapy with certolizumab pegol for Crohn's disease. *N Engl J Med* 2007; 357: 239–250.
11. Markowitz J, Grancher K, Kohn N, et al. A multicenter trial of 6-mercaptopurine and prdenisone in children with newly diagnosed Crohn's disease. *Gastroenterology* 2000; 119: 895–902.
12. Arnott IDR, McNeill G and Satsangi J. An analysis of factors influencing short-term and sustained response to infliximab treatment for Crohn's disease. *Aliment Pharmacol Ther* 2003; 17: 1451–1457.
13. Vermeire S, Loius E, Carbonez A, et al. Demographic and clinical parameters influencing the short-term outcome of anti-tumor necrosis factor (infliximab) treatment in Crohn's disease. *Am J Gastroenterol* 2002; 97: 2357–2363.
14. Parsi MA, Achkar JP, Richardson S, et al. Predictors of response to infliximab in patients with Crohn's disease. *Gastroenterology* 2002; 123: 707–713.
15. Su C and Lichenstein GR. Are there predictors of Remicade treatment success or failure? *Adv Drug Deliv Rev* 2005; 57: 237–245.
16. Reinish W, Wang Y, Oddens BJ, et al. C-reactive protein, an indicator for maintained response or remission to infliximab in patients with Crohn's disease: a post-hoc analysis from ACCENT I. *Aliment Pharmacol Ther* 2012; 35: 568–576.
17. Vermeire S, Van Assche G and Rutgeerts P. Role of genetics in prediction of disease course and response to therapy. *World J Gastroenterol* 2010; 16: 2609–2615.
18. Roberts RL and Barclay ML. Current relevance of pharmacogenetics in immunomodulation treatment of Crohn's disease. *J Gastroenterol Hepatol* 2012; 27: 1546–1554.
19. Mascheretti S, Hampe J, Croucher PJ, et al. Response to infliximab treatment in Crohn's disease is not associated with mutations in the CARD15 (NOD2) gene: an analysis in 534 patients from two multicenter, prospective GCP-level trials. *J Gastroenterol Hepatol* 2002; 12: 509–515.
20. Mascheretti S, Hampe J, Kuhbacher T, et al. Pharmacogenetic investigation of the TNF/TNF receptor system in patients with chronic active Crohn's disease treated with infliximab. *Pharmacogenomics J* 2002; 2: 127–136.
21. Vermeire S, Louis E, Rutgeerts P, et al. NOD2/CARD15 does not influence response to infliximab in Crohn's disease. *Gastroenterology* 2002; 123: 106–111.
22. Potocnik U, Ferkolj I, Glavac D, et al. Polymorphisms in multidrug resistance 1 (MDR1) gene are associated with refractory Crohn disease and ulcerative colitis. *Genes Immun* 2004; 5: 530–539.
23. Mendoza JL, Urcelay E, Lana R, et al. MDR1 polymorphism and response to azathioprine therapy in patients with Crohn's disease. *Inflamm Bowel Dis* 2007; 13: 585–5890.
24. Palmieri O, Latiano A, Valvano R, et al. Multidrug resistance 1 gene polymorphism are not associated with inflammatory bowel disease and response to therapy in Italian patients. *Aliment Pharmacol Ther* 2005; 22: 1129–1138.
25. Cucchiara S, Latiano A, Palmieri O, et al. Polymorphisms of tumor necrosis alpha but not MDR1 influence response to medical therapy in pediatric onset inflammatory bowel disease. *J Pediatr Gastroenterol Nutr* 2007; 44: 171–179.
26. Duerr RH, Taylor KD, Brant SR, et al. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. *Science* 2006; 314: 1461–1463.
27. Jurgens M, Laubender RP, Hartl F, et al. Disease activity, ANCA, and IL–23R genotype status determine early response to infliximab in patients with ulcerative colitis. *Am J Gastroenterol* 2010; 105: 1811–1819.
28. Jurgens M, Brand S, Laubender RP, et al. The presence of fistulas and NOD2 homozygosity strongly predict intestinal stenosis in Crohn's disease independent of IL23R genotype. *J Gastroenterol* 2010; 45: 721–731.
29. Hlavaty T, Pierik M, Henckarerts L, et al. Polymorphisms in apoptosis genes predict response to infliximab therapy in luminal and fistulizing Crohn's disease. *Aliment Pharmacol Ther* 2005; 22: 613–616.
30. Hlavaty T, Ferrante M, Henckarerts M, et al. Predictive model for the oucome of infliximab therapy in Crohn's disease based on apoptotic pharmacogenetic index and clinical predictors. *Inflamm Bowel Dis* 2007; 13: 372–379.
31. Nys K, Agostinis P, Vermeire S, et al. Autophagy: a new target or an old strategy to the treatment of Crohn's disease? *Nat Rev Gastroenterol Hepatol* 2013; 10: 395–401.
32. Duraes C, Machado JC, Portela F, et al. Phenotype-genotype profiles in Crohn's disease predicted by genetic markers in autophagy related genes (GOIA study II). *Infl Bowel Dis* 2013; 19: 230–239.
33. Lennard-Jones JE. Classification of inflammatory bowel disease. *Scand J Gastroenterol Suppl* 1989; 225: 92–99.
34. Satsangi J, Silverberg MS, Vermeire S, et al. The Montreal classification of inflammatory bowel disease: controversies, consensos, and implications. *Gut* 2006; 55: 749–753.
35. Feagan BG, Lemann M, Befrits R, et al. Recommendations for the treatment of Crohn's disease with tumor necrosis factor antagonist: an expert consensus report. *Inflamm Bowel Dis* 2012; 18: 152–160.
36. Ordas I, Feagan BG and Sandborn WJ. Early use of immunosupressives or TNF antagonists for the treatment of Crohn's disease: time for a change. *Gut* 2011; 60: 1754–1763.
37. D'Haens GR. Top-down therapy for IBD: rationale and requisite evidence. *Nat Rev Gastroenterol Hepatol* 2010; 7: 86–92.
38. Colombel JF, Sandborn WJ, Reinisch W, et al. SONIC study Group. Infliximab, azathioprine, or combination therapy for Crohn's disease. *N Engl J Med* 2010; 362: 1383–1395.
39. D'Haens G, Baert F, van Assche G, et al. Early combined immunosuppression in patients with newly diagnosed Crohn's disease: an open randomized trial. *Lancet* 2008; 371: 660–667.
40. Allez M, Lemann M, Bonnet J, et al. Long-term outcome of patients with active Crohn's disease exhibiting extensive and deep ulcerations at colonoscopy. *Am J Gastroenterol* 2002; 97: 947–953.

41. Esters N, Vermeire S, Joossens S, et al. Serological markers for prediction of response to anti-tumor necrosis factor treatment in Crohn's disease. *Am J Gastroenterol* 2002; 97: 1458–1462.
42. Taylor KD, Plevy SE, Yang H, et al. ANCA pattern and LTA haplotype relationship to clinical responses to anti-TNF antibody treatment in Crohn's disease. *Gastroenterology* 2001; 120: 1347–1355.
43. Su C, Lichtenstein GR, Krok K, et al. A meta-analysis of the placebo rates of remission and response in clinical trials of active Crohn's disease. *Gastroenterology* 2004; 126: 1257–1269.

Original article

IL23R polymorphisms influence phenotype and response to therapy in patients with ulcerative colitis

Marília L. Cravo[a,c], Paula A. Ferreira[d], Patricia Sousa[e], Paula Moura-Santos[c,e], Sónia Velho[b], Lurdes Tavares[e], João R. de Deus[g], Paula Ministro[h], Paula Peixe[f], Luis A. Correia[c,e], José F. Velosa[c,e], Rui F. Maio[a] and Miguel Brito[d]

Objective **We aimed to identify the clinical and genetic [IL23 receptor (IL23R) single nucleotide polymorphisms (SNPs)] predictors of response to therapy in patients with ulcerative colitis.**

Patients and methods **A total of 174 patients with ulcerative colitis, 99 women and 75 men, were included. The mean age of the patients was 47±15 years and the mean disease duration was 11±9 years. The number of patients classified as responders (R) or nonresponders (NR) to several therapies was as follows: 110 R and 53 NR to mesalazine (5-ASA), 28 R and 20 NR to azathioprine (AZT), 18 R and 7 NR to infliximab. Clinical and demographic variables were recorded. A total of four SNPs were studied: IL23R G1142A, C2370A, G43045A, and G9T. Genotyping was performed by real-time PCR using Taqman probes.**

Results **Older patients were more prone to respond to 5-ASA ($P=0.004$), whereas those with pancolitis were less likely to respond to such therapies ($P=0.002$). Patients with extraintestinal manifestations (EIMs) were less likely to respond to 5-ASA ($P=0.001$), AZT ($P=0.03$), and corticosteroids ($P=0.06$). Carriers of the mutant allele for IL23R SNPs had a significantly higher probability of developing EIMs ($P<0.05$), a higher probability of being refractory to 5-ASA ($P<0.03$), but a higher likelihood of responding to AZT ($P=0.05$). A significant synergism was observed between IL23R C2370A and EIMs with respect to nonresponse to 5-ASA ($P=0.03$).**

Conclusion **Besides extent of disease and age at disease onset, the presence of EIMs may be a marker of refractoriness to 5-ASA, corticosteroids, and AZT. IL23R SNPs are associated both with EIMs and with nonresponse to 5-ASA and corticosteroids.** ***Eur J Gastroenterol Hepatol*** **26:26–32 © 2013 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

European Journal of Gastroenterology & Hepatology 2014, **26**:26–32

Keywords: extraintestinal manifestations, IL23R single nucleotide polymorphisms, predictors of response to therapy, ulcerative colitis

[a]Gastroenterology Service, [b]Dietetics Service, Beatriz Angelo Hospital, Loures, [c]Faculty of Medicine, Lisbon University, [d]Department of Natural Sciences, School of Health Technologies, [e]Gastroenterology Service, Santa Maria Hospital, [f]Gastroenterology Service, Egas Moniz Hospital, Lisbon, [g]Gastroenterology Service, Fernando da Fonseca Hospital, Amadora and [h]Gastroenterology Service, S. Teotónio Hospital, Viseu, Portuga

Correspondence to Marília L. Cravo, MD, PhD, Gastroenterology Service, Beatriz Angelo Hospital, Avenue Carlos Teixeira #3, Loures 2774-514, Portugal Tel: +351 919439192; fax: +351219847209; e-mail: marilia.cravo@sapo.pt

Received 24 July 2013 **Accepted** 12 September 2013

Introduction

The majority of patients with ulcerative colitis (UC) respond to therapy with mesalamine either orally and/or topically. For nonresponders, either immunosuppressants (IS) and/or anti-TNF agents are recommended, although the responses to these agents are less well documented than in patients with Crohn's disease (CD) [1]. Previous studies have been carried out aiming at identifying patients with a poor prognosis in whom aggressive therapies could be started earlier. Most of these studies focused on clinical, endoscopic, or histologic parameters. Extensive colitis, severe disease activity, younger age, and female sex have been associated with poor outcome in most population-based studies [2–5]. The presence of basal plasmacytosis and a Geboes score of at least 3.1 were associated recently with a greater chance of disease relapse [6].

A different issue from predicting patients with a poor prognosis is to predict response to therapy. Although patients with poor clinical prognostic indicators are usually those proposed to start more aggressive therapies, to prevent complications, this does not necessarily relate with response to therapy.

Most studies have focused on predictors of response to infliximab [7–9]. As most patients are initially treated with mesalazine (5-ASA) compounds and respond to such therapies, we believe that it would be clinically more useful to have early indicators of nonresponse to these compounds. In these patients, a more aggressive therapeutic strategy could be started early on. This is even more relevant as recent papers propose that UC should also be considered as a progressive disease, where structural damage of the intestinal wall may ensue, thereby stressing the need for early effective therapy to prevent it [10–13].

Besides clinical factors, serological and genetic predictors have also been studied in UC, although far less than in

0954-691X © 2013 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/MEG.0000000000000004

CD [13,14]. The IL23 receptor (IL23R) gene has been identified as a susceptibility gene both for CD and for UC [14–16]. The IL23R gene is mapped to chromosome 1p32.1-p31.2, and the protein encoded by this gene is a subunit of the receptor for IL23A/IL23. IL23 is produced by bacteria-stimulated antigen-presenting cells in response to microbial stimulation and has been shown to promote expansion of CD4+ T helper 17 cells [15,16] and to induce the production of Th17-type cytokines by non-T cells/innate cells. Th17 cytokines are now considered crucial factors for enhancing the effector phase of T-cell responses that cause tissue inflammation and damage in the gut. In several models of colitis, it has been shown that IL23 is the major driver of intestinal inflammation [17,18]. Furthermore, genome-wide association studies of large cohorts of patients with inflammatory bowel disease (IBD) and healthy controls subsequently identified several single nucleotide polymorphisms (SNPs) in the IL23R gene locus associated with either susceptibility or resistance to IBD [15,19]. So far, few studies have explored an association between IL23R SNPs and phenotype and/or response to therapy in patients with UC. Previous authors [7,9] reported that ANCA seronegativity and the IL23R genotype were predictors of early response to infliximab. Jurgens *et al.* [7] found that carriers of IBD risk-increasing IL23R variants were more likely to respond to infliximab as compared with those homozygous for IBD risk-decreasing IL-23R variants. Besides predicting response to infliximab, the value of these SNPs in predicting response to 5-ASA compounds and/or azathioprine has not been studied so far.

The aim of the present study was to identify clinical and specific IL23R SNPs as predictors of response to therapy in patients with UC.

Patients and methods

This was a multicenter study with participating hospitals from central Portugal. Informed consent was obtained from all patients entering the study, which was approved by the scientific and ethical committees of the several participating hospitals.

Phenotypic characteristics retrospectively collected from charts included demographic data, age at disease onset, disease extent, time of follow-up, smoking habits, presence of extraintestinal manifestations (EIMs), and previous therapies. All phenotypic data were collected in a blinded manner to the results of the genotypic data. Patients were selected to enter the study if a definitive classification in terms of response to a specific drug could be obtained clearly after reviewing the chart and interviewing the patient at the time of entering the study. Patients were considered responders if they presented a long-term sustained remission, defined as a stool frequency of 3 or less with no bleeding and no urgency, lasting at least 1 year after a specific therapy was started, not needing steroids, surgery, or escalation of therapy. Patients requiring the addition or switching to other therapies, corticosteroids, or surgery before 1 year were considered nonresponders. If a clear distinction between these two scenarios was not possible for a specific drug, the patient was not considered, at least for this drug.

The decision to switch therapy was made by the treating physician. Remission was recorded after a new therapy was started, until the drug was discontinued, or until the end of follow-up. Biologic parameters such as CRP levels or endoscopic response were not used to classify patients as responders or nonresponders. For mesalamine, patients received for induction therapy 4.8 g/day or equivalent together with topic therapy (enemas and/or suppositories) as needed to control rectal bleeding. Once remission was achieved, the maintenance dosage was decreased to 2.4 g/day according to ECCO recommendations [1]. If they relapsed, patients were treated in the same way. Therapy optimization was not considered nonresponse. Steroids were started if remission was not obtained after 4–6 weeks of therapy with mesalamine or if disease activity was considered severe. Only short-term response (1 month) was considered. Steroid dependence was defined as recurrent flare-up on withdrawal of glucocorticoids or the need for glucocorticoid treatment twice within 6 months. Patients were considered refractory to steroids when no remission was obtained with a dose of 1 mg/kg during a period of at least 4 weeks. Steroid dependency or nonresponse to mesalamine was considered an indication to escalate therapy to 6-mercaptopurine (1 mg/kg/day) or azathioprine (2.0–2.5 mg/kg/day). Nonresponse to purine analogues after 10–12 weeks was considered an indication to infliximab. None of the patients included in the present study received adalimumab.

DNA extraction and genotyping

Blood samples were taken from all the study participants and genomic DNA was isolated from peripheral blood using the DNA blood mini kit from Qiagen (Hilden, Germany) according to the manufacturer's guidelines. A total of four SNPs were studied: IL23R G1142A (rs11209026), C2370A (rs10889677), G43045A (rs 1004819), and G9T (rs1884444). These SNPs were chosen because previous studies had found either an association with increased or decreased susceptibility and/or response to infliximab [7,18]. All genotypes were determined using real-time PCR methods with TaqMan Pre-Designed SNP Genotyping Assays (Applied Biosystems, Grand Island, New York, USA). For the genotype analysis, the target fragments were amplified in a 20 µl reaction mixture containing 10 µl TaqMan Universal PCR Master Mix, 1 µl primers, 5 µl MilliQ water, and 4 µl DNA. Real-time PCR using an iCycler IQ Multicolor Real-Time PCR Detection

European Journal of Gastroenterology & Hepatology 2014, Vol 26 No 1

System (Bio-Rad, Hercules, California, USA), was carried out as follows: 10 min of the initial step at 95°C, 50 cycles of 15 s, and 1 min at 92°C and 60°C, respectively.

Statistical analysis

Statistical analysis was carried out using SPSS (version 14.0; SPSS Inc, Chicago, Illinois, USA) and SNPassoc 1.6 package in R software (Center for Genomic Regulation, Unit of Biostatistics and Bioinformatics Epidemiology Service, Bellvitge Biomedical Research Institute, Catalan Institute of Oncology, Barcelona, Spain). Data were expressed as mean ± SD, number of participants and percentage, or as odds ratio (OR) and 95% confidence interval (CI). Primary analyses were carried out using the χ^2-test and univariate analysis. The association between outcome and SNPs was analyzed using the SNPassoc library (Center for Genomic Regulation, Unit of Biostatistics and Bioinformatics Epidemiology Service, Bellvitge Biomedical Research Institute, Catalan Institute of Oncology). Different inheritance models (dominant, recessive, log-add, and overdominant) were considered and were presented as eligible. Haplotype analysis was carried out for the four IL23R SNPs. Statistical significance was established for P less than 0.05. Differences in genotypic and allelic frequencies and Hardy–Weinberg tests were performed using GENEPOP Web version 4.0.10 program (Michel Raymond and Francois Rousset at Laboratiore de Genetique et Environment, Montpellier, France). To obtain the exact P value of Hardy–Weinberg equilibrium, the Markov chain method [20] with a dememorization number of 1000, 100 batches, and 1000 iterations per batch was used. The P value returned by this method is calculated as the sum of the probabilities of all tables and its SE. Genotypic frequencies are under Hardy–Weinberg equilibrium when the P value is more than 0.001.

Results

A total of 174 patients entered the study; the median follow-up was 3.9 years. The clinical characteristics and percentages of responders to several therapies are shown in Table 1. Allelic and genotypic frequencies for all the studied SNPs are shown in Table 2. The genotypic frequencies of all SNPs did not deviate significantly from those expected under Hardy–Weinberg equilibrium ($P > 0.001$).

The association between clinical variables and response to several drugs was analyzed; only a significant or near-significant associations are shown in Table 3.

We observed that older patients and those diagnosed after the age of 40 responded better to 5-ASA compounds. In contrast, duration of disease for more than 5 years was a negative predictor of response both for 5-ASA and for azathioprine, although the latter did not reach statistical significance ($P = 0.008$ and 0.07, respectively). Disease extent negatively influenced response to 5-ASA and azathioprine, with patients with pancolitis showing poorer responses (Table 3). With respect to EIMs, we observed that it was a negative predictor of response to 5-ASA, corticosteroids, and azathioprine ($P = 0.001$, 0.06, and 0.02, respectively) but it seemed to positively influence response to biologics, although not significantly (OR 8.0, 95% CI 0.72–88.22; $P = 0.09$).

Interestingly, previous therapies also seemed to influence response to several drugs. Thus, previous users of corticosteroids, azathioprine, and/or biologics responded better to 5-ASA. The same was observed for corticosteroids and azathioprine, suggesting that the use of IS or biologics may allow recapture of patients to respond to therapies to which they were previously nonresponsive.

Table 4 shows the associations between IL23R SNPs and clinical characteristics including response to therapy. We observed that carriers of IL23R C2370A and IL23R_G4305A alleles were at an increased risk of showing EIMs (Table 4). No significant association

Table 1 Demographic and clinical data of patients included in the study

Variables	*N* (%)
Sex	
Male	75 (43.1)
Female	99 (56.9)
Mean age (years)	47.21±15.9
Mean duration of disease (years)	10.55±9.2
Median duration of follow-up (years)	3.9±3.2
Age of disease onset	
A1	8 (4.62)
A2	93 (53.76)
A3	72 (41.62)
Extent	
E1	29 (16.67)
E2	60 (34.48)
E3	85 (48.85)
Previous surgery	
Yes	2 (1.15)
No	172 (98.85)
Smoking habits	
Yes	12 (6.88)
No	137 (78.74)
Unknown	25 (14.37)
Extraintestinal manifestations	
Yes	35 (20.12)
No	139 (79.89)
Response to therapy	
5-ASA	
Yes	110 (68/64)[b]
No	53 (32)
Corticosteroids	
Yes	52 (64/30)[b]
No	29 (36)[a]
Azathioprine	
Yes	28 (58/16)[b]
No	20 (42)
Infliximab	
Yes	18 (72/10)[b]
No	7 (28)

5-ASA, mesalazine.
[a]Response to corticosteroids refers to short-term (1 month) response.
[b]% of responders to this specific drug and % of responders from total of patients.

Table 2 Genotypic frequencies, allelic frequencies, and Hardy–Weinberg equilibrium

Polymorphism	Genotypic frequencies		Allelic frequencies	Hardy–Weinberg equilibrium
IL23R G1142A				
GG	0.718			P=0.0283
GA	0.282	G	0.859	
AA	0	A	0.141	
IL23R C2370A				
CC	0.448			P=0.7386
CA	0.431	C	0.664	
AA	0.121	A	0.336	
IL23R G9T				
TT	0.201			P=0.0507
TG	0.580	T	0.491	
GG	0.218	G	0.509	
IL23R C/T				
CC	0.448			P=0.1836
CT	0.408	C	0.652	
TT	0.144	T	0.348	

Table 3 Association between clinical characteristics and response to therapy

	5-ASA	Corticosteroids	Azathioprine	Biologics
N (%)	163 (94)	81 (47)	48 (28)	25 (14)
R/NR	110/53	52/29	28/20	18/7
Age (OR)	1.03			
95% CI	1.00–1.05			
P values	0.004			
Age at Dx (OR)	1.04			
95% CI	1.01–1.06			
P value	0.002			
Age>40 yrs (OR)	2.36			
95% CI	1.21–4.57			
P value	0.01			
Duration>5 years (OR)	0.37		0.26	
95% CI	0.17–0.77		0.05–1.16	
P value	0.008		0.07	
Disease extent				
E2 (OR)	0.31			
95% CI	0.09–1.05			
P value	0.06			
E3 (OR)	0.15		0.18	
95% CI	0.04–0.49		0.03–0.99	
P value	0.002		0.05	
EIMs (OR)	0.25	0.35	0.18	8.0
95% CI	0.10–0.56	0.11–1.06	0.04–0.76	0.72–88.22
P value	0.001	0.06	0.02	0.09
Previous Cx (OR)	5.79			
95% CI	2.78–12.04			
P value	<0.001			
Previous AZT (OR)	77.40	8.92		
95% CI	21.87–273.94	3.14–25.35		
P value	<0.001	<0.001		
Previous IFX (OR)	15.57	19.12	10.19	
95% CI	3.41–71.16	3.91–93.48	2.30–45.04	
P value	<0.001	<0.001	0.002	

Significant or near-significant results are shown.
OR<1.00, less responsive; OR>1.00, better response.
AZT, azathioprine; CI, confidence interval; Cx, corticosteroids; Dx, diagnosis; EIMs, extraintestinal manifestations; IFX, infliximab; *N*, number of patients; NR, nonresponders; OR, odds ratio; R, responders.

was observed with disease extension or other phenotypic characteristics. With respect to response to therapy, we observed that IL23R_C2370A negatively influenced response to 5-ASA and corticosteroids, whereas homozygous carriers for IL23R_G9T were more likely to respond to azathioprine.

We also examined whether there was any interaction between clinical and genetic predictors of response. We observed that IL23R_C2370A CA carriers with EIMs had a significantly lower chance of responding to 5-ASA compounds (OR 0.12, 95% CI 0.018–0.86; $P = 0.03$).

Discussion

Compared with CD, the individual natural history of patients with UC is harder to predict than in CD [21,22] and the relevance of clinical predictors has not been validated in independent cohorts [13]. Recent papers strongly suggest that UC, as CD, should be considered as progressive diseases resulting in cumulative bowel damage, thereby affecting bowel function and the patient's quality of life [10–12].

As most patients with UC respond to 5-ASA-based therapies, clinicians should focus on early identification of nonresponders to these therapies as these should be started on more aggressive and effective therapies as early as possible. Most series published so far identified young age, female sex, and extensive colitis as poor prognostic markers, theoretically but not necessarily, with less probability of responding to therapy with 5-ASA only [2–4]. Serologic and genetic predictors have been far less explored in UC than in CD and only a few clinical settings have been investigated [13]. In a recent study carried out in 94 children with IBD, the authors identified pANCA positivity and a diagnosis of UC as predictors for a primary nonresponse to infliximab [7]. Genetic markers are more attractive as they are already present at the onset of the disease and remain stable over time, which is not the case for clinical and serologic parameters. In the present study, we focused on IL23R SNPs because there is accumulating evidence that IL23 cytokine is essential to drive the chronic intestinal inflammation observed in IBD, namely UC. IL23 is produced by activated dendritic cells and macrophages present in the gut mucosa in response to microbial stimulation [17]. Although initially found to support the expansion and maintenance of CD4+ T helper 17 cells, IL23 is now recognized as having multiple effects on the immune response, including restraining Foxp3+ regulatory T-cell activity and inducing the expression of Th17-type cytokines from non-T-cell sources [17]. Certainly related to this diversity and multiplicity of upstream and downstream effects, certain of these SNPs confer a protective effect against developing IBD [19], whereas others increase the susceptibility to develop IBD [7,15]. Furthermore, there are significant ethnic differences, with some variants increasing the risk of developing IBD in Whites but not in Asian individuals [18]. These apparent discrepancies may be explained by the

European Journal of Gastroenterology & Hepatology 2014, Vol 26 No 1

Table 4 Association between IL23 R genetic SNPs with extraintestinal manifestations and response to therapy

	IL23R C2370A		IL23R G9T		IL23R C/T	
	CA	AA	TG	GG	CT	TT
EIMs (OR)	2.62	3.21			3.05	3.34
95% CI	1.08–6.31	0.95–10.77			1.24–7.49	1.04–10.66
P value	0.03	0.05			0.01	0.04
Response to 5-ASA (OR)		0.32				
95% CI		0.11–0.92				
P value		0.03				
Response to Cx (OR)		0.19				
95% CI		0.04–0.84				
P value		0.02				
Response to AZT (OR)				11.8		
95% CI				1.0–139		
P value				0.05		

Significant or near-significant results are shown.
OR<1.00, less responsive; OR>1.00, better response.
5-ASA, mesalazine; AZT, azathioprine; CI, confidence interval; Cx, corticosteroids; EIMs, extraintestinal manifestations; OR, odds ratio; SNP, single nucleotide polymorphism.

fact that IL23R contributes to colitis pathogenesis through various pathways that may be tackled in a number of different ways, thereby resulting in various types of responses. The exact mechanism by which the studied polymorphisms exert their effect on disease pathogenesis and on metabolism and mechanism of action of drugs is still not known. When we consider SNPs affecting coding regions, such as the IL23R G1142A or G9T polymorphisms, we can hypothesize changes in protein structure, namely an effect on the helical structure of the protein product [23] and consequently modifications in receptor affinity. In what concerns the SNP IL23R C2370A, located in the 3′ untranslated region, the latter may be associated with RNA stability or even translation efficiency, which in turn lead to changes in protein expression. Finally, the IL23R G43045A with intronic localization could be associated with RNA splicing or control of transcription, and indirectly affect RNA stability or gene expression. However, we cannot exclude that these polymorphisms could be in linkage with other variants in genes located nearby, responsible for UC pathogenesis and/or disease phenotype. Finally, the reason why certain of these variants are associated with different responses to therapy is probably through the modulation of the phenotype that they produce as opposed to a direct interference with drug metabolism.

In the present study, besides clinical and genetic predictors of response to infliximab, we also focused on predictors of nonresponse to other therapies including 5-ASA and azathioprine, which is certainly useful in clinical practice, as only a minority of patients will need escalation to biologics. We also decided to use long-term, more than 1-year, response, because we believe that this concept is clinically more relevant. Thus, a patient who responded to 5-ASA, but before 1 year relapsed with the need for corticosteroids or escalation of therapy, was considered a nonresponder to 5-ASA. Early institution of IS and/or biologic therapy in these patients could be a way of maintaining the patient in remission and avoiding the complications of undertreated disease.

In the present study, we examined whether clinical and/or IL23R SNPs, alone or in combination, could be used as predictors of response to several drugs used in the treatment of UC. Similar to previous series, over 60% of patients responded to 5-ASA compounds. We had estimated that at least 40% of patients would be refractory to 5-ASA and would require azathioprine and/or biologics. As a step-up approach was followed in all instances of patients not responding to 5-ASA, most of these patients were started on azathioprine, 58% of whom responded to this therapy. Thus, only 25 of 174 patients ultimately required biologics, which may preclude some statistical significance.

Consistent with previous studies [2–5,24,25], we observed that older patients responded better to 5-ASA, whereas no influence was observed in terms of probability of response to azathioprine or biologics. Duration of disease for more than 5 years was associated with a lower probability of response both for 5-ASA and for azathioprine. In agreement with previous studies, disease extent [2–5,24,25] was also a negative predictive factor for responding to 5-ASA and azathioprine but not to steroids or biologics. EIMs were strongly associated with poor response to 5-ASA, steroids, and azathioprine, whereas a nonsignificant but positive association was observed in terms of biologics. To our knowledge, this has not been clearly reported before. EIMs are among the clinical risk factors that may be associated with disease extent and severity [26], and a recent review on this topic [27] suggests that early aggressive therapy may be required for treating several EIMs to prevent chronic damage. According to our results, together with young age of disease onset and greater disease extent, the presence of EIMs may predict nonresponse to 5-ASA and need for aggressive therapy.

A very interesting association was also observed with previous therapies. Thus, previous users of steroids, azathioprine, or biologics were more likely to respond to 5-ASA. This recapture phenomenon is frequently observed in clinical practice. Similarly, previous users of azathioprine and biologics had a greater chance of responding to corticosteroids and that of biologics with a greater likelihood of responding to azathioprine. These observations suggest that patients who did not respond to 5-ASA and required escalation of therapy, after remission was achieved, may be maintained on 5-ASA.

With respect to IL23R SNPs, these have been described as susceptibility genes but to our knowledge there are no previous reports on whether these SNPs influence

phenotypic manifestations in UC patients. A recent study by a German group suggests that homozygous carriers of IL23R variants that increase the risk of developing IBD are more likely to respond to infliximab [7]. In the present study, we found that IL23R SNPs were associated with a significantly increased risk of developing EIMs, with a lower chance of responding to 5-ASA and corticosteroids but with a higher chance of responding to azathioprine. No association was found between these SNPs and response to infliximab, which may be related to the low number of patients treated with this drug. We also found a significant interaction between the IL23R_-C2370A allele and the presence of EIM in predicting nonresponse to 5-ASA. This is consistent with these two factors, alone or in combination, being early indicators of need for azathioprine and/or infliximab therapy in agreement with a previous study published by Jurgens *et al.* [7]. According to our data, older patients, with limited disease, not showing EIMs and not carrying IL23R variants are more prone to respond to 5-ASA compounds. The effect of IL23R variant alleles may be independent or because of the fact that they are more often associated with EIM, which is also a negative predictor for responding to 5-ASA compounds.

This study had some limitations that need to be addressed. A major drawback is the fact that response to drugs was evaluated retrospectively from data recorded in the charts. The decision to change a drug was made according to the treating physician, which might not be uniform across the several centers involved in the study. Nonetheless, the numbers obtained for response to several drugs are close to the ones reported in the literature, thereby suggesting similar policies of treatment. In our opinion, evaluating the response to a specific drug after the long-term evolution and whether the patient required therapy escalation after a transitory response that lasted less than 1 year is clinically more relevant than evaluating short-term responses. Another drawback of this study was that disease activity was not recorded on admission and it is plausible that this influenced therapeutic decisions. However, our main purpose was to identify early predictors of response and not prognosis and a conventional step-up approach was followed in the large majority of patients. Finally, it would be interesting to have a better characterization of the type of EIMs to determine whether they all had the same prognostic value.

Conclusion

In the present study, we could identify a number of clinical variables that could predict nonresponse to 5-ASA compounds, namely, the existence of EIMs. The latter were significantly more frequent in IL23R SNPs carriers, which was also associated with poor response to 5-ASA but to a higher probability of responding to azathioprine. These findings might be valuable in clinical practice, although further confirmation is still needed.

Acknowledgements

This work was funded by a grant from GEDII 2009-2012.

Conflicts of interest

There are no conflicts of interest.

References

1 Dignass A, Lindsay JO, Sturn A, Sturm A, Windsor A, Colombel JF, *et al.* Second European evidence-based consensus of the diagnosis and management of ulcerative colitis: current management. *J Crohns Colitis* 2012; **6**:991–1030.
2 Langholz E, Munkholm P, Davidsen M, Binder V. Course of ulcerative colitis: analysis of changes in disease activity over years. *Gastroenterology* 1994; **107**:3–11.
3 Hoie O, Wolters F, Riis L, Solberg C, Bernklev T, Odes S, *et al.* Ulcerative colitis: patients characteristics may predict 10-year disease recurrence in a European-wide population based cohort. *Am J Gastroenterol* 2007; **102**:1692–1701.
4 Solberg IC, Lygren I, Janhsen J, Aadland E, Høie O, Cvancarova M, *et al.* Clinical course during the first 10 years of ulcerative colitis: results from a population-based inception cohort (IBSEN Study). *Scand J Gastroenterol* 2009; **44**:431–440.
5 Portela F, Magro F, Lago P, Cotter J, Cremers I, de Deus J, *et al.* Ulcerative colitis in a Southern European country: a national perspective. *Inflamm Bowel Dis* 2010; **16**:822–829.
6 Bessissow T, Lemmens B, Ferrante M, Bisschops R, Van Steen K, Geboes K, *et al.* Prognostic value of serologic and histologic markers on clinical relapse in ulcerative colitis patients with mucosal healing. *Am J Gastroenterol* 2012; **107**:1684–1692.
7 Jurgens M, Laubender RP, Hartl F, Weidinger M, Seiderer J, Wagner J, *et al.* Disease activity, ANCA, and IL-23R genotype status determine early response to infliximab in patients with ulcerative colitis. *Am J Gastroenterol* 2010; **105**:1811–1819.
8 Dubinsky MC, Mei L, Friedman M, Dhere T, Haritunians T, Hakonarson H, *et al.* Genome wide association predictors of anti-TNFα therapeutic responsiveness in pediatric inflammatory bowel disease (IBD). *Inflamm Bowel Dis* 2010; **16**:1357–1366.
9 Ferrante M, Vermeire S, Katsanos K, Noman M, Van Assche G, Schnitzler F, *et al.* Predictors of early response to infliximab in patients with ulcerative colitis. *Inflamm Bowel Dis* 2007; **13**:123–128.
10 Torres J, Billioud V, Sachar DB, Peyrin-Biroulet L, Colombel JF. Ulcerative colitis as a progressive disease: the forgotten evidence. *Inflamm Bowel Dis* 2012; **18**:1356–1363.
11 Ochsenkuhn T, D'Haens G. Current misunderstandings in the management of ulcerative colitis. *Gut* 2011; **60**:1294–1299.
12 Torres J, Billioud V, Peyrin-Biroulet L, Colombel JF. Ulcerative colitis as a sole mucosal disease: another misunderstanding (Letter). *Gut* 2012; **61**:633.
13 Beaugerie L. Clinical, serological and genetic predictors of inflammatory bowel disease course. *World J Gastroenterol* 2012; **18**:3806–3813.
14 Vermeire S, Van Assche G, Rutgeerts P. Role of genetics in prediction of disease course and response to therapy. *World J Gastroenterol* 2010; **16**:2609–2615.
15 Duerr RH, Taylor KD, Brant SR, Rioux JD, Silverberg MS, Daly MJ, *et al.* A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. *Science* 2006; **314**:1461–1463.
16 Sarra M, Pallone F, MacDonald T, Monteleone G. IL-23/IL-17 axis in IBD. *Inflamm Bowel Dis* 2010; **16**:1808–1813.
17 Morrison PJ, Ballantyne SJ, Kullberg MC. Interleukin-23 and T-helper 17-type responses in intestinal inflammation: from cytokines to T-cell plasticity. *Immunology* 2011; **133**:397–408.
18 Kim SW, Kim ES, Moon CM, Park JJ, Kim TI, Kim WH, Cheon JH. Genetic polymorphisms of IL23R and IL-17A and novel insights into their associations with inflammatory bowel disease. *Gut* 2011; **60**:1527–1536.
19 Dubinski MC, Wang D, Picomell Y, Wrobel I, Katzir L, Quiros A, *et al.* IL-23 receptor (IL-23R) gene protects against pediatric Crohn's disease. *Inflamm Bowel Dis* 2007; **13**:511–515.
20 Guo SW, Thompson EA. Performing the exact test of Hardy–Weinberg proportion for multiple alleles. *Biometrics* 1992; **48**:361–372.
21 Dignass A, Eliakim R, Magro F, Maaser C, Chowers Y, Geboes K, *et al.* Second European evidence-based consensus on the diagnosis and management of ulcerative colitis: definitions and diagnosis. *J Crohns Colitis* 2012; **6**:965–990.

European Journal of Gastroenterology & Hepatology 2014, Vol 26 No 1

22 Cosnes J, Gower-Rousseau C, Seksik P, Cortor A. Epidemiology and natural history of inflammatory bowel diseases. *Gastroenterology* 2011; **140**: 1785–1794.
23 Safrany E, Szabo M, Szell M, Kemeny L, Sumegi K, Melegh BI, *et al*. Difference of interleukin-23 receptor gene haplotype variants in ulcerative colitis compared to Crohn's disease and psoriasis. *Inflamm Res* 2013; **62**:195–200.
24 Bitton T, Peppercorn MA, Antonioli DA, Niles JL, Shah S, Bousvaros A, *et al*. Clinical, biological, and histologic parameters as predictors of relapse in ulcerative colitis. *Gastroenterology* 2001; **12**:13–20.
25 Romberg-Camps MJ, Dagnelie PC, Kester AD, Hesselink-van de Kruijs MA, Cilissen M, Engels LG, *et al*. Influence of phenotype at diagnosis and of other potential prognostic factors on the course of inflammatory bowel disease. *Am J Gastroenterol* 2009; **104**:371–383.
26 Farmer RG, Easley KA, Rankin GB. Clinical patterns, natural history, and progression of ulcerative colitis. A long term follow-up of 1116 patients. *Dig Dis Sci* 1993; **38**:1137–1146.
27 Veloso FT. Extraintestinal manifestations of inflammatory bowel disease: do they influence treatment and outcome. *World J Gastroenterol* 2011; **17**:2702–2707.

Is it personalized therapy around the corner for IBD patients? A review and a perspective from a Portuguese population.

Running title - Personalized therapy for IBD patients: a review and a perspective from a Portuguese population.

AUTORS

Paula Ferreira, BSc[1], Marília Cravo, MD, PhD[2,3] and Miguel Brito, BSc, PhD [1].

AFFILIATION

1- Escola Superior de Tecnologias da Saúde de Lisboa, Lisboa, Portugal.

2- Serviço de Gastrenterologia do Hospital Beatriz Ângelo, Loures, Portugal.

3- Faculdade de Medicina da Universidade de Lisboa, Lisboa, Portugal

CORRESPONDING AUTHOR

Paula Ferreira

ESTeSL - Escola Superior de Tecnologia da Saúde de Lisboa

Av. D. João II, Lote 4.69.01

1990 - 096 Lisboa, Portugal

Telephone number: (351) 218 980 400, Extension code 616

Fax number: (351) 218 980 460

Email address: paula.ferreira@estesl.ipl.pt

ABSTRACT

Inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC), are chronic intestinal inflammatory disorders whose pathogenesis isn't fully understood and are defined by remissions and exacerbations. Until nowadays there are no medical cures available.

The importance of the genetic factors in the determination of susceptibility to IBD has been described in several epidemiologic and linkage studies, mainly through the use of genome-wide association studies (GWAS) that permitted the knowledge of a number of susceptibility loci to both diseases.

Once that many inflammatory diseases share common risk alleles, it will be beneficial to use different disease pathways for pharmacogenetic studies, which will allow the development of new treatments more effective and more economical attractive.

The purpose of this article is to make a review about the importance of genetics and susceptibility genes in IBD and its use as predictors to individualized therapy. To achieve it, we will approach the conventional therapy and its advantages/disadvantages, describe genetic susceptibility to IBD, identify genetic predictors to response to therapy and analyze the applicability of this knowledge to a Portuguese population study in order to step forward to the ultimate purpose of personalized medicine.

KEYWORDS: Inflammatory bowel diseases, therapy, genetic predictors, pharmacogenetics, personalized medicine.

INTRODUCTION

It is common knowledge that both environmental and host genetic factors are determining in inflammatory bowel diseases (IBD) susceptibility, disease behavior and response to therapy.[1]

The genetic basis of Crohn's disease (CD) is clinically based on the previous described monozygotic twin concordance rate of almost 50% and the positive family history association between several patients.[2] The first relevant discoveries were the mutations in the NOD2 gene in the IBD1 locus on chromosome 16 that emphasized the importance of innate immunity in CD pathogenesis.[2] Following, genome-wide association studies (GWAS) presented the association of CD and autophagy genes, identifying strongest associations with ATG16L1 and IRGM.[2] Regarding Ulcerative Colitis (UC) it was described that the interleukin-23 receptor gene (IL23R) and the interleukin-12β gene (IL12B) were significantly associated and GWA studies identified associations with the actin-related protein 2/3 complex subunit 2 gene (ARPC2), the interleukin-10 gene (IL10) and with regions on chromosomes 1p36 and 12q15.[3]

Inflammatory bowel diseases are chronic relapsing diseases,[4] being CD and UC the two main clinical presentations with differences regarding disease extension, localization, behavior and the occurrence of extraintestinal manifestations (EIM). The precise etiology of IBD is unknown but both environmental factors and genetic susceptibility are involved.[5,6] Nowadays it's known that extensive bowel damage in both CD and UC is caused by defects in the innate immune system that consequently triggers an exaggerated adaptive immune response.[7,8] For CD the localization is somewhat stable, but the disease type can alternate over time from an inflammatory pattern to a stricturing or penetrating disease, causing a problematic disease course.[9] Identifying the location of UC is extremely advantageous for both patients risk profile and early determination of the most effective therapy suitable for each patient phenotype.[10,12]

Guidelines for CD therapeutic recommend initiating treatment with mesalamine and systemic corticosteroids, pursued by azathioprine, and finally anti-TNF therapies for patients in whom conventional therapies have failed.[13]

In UC the main concern is to induce a steroid-free remission, and for this reason, its important to choose the most effective treatment based on severity, localization and course of the disease. For proctitis, is recommended topical therapy with 5-aminosalicylic acid (5-ASA) compounds, while for more extensive or severe disease its use oral and local 5-ASA compounds and corticosteroids. In case of non-response, patients require hospitalization for intravenous steroids and calcineurin inhibitors, tumor necrosis factor-α antibodies or immunomodulators when refractory occurs.[14,15]

In this review we intend to approach thematics as genetic susceptibility to IBD, existent therapeutic, genetic parameters as predictors for therapy response including a perspective from a clinical study in a Portuguese population and, finally, the importance of genetic in personalized medicine and challenges for the future to come.

GENETIC SUSCEPTIBILITY RELATED TO IBD

Although clinical parameters have some predictive value for prognosis and guiding treatment strategies in CD, the search for genetic polymorphisms and cytokine profiles has led to potential predictors of prognosis and thus the identification of patients who should received more aggressive therapy early on (top-down).

Before GWAS studies, the NOD2, IBD5, and HLA class II were the most studied associations with IBD.[16,17]

The NOD2 gene associations primarily described demonstrated that it was somehow related to CD pathogenesis, mostly due to host responses in intracellular bacterial processing.[16] Recent CD associations with the ATG16L1 gene and IRGM gene region have been presented, noticing that for both NOD2 and ATG16L1 genes, the association only appears related to CD.[16] With great importance is also the IL23 pathway in IBD pathogenesis, with multiple described associations within the IL23R gene to IBD and more moderate associations with the IL12B and PTPN2 genes.[16] Also relevant known associations with potentially different patterns of relation in the

IL23R and major histocompatibility complex regions allows new insight in important disease-modifying regions that may permit the establishment of differences between CD and UC.[16]

In 2006, beyond NOD2 and IBD5, three high-density GWAS and one non-synonymous SNP scan identified new 11 CD susceptibility loci.[18] Since 2008 until date, it was reported 99 risk loci: 71 CD-associated loci and 47 associated with UC, with 28 loci shared between the two diseases, which are involved in several pathways important to microbial recognition, autophagy, inflammatory response, epithelial barrier maintenance, metabolism and endoplasmic reticulum stress responses.[18,19]

NOD2

The first IBD gene, NOD2 (for nucleotide-binding oligomerization domain containing 2; previously known as caspase recruitment domain protein 15/CARD15), located on chromosome 16q12 within the IBD1 region, was identified in 2001 through association mapping of one of these linkage regions.[20] Three mutations of the gene (Arg702Trp, Gly908Arg, and Leu1007fsinsC) are described as cause for reduction or loss of NOD2 function.[17,19] Its reported that a 2.4-fold increased risk of CD occurs in heterozygous carriers of the major risk allele, while a 17.1-fold increased risk occurs in homozygous or compound heterozygous individuals of European decent and no association has been observed in Asian or sub-Saharan African populations.[19] Because of complexity and multifactorial nature of disease onset, associations with NOD2 variants simple refers to patients with an earlier age of onset, ileal location and stricture formation, and not determining of disease cause.[19] Recent studies have linked a novel risk gene interaction between NOD2 and ATG16L1 that may help in the understanding of IBD susceptibility, which is thought to be due to a dysregulation of a central pathway, instead of a dysfunction of one specific gene.[19] The mechanism of autophagy induction by NOD2 that can be observed in multiple cell types, such as epithelial cells, depends on ATG16L1 expression and relies on the autophagic response initiated by ATG16L1 existing in the bacterial entry sites in the plasma membrane.[19]

In the IBD5 risk haplotype was identified within a larger linkage region on chromosome 5q31, single nucleotide polymorphisms (SNPs) genes from prolyl 4-hydroxylase (P4HA2), interferon regulatory factor 1 (IRF1) and organic cation transporter (OCTN) 1 and OCTN2, but further studies are needed.[3]

Interleukin 23 receptor (IL23R)

IBD susceptibility has been associated with several genes in the Th17 pathway, with major focus on the IL23R gene on chromosome 1p31, due to its significantly high association with disease development and involvement in other chronic inflammatory diseases.[21]

Also the importance of the IL23 pathway has been emphasized by its associations with variants in IL12B, which encodes the p40 subunit shared between IL12 and IL23, CD and UC susceptibility and several other components of the Th17 pathway, namely STAT2 and JAK3 that are also associated with UC, the chemokine receptor CCR6 and co-stimulatory molecule ICOS-L27.[18]

Major attention has also been pointed to the IL23/IL12 because of its determining role in naïve T cells differentiation into effector Th1 cells (driven by IL12) or Th17 cells (driven by IL23).[18]

The genome wide association study by Duerr *et al* presented significantly important associations between variants in the IL23R gene and CD, which allowed the identification of an uncommon coding variant, rs11209026 (Arg381Gln), that confers strong protective effect against CD.[22] These findings are interesting due to the participation of IL23 in the IL23/IL17 axis, its influence as a proinflammatory mediator and the possibility of its use as a therapeutic target of autoimmune and chronic inflammatory diseases such as CD.[22]

Unlike NOD2, IL23R variants don't present an association with CD behavior and location and it haven't been reported that the IL23R genotype influenced age of onset, need for surgery or association with family history.[22]

Apoptosis

The Fas gene, in the chromosome 10q24.1, presents a single nucleotide substitution at the -670 position that probably exerts an effect on the level of transcription of the Fas protein due to its location at the consensus sequence site, the gamma interferon activation site (GAS), that may bind to transcription factors such as signal transducers and activator of transcription (STAT).[23] This gene is a member of the tumor necrosis factor superfamily, is possibly involved in autoimmune diseases and inflammatory disorders and its described as a mediator of apoptosis when cross-linked with agonistic anti-Fas antibody or Fas ligand (FasL).[23]

FasL (1q23) is a key apoptosis inducing ligand of the TNF family of death factors.[24] It has been pointed a threefold increased binding capacity to the CAAT enhancer protein by the carriers of the C allele of FasL -843C/T polymorphism, and consequently a threefold higher expression of FasL, leading to an increase of active cells expressing Fas apoptosis. For these reasons, a less severe phenotype due to a more susceptibility to apoptosis will occur in wild type carriers.[24]

Deregulation of caspase activity has been correlated with several human diseases, including IBD and colorectal cancer, namely caspase-8 that controls the death of intestinal epithelial cells in patients with Crohn's disease and appears to be involved in mucosal inflammation and caspase-9 involved in the sequential activation of caspases determining in cell apoptosis.[25]

Peroxisome proliferator-activated receptor gamma (PPARG) (3p25) encodes a member of the peroxisome proliferator activated receptor (PPAR) subfamily of nuclear receptors, is responsible for the inhibition of NFkB activity and their polymorphisms were related to Crohn's disease.[24]

It has been described a polymorphism in the PPARG gene, exon 1 (CCA-GCA, producing a Pro/Ala substitution at codon 12). This described conformational change in the protein is associated with a more aggressive and active phenotype in the wild type carriers, which also present a less inhibition of NFkB pathway.[24]

Autophagy

In 2007, a 2-fold disease risk increases in individuals homozygous for the risk allele of a SNP in the coding region of the ATG16L1 gene (rs2241880) was identified by GWAS and was further reported that the linkage of this variant to IBD is more significantly for CD patients with ileal disease.[19]

Other discoveries were immunity-related guanosine triphosphatases (IRGs), with two SNPs (rs13361189 and rs4958847) flanking the coding region of IRGM, that are an important family of proteins involved in the elimination of different intracellular pathogens in most mammals, ULK1, presenting a single identified SNP (rs12303764) significantly associated with CD, is a component of an essential protein complex involved in autophagy initiation and, finally LRRK2 (leucine-rich repeat kinase 2), with a CD-associated SNP (rs1175593) located upstream of the coding sequence of LRRK2, is an important participant in the autophagic equilibrium maintenance and is near two relevant autophagy proteins (p62 and LC3).[19]

It has been approached in different studies the importance of ATG16L1 as a risk locus, mainly through the analysis of its polymorphisms interactions with NOD2 and IL23R susceptibility variants, once it hasn't been quiet understood if it depends on NOD2 or IL23R status or it just occurs for particular IBD subgroups.[26]

Recently, it also has been reported that the risk variant of ATG16L1 is important to IL1B, IL6 and TNFA production in CD and influences the induction of autophagy, specifically after NOD2 engagement.[27] It was also demonstrated that it is possible to predict response to anti-TNF therapy in patients with CD through gene polymorphisms, once it has been confirmed that patients with mutated NOD2/ATG16L1-combined genotypes are more frequently submitted to an enhanced anti-TNF therapy.[27]

Other polymorphisms

The occurrence of an enhanced chronic inflammatory response during the development of CD has led to the identification, through genetic mapping studies, of several polymorphisms in the

TNFA gene and the IL1 gene cluster.[28] The TNFA gene, on chromosome 6 in a region containing the IBD3 locus, present some polymorphisms in the TNFA promoter that have been suggested as implicated in CD susceptibility, such as TNFA -308 which is the strongest association described, TNFA -238, TNFA -376, and TNFA -1031 that play a role in the transcription rate of TNFA.[29]

Studies in lymphotoxin-α, namely LTA +250, a G to A transition in the first intron of LTA, have suggested an association with increased TNFA production both *in vitro* and *in vivo* for carriers of the A allele, but further conclusions are needed. This locus has been pointed out due to its relation with many inflammatory conditions. An association of linkage disequilibrium has been verified between the TNFA -308G allele and the LTA +250 A allele.[29]

Interleukin 1 (IL1), a potent proinflammatory cytokine, belongs to the IL1 family that includes the agonists IL1A and IL1B and the IL1 receptor antagonist (IL1RA), with IL1B crucial in the process of inflammatory response.[28, 29] The studied polymorphism IL1B-511 C/T and the IL1RA variable number of 86-pb tandem repeats (VNTR) alleles, are associated with increased levels of IL1B production, which suggest a role in the development of IBD.[28]

Interleukin 6 (IL6) is a multifunctional cytokine involved in inflammatory response and differentiation and activation of macrophages and T cells that presents several SNPs within the promoter, with the most studied suggesting that the wild-type carriers of G allele of IL6 -174 C/G polymorphism possesses an enhance production of IL6 compared with the other carriers.[29,30]

Currently major focus has been driven on the multidrug resistance 1 (MDR1) gene, which is composed of 28 exons and is 209 kilobases in length with 29 SNPs described,[31] with the most relevant SNPs known being the C3435T in exon 26 and G2677T/A in exon 21. The latter SNP originates 2 distinct amino acid changes, namely 893Ser (G2677T) or the much rarer 893Thr (G2677A).[31,32] These two SNPs are thought to be important in determination of UC extension, like has been confirmed by Ho *et al.*, CD severity and susceptibility to IBD, once it has been reported that they may lie in linkage disequilibrium.[31,32,33] It has been emphasized the importance for a role of the MDR1 gene in IBD pathogenesis,[34] since encodes the ATP-dependent membrane efflux transporter P-glycoprotein-1 (PgP), whose gene product pgp-170 when highly

expressed in intestinal epithelium plus its constitutive levels in the gut suggests a protection against xenobiotics, including bacterial products and, finally, due to its location within a region of suggestive IBD linkage on chromosome 7q.[32,34]

Analysis of susceptibility genes in a Portuguese population

Important genetic polymorphisms in a variety of antigen recognition pathways, proinflammatory cytokines and antiinflammatory cytokines have been identified because of its influences on the inflammatory response.[29]

Accordingly, major attention has been paid to agents able to reduce the secretion of proinflammatory cytokines in the research for the treatment of this disease. In our study[30], we have examined in 116 controls and 99 patients with CD, seven SNPs in IL1, TNFA, LTA and IL6 genes for its influence in modifying the susceptibility for CD and disease activity and we concluded that wild-type carriers of G allele for the IL6 − 174G/C polymorphism had a six-fold higher risk for CD, whereas the carriers of T allele for the TNFA − 857C/T polymorphism were associated with more active disease.[30]

Other fundamental mechanism that occurs in CD is resistance to apoptosis, namely of T-cells, which certainly contributes to perpetuate inflammation in the intestinal mucosa. For these reason is suspected that SNPs in various genes, namely apoptotic ones, may explain not only the heterogeneous phenotypes but also the different responses to similar treatments. In our study[24], Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP) techniques were used to analyze CASP9 +93C/T, FasL -843C/T, PPARG +161C/T and Pro12Ala SNPs in 99 patients with CD and 116 healthy controls. We didn't observe any significant differences in odds ratio concerning the risk of developing CD or predisposition for a more active phenotype, as well as no significant association between SNPs analyzed and disease location, phenotype, age of disease onset and other characteristics of disease.[24]

THERAPEUTIC IN INFLAMMATORY BOWEL DISEASES

The big challenge clinicians face when treating IBD patients is that the course of the disease in the years following diagnosis is difficult to predict. For this reason, the main goals to attain with the therapeutic are: relief of the symptoms, upgrade of the patient's quality of life, maintenance of the nutritional status, deep remission and mucosal healing.[35] Several studies are identifying a range of clinical, serologic, and genetic predictors that might be able to solve this question in the future.[36]

At present, however, clinicians must select treatments based on clinical criteria and on the existent therapies, namely:

Aminosalicylates

Mesalazine [5-aminosalicylic acid (5-ASA)] is mostly used for the treatment of mild to moderate ulcerative colitis, which embraces the majority of patients with this disease, while for CD it is reported a much more limited role.[11] This first line of treatment has been used over 30 years in the treatment of IBD, once it is a highly effective, safe, and well-tolerated drug.[10,11,37] Over the years several formulations have been developed and nowadays exists as oral formulations that differ in their delivery mechanisms and newer drugs that allow direct release in specific regions of the gastrointestinal tract allowing a more convenient dosing form and schedule.[11]

Corticosteroids

Corticosteroids (CS) are potent inhibitors of T cell activation and cytokine secretion, which leads to its use as the first-line conventional therapy for patients with active CD of moderate to severe activity with an efficacy range from 48% to 92%.[38,39,40] It has been reported a prolonged steroid response in 44% of patients with CD, steroid dependency in 36%, and steroid refractory in 20%, while failure to response leads to indication for surgery in as many as 20% of UC patients and approximately 50% of CD patients.[38,40,41]As side effects it has been referred systemic action and inhibition of endogenous adrenal function.[39]

Immunossupressors

Thiopurine drugs, azathioprine (AZA) and mercaptopurine (MP) are the principally treatment for steroid-dependent CD, due to its efficacy in maintaining remission of the disease induced by steroids.[42] It was also disclosed a clear steroid sparing effect in active or quiescent CD with AZA/MP therapy, however only 40% to 50% of patients achieve a complete, steroid-free remission with AZA.[42] The effectiveness of thiopurine therapy in UC and CD is still uncertain, because of the limited number of studies existing and the knowledge that surgical cure of UC is theoretically possible.[43]

Other immunosupressors have been used when thiopurines fail, namely, methotrexate, effective for induction and maintenance of remission; cyclosporine IV, with good results in the treatment of corticoresistant UC patients, with variable remission rates from 50-80%, nevertheless it's necessary to combine with AZA or 6-MP for maintenance of remission up until 5 years in 60% of the cases and finally, tacrolimus, with a mechanism of action and toxicity similar to cyclosporine, but more powerful, presents rates of efficacy in the treatment of corticoresistant UC patients nearly as the same as cyclosporine and it's not applicable in CD.

Biologics

The realization of the importance of TNF in the pathogenesis of CD has lead to the appearance of biologic therapy.[5,44] These knowledge pointed to the development of biological agents for treating IBD capable of targeting a specific event of the inflammatory cascade, induce and maintain a sustained remission, be well tolerated and induce no immunogenicity.[4] The anti-TNF therapy commonly used reduces reliance or dependence on corticosteroid- based therapies and avoids corticosteroid- associated adverse effects.[44]

Currently, there are four anti-TNF agents (infliximab, adalimumab, certolizumab pegol and golimumab) approved by the US Food and Drug Administration (FDA) and European Medicines Agency (EMA)[45] for use in patients with moderate to severe CD for whom conventional therapy has failed. [46] Infliximab (IFX), the first anti-TNF agent developed, is a chimeric mouse/human

monoclonal IgG1 antibody composed of 75% human and 25% murine sequences characterized by its high specificity for and affinity to TNFA, that neutralizes the biologic activity of TNFA by inhibiting the binding with its receptors.[47] Adalimumab (ADA) is a fully human recombinant IgG1 monoclonal antibody against TNFA that appears as an alternative for patients loosing response to infliximab, with its use approved for luminal CD. This therapy major advantage for UC treatment is its use as a subcutaneous drug, which is very important principally for patients with difficult venous access.[47] Certolizumab pegol (CDP-870) (CPZ), therapy subcutaneously administered, is a pegylated humanized fragment antigen binding (Fab) that binds TNFA.[33] Golimumab (Simponi®) is a relatively new human monoclonal anti-TNF IgG1 antibody, that emerges as an important new subcutaneous therapeutic option for the treatment of moderately to severity active UC in adults.[45]

Since biologics are frequently given to patients refractory to former treatments, it is frequently observed the development of an antibody response against biological drugs due to cumulative toxicities, with the immunogenicity different for each drug.[7,48]

Combined Therapy

Data now show that combined therapy with an anti‑TNF agent and an immunosuppressant is the most effective strategy for treating CD.[36,49] However, it is reasonable to propose that a patient with mild disease and no criteria that would predict increased disease severity, could likely be treated effectively with a less intensive strategy.[36]

The goal of combined therapy in patients with CD is to induce both deep remission and full healing of the transmural inflammatory process that occurs in CD in order to avoid complications of CD, surgeries and disability linked to surgery.[36] Unfortunately, it is still debatable long-term safety, namely opportunistic infections or increased risk for certain neoplasms.[48]

GENETIC PARAMETERS AS PREDICTORS FOR THERAPY RESPONSE

The clinical course of CD and UC differs enormously between patients which is problematic to the design of the treatment, although there are some known clinical parameters that help to predict a mild or more severe outcome, however they are variable over time and very subjective.[50]

Genetic markers are emerging as powerful tools for patients stratification once they are stable over time and not suitable for subjective interpretation, although further studies are needed for its use in a regular basis.[50]

Recent studies have highlighted the associations between genetics and clinical features of IBD, namely disease location, behavior, natural history and response, and side effects of drug therapy.[51]

Pharmacogenetics and genetic parameters

Pharmacogenetics permits not only the explanation of interindividual variability in drug response, but also prediction of efficacy and adverse drug events in different patients.[52] Its ultimate goal is the recognition of genetic predictors of drug response with the purpose of development of prospective genetic tests that permit the identification of patients at risk of non-response or of developing an adverse effect before the initiation of the treatment, that usually results from allelic variants in genes involved in the uptake, distribution, metabolism, transport, receptor and target of the drug. [53,54]

The major focus in pharmacogenetics research has been on allelic variants in drug-metabolizing enzymes (DMEs).[52,54]

Drug-metabolizing enzymes (DMEs).

During the course of the disease the majority of CD patients is treated with immunossupressors, being most commonly used azathioprine and 6-mercaptopurine and methotrexate less commonly, although is observed wide variability in interindividual response in terms of efficacy and toxicity.[53] For these reasons, emphasis has been made in

pharmacogenetic research that aimed at predicting response to treatment, in order to describe individualizing drug type and dose for each patient, with thiopurine analogues studies being the ones with most relevant results.[53] It is known that after absorption AZA is rapidly converted to 6-MP by a nonenzymatic reaction, where three enzymes compete for its metabolization: hypoxanthine guanine phosphoribosyltransferase initiates the production of 6-thioguanine nucleotides (6-TGNs), involved in the therapeutic and toxic hematologic effects of thiopurines, whereas xanthine oxidase (XO) and thiopurine S-methyltransferase (TPMT) control the production of 6-TGNs by converting 6-MP to 6 thioruric acid and 6-methylmercaptopurine, respectively.[51]

The enzyme TPMT that metabolizes azathioprine presents two wild-type TPMT alleles (TPMT*1 and TPMT*1S) and 16 variant alleles with low enzymatic activity (TPMT*2, *3A, *3B, *3C, *3D, *4-15) described, with mutations on its gene resulting in lower TPMT enzyme activity.[6]

In clinical practice, its commonly genotyped TPMT variants and measured TPMT enzyme activity with the purpose of identifying patients with high TPMT activity that metabolize 6-mercaptopurine to 6-methyl-MP and therefore may be resistant to treatment with thiopurine drugs, once genotypes do not fully correlate with the enzyme activity, especially in the case of wild-type (some patients will have reduced TPMT activity) or heterozygous (some will have a normal TPMT activity) individuals.[50]

Multidrug resistance 1 (MDR1) gene

It has been shown that polymorphisms in MDR1 gene control, in part, the expression and efflux efficiency of Pgp, fact that have been described in several studies based on the initial observation that homozygous carriers of the T allele for the MDR1 3435 polymorphism present a lower intestinal Pgp expression and therefore drug absorption from the gastrointestinal (GI) tract should be higher and result in increased plasma levels .[32,55] Concerning MDR1 G2677T/A polymorphism it has been reported an enhanced Pgp-170 activity in carriers of the T allele (Ser893).[56] In a study, Farrell *et al* observed an association between high Pgp-170 expression

and UC patients with severe glucocorticoid-resistant disease and that high peripheral blood lymphocyte Pgp-170-expressing patients are more likely to require steroids, what corroborates the also described association of homozygous carriers for the T allele with severe UC.[56]

Also Pgp and MDR expression were shown to be significantly higher in CD and UC patients requiring surgery due to failure of medical therapy, fact that can be supported by the observation that glucocorticoid resistance was found to be associated with prior bowel resection, perianal disease and a high initial Crohn's disease activity index (CDAI).[54] The homozygous carriers of the T allele for the polymorphism MDR1 3435 C/T were associated with extensive UC, which can be supported by an reported association between steroid refractoriness and the 3435 TT genotype, but the TT genotype is associated with lower expression of MDR1 and Pgp170.[32] Other study by Potocnik *et al* reported an association between SNPs in introns 13 and 16 of the MDR1 gene and CS-refractory in CD and UC, while the polymorphism C3435T in exon 26 was associated with significant or complete CS tapering by Leuven *et al*.[50] It was also suggested that these MDR1 polymorphisms although increasing disease susceptibility for CD and UC, may also be involved in the modulation of response to immunossupressors, once it was described in a study the occurrence of a higher frequency of 2677T/3435T haplotype in azathioprine non-responder CD patients.[32]

TNF and TNF receptor pathway

Novel therapeutic strategies have been developed involving the TNF family because of its participation on stimulating its own and others cytokine production, enhancing the expression of adhesion molecules and neutrophil activation and its involvement as a costimulator of T-cell activation and antibody production by ß cells.[52] The interest on these therapy relies on the blockage of the interaction between TNFA and the accessory TNF cell-surface receptors, important in the pathogenesis of IBD, apoptosis cell proliferation and differentiation.[52,57]

It has been already identified different SNPs in the TNFA promoter region that influence its gene expression, namely -238G/A that is associated with lower production of TNFA in patients

with UC and -308G/A that is associated with enhanced TNFA production in cells *in vitro* and in patients with CD in vivo.[57]

Despite the major improvement in quality of life of IBD patients with the use of monoclonal antibodies to TNF, it should be noticed the high economical costs of this therapy, as well as its side effects.[50] Nevertheless more than 75% of patients are responsive, but resistance still occurs and it was of great benefit if early response could be accurately predicted in order to optimize management of the disease.[50]

When comparing allele and genotype frequencies regarding response to IFX or ADA treatment it was not significant in the TNFA -238G/A promoter SNP study, but it was reported in a TNFA -308G/A promoter SNP study that a higher frequency of the -308A allele and -308GA genotype occurs in no responders to anti-TNF treatment opposing to responders patients ($P<0.05$).[57]

Other known aspect of infliximab is its ability to induce apoptosis of activated T lymphocytes, fact that was studied by Hlavaty *et al* in a population of luminal or fistulizing CD patients and that led him to the observation that in luminal CD, heterozygous individuals for the FasL -843 C/T polymorphism presented a 74.7% versus 38.1% response rate in homozygous carriers for the T allele ($P < 0.01$, OR = 0.11, 95% CI 0.08-0.56), what can be overthrown by concomitant use of AZA.[6,50] For the homozygous carriers of the T allele for the CASP9 93 C/T SNP it was reported a positive response to IFX in opposition to the remaining 66.7% of patients with the CC and CT genotypes ($P = 0.04$, OR = 1.50, 95% CI 1.34-1.68).[6,50]

THERAPY IN CLINICAL CASES: A PERSPECTIVE FROM A PORTUGUESE POPULATION

In the past years our group worked with the purpose of identifying clinical and genetic predictors of response to therapy in IBD, which may become of potential utility in clinical practice. In 2013 and 2014 we published the results driven from these multicenter studies with participating Hospitals from Central Portugal, were we analyzed clinical parameters characteristic from both diseases and polymorphisms in MDR1, inflammation, apoptosis and

autophagy genes. In both studies informed and approved by the Scientific and Ethical committees consent was obtained from all patients entering the study.

Here we intend to emphasize the main clinical and genetic predictors obtained from both studies.

Application of therapy in a Crohn's disease population in Portugal[58]

A total of 242 CD patients were eligible to enter the study; mean follow-up period was 2.5 years. In terms of percentage of responders from total patients to different therapies we have 12% responders to 5'-ASA, 44% responders to corticosteroids, 41% responders to azathioprine and 33% responders to infliximab (Fig.1).

Our results showed that in terms of clinical parameters age and previous surgery were identified as predictors. We found a better response to 5-ASA and to azathioprine in older patients while younger ones responded better to biologicals and that previous surgery negatively influenced response to 5-ASA compounds, but favoured response to azathioprine.[58] In respect to genetic predictors, we identified a relation between autophagy ATGL16L1 SNP and better response to corticosteroids and CASP9 C93T SNP presented a lower chance of responding both to corticosteroids and to azathioprine. MDR1 C3435T SNP related to a higher chance of responding to azathioprine, while MDR1 G2677T/A SNP presented a better response to azathioprine, but a lower chance of responding to biologicals.[58]

In terms of response to the switching of the therapy when it became necessary and in regard to corticosteroids we observed that previous therapy with biologicals was a negative predictor of response to both corticosteroids and to azathioprine.[58]

Application of therapy in a Ulcerative Colitis disease population in Portugal[59]

A total of 174 patients entered the study; the median follow-up was 3.9 years. In terms of percentage of responders from total patients to different therapies we have 64% responders to 5'-ASA, 30% responders to corticosteroids, 16% responders to azathioprine and 10% responders to infliximab (Fig.2).

Several clinical predictors were identified, namely age, age at diagnosis, duration of disease, disease extension and EIM. We observed that older patients and those diagnosed after the age 40 responded positively to 5-ASA compounds and patients with duration of disease for more than 5 years presented a negative predictor of response both for 5-ASA and for azathioprine, although the latter did not reach statistical significance. Disease extent negatively influenced response to 5-ASA and azathioprine, with patients with pancolitis showing poorer responses. With respect to EIM, we observed that it was a negative predictor of response to 5-ASA, corticosteroids, and azathioprine but it seemed to positively influence response to biologics, although not significantly.[59] In terms of genetic predictors we found that IL23R C2370A and G9T SNPs are associated both with EIM, while IL23R C2370A SNP is associated with nonresponse to 5-ASA and corticosteroids and IL23R G9T SNP is more likely to respond to azathioprine.[59]

Previous therapies also seemed to influence response to several drugs, since previous users of corticosteroids, azathioprine, and/or biologics responded better to 5-ASA, which was also observed for corticosteroids and azathioprine.[59]

These observations need to be confirmed in future studies.

PERSONALIZED MEDICINE: IS THE FUTURE ON GENETICS?

Instead of searching for unique treatment that can be applied to all patients, individualize therapy seems to be the solution for the future.[60] To date clinical characteristics of disease as age stratification, disease location and extension, serologic parameters, site of inflammation, severity and course of disease all have potential to predict disease progression and complication, and thus contribute to the physician's individualized plan of treatment, but it is now recognized the importance and influence of individual patient's genetic background.[15,27,33]

In the recent years has emerged the concept of pharmacogenetics, described as the study of association between variability in drug response and genetic variation, with the purpose of discriminating the appropriate therapy regarding a patient's specific genetic background and promote efficacy and drug safety rates.[50,51]

The major objective of the pharmacogenetic studies has been the establishment of associations between genetic variation and response and side effects of known IBD therapies, once its common knowledge that differences in drug response are related to functional differences in a gene product encoded by different alleles of the same gene.[51]

But genetics can't explain everything, including the fact that 20% to 30% of IBD patients are refractory to any therapy despite optimal dose and duration, side effects and drugs toxicity are variable and disease duration, severity, behavior and concomitant therapies may all influence the response to a drug.[40,50] Other evidence is that heterogeneity in drug effects is due to genetic polymorphisms in drug metabolizing enzymes that affect active drug concentrations together with drug receptor genetic variants.[50]

Quality of life (QoL) of IBD patients is a major concern for the physicians and therefore its important to considerate the patient's response to a drug, both the therapeutic and side effects.[14] This achievement is possible if in the future would be consider the analysis whether earlier and more effective treatment of CD would influence disease activity and long-term outcomes for patients.[44]

With appropriate therapies and treatment regimens, deep remission is currently achievable in a minority of patients, and because of that, the future of IBD treatment is to ensure that all patients achieve this goal.[61]

Although much progress has been attained, it is expectable that in the near future aspects as new therapies with considerable benefits, less side effects and fewer costs will be obtained through patients genetic background studies in order to predict their response to a given drug.[60]

CHALLENGES FOR THE FUTURE

The potential clinical relevance of identification and utilization of clinical and genetic predictors of response to several therapies available to treat IBD is to gather valuable information for physicians in order to help them assess the initial response to the different

therapies and allow the success of personalized therapy and the achievement of better quality of life for the patients.

Given the importance of mucosal healing it will most certainly be of great importance a patient's mucosal gene signature in order to achieve personalized medicine once it will allow the identification of therapy for each individual.[62]

It is expected that biological therapies for IBD will be developed and more selectively used, accordingly to the application of personalized benefit/risk analysis for each drug through the use of reliable biomarkers and tissue signatures.[45]

New biologic treatments are currently in development for IBD and they mostly target leukocyte trafficking and proinflammatory cytokines, such as IL6, IL17, IL18, and IL21.[45]

It was recently reported other means of blocking TNF like anti-TNF vaccination, TNF gene silencing with small interfering RNA and TNF-neutralizing nanobodies.[62]

Moreover, a variety of small molecules that selectively inhibit signaling molecules, including protein kinase C and NFkß are currently under further development. Finally, approaches such as appendectomy, stem-cell therapies and fecal transplantation are also being assessed in controlled trials, aiming to target the pathophysiological basis of IBD.[45]

Regarding MDR1 gene it is enhanced the importance of diagnostic tests for the discrimination of MDR1 alleles with the goal of CD therapy improvement.[45]

Over the next decade are expected advances in drug action knowledge, appearance of new drugs targets and a greater understanding of the genetic factors that determine drug response. In summary, it is desirable the use of pharmacogenetic as a daily clinical practice in association with the commonly use methods for choosing drugs and selecting dosing regimens applied nowadays.

FUNDING

This research was supported by Grupo de Estudo da Doença Inflamatória Intestinal (2009-2012).

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

REFERENCES

1. Satsangi J, Morecroft J, *et al.* Genetics of inflammatory bowel disease: scientific and clinical implications. *Best Pract Res Clin Gastroenterol* 2003; **17**(1): 3-18.

2. Brain O, Cooney R, *et al.* Functional consequences of mutations in the autophagy genes in the pathogenesis of Crohn's disease. *Inflamm Bowel Dis* 2012; **18**(4): 778-781.

3. Budarf ML, Labbe C, *et al.* GWA studies: rewriting the story of IBD. *Trends Genet* 2009; **25**(3): 137-146.

4. Reenaers C, Louis E, *et al.* Current directions of biologic therapies in inflammatory bowel disease. *Therap Adv Gastroenterol* 2010; **3**(2): 99-106.

5. Cottone M and Criscuoli V. Infliximab to treat Crohn's disease: an update. *Clin Exp Gastroenterol* 2011; **4**: 227-238.

6. Pierik M, Rutgeerts P, *et al.* Pharmacogenetics in inflammatory bowel disease. *World J Gastroenterol* 2006; **12**(23): 3657-3667.

7. Rutgeerts P, Vermeire S, *et al.* Biological therapies for inflammatory bowel diseases. *Gastroenterology* 2009; **136**(4): 1182-1197.

8. Hlavaty T, Pierik M, *et al.* Polymorphisms in apoptosis genes predict response to infliximab therapy in luminal and fistulizing Crohn's disease. *Aliment Pharmacol Ther* 2005; **22**(7): 613-626.

9. Wenger S, Nikolaus S, *et al.* Predictors for subsequent need for immunosuppressive therapy in early Crohn's disease. *J Crohns Colitis* 2012; **6**(1): 21-28.

10. Bokemeyer B, Hommes D*, et al.* Mesalazine in left-sided ulcerative colitis: efficacy analyses from the PODIUM trial on maintenance of remission and mucosal healing. *J Crohns Colitis* 2012; **6**(4): 476-482.

11. Williams CPR, Ghosh S, *et al.* Optimizing clinical use of mesalazine (5-aminosalicylic acid) in inflammatory bowel disease. *Therap Adv Gastroenterol*. 2011; **4**(4): 237–248.

12. Allez M and Lemann M. New directions and new strategies in inflammatory bowel diseases. *Gastroenterol Clin Biol* 2009; **33 Suppl 3**: S121-122.

13. Colombel JF, Sandborn WJ, *et al.* Infliximab, azathioprine, or combination therapy for Crohn's disease. *N Engl J Med* 2010; **362**(15): 1383-1395.

14. Lawlor G and Moss AC. Cause for controversy? Infliximab in the treatment of ulcerative colitis: an update. *Clin Exp Gastroenterol* 2009; **2**: 149-161.

15. Meier J and Sturm A. Current treatment of ulcerative colitis. *World J Gastroenterol* 2011; **17**(27): 3204-3212.

16. Cho JH and Weaver CT. The genetics of inflammatory bowel disease. *Gastroenterology* 2007; **133**(4): 1327-1339.

17. Brant SR. Promises, Delivery, and Challenges of Inflammatory Bowel Disease Risk Gene Discovery. *Clinical Gastroenterology and Hepatology* 2013; **11**: 22–26.

18. Stappenbeck TS, Rioux JD, *et al.* Crohn disease: a current perspective on genetics, autophagy and immunity. *Autophagy* 2011; **7**(4): 355-374.

19. Kabi A, Nickerson KP, *et al.* Digesting the genetics of inflammatory bowel disease: insights from studies of autophagy risk genes. *Inflamm Bowel Dis* 2012; **18**(4): 782-792.

20. Ardizzone S, Maconi G, *et al.* Multidrug resistance 1 gene polymorphism and susceptibility to inflammatory bowel disease. *Inflamm Bowel Dis* 2007; **13**(5): 516-523.

21. Pidasheva S, Trifari S, *et al.* Functional studies on the IBD susceptibility gene IL23R implicate reduced receptor function in the protective genetic variant R381Q. *PLoS One* 2011; **6**(10): e25038.

22. Li Y, Mao Q, *et al.* Interleukin-23 receptor genetic polymorphisms and Crohn's disease susceptibility: a meta-analysis. *Inflamm Res* 2010; **59**(8): 607-614.

23. Xia B, Yu YH, *et al.* Association of Fas-670 gene polymorphism with inflammatory bowel disease in Chinese patients. *World J Gastroenterol* 2005; **11**(3): 415-417.

24. Ferreira P, Cravo M, *et al.* Fat intake interacts with polymorphisms of Caspase9, FasLigand and PPARgamma apoptotic genes in modulating Crohn's disease activity. *Clin Nutr* 2010; **29**(6): 819-823.

25. Becker C, Watson AJ, *et al.* Complex roles of caspases in the pathogenesis of inflammatory bowel disease. *Gastroenterology* 2013; **144**(2): 283-293.

26. Marquez A, Nunez C, *et al.* Role of ATG16L1 Thr300Ala polymorphism in inflammatory bowel disease: a Study in the Spanish population and a meta-analysis. *Inflamm Bowel Dis* 2009; **15**(11): 1697-1704.

27. Chen QQ, Wan J Select a suitable treatment strategy for Crohn's disease: Step-up or Top-down. *EXCLI Journal* 2014; **13**: 111-122.

28. Cummings SA and Rubin DT The complexity and challenges of genetic counseling and testing for inflammatory bowel disease. *J Genet Couns* 2006; **15**(6): 465-476.

29. Waterer G.W and Wunderink RG Science review: Genetic variability in the systemic inflammatory response. *Crit Care* 2003; **7**(4): 308-314.

30. Guerreiro CS, Ferreira P, *et al.* Fatty acids, IL6, and TNFalpha polymorphisms: an example of nutrigenetics in Crohn's disease. *Am J Gastroenterol* 2009; **104**(9): 2241-2249.

31. Ho GT, Nimmo ER, *et al.* Allelic variations of the multidrug resistance gene determine susceptibility and disease behavior in ulcerative colitis. *Gastroenterology* 2005; **128**(2): 288-296.

32. Mendoza JL, Urcelay E, *et al.* MDR1 polymorphisms and response to azathioprine therapy in patients with Crohn's disease. *Inflamm Bowel Dis* 2007; **13**(5): 585-590.

33. Herrlinger KR, J. D. Review article: interactions between genotype and response to therapy in inflammatory bowel diseases. *Aliment Pharmacol Ther.* 2006; **24**(10): 1403-1412.

34. Huebner C, Browning BL, *et al.* Genetic analysis of MDR1 and inflammatory bowel disease reveals protective effect of heterozygous variants for ulcerative colitis. 2009; **15**(12): 1784-1793.

35. Reenaers C, Belaiche J, *et al.* Impact of medical therapies on inflammatory bowel disease complication rate. *World J Gastroenterol* 2012; **18**(29): 3823-3827.

36. Colombel JF. When Should Combination Therapy for Patients with Crohn's Disease Be Discontinued? *Gastroenterol Hepatol (N Y)* 2012; **8**(4): 259–262.

37. Feagan BG and MacDonald JK. Once daily oral mesalamine compared to conventional dosing for induction and maintenance of remission in ulcerative colitis: a systematic review and meta-analysis. *Inflamm Bowel Dis* 2012; **18**(9): 1785-1794.

38. Farrell RJ and Kelleher D. Glucocorticoid resistance in inflammatory bowel disease. *J Endocrinol* 2003; **178**(3): 339-346.

39. Lemann M, Mary JY, *et al.* Infliximab plus azathioprine for steroid-dependent Crohn's disease patients: a randomized placebo-controlled trial. *Gastroenterology* 2006; **130**(4): 1054-1061.

40. Faubion WA, Jr., Loftus EV, Jr., *et al.* The natural history of corticosteroid therapy for inflammatory bowel disease: a population-based study. *Gastroenterology* 2001; **121**(2): 255-260.

41. Orland, A, Renna S, *et al.* Adalimumab in steroid-dependent Crohn's disease patients: prognostic factors for clinical benefit. *Inflamm Bowel Dis* 2012; **18**(5): 826-831.

42. Camus M, Seksik P, *et al.* Long-term outcome of patients with Crohn's disease who respond to azathioprine. *Clin Gastroenterol Hepatol* 2013; **11**(4): 389-394.

43. Gisbert JP, Linares PM, *et al.* Meta-analysis: the efficacy of azathioprine and mercaptopurine in ulcerative colitis. *Aliment Pharmacol Ther* 2009; **30**(2): 126-137.

44. Schreiber S. Certolizumab pegol for the treatment of Crohn's disease. *Therap Adv Gastroenterol* 2011; **4**(6): 375-389.

45. Lowenberg M and D'Haens G. Novel targets for inflammatory bowel disease therapeutics. *Curr Gastroenterol Rep* 2013; **15**(2): 311.

46. Rubin DTU and Sederman R. Response to Biologic Therapy in Crohn's Disease Is Improved with Early Treatment: An Analysis of Health Claims Data. *Inflamm Bowel Dis* 2012; **18**: 2225–2231.

47. Garcia-Bosch O, Gisbert JP, *et al.* Observational study on the efficacy of adalimumab for the treatment of ulcerative colitis and predictors of outcome. *J Crohns Colitis* 2013; **7**(9): 717-722.

48. Panaccione R. Optimal use of biologics in the management of Crohn's disease. *Therap Adv*

Gastroenterol. 2010; **3**(3): 179–189.

49. Panaccione R, Gosh S, Middleton S *et al*. Combination Therapy With Infliximab and Azathioprine Is Superior to Monotherapy With Either Agent in Ulcerative Colitis. *Gastroenterology* 2014; **146**: 392–400.

50. Vermeire S, Van Assche G, *et al.* Role of genetics in prediction of disease course and response to therapy. *World J Gastroenterol* 2010; **16**(21): 2609-2615.

51. Ahmad T, Tamboli CP, *et al.* Clinical relevance of advances in genetics and pharmacogenetics of IBD. *Gastroenterology* 2004; **126**(6): 1533-1549.

52. Kooloos WM, Jong DJ, *et al.* Potential role of pharmacogenetics in anti-TNF treatment of rheumatoid arthritis and Crohn's disease. *Drug Discov Today* 2007; **12**(3-4): 125-131.

53. Roberts, RL and Barclay ML. Current relevance of pharmacogenetics in immunomodulation treatment for Crohn's disease. *J Gastroenterol Hepatol* 2012; **27**(10): 1546-1554.

54. Mascheretti S, Croucher PJ, *et al.* Pharmacogenetics of inflammatory bowel disease. *Best Pract Res Clin Gastroenterol* 2004; **18**(3): 597-609.

55. Huebner C, Browning BL, *et al.* Genetic analysis of MDR1 and inflammatory bowel disease reveals protective effect of heterozygous variants for ulcerative colitis. *Inflamm Bowel Dis* 2009; **15**(12): 1784-1793.

56. Onnie CM, Fisher SA, Pattni R *et al*. Associations of Allelic Variants of the Multidrug Resistance Gene (ABCB1 or MDR1) and Inflammatory Bowel Disease and Their Effects on Disease Behavior: A Case-control and Meta-analysis Study. *Inflamm Bowel Dis* 2006; **12**: 263–271.

57. Lopez-Hernandez R, Valdes M, Campillo JA *et al*. Genetic polymorphisms of tumour necrosis factor alpha (TNF-a) promoter gene and response to TNF-a inhibitors in Spanish patients with inflammatory bowel disease. *International Journal of Immunogenetics* 2014; **41**: 63–68.

58. Cravo M, Ferreira P, Sousa P *et al*. Clinical and genetic factors predicting response to therapy in patients with Crohn's disease. *United European Gastroenterology Journal* 2014; **2**(1): 47–56.

59. Cravo ML, Ferreira PA, et al. IL23R polymorphisms influence phenotype and response to

therapy in patients with ulcerative colitis. *Eur J Gastroenterol Hepatol* 2014; **26**(1): 26-32.

60. Kalow W. Pharmacogenetics and pharmacogenomics: origin, status, and the hope for personalized medicine. *Pharmacogenomics J* 2006; **6**(3): 162-165.

61. Travis S, Feagan BG, et al. The future of inflammatory bowel disease management: combining progress in trial design with advances in targeted therapy. *J Crohns Colitis* 2012; **6 Suppl 2**: S250-259.

62. D'Haens GR, Sartor RB, et al. Future directions in inflammatory bowel disease management. *J Crohns Colitis* 2014; **8**(8): 726-34.

CHAPTER IV – DISCUSSION

Crohn's disease is a heterogeneous disease that presents several differences regarding disease severity, location, behaviour and extraintestinal manifestations that may influence therapeutic outcome (Pierik *et al.* 2006).

It is mostly a disease from the western industrialized countries, which enhances the importance of one or more environmental factors involved in its pathogenesis, with higher attention paid to diet, and the influence of genetic factors.

For the disease control with appropriate therapy it is important to identify clinical or genetic predictors for a more aggressive phenotype and try to target it. So far, clinical predictors have been the most studied ones, allowing the identification of age at diagnosis, disease location and smoking as the most significant parameters (Louis *et al.* 2010). Moreover, other predictors such as serologic markers and genetic tests are available, but none of them is predictive enough to be use alone (Yarur *et al.* 2011). More studies are recommended to assess clinical and genetic predictors to improve treatment decisions and patients outcomes (Parkes *et al.* 2014) (Nunes *et al.* 2013).

With the aim of embracing as many as possible all of the questions regarding the complexity involving Crohn's disease, we have been developing a working project for the past years. Those projects intended to clarify some of the known results applied to the Crohn's disease and Ulcerative colitis Portuguese populations. In our work, we have approached several themes involving different important components of the disease, namely cytokines associated with the inflammatory pathway, biologic processes such as apoptosis and autophagy, clinical and genetic predictors, nutrition and response to therapy. Our goal was to comprehend more the IBD development and management in order to contribute with some guidelines that could provide a higher quality of life for the patients.

IV.1 ASSOCIATIONS OF CLINICAL AND GENETIC PARAMETERS

Several studies have demonstrated that clinical factors such as age at diagnosis, disease extent, disease location and behaviour at diagnosis are predictive for the development of a more aggressive disease and could be used by physicians to delineate therapeutic strategies (Louis *et al.* 2009). While some clinical features show significant associations with adverse prognosis, they are usually described retrospectively and many features lack standardization, which leads to a heterogeneity that difficult the use of clinical data as predictors for therapy treatment in CD patients (Tamboli *et al.* 2011*).*

Initially, we started to look for clinical and genetic associations with Crohn's disease that were related to the inflammatory pathway, such as pro or antiinflammatory cytokines, and its contribution to the risk of developing CD or modifying disease activity. For that, we analyzed 7 polymorphisms in 5 cytokines genes (*IL1β, IL1RN, TNFα, LTα e IL6*) by Polimerase chain reaction (PCR) and PCR/ Restriction fragment length polymorphism (RFLP) in a population of 78 individuals with Crohn's disease and 102

control individuals (Ferreira 2006). As significant results in this work, we refer among the genetic factors the *TNFα* -857C/T e *IL6* -174G/C polymorphisms as risk factors in disease's development, with a four times higher risk for TT genotype individuals [OR (95%CI) 4.14 (0.81-21.24)] and a ten times greater risk for CC genotype individuals [OR (95%CI) 10.39 (2.22-48.77)], respectively, and the *TNFα* -308G/A polymorphism as a protective factor in disease's development, with a three times greater protection for AA genotype individuals [OR (95%CI) 0.31 (0.01-6.97)]. Among the identification of associations between polymorphisms and disease's aggressiveness, we can refer *TNFα* -857C/T polymorphism as a risk factor to a high disease's activity, with a three times greater risk for the TT genotype individuals [OR (95%CI) 2.68 (0.41-17.51)] and *IL1β* +3953C/T and *LTα* +252A/G polymorphisms as protective factors associated with a less aggressive disease, with a four times greater protection for TT genotype individuals [OR (95%CI) 0.23 (0.01-4.71)] and a six times greater protection for GG genotype individuals [OR (95%CI) 0.16 (0.01-3.10)], respectively.

Regarding Crohn's disease pathogenesis very little is known other than the perpetuation of the inflammation it's probably due to the imbalance between pro and antiinflammatory cytokines as well as the resistance of the inflammatory cells to apoptotic stimuli (Louis *et al.* 1996) (Souza *et al.* 2005).

With this in mind we tried to disclose the associations with apoptosis, once it's established that resistance to apoptosis is one major defect in a multifactorial disease such as Crohn's disease. For that, we analysed by PCR/RFLP the *Casp9* +93C/T, *FasL* -843C/T and peroxisome proliferator-activated receptor gamma (*PPARγ*) +161C/T and Pro12Ala SNP in a population of 99 individuals with Crohn's disease and 116 control individuals. As results, we have seen that none of the polymorphisms analyzed influenced disease susceptibility and/or activity, as can be seen in the paper 1 in chapter II.

Another important theme to uncover related to CD management is the phenotype-genotype relations in association with response to therapy. Although determination of clinical phenotype remains complex, ongoing efforts are being made to standardize a clinical classification scheme for IBD (Tamboli *et al.* 2011). Its usefulness to physicians is the identification of clinical and genetic predictors that allows them to apply the appropriate therapy for each specific individual based on its clinical and genetic profile background. Several studies have been developed along the years with major focus in genetic polymorphisms involved in drug transporters, proinflammatory cytokines, apoptosis and autophagy, among others important pathways.

In order to contribute to this purpose, we developed a multicenter study, as can be seen in paper 2 in chapter III, were we have analyzed several SNPs in *MDR1*, *IL23R*, *Casp9*, *Fas*, *FasL* and *ATG16L1* genes by real-time PCR in 242 CD patients from several participating hospitals from Central Portugal. As results, we have seen that polymorphic allele carriers for *IL23R* G9T and C2370A SNPs had less frequently upper GI involvement as compared to wild-type carriers [OR (95%CI) 0.4 (0.02-0.82), p=0.008] and [OR (95%CI) 0.25 (0.06-0.86), p=0.03], respectively. Also, individuals with the TT genotype for the *FasL* C844T SNP exhibited more often an inflammatory behaviour (B1) [OR (95%CI) 0.38 (0.18-0.82), p=0.014]. No other significant associations were observed for the remaining polymorphisms and disease characteristics, what was expected at least for the *MDR1* G2677T/A polymorphism once it has been already described (Ardizzone *et al.* 2007).

Afterward a similar project was designed for the identification of clinical and genetic predictors and its association with response to therapy in Ulcerative colitis patients, for us to have a large perspective that embraces the totality of inflammatory bowel diseases. Therefore, we developed a multicenter study, as can be seen in paper 3 in chapter III, were we have analyzed four SNPs in *IL23R* gene, namely G1142A, C2370A, G43045A and G9T, by real-time PCR in 174 CD patients from several participating hospitals from Central Portugal. As results, we observed that carriers of *IL23R* C2370A and *IL23R* G4305A alleles were at increased risk of presenting extraintestinal manifestations. No other significant association was observed between another phenotypic characteristics and genetic polymorphisms in *IL23R* gene.

IV.2 NUTRITION EFFECTS ON CROHN'S DISEASE PATIENTS

Nutritional support is a vital component of the management of patients with CD.

Through time and with the evolution in modern science and knowledge, emerges in the 21s century the study of how nutrition and genetic could relate and how these association influence CD disease course in order to obtain guidelines that would be given by physicians to patients to live a more healthy and long life and avoid multiples therapies and illness.

Nowadays, we know that dietary patterns could influence disease risk by modifying specific pathways involved in disease course or activity, nutrients are able to influence direct or indirectly gene expression and the effects of diet on disease depend on individual genetic susceptibility profile.

Based on these assumptions, nutrigenetics tends to identify and characterized the different human genetic variations responsible for nutrients metabolism alteration, leading to the understanding of how individual genetic profile influence response to diet.

The main goal to be achieved is the personalization of diet to each CD patient based on the individual genetic identity trough the use of specific nutritional guidelines, which will lead to the use of nutritional therapy.

Although it's an emerging field, further studies are needed to allow nutritional therapy to be use as current clinical practice in CD.

With this in mind our group developed two studies to approach this thematic and verifiy if any significant results would be obtained.

In our earliest study (Guerreiro *et al.* 2009), we approached the nutritional question in a population of 78 individuals with Crohn's disease and 102 control individuals, where we applied a Semiquantitative Food Frequency Questionnaire (FFQ). In what concern the nutrition factors in association with polymorphisms and disease's aggressiveness, we can refer glicids, lipids, saturated, monounsaturated and polyunsaturated fats as risk factors to increase the disease's aggressiveness when consumed in higher quantities for the majority of the polymorphisms studied, except for *TNFα* -308G/A, *LTα* +252A/G and *TNFα* -857C/T polymorphisms, where we couldn't established any significant association between disease's aggressiveness and genetic and nutrition factors. The association between fat, particularly saturated fat and inflammation, has been previously reported

once it increases monocyte production of TNFα, IL1β and IL6 among other proinflammatory cytokines (Mozaffarian *et al.* 2004). Contrarily, high intakes of ω-3 fatty acids were shown to diminish circulating levels of proinflammatory cytokines (Tucker 2007). We also observed that the detrimental effect of a greater intake of saturated or monounsaturated fat was higher in individuals with the variant allele for the *TNFα* -857C/T and *IL6* -174G/C polymorphisms, with the first polymorphism also being referred as a promoter factor in the presence of a diet poor in ω-3 fatty acids. In summary, with this study, we gained knowledge that the *IL6* -174G/C polymorphism was the genetic factor with more associations with disease's aggressiveness and nutrition factors and that differences in the type of dietary fat may be important in modulating intestinal inflammation, since we have seen that a diet rich in monounsaturated fat seems to be associated with a more active disease and ω-3 polyunsaturated fatty acids may have a protective role in CD in opposition to ω-6 polyunsaturated fatty acids.

Another topic of interest is the previous described basic pathogenic defect of resistance to apoptosis, namely of T-cells, that characterizes CD and leads to a perpetuated inflammation in the intestinal mucosa. Previously, polymorphisms in apoptotic genes have been associated with heterogeneous phenotypes and different responses to CD therapies (Hlavaty *et al.* 2005). With these assumptions, we explored the associations between the *Casp9* +93C/T, *FasL* -843C/T and *PPARγ* +161C/T and Pro12Ala SNP and dietary fat intake in a population of 99 individuals with Crohn's disease and 116 control individuals, since previous studies have shown that dietary fat has the ability of affecting cellular kinetics by interfering with important processes such as apoptosis induction, cell proliferation and cell differentiation (Llor *et al*. 2003).

In our study, here described in the paper 1 in chapter II, we verified that a high intake of total, saturated and monounsaturated fats and a higher ratio of ω-6/ω-3 polyunsaturated fatty acids were associated with a more active phenotype ($p<0.05$), which is in accordance with the fact that the combination of both ω-6 and ω-3 polyunsaturated fatty acids are associated with lowest levels of inflammation as suggested before (Pischon *et al.* 2003). We observed a more detrimental effect of a high intake of total and trans fat in wild type carriers of the *Casp9* +93C/T polymorphism [OR (95% CI) 4.64 (1.27-16.89), p=0.020] and [OR (95% CI) 4.84 (1.34-17.50), p=0.016], which led us to hypothesize that wild type carriers might exhibit more resistance to apoptosis and therefore, there would be a synergism between two potentially harmful factors. However, this may not be as clear as that since we have observed that high intake of saturated and monounsaturated fat is already associated to a more active phenotype by itself and because it presents comparable effects on wild type and polymorphic allele carriers. For *PPARγ* Pro12Ala SNP, we also observed that a high intake of saturated and monounsaturated fat was associated to a more active disease in wild type carriers [OR (95% CI) 4.21 (1.33-13.26), p=0.014] and [OR (95% CI) 4.37 (1.52-12.51), p=0.006], what is in conformity with previous studies (Debril *et al.* 2001) (Gong *et al*. 2005). Those studies shown that the Pro→Ala change may cause a conformational change in the protein and consequently patients wild type carriers of this polymorphism would have less inhibition of NFκβ pathway, which is known to be part of a central signalling pathway that stimulates the transcription of multiple genes that encode proinflammatory molecules, and therefore would exhibit a more aggressive and active phenotype. Finally, a high intake of ω-6 polyunsaturated fatty acids was associated with a more active disease in

wild type carriers for the *FasL* -843C/T polymorphism [OR (95% CI) 5.15 (1.07-24.74), p=0.041], which it's not in congruity with previous studies. It is known that the carriers of the C allele for this polymorphism have a threefold increased binding capacity to the CAAT enhancer protein and subsequently a threefold higher expression of FasL that leads to an increase of apoptosis of the active cells which are expressing Fas and consequently, wild type carries would be more susceptible to apoptosis and, theoretically, exhibit a less severe phenotype (Hlavaty *et al.* 2005) (Wu *et al.* 2003). These opposite results may be explained, first of all by recalling that interactions between nutrients and genes may be extremely complex, but also reminding the existence of two main pathways of apoptosis, the extrinsic pathway which is between others Fas dependent and the intrinsic pathway, and therefore we may hypothesize that ω-6 polyunsaturated fatty acids influence apoptosis by interfering in the intrinsic pathway, among others hypotheses.

To our knowledge these were the first studies that examining the interactions between polymorphisms of proinflammatory cytokine genes and apoptosis genes and the type of fat intake in modulating disease activity in patients with Crohn's disease.

It has been observed that the interaction of dietary components with the host's mucosa and alteration of resident intestinal gut microbiome provides new insights into its mechanism of action in IBD pathogenesis. Changes in dietary intake of food components (e.g., fatty acids, carbohydrates, proteins and peptides, prebiotics, and probiotics) modulate gene expression in host intestine, as well as in liver, adipose tissue, and muscle and change the intestinal gut microbiome composition (Fergunson 2013).

The introduction of the concept of nutrigenetics as a routine tool to assess the management of CD is not a reality, but has taken in consideration the known pathways suggested to be involved and used them for specific dietary guidelines based in the previously described genes. The main goal is to achieve personalized nutrition to guarantee disease prevention and treatment.

IV.3 PHENOTYPE-GENOTYPE RELATIONS IN ASSOCIATION WITH RESPONSE TO THERAPY

Crohn's disease and ulcerative colitis are chronic disabling inflammatory bowel diseases. The treatment of IBD has focused on the management of symptoms but is becoming more resolute on changing the course of the disease and its complications in the long-term (Magro *et al.* 2012).

The main goals in treating Crohn's disease involve healing the intestinal mucosa, prevent CD complications, hospitalization and surgery, induce and maintain remission, improve patient's quality of life and minimize applied therapeutic toxicity (Panaccione *et al.* 2012).

In a heterogenic disease like CD, treatment response may vary depending on several factors such as duration of disease, disease behaviour and severity as well as those related with individual genetic background and polymorphisms in particularly drug metabolizing enzymes or target proteins (Pierik *et al.* 2006).

Pharmacogenetics appears as the study of the existing associations between variability in drug response and/or drug toxicity and genetic polymorphisms, aiming to a more efficacious and safe applicability of appropriate therapeutic to each CD patient based on his specific genetic profile (Pierik *et al.* 2006).

During our project work, it has come to our attention the growing interest in finding clinical and/or genetic predictors for the susceptibility or development of CD, in order to apply appropriate therapeutic to individual patients. Consequently, we have designed two different retrospective studies to identify clinical or genetic predictors for IBD and their association with response to therapy.

We have introduced this thematic with a multicenter study, as can be seen in paper 2 in chapter III, were we have analyzed several SNPs in *MDR1*, *IL23R*, *Casp9*, *Fas*, *FasL* and *ATG16L1* genes by real-time PCR in 242 CD patients from several participating hospitals from Central Portugal, in order to identify the associations between clinical characteristics, polymorphisms and response to the commonly used therapy in CD management, namely 5'ASA, corticosteroids, azathioprine and biological therapies. For clinical predictors we have identified that older patients responded better to 5'ASA and to AZA [OR (95%CI) 1.07 (1.02-1.14), p=0.003] and [OR (95%CI) 1.03 (1.01-1.06), p=0.01], respectively, while younger ones responded better to biologics [OR (95%CI) 0.95 (0.90-1.00), p=0.06], previous surgery negatively influenced response to 5'ASA [OR (95%CI) 0.25 (0.05-0.96), p=0.05], but favoured response to AZA [OR (95%CI) 2.1 (1.04-4.49), p=0.04] and, finally we observed that patients with perianal involvement had a worse response to corticosteroids [OR (95%CI) 0.32 (0.14-0.72), p=0.006]. Regarding genetic predictors we observed that homozygotes TT for *Casp9* C93T SNP had a lower change of responding both to corticosteroids [OR (95%CI) 0.23 (0.36-0.88), p=0.03] and azathioprine [OR (95%CI) 0.08 (0.01-0.51), p=0.02] and individuals TT genotype for the *MDR1* C3435T SNP had a higher chance of responding to azathioprine [OR (95%CI) 2.38 (1.13-5.02), p=0.01]. Carriers for the polymorphic allele of *MDR1* G2677T/A SNP responded better to AZA [OR (95%CI) 1.89 (0.94-3.81), p=0.07], but have a lower chance of responding to biologics [OR (95%CI) 0.31 (0.08-1.07), p=0.07], which became significant after adjusting for gender [OR (95%CI) 0.75 (0.24-0.63), p=0.05]. The results obtained for the association between *MDR1* gene SNPs and response to azathioprine are in disagreement with a previous study that investigated the influence of G2677T/A and C3435T *MDR1* gene polymorphisms on the efficacy of azathioprine in inducing remission in CD patients, where it was observed higher frequencies of the 2677TT and 3435TT genotypes and the 2677T/3435T haplotype in CD patients that did not respond to azathioprine (Mendoza *et al*. 2007).

In the present study, we decided to use long-term response (more than 1 year) because we believe that this concept is clinically more relevant. We have seen that 12% of the population responded better to 5'ASA therapy and, although it's a low number of patients, emphasizes the importance of identifying those patients who respond better just with less aggressive and toxic therapies, since a systematic review of clinical trials (Su *et al*. 2004) have demonstrated that 18% of patients entered remission with placebo alone, which may suggest that in our population these patients could be treated with 5'ASA compounds alone. The identification of surgery as a negative predictor of response to 5'ASA, but a positively one for response to azathioprine, is clinically very important once suggests to the physician that after the resection of the diseased segment the patient should start

immunosuppression therapy instead of milder therapies as 5'ASA, ending with commonly doubts about which treatment to choose after surgery. The results obtained for the genetic predictors associated with response to therapy gave us some important guidelines that might be relevant in clinical decisions, nevertheless further studies in bigger populations are needed.

The understanding of Crohn's disease is nowadays more explored than Ulcerative colitis, since it's more difficult to predict disease course in UC. In routine clinical practice physicians treat UC patients with 5'ASA therapies because of its treatment success and therefore the main goal to achieve in studies trying to unravel the most appropriate therapies to treat UC would be to identify whose patients are 5'ASA nonresponders and should be started on more aggressive and effective therapies as early as possible.

For this reason, a similar project was designed for the identification of clinical and genetic predictors and its association with response to 5'ASA, azathioprine, corticosteroids and biologic therapies in Ulcerative colitis patients, for us to have a large perspective that embraces the totality of inflammatory bowel diseases. Therefore, we developed a multicentre study, as can be seen in paper 3 in chapter III, were we have analyzed four SNPs in *IL23R* gene, namely G1142A, C2370A, G43045A and G9T, by real-time PCR in 174 CD patients from several participating hospitals from Central Portugal. As results, we have seen that older patients and those diagnosed after the age 40 responded better to 5'ASA [OR (95%CI) 1.03 (1.00-1.05), p=0.004] and [OR (95%CI) 2.26 (1.21-4.57), p=0.01], respectively, in opposition to duration of disease for more than 5 years that was a negative predictor of response for both 5'ASA and azathioprine, although the latter did not reach statistical significance [OR (95%CI) 0.37 (0.17-0.77), p=0.008] and [OR (95%CI) 0.26 (0.05-1.16), p=0.07], respectively. Patients with pancolitis presented poorer responses to 5'ASA and azathioprine [OR (95%CI) 0.15 (0.04-0.49), p=0.002] and [OR (95%CI) 0.18 (0.03-0.99), p=0.05], respectively. Previously works have shown that young age, female sex and extensive colitis presented less probability of responding to 5'ASA therapy (Langholz *et al.* 1994) (Hoie *et al*. 2007) (Solberg *et al.* 2009). In our case, none of these predictors were identified. Regarding extraintestinal manifestations, we observed that it was a negative predictor of response to 5'ASA, corticosteroids and AZA [OR (95%CI) 0.25 (0.10-0.56), p=0.001], [OR (95%CI) 0.35 (0.11-1.06), p=0.06] and [OR (95%CI) 0.18 (0.04-0.76), p=0.02], respectively, but it seemed to positively influence response to biologics, although not statistically significant [OR (95%CI) 8.00 (0.72-88.22), p=0.09]. To our knowledge the results from extraintestinal manifestations weren't clearly reported before. All that is published refers that EIM are among the clinical risk factors that may be associated with disease extent and severity (Farmer *et al.* 1993), and a recent review (Veloso 2011) suggests that early aggressive therapy may be required for treating several EIMs in order to prevent chronic damage. This suggestion is in accordance with our results since it was shown that EIM was a negative predictor of response to 5'ASA, corticosteroids and AZA, therefore suggesting the use of more aggressive therapeutic like biologic therapy.

In reference to genetic predictors to UC very less is known compared to CD, once only a few clinical settings have investigated it (Beaugerie & Sokol 2012). In our work, we studied the associations of *IL23R* gene polymorphisms with response to therapy, since it's recognized that IL23 cytokine is essential to drive the chronic intestinal inflammation in IBD, particularly in UC (Morrison *et al.* 2011),

and that *IL23R* gene variants contributes to colitis pathogenesis through several pathways, thereby resulting in different types of responses (Safranny *et al.* 2013). In what concerns genetic predictors we have seen that individuals with the AA genotype for the *IL23R* C2370A SNP negatively influenced the response to 5'ASA and corticosteroids [OR (95%CI) 0.32 (0.11-0.92), p=0.03] and [OR (95%CI) 0.19 (0.04-0.84), p=0.02], respectively, while individuals with the GG genotype for the *IL23R* G9T SNP were more likely to respond to azathioprine [OR (95%CI) 11.8 (1.00-139.0), p=0.05]. Early indicators of need of azathioprine therapy were already described in previous studies (Jurgens *et al.* 2010).

Once again we decided to use long-term response (more than 1 year) because we believe that this concept is clinically more relevant. As was expected from previous works, over 60% of our UC patients responded positively to 5'ASA compounds while the other 40% that were refractory to 5'ASA were started on azathioprine, and from this latter group 58% of them responded well to azathioprine while the others ultimately required biologic therapy. Regarding clinical predictors none of the previously described were found but new ones rise, such as older patients and those diagnosed after the age 40 responded better to 5'ASA, duration of disease for more than 5 years was a negative predictor of response for both 5'ASA and azathioprine, although the latter did not reach statistical significance. Patients with pancolitis presented poorer responses to 5'ASA and azathioprine and finally, extraintestinal manifestations was a negative predictor of response to 5'ASA, corticosteroids and AZA, but it seemed to positively influence response to biologics, although not statistically significant. For genetic predictors we identified *IL23R* C2370A SNP as a poorer responder to 5'ASA and corticosteroids therapies, while *IL23R* G9T SNP is most likely associated with a positive response to azathioprine, fact that is supported by previous indications that encourage an early use of azathioprine therapy. All these findings might be extremely important in clinical practice, nevertheless further studies are still needed.

All of the previous results described have shown us the importance of researching in the different existing fields of knowledge that are available for IBD and the ultimate purpose of determining clinical and genetic predictors to response to therapy that would guide physicians to the concept of personalized medicine. This necessity has led us to write a review article, as can be seen in paper 4 in chapter III, where we tried to approach the importance of the genetic factors in the determination of susceptibility to IBD, the existing conventional therapies and its advantages/disadvantages, the identification of genetic predictors of response to therapy, including a perspective from the studies we have developed in a Portuguese population and, finally, the importance of genetic in personalized medicine and challenges for the future to come.

Long before GWA studies, the *NOD2* (chromosome 16q12), *IBD5* (chromosome 5q31) and *HLA* class II (chromosome 6p21) associations with IBD were the most studied ones, regarding the identification of susceptibility genes to CD (Cho & Weaver 2007) (Brant 2013). More recently, we have seen emerging important data on the associations with *ATG16L1* (chromosome 2q37), *IRGM* (chromosome 5q33) and *IL23R* (chromosome 1p31) as susceptibility genes to CD (Stappenbeck *et al.* 2011). Many other genes have been studied along the years, namely apoptotic genes like *Fas*, *FasL*, *Casp9*, inflammatory response related genes like *TNF*α, *LT*α, *IL1*, *IL6* and drug response genes like

MDR1, but in any manner they don't appear as risk disease genes (Waterer & Wunderink 2003) (Ho *et al.* 2005).

Genetic markers are emerging as powerful tools for patients stratification once they are stable over time and not suitable for subjective interpretation, but further studies are needed for its use in regular basis (Vermeire *et al.* 2010). Recent advances in this area have led to the concept of pharmacogenetics that permits not just the explanation of interindividual variability in drug response, but most importantly the prediction of efficacy and adverse drug events in different patients before the initiation of the treatment. The major focus on pharmacogenetics research has been on allelic variants in drug-metabolizing enzymes (DMEs), but other genetic locus have gain relevance such as *MDR1* gene and the TNF/ TNF receptor pathway (Mascheretti *et al*. 2004).

With all the scientific advances in this field, individualize therapy seems to be the solution for the future. Nevertheless it's important to remember that clinical and genetic parameters can't explain everything, including the fact that 20-30% of IBD patients are refractory to any therapy despite optimal dose and duration, side effects and drugs toxicity are variable and disease duration, severity, behaviour and combined therapies may all influence the response to a therapy (Mascheretti *et al.* 2004). The achievement of a better quality of life for CD patients should, accordingly with the previously mention, consider the analysis whether earlier and more effective treatment would influence disease activity and long-term outcomes for patients.

This type of studies and the predictors that we identified here will help a better prediction of disease outcome and the possibility to stratified patients in subgroups that will allow the correct application of the most efficacious therapy for each case, minimizing toxicity and costs.

IV.4 REFERENCES

Ardizzone S, Bianchi V *et al.* (2007). "Multidrug resistance 1 gene polymorphism and susceptibility to inflammatory bowel disease." Inflamm Bowel Dis **13**(5): 516-523.

Beaugerie L, Sokol H (2012). "Clinical, serological and genetic predictors of inflammatory bowel disease course." World J Gastroenterol. **18**(29): 3806-3813.

Brant SR (2013). "Promises, delivery, and challenges of inflammatory bowel disease risk gene discovery." Clin Gastroenterol Hepatol **11**(1): 22-26.

Cho JH and Weaver CT (2007). "The genetics of inflammatory bowel disease." Gastroenterology **133**(4): 1327-1339.

Debril MB, Renaud JP *et al.* (2001). "The pleiotropic functions of peroxisome proliferator-activated receptor gamma." J Mol Med (Berl) **79**(1): 30-47.

Farmer RG, Easley K *et al.* (1993). "Clinical patterns, natural history, and progression of ulcerative colitis. A long-term follow-up of 1116 patients." Dig Dis Sci **38**(6): 1137-1146.

Ferguson LR (2013). "Nutrigenetics, nutrigenomics and inflammatory bowel diseases." Expert Rev Clin Immunol. **9**(8): 717-726.

Ferreira P (2006). Doença de Crohn - Factores genéticos e nutricionais. Departamento de Engenharias e Ciências. Lisboa, Universidade Lusófona de Humanidades e Tecnologias. **Licenciatura:** 104.

Gong Z, Xie D *et al.* (2005). "The PPAR{gamma} Pro12Ala polymorphism and risk for incident sporadic colorectal adenomas." Carcinogenesis. **26**(3): 579-585.

Guerreiro CS, Ferreira P *et al.* (2009). "Fatty acids, IL6, and TNFalpha polymorphisms: an example of nutrigenetics in Crohn's disease." Am J Gastroenterol. **104**(9): 2241-2249.

Hlavaty T, Pierik M *et al.* (2005). "Polymorphisms in apoptosis genes predict response to infliximab therapy in luminal and fistulizing Crohn's disease." Aliment Pharmacol Ther **22**: 613–626.

Ho G, Nimo ER *et al.* (2005). "Allelic variations of the multidrug resistance gene determine susceptibility and disease behaviour in ulcerative colitis." Gastroenterology **128**(2): 288-296.

Höie O, Wolters F *et al.* (2007). "Ulcerative Colitis: Patient Characteristics May Predict 10-Yr Disease Recurrence in a European-Wide Population-Based Cohort." The American Journal of Gastroenterology **102**: 1692–1701.

Jürgens M, Laubender RP *et al.* (2010). "Disease activity, ANCA, and IL23R genotype status determine early response to infliximab in patients with ulcerative colitis." Am J Gastroenterol. **105**(8): 1811-1819.

Langholz E, Munkholm P *et al.* (1994). "Course of ulcerative colitis: analysis of changes in disease activity over years." Gastroenterology **107**(1): 3-11.

Llor X, Pons E *et al.* (2003). "The effects of fish oil, olive oil, oleic acid and linoleic acid on colorectal neoplastic processes." Clin Nutr **22**(1): 71-79.

Louis E, Satsangi J *et al.* (1996). "Cytokine gene polymophisms in inflammatory bowel disease " Gut. **39**(5): 705–710.

Louis E, Belaiche J, Reenaers C (2009). "Tailoring the Treatment to the Individual in Crohn's Disease." Therap Adv Gastroenterol **2**(4): 239-244.

Louis E, Belaiche J, Reenaers C (2010). "Do clinical factors help to predict disease course in inflammatory bowel disease?" World J Gastroenterol. **16**(21): 2600-2603.

Magro F, Cravo M, Lago P *et al.* (2012) Portuguese consensus on the best practice for the management of inflammatory bowel disease: IBD ahead 2010 meeting results. GE Jornal Português de Gastrenterologia **19**(4): 190-198.

Mascheretti S, Croucher PJ, Schreiber S (2004). "Pharmacogenetics of inflammatory bowel disease." Best Practice & Research Clinical Gastroenterology **18**(3): 597–609.

Mendoza JL, Urcelay E *et al.* (2007). "MDR1 polymorphisms and response to azathioprine therapy in patients with Crohn's disease." Inflamm Bowel Dis. **13**(5): 585-590.

Morrison PJ, Ballantyne SJ *et al.* (2011). "Interleukin-23 and T helper 17-type responses in intestinal inflammation: from cytokines to T-cell plasticity." Immunology **133**(4): 397-408.

Mozaffarian D, Pischon T *et al.* (2004). "Dietary intake of trans fatty acids and systemic inflammation in women." Am J Clin Nutr **79**(4): 606-612.

Nunes T, Etchevers MJ *et al.* (2013). "Does smoking influence Crohn's disease in the biologic era? The TABACROHN study." Inflamm Bowel Dis. **19**(1): 23-29.

Panaccione R, Hibi T *et al.* (2012). "Implementing changes in clinical practice to improve the management of Crohn's disease." J Crohns Colitis **6 Suppl 2**: S235-242.

Parkes GC, Whelan K *et al.* (2014). "Smoking in inflammatory bowel disease: impact on disease course and insights into the aetiology of its effect." J Crohns Colitis **8**(8): 717-725.

Pierik M, Rutgeerts P *et al.* (2006). "Pharmacogenetics in inflammatory bowel disease." World J Gastroenterol. **12**(23): 3657-3667.

Pischon T, Hankinson SE *et al.* (2003). "Habitual dietary intake of n-3 and n-6 fatty acids in relation to inflammatory markers among US men and women." Circulation. **108**(2): 155-160.

Safrany E, Szabo M *et al.* (2013). "Difference of interleukin-23 receptor gene haplotype variants in ulcerative colitis compared to Crohn's disease and psoriasis." Inflamm Res. **62**(2): 195-200.

Solberg IC, Lygren I *et al.* (2009). "Clinical course during the first 10 years of ulcerative colitis: results from a population-based inception cohort (IBSEN Study)." Scand J Gastroenterol **44**(4): 431-440.

Souza HS, Tortori CJ *et al.* (2005). "Apoptosis in the intestinal mucosa of patients with inflammatory bowel disease: evidence of altered expression of FasL and perforin cytotoxic pathways." Int J Colorectal Dis. **20**(3): 277-286.

Stappenbeck TS, Rioux JD *et al.* (2011). "Crohn disease: a current perspective on genetics, autophagy and immunity." Autophagy **7**(4): 355-374.

Su C, Lichtenstein GR *et al.* (2004). "A meta-analysis of the placebo rates of remission and response in clinical trials of active Crohn's disease." Gastroenterology **126**(5): 1257-1269.

Tamboli CP, Doman DB *et al.* (2011). "Current and future role of biomarkers in Crohn's disease risk assessment and treatment." Clin Exp Gastroenterol **4**: 127-140.

Tucker KL (2007). "Assessment of usual dietary intake in population studies of gene-diet interaction." Nutr Metab Cardiovasc Dis **17**(2): 74-81.

Veloso FT (2011). "Extraintestinal manifestations of inflammatory bowel disease: Do they influence treatment and outcome?" World J Gastroenterol **17**(22): 2702-2707.

Vermeire S, Van Assche G *et al.* (2010). "Role of genetics in prediction of disease course and response to therapy." World J Gastroenterol **16**(21): 2609-2615.

Waterer GW and Wunderink RG (2003). "Science review: Genetic variability in the systemic inflammatory response." Crit Care **7**(4): 308-314.

Wu J, Metz C *et al.* (2003). "A novel polymorphic CAAT/enhancer-binding protein beta element in the FasL gene promoter alters Fas ligand expression: a candidate background gene in African American systemic lupus erythematosus patients." J Immunol. **170**(1): 132-138.

Yarur AJ, Strobel SJ *et al.* (2011). "Predictors of aggressive inflammatory bowel disease." Gastroenterol Hepatol (N Y) **7**(10): 652-659.

CHAPTER V – FINAL REMARKS AND FUTURE PERSPECTIVES

Crohn's disease is a chronic relapsing disease with high phenotype heterogeneity and no cure available so far. Its precise etiology remains unknown, but is certain the involvement of clinical, genetic and environmental factors.

For the last decades relevant advances have been made in the fields of clinical, genetics, immunology, microbiology and therapy in IBD, that have led us to a better understanding of a complex disease such as Crohn's disease.

In line with these advances, our group have approached distinctive areas of study related with Crohn's disease and more lately to Ulcerative colitis and Inflammatory bowel diseases globally. With these purposes we designed a study that included the inflammatory response pathways, biologic processes as apoptosis and autophagy, nutrigenetics, therapeutic and pharmacogenetics components on a Portuguese population from different participating hospitals. To achieve it we initiated clinical data collection, genetic analysis with PCR/RFLP and real-time PCR techniques of relevant gene polymorphisms, study of diet patterns and response to therapies data collection.

All of the aims proposed in this dissertation were achieved through the analysis of data collected, study of important phenotype-genotype associations for CD susceptibility and/or development, investigation of the effects of dietary patterns in disease activity and search of phenotype-genotype relations in association with response to therapy.

Throughout our work the main purpose was the identification of guidelines that would possibly be used by physicians to stratified patients in order to receive personalized therapy accordingly to their disease prognosis, looking at it as a contribution to a step forward into individualized CD treatment. Several results were obtained along the way, but it's pertinent to emphasized the importance of:

- TNFα, LTα, IL1 and IL6 cytokines as associated with disease aggressiveness and development;
- *FasL* apoptotic gene as related to disease behaviour;
- IL23R cytokine associated with a greater risk of developing EIM in UC patients;
- glicids, lipids, saturated, monounsaturated and polyunsaturated fats as risk factors to increase the disease's aggressiveness when consumed in higher quantities for the majority of the polymorphisms studied;
- identification of clinical predictors such as patients age, surgery and perianal involvement and genetic predictors like *Casp9* and *MDR1* gene polymorphisms in association with response to therapy for Crohn's disease;
- description of clinical predictors such as patients age, age at diagnosis, duration of disease, pancolitis and extraintestinal manifestations and genetic predictors like *IL23R* gene polymorphisms in association with response to therapy for Ulcerative colitis;

Our study has revealed some important guidelines, but further studies are still needed for the success of personalized therapy and the achievement of a better quality of life for CD patients. For these reasons, some new goals for our study could be drawn, namely the verification of the obtained

guidelines in a greater population; the identification by testing in cell cultures with commercial cell lines of the best therapy, among the studied ones, that should be applied to specific groups of patients divided based on their phenotype-genotype associations; the analysis of emerging important gene polymorphisms that have been recently related to CD and the study of its association with phenotype and response to therapy and, lastly with the importance that autophagy has gained in the regulation of immune and/or inflammatory responses, it may be interesting to study the modulation of this signalling pathway to restore and control the imbalance inflammatory responses in CD patients.

Nowadays, several hypotheses have been suggested and are being exploited worldwide, namely the use of pretreatment genetic screening based in a combination of genetic tests, serologic markers and determined disease characteristics in order to select an optimal dosage and schedule a more effective, safer and less expensive therapeutic directed to each patient; mucosal gene signature given the importance of mucosal healing in CD; identification of reliable biomarkers and tissue signatures; development of new biologic treatments that mostly target leukocyte trafficking and proinflammatory cytokines such as IL6, IL17, IL18 and IL21; blockage of TNF with anti-TNF vaccination, *TNF* gene silencing with small interfering RNA and TNF-neutralizing nanobodies; development of a variety of small molecules that selectively inhibit signalling molecules; diagnostic tests for the discrimination of *MDR1* gene alleles and, finally, approaches like stem-cell therapies and fecal transplantation.

There is currently available several genetic information to improve drug therapy and, therefore, significant development is expected to occur throughout the next decades in accordance with the emerging of new techniques, medical availability, new knowledge that will allow individual genetic background to be studied for prediction of response to therapy.

The future will rely in the application of pharmacogenetics and nutrigenetics guidelines in daily clinical practice for Crohn's disease management, in order to induce and maintain remission through appropriate therapeutic and diet patterns that ultimately will contribute to a better quality of life for CD patients.