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Single Nucleotide Polymorphisms of some Cytokines in Inflammatory Bowel Disease of Iraqi Patients

A dissertation Submitted

By

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

سَنُرِيهِمْ آيَاتِنَا فِي الْآفَاقِ وَفِي
أَنفُسِهِمْ حَتَّىٰ يَتَبَيَّنَ لَهُمْ أَنَّهُ
الْحَقُّ ۗ أَوَلَمْ يَكْفِ بِرَبِّكَ أَنَّهُ
عَلَىٰ كُلِّ شَيْءٍ شَهِيدٌ (53)

صدق الله العظيم

(من سورة فصلت الآية 53)

Dedication

To...They illumine the darkness of my life, light of my eyes
Mom and Dad**.**

To... those who cherish them and their proud in this world, my brothers and sisters.

To...My dear to the spirit of the martyr Mohammed, who never forget him.

To... my Small family.

To ... all my friends

To... all those who helped me and taught me and good people

Researcher

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Summary

Inflammatory bowel disease (IBD) is a world healthcare problem that involves two major forms: Crohn's disease (CD) and ulcerative colitis (UC). Immunogenetic predisposition is one of the risk factors for the disease, and cytokines are among these factors. Therefore, the association between 13 SNPs (single nucleotide polymorphisms) of cytokine and cytokine receptor genes (*IL1A*, *IL1B*, *IL1R1*, *IL1RN*, *IL2*, *IL4*, *IL4R*, *IL6*, *IL10*, *IL12B*, *IFNG*, *TNF* and *TGFB1*) and IBD was determined in samples of Iraqi Arab patients (34 CD and 66 UC). The patients were referred to the Gastrointestinal Tract Unit at Al-Kindy Teaching Hospital, Al-Yarmouk Teaching Hospital and Al-Zuafrania General Hospital in Baghdad for diagnosis and treatment during the period August 2013 - October 2014. A control sample of 43 individuals was also included.

The following results were reached by the study:

1. Interleukin-1 alpha gene ($IL1A_{-889}$): frequencies of CC genotype and *C* allele were significantly increased in CD (58.8 and 73.5%, respectively) and UC (54.6 and 71.2%, respectively) patients compared to controls (25.5 and 40.7%, respectively). In contrast, TT genotype and *T* allele frequencies were significantly decreased in CD (11.8 and 26.5%, respectively) and UC (12.1 and 28.8%, respectively) patients compared to controls (44.2 and 59.3%, respectively).
2. Interleukin-1 beta gene ($IL1B_{-511}$): Frequency of TC genotype was significantly increased in UC patients compared to controls (63.6 *vs*. 39.5%; P = 0.018), while CC genotype frequency was decreased (6.1 *vs*. 32.2%; P = 0.061). No variation was observed in CD patients.
3. Interleukin 1 receptor type 1 gene ($IL1R1_{\text{pstl 1970}}$): Frequencies of TT genotype and *T* allele (47.1 and 67.7%, respectively) were significantly increased (P = 0.026 and $8.6*10^{-5}$, respectively) in CD patients compared to controls (20.9 and 34.97%, respectively). In contrast, CC genotype (11.8 *vs*. 51.2%) and allele *C* (32.4 *vs*. 65.1%) frequencies were significantly decreased (P = $2.8*10^{-4}$ and $8.6*10^{-5}$, respectively) in CD patients. For UC, the patients demonstrated

significant increased frequencies of TC genotype (48.5 *vs*. 27.9%; P = 0.045) and T allele (63.6 *vs*. 34.9%; P = $5.1*10^{-5}$) compared to controls. As in CD, UC patients also demonstrated significant decreased frequencies of CC genotype (12.1 *vs*. 51.2%; P = $1.7*10^{-5}$) and *C* allele (36.4 *vs*. 65.1%; P = $8.6*10^{-5}$).

4. Interleukin 2 gene ($IL2_{+166}$): Among CD patients, frequencies of TT genotype (41.2 *vs.* 6.9%) and *T* allele (64.7 *vs.* 43.0%) were significantly increased in patients compared to controls (P = 0.001 and 0.009, respectively). In contrast, TG genotype (47.1 *vs*. 72.1%; P = 0.035) and *G* allele (35.3 *vs.* 56.9%; P = 0.009) frequencies were significantly decreased. Almost, similar observations were made in UC patients.
5. Interleukin 4 gene ($IL4_{-590}$): it was observed that frequencies of TT genotype (52.9 *vs*. 11.6%; P = $1.2*10^{-4}$) and *T* allele (70.6 *vs*. 24.4%; P = $1.6*10^{-8}$) were significantly increased in CD patients compared to controls. In contrast, CC genotype (11.8 *vs*. 62.8%, P = $5.6*10^{-6}$) and *C* allele (29.4 *vs*. 75.6%; P = $1.6*10^{-8}$) frequencies were significantly decreased. In the case of UC, frequencies of TC genotype (69.7 *vs*. 25.6%; P = $1.1*10^{-5}$) and *T* allele (62.1 *vs*. 24.4%; P = $4.6*10^{-8}$) were significantly increased in patients, while CC genotype (3.0 *vs*. 62.8%; P = $2.5*10^{-12}$) and *C* allele (37.9 vs. 75.6%; P = $1.6*10^{-8}$) frequencies were significantly decreased in patients.
6. Interleukin 4 receptor gene ($IL4R_{+1902}$): frequencies of GG genotype (47.1 *vs.* 11.6%) and *G* allele (55.9 *vs.* 37.2%) were significantly increased in CD patients compared to controls (P = 0.001 and 0.023, respectively). In contrast, GA genotype (17.7 *vs*. 51.1%; P = 0.004) and *A* allele (44.1 *vs.* 62.8%; P = 0.023) frequencies were significantly decreased. In UC patients, frequencies of GG genotype (57.6 *vs.* 11.6%) and *G* allele (75.8 *vs.* 37.2%) were significantly increased (P = $1.0*10^{-6}$ and $1.9*10^{-8}$, respectively); while frequencies of AA genotype (6.1 *vs.* 37.2%; P = $7.0*10^{-5}$) and *A* allele (24.2 *vs*. 62.8%; P = $1.9*10^{-8}$) were significantly decreased.
7. Interleukin 6 gene ($IL6_{+565}$): Comparing patients to controls revealed that GG genotype frequency was significantly increased in CD (70.6 *vs*. 13.9%; P = $1.5*10^{-8}$) and UC (69.7 *vs*. 13.9%; P = $4.4*10^{-7}$) patients, and a similar increased

frequency of *G* allele was observed. In contrast, the GC genotype frequency was significantly decreased in CD (23.5 *vs*. 76.7%; P = $3.7*10^{-6}$) and UC (24.2 *vs*. 76.7%; P = $7.0*10^{-6}$) patients.

8. Interleukin 12B gene ($IL12B_{-1188}$): Frequency of *A* allele was significantly increased in CD patients compared to controls (67.7 *vs*. 47.7%; P = 0.015), while *C* allele was significantly decreased (32.4 *vs*. 52.3%; P = 0.015). For UC, the heterozygous genotype AC showed a significant increased frequency in patients compared to controls (66.7 *vs*. 30.2%; P = $3.6*10^{-4}$), while CC genotype frequency was significantly decreased (6.1 *vs*. 37.2%; P = $7.0*10^{-5}$).
9. Interferon gamma gene ($IFNG_{+874}$): The AT genotype showed a significant increased frequency in CD patients compared to controls (58.8 *vs*. 30.2; P = 0.020), while TT genotype frequency was significantly decreased (11.8 *vs*. 39.5%; P = 0.009). For UC, AA genotype (54.6 *vs*. 30.2%; P = 0.018) and *A* allele (74.2 *vs*. 45.4; P = $2.7*10^{-5}$) frequencies were significantly increased in patients. In contrast, TT genotype (6.1 *vs*. 39.5%; P = $2.8*10^{-5}$) and *T* allele (25.8 *vs*. 54.7%; P = $2.7*10^{-5}$) frequencies were significantly decreased.
10. Tumor necrosis factor alpha gene (TNF_{-308}): Comparing CD patients to controls revealed no significant variation, while UC patients demonstrated a significantly (P = 0.006) increased frequency of *G* allele (71.2 *vs*. 52.3%) and a decreased frequency of *A* allele (28.8 *vs*. 47.7%).
11. Interleukin 1 receptor antagonist ($IL1RN_{\text{maspl }11100}$), interleukin 10 gene ($IL10_{-1082}$) and Transforming growth factor beta ($TGFB1_{\text{codon }25}$) genes: Comparing patients to controls revealed no significant variations in genotype or allele frequencies.

The presented results of the 13 cytokine SNPs in CD and UC patients are the first report in Iraqi patients, and their findings highlighted the role of these SNPs in etiopathogenesis of both groups of IBD, and paved the way for further investigations to determine the role cytokine gene polymorphisms in susceptibility to IBD or their protective effects.

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List of Abbreviations

CD	Crohn's disease
CTS-PCR	Collaborative transplant study-polymerase chain reaction
CGPs	Cytokine gene polymorphisms
EF	Etiological fraction
EGF	Epidermal growth factor
ESR	Erythrocyte sedimentation rate
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte monocyte-colony stimulating factor
GWAS	Genome-wide association studies
Hb	Hemoglobin
HLA	Human leukocyte antigen
HWE	Hardy-Weinberg equilibrium
IBD	Inflammatory bowel disease
IFN	Interferon
IFN-γ	Interferon-gamma
IL	Interleukin
IL-1R	Interleukin type I receptor
IP-10	Interferon gamma-induced protein-10
kDa	Kilodalton
LAP	latency-associated peptide
LSD	Least significant difference
LPS	Lipopolysaccharide
MCP	Monocyte chemo-attractant protein
MCP-1	monocyte chemoattractant protein-1
MHC	Major histocompatibility complex
MIP1	Macrophage-inflammatory protein-1
NK	Natural killer
N.S.	Not Significant
NSAID	Non-steroidal anti-inflammatory drug

NOD2	nucleotide-binding oligomerization domaincontaining 2 gene
OD	Optical density
P	Probability
pANCA	Perinuclear anti-neutrophil cytoplasmic antibody
PCR	polymerase chain reaction
PCR-SSP	Polymerase chain reaction-specific sequence primer
PET	Position emission tomography
PF	Preventive fraction
PGE2	Prostaglandin E2
PRRs	pattern recognition receptors
ROS	Reactive oxygen species
rpm	Revolution per minute
RR	Relative risk
S.E.	Standard error
SLE	systemic lupus erythematosus
SNP	Single nucleotide polymorphisms
SPECT	Single photon emission computed tomography
SPSS	Statistical package for social sciences
STAT	Signal transducer and activator of transcription
TGFB	Transforming Growth Factor Beta Gene
Th	T-helper
TLC	total leukocyte count
TLRs	toll- like receptors
TNF-α	Tumor necrosis factor alpha
Treg	T regulatory
UC	Ulcerative colitis

Chapter One
Introduction

Chapter One
Introduction

1.1 Introduction

Inflammatory bowel disease (IBD), classified as autoimmune disease, is chronic inflammatory conditions that occur in gastrointestinal tract. The incidence of IBD is relatively high in developed countries. In the United States and Europe, as many as 1.4 million and 2.2 million persons respectively have be recorded to have IBD, and in recent years, the incidence rate continued to rise in Asia, South America, Pacific region and many other developing countries (Mansour-Ghanaei *et al*., 2015). The major two types of IBD are ulcerative colitis (UC) and Crohn's disease (CD). The first is restricted to rectum and colon and invading epithelial lining of the gut, while CD can affect any part of the gastrointestinal tract and it causes transmural lesions (Singh *et al*., 2015). Etiologically, it is widely accepted that several factors, such as genetic, environmental, immunological factors and their interaction contribute to the onset of IBD. In 2001, NOD/ CARD15 gene was identified as the first susceptibility gene to CD which provided approaches to identify potential IBD genes. Since then, many genes involving in the initiation and evolution of IBD have been identified as its susceptible genes, and among them, interleukin-10 (IL-10) gene polymorphisms is widely investigated, as well as other cytokine genes (Ek *et al*., 2014; Trifunović *et al*., 2015).

Cytokines are potent immunomodulatory molecules with important roles in immune responses to foreign elements, such as microorganisms or transplants. Several studies have reported the role of cytokine gene polymorphisms in transplant rejection, and autoimmune and malignant diseases (Nadeem *et al*., 2015). Cytokines act as factors for immunocyte

activation, differentiation and function. There are also many reports about cytokine gene polymorphisms and cytokines production influencing the balance of the immune response. Most polymorphisms have been reported to be of the single nucleotide polymorphism (SNP) type or microsatellite polymorphism, but other types such as insertion and deletion have also been observed (Connelly *et al*., 2015). Therefore, the study of cytokine gene polymorphisms has considered as a useful tool for anthropological analysis and for the prediction of genetic susceptibility to diseases in certain populations and IBD has also been a target for such analyses. Alterations in the regulation of several cytokines have been implicated in IBD (CD and UC), cytokine gene polymorphisms have also known to affect the level of gene expression in the patients (López-Hernández *et al*., 2015). In a recent Iraqi study, 10 cytokines (IL-2, IL-4, IL-8, IL-10, IL-12, IL-17A, IL-31, IL-33, IP-10 [Interferon gamma-induced protein] and IFN-γ) were evaluated in the sera of CD and UC patients, and down-regulation and up-regulation of some of these cytokines were observed, and it was suggested that these differences might have a genetic background (Saake, 2014).

1.2 Aims of Study

The present study aimed to determine the association between the polymorphisms of 13 cytokine and cytokine receptor genes (*IL1A*, *IL1B*, *IL1R1*, *IL1RN*, *IL2*, *IL4*, *IL4R*, *IL6*, *IL10*, *IL12B*, *IFNG*, *TNF* and *TGFB1*) and the two types of IBD (UC and CD) in samples of Iraqi patients. Such association study may help to understand the immunogenetic predisposition for the development of both types of IBD, as well as their protective effects can also be determined.

Chapter Two
Review of Literature

Chapter Two
Review of Literature

2.1 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) has been regarded as a world healthcare problem that is presented with a global sustained increasing incidence. Two major forms are recognized as IBD; Crohn's disease (CD) and ulcerative colitis (UC), which represent distinct chronic bowel inflammatory disorders (Mansour-Ghanaei *et al*., 2015). The former can cause inflammation of a transmural type and affect any part of the gastrointestinal tract in a non-continuous type, but most commonly it affects the terminal ileum or the perianal region, and commonly associated with clinical complications like abscesses, fistulas and strictures. By contrast, UC inflammatory lesions are continuous and restricted to the mucosa of large intestine, involving rectum and a variable portion of colon (Abraham *et al*., 2009). The etiology of IBD is largely unknown, but recent investigations highlighted that host genetic susceptibility, external environment, intestinal microbiota and immune responses are functionally involved and integrated in IBD pathogenesis (Zhang and Li, 2014; Trifunović *et al*., 2015).

2.1.1 Genetic Factors

Studies comparing the prevalence of IBD among different ethnic groups suggest a genetic tendency. Inflammatory bowel disease has been observed to occur two to four times greater in Jewish population as compared with other ethnic groups. Other epidemiologic studies have shown higher rates in Whites and lower rates in African Americans, while the lowest rates have been recorded in Asians (Ahmad *et al.*, 2009).

The prevalence of IBD is also increased in relatives of those who have CD and UC. A Danish study found that the risk increases 2 to 13 times for offspring of patients who have IBD as compared with the general population. For patients who have UC, the occurrence of IBD in their offspring was 6.26%, while for patients who have CD; the occurrence was 9.2%. Accordingly, the genetic risk has been suggested to be higher for CD than UC (Halme *et al.* 2006). Twin studies have further confirmed the genetic etiology of IBD. In, CD, the concordance rate among monozygotic twins had a range of 20 - 50%, compared to approximately 10% in dizygotic twins; emphasizing a definite genetic component. In UC, a less concordance rate (16%) was reported in monozygotic twins, but it was still higher than that (4%) of dizygotic twins (Bengtson *et al.*, 2010; Ng *et al.*, 2012).

However, there have been great technological advances in the understanding of genetic factors that contribute to IBD etiology, which include DNA analysis and sequencing and the employment of multinational databases. These advances allowed for the achievement of genome-wide association studies (GWAS) that identified different single nucleotide polymorphisms (SNPs) and defined their role in IBD. Based on these studies, the number of IBD-associated gene loci has been brought to163, which are distributed as 110 shared loci for both diseases, 30 loci as CD specific and 23 loci as UC associated. An understanding of gene loci that are shared by UC and CD may provide a pathway to elucidate the common pathogenesis involved in both forms of IBD (Jostins *et al*., 2012).

The modern genetic research era of IBD began with the discovery of the first susceptibility gene for CD, which was nucleotide-binding oligomerization domaincontaining 2 gene (*NOD2*) CD (Ogura *et al*., 2001). The protein coded by *NOD2* gene was described as an

intracellular receptor that recognizes the muramyl dipeptide (MDP), which is a conserved motif present in Gram-positive and -negative bacteria peptidoglycan (Inohara *et al*., 2003). A stimulation of MDP can induce autophagy, and by which bacterial replication and antigen presentation is controlled, and in addition, it modulates innate and adaptive immune responses (Shaw *et al*., 2011). It has been demonstrated that NOD2 is involved in distinct MDP-independent pathways such as T-cell response regulation, and the association between CD and NOD2 has been confirmed at the level of genome-wide significance (Sabbah *et al*., 2009).

Genetic analyses have declared further for an indispensable role of autophagy in IBD immune responses, and recorded two autophagy-related genes; *ATG16L1* and *IRGM* (McCarroll *et al*., 2008). Autophagy has been considered as important regulator of intracellular homeostasis that contributes to the degradation and recycling of cytosolic contents and organelles, as well as, it involved in resistance against infection and clearing of intracellular microbes (Khor *et al*., 2011). It has been found that ATG16L1 is necessary for all forms of autophagy, and moreover, the coding mutation T300A has been associated with a CD increased risk (Kuballa *et al*., 2008). The second gene (*IRGM*) codes for a protein belong to immunity-related GTPase family, and CD-associated *IRGM* SNPs has been associated with a reduced protein expression. In addition, epithelial cells and dendritic cells having *ATG16L1* and *NOD2* variants showed defects in antibacterial autophagy (Travassos *et al*., 2010).

With further applications of GWAS and SNPs, a significant association between IBD and *IL23R* gene has also been observed. Such gene codes for a receptor for the pro-inflammatory cytokine IL-23, which is involved in the generation of Th17 cells (Duerr *et al*., 2007). The Th17 and IL-23 pathway has been well depicted in IBD

pathogenesis, with the identification of susceptibility gene loci (*IL23R*, *IL12B*, *JAK2* and *STAT3*) that are involved in both UC and CD (Anderson *et al*., 2011). Variants in *IL12B*, which encodes the p40 subunit of IL-12, have been further associated with IBD, and in addition, a malfunction in IL-10 have also been associated with both forms of IBD (CD and UC) (Tremelling *et al*., 2007).

In addition, immunogenetic predisposition has also been implicated in etiopathogenesis of IBD, and in this context, two genetic polymorphic systems were mostly encountered; human leukocyte antigen (HLA) and cytokines. HLA genes have been identified as the first of the most significant regions of the genome with respect to IBD susceptibility, especially CD. In particular, HLA-DR and HLA-DQ genes, part of the HLA class II subgroup, which encode heterodimeric peptide-binding proteins and proteins that modulate peptide loading, have been associated with IBD susceptibility (Ad'hiah *et al*., 2008; Lees and Satsangi, 2009). Cytokine genetic polymorphisms have also been associated with IBD, especially if we consider that such polymorphisms in the cytokine gene regulatory regions have been correlated with cytokine secretion (Warle *et al*., 2008). A wealth of data have indicated the importance of genetic background in regulating the cytokine network in IBD, and in fact, polymorphisms of cytokine/cytokine receptor genes have been shown to be associated with the development of IBD; implicating their role in determining the risk or protection from the disease expression (Andersen *et al*., 2010; Palmieri *et al*., 2010; Kim *et al*., 2011). This subject will be reviewed later in more details.

2.1.2 Environmental Factors

It is a well-established fact that environmental factors play an important role in IBD pathogenesis, and a number of environmental

factors have been regarded as risk factors for IBD; including smoking, diet, drugs, geography, social stress, and psychological element (Loftus, 2004). However, smoking has been observed as the most environmental prompter for IBD. It was in 1982 when an inverse association between UC and smoking was reported, and subsequent studies have sustained the protective effect of heavy smoking on UC development (Cosnes, 2008). In contrast, smoking has been associated with an increased risk to develop CD (Birrenbach and Böcker, 2004).

Vitamin D is a further environmental factor, and Leslie *et al*. (2008) noticed that vitamin D deficiency was commonly observed in IBD patients and suggested that low vitamin D can increased the risk of IBD. The role of aspirin and nonsteroidal anti-inflammatory drugs (NSAIDs) has also been recognized, and Ananthakrishnan ,(2013) found that high dose, prolonged duration use and frequent use of NSAIDs was associated with an increased risk of CD and UC. A more recent study suggested that antibiotics are important environmental factor that influences influencing the risk of IBD through their effects on intestinal flora (Shaw *et al*., 2010).

Stress has long been considered to play a role in CD and UC pathogenesis, and Bitton *et al*. (2008) suggested that individuals with lower stress levels had a reduced risk of IBD disease onset. In addition to psychological stress, epidemiological surveys highlighted that air pollution may contribute to CD and UC risk, and the increasing incidence of IBD in developing countries that parallels industrialization development may confirm such disease-associated risk (Thia *et al*., 2008). Such air pollution associated risk has been explained in the ground that air pollution is associated with an augmentation in circulating polymorphonuclear leukocytes and serum cytokines, which may contribute to IBD pathogenesis (Kaplan *et al*., 2010).

2.1.3 Role of Microbiota

The gut microbiome of human consists of nearly 1150 bacterial species, with each individual host having approximately 160 species, and it has been established that there is an association between microbiome changes and IBD risk (Qin *et al*., 2010). Therefore, the gut flora has been the focus of studies in CD and UC patients in inflamed and non-inflamed regions. These studies reported that there is a significant reduction in biodiversity of fecal microbiome in CD and UC patients compared to controls (Joossens *et al*., 2011). A further observation found that the microbiota is unstable in IBD patients (Andoh *et al*., 2011). In healthy intestine, two phyla (Firmicutes and Bacteroidetes) predominate and have their contribution to the production of epithelial metabolic substrates. In contrast, microbiota is characterized by a relative lack of these two phyla, and instead an over-representation of enterobacteria in CD has been recorded, while, a reduction in *Clostridium* spp. and an increase in *Escherichia coli* have been found in UC (Martinez *et al*., 2008). In addition, it has been observed in IBD patients (particularly CD), that there is a dramatic increase in bacteria that is associated with the colonic adherent mucus layer (Johansson *et al*., 2008), and a consistent increase in mucosa-associated *E. coli* and reduction in Firmicutes have been reported UC patients. Also, there is strong evidence that depicts an increase in mucosa-associated *E. coli* in ileum and colon of CD patients, and their presence within the granulomas has been associated with a pathogenic role in patients (Ryan *et al*., 2004). An adherent and invasive phenotype of *E. coli* has also been observed in CD patients, which was pictured by bacterial invasion into epithelial cells and replication within macrophages, and it was associated with granuloma formation (Meconi *et al*., 2007).

2.1.4 Immune response

The interaction of the above three etiological factors (genetics, environment and microbiota) will certainly modulate the immune response, especially mucosal immunity, that is involved in IBD pathogenesis, which has been dominated by studies of mucosal immunity, especially the T lymphocyte response (Zhang and Li, 2014). The available information suggests that a dysfunction in innate and adaptive immune responses is involved in the aberrant intestinal inflammatory response in IBD patients, and most studies that focused on adaptive immune response has led to the conclusion that the two forms of IBD are clearly distinct types of gut inflammation: CD is driven by a Th1 response, while UC has been associated with a Th2 response (Cobrin and Abreu, 2005; Targan and Karp, 2005).

2.1.4.1 Innate Immune Response

The innate immune response (first line of defense against pathogens) is achieved by a variety of cells that include epithelial cells, neutrophils, dendritic cells, monocytes, macrophages and natural killer cells, and initiated by microbial antigens recognition via PRRs (pattern recognition receptors) including TLRs (toll- like receptors) on surface of cell and receptors in the cytoplasm (NOD-like receptors) (Abreu *et al*., 2005). Investigations in this context have demonstrated that cells mediating innate immunity and TLRs and NOD proteins expression and function are significantly deviated in IBD patients. Among these, accumulation of mucosal neutrophils and their production of IL-1β and IL-8 in response to trauma were reduced in CD patients but not UC patients (Marks *et al*., 2006). In addition, GWAS presented evidence that *NOD2* gene is mutated, and such mutations have been associated with CD, and are responsible for the defective ability in the gut to respond to

lipopolysaccharides. Such defect has been suggested that may account for IBD susceptibility (Bonen *et al*., 2003). Such altered response might enhance pathogenic microbial invasion in CD patients and may also cause a reduction in the production of antibacterial agents (Wehkamp *et al*., 2004). Further studies suggested that *NOD2* mutations that are associated with a functional loss may consequence in a reduced inhibition of TLR2 stimulation, which leads to inflammatory pathways activation and excessive Th1 immune responses (Abraham *et al*., 2006). Therefore, production of cytokines is altered and one of them is IL-23, which is a key cytokine involved in innate and adaptive immunity and exerts a central role in driving early immune responses against microbes. Investigations have demonstrated that *IL23R* SNPs are associated with both forms of IBD (CD and UC); an observation that suggests that IL-23 may represent an inflammatory molecule that has a role in a chronic intestinal inflammation, and by this pathway IL-23 may contribute to IBD pathogenesis at early disease (Takatori *et al*., 2009).

2.1.4.2 Adaptive Immune Response

Most investigations in the last two decades have spotted the role of abnormal adaptive immune responses in IBD pathogenesis, and such spot has ultimately led to the concept that the CD and UC represent clearly distinct forms of IBD that involves gut inflammation: CD has long been regarded to be driven by a Th1 response, while UC has been associated with Th2 response (Geremia *et al*., 2014). T regulatory (Treg) and Th17 cells have also been involved in inflammatory response of gut in IBD (Zhang and Li, 2014). The functional communication between these cells is mainly mediated by cytokines (Figure 2-1), which were the main concern of present study, but in terms of their genetic polymorphisms.

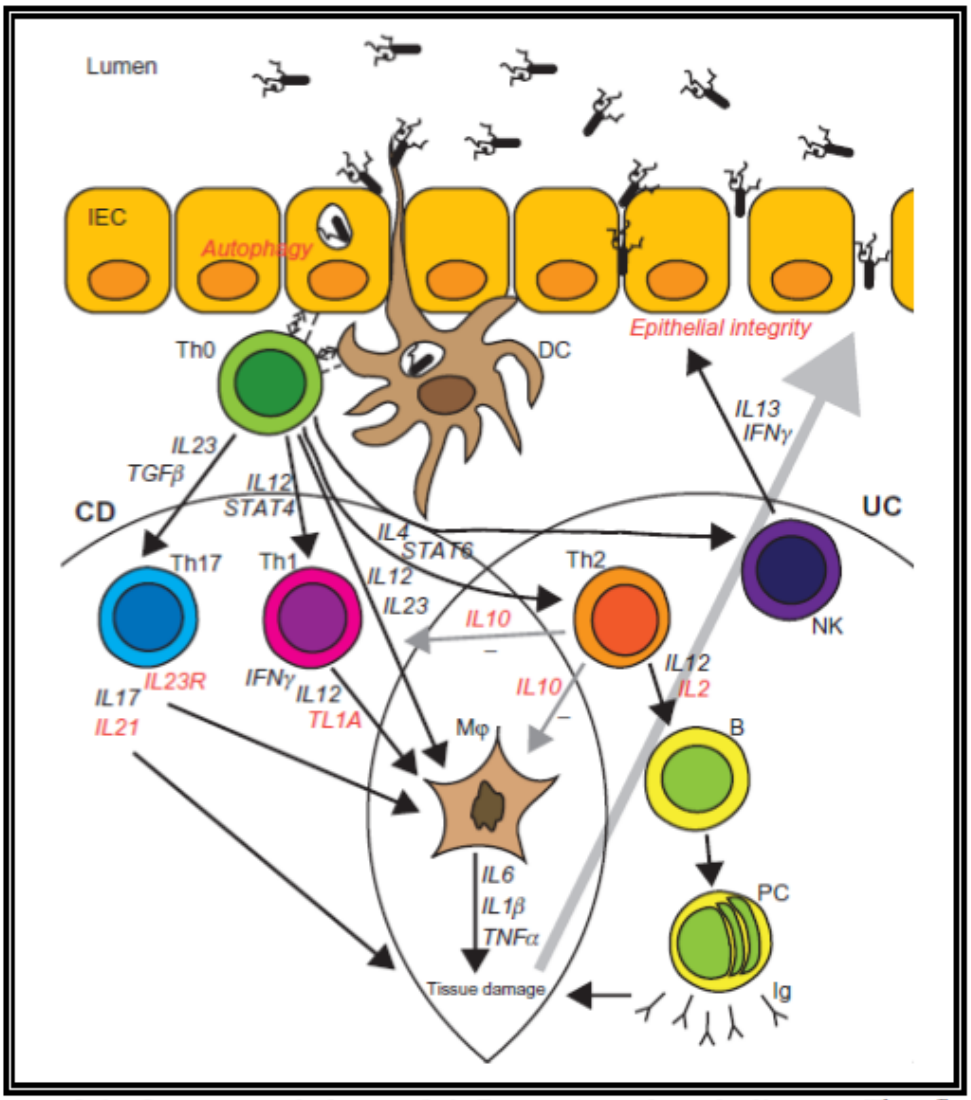

Figure 2-1: **Immunopathology of inflammatory bowel disease**. The figure schematically shows the pathomechanisms for Crohn's disease (CD: left) and ulcerative colitis (UC: right) from the initial stimulation by the intestinal microflora (top) to the tissue damage (bottom). The intestinal microflora is sampled by dendritic cells and invaded intestinal epithelial cells that, after autophagy, present antigen to Th0 cells. These T helper cells proliferate and differentiate to Th 17 or Th 1 in CD. Through direct effector functions of these pathways and through stimulation of pro-inflammatory activity of macrophages, these pathways lead to tissue damage. In UC, Th 2 cells stimulate B cell differentiation and NK cells proliferate that lead to direct (effector functions of the individual pathways) and indirect (through stimulation of macrophages) tissue damage. Lumen: the bowel lumen, IEC: intestinal epithelial cell, Tho: undifferentiated T-helper cell, DC: dendritic cell, Th: T-helper cell, NK: natural killer cell, Mφ: macrophage, B: B cell, PC: plasma cell, IL: interleukin, TGFβ: tumor growth factor beta, STAT: signal transducer and activator of transcription, IL23R: interleukin 23 receptor, TL1A: TNF-like ligand 1A, TNFα: tumor necrosis factor alpha, Ig: immunoglobulin (Festen *et al*., 2013).

Among these cytokines that have been extensively studied in IBD are those produced by Th1and Th2 lymphocytes. The former are induced

by IL-12 to produce mainly IL-2 and IFN-γ, while Th2 cells are signatured by producing IL-4, IL-5 and IL-13 (Maerten *et al*., 2004). An abnormal Th1 immune response has been suggested to cause the intestinal inflammation in CD and triggered by increased mucosal levels of IL-12, and in addition, macrophage-derived IL-12 has been presented to be preferentially over-expressed in CD patients (Schmidt *et al*., 2005). Mucosal T lymphocytes from CD patients have also been shown to produce higher levels of IL-2 and IFN-γ than T lymphocytes from UC patients or controls, but UC patients produced more IL-5 than CD patients (Ito *et al*., 2005; Sventoraityte *et al*., 2008). Further investigations confirmed that T cells from CD patients produced more IFN-γ than cells of UC and controls, and a similar observation was made regarding NK T cells (Fuss *et al*., 2004; Heller *et al*., 2005). Crohn's disease has therefore been suggested to be characterized by a Th1 immune response, while UC has been considered as a Th2-mediated disease, with excessive production of IL-5 and IL-13(Di Sabatino *et al*., 2012).

Regulatory T cells have also been defined to have a role in pathogenesis of IBD via their production of the anti-inflammatory cytokines IL-10 and TGF-β, and they are suggested to be involved in the maintenance of gut mucosal homeostasis by suppressing abnormal immune responses against normal flora or dietary antigens in the gut. (O'Garra and Vieira, 2004). In this regard, it has been reported that Treg cells demonstrate a potent anti-inflammatory action in experimental colitis, and in addition, they were found to be depleted from the peripheral blood of active IBD patients compared to quiescent IBD patients or controls (Fantini *et al*., 2006; Chamouard *et al*., 2009). In agreement with such findings, defects in the function of IL-10 have also been associated with CD and UC, and an up-regulation of IL-10

produced by cultured monocytes from IBD patients was found (Sventoraityte *et al*., 2008). Therefore, IL-10 production by Treg cells has been regarded as important cytokine in the pathogenesis of IBD (Műzes *et al*., 2012).

2.2 Cytokine Gene Polymorphisms

Cytokines are low-molecular-weight soluble protein messengers that are involved in all aspects of innate and adaptive immune responses, including cellular growth and differentiation, inflammation, and repair. They act in an antigen-nonspecific manner and are involved in a wide array of biological activities ranging from chemotaxis to activation of specific cells and induction of broad physiologic changes (Mehta, 2006). A large number of cytokines have been identified, and many are crucial in regulating lymphocyte development and in determining the types of immune responses evoked by specific responses (Doan *et al*., 2008). Cytokines are produced mainly by immune cells that facilitate communication between cells, stimulate the proliferation of antigen-specific effector cells, and mediate local and systemic inflammation using three pathways: autocrine, paracrine and endocrine (Sanchez-Muñoz *et al*., 2008). However, these actions might be subjected to the influence of genetic polymorphisms of cytokine and cytokine receptor genes (Kaur and Mehra, 2012).

Genetic polymorphism can be defined as the existence together of many forms of DNA sequences at a locus within the population, or a discontinuous genetic variation that results in different forms or types of individuals among the members of a single species (Dai *et al*.,2008). Genetic polymorphism promotes diversity within a population. It often persists over many generations because no single form has an overall advantage or disadvantage over the others regarding

natural selection. A common example is the different allelic forms that give rise to different blood types in humans (Riha *et al.*, 2004).

With the recent knowledge on cytokine gene polymorphisms (CGPs), differences between individuals have been discovered that influence not only cytokine gene expression, but also susceptibility to diseases, their progression, severity, and clinical outcomes. Ethnic differences observed in patterns of CGPs at the population level are mainly a result of natural selection imposed by microbiota, environmental factors, and complex host-pathogen interactions. These correlate with population-based variations in the ability to mount an immune response, and data generated over the years has identified several important single nucleotide polymorphisms (SNPs) in various cytokine and cytokine receptor genes that are important markers not only for better understanding of etiology and pathology of a disease, but also as potential biomarkers of disease susceptibility and severity (Fang *et al.*, 2010; Fareed and Afzal, 2013).

Genetic polymorphisms have therefore emerged in recent years as important determinants of disease susceptibility and severity. Polymorphisms are naturally occurring DNA sequence variations, which differ from gene mutations in that they occur in the normal healthy population and have a frequency of at least 1%. Approximately 90% of DNA polymorphisms are SNPs due to single base substitutions. Others include insertion/deletion polymorphisms, minisatellite and microsatellite polymorphisms (Ulger *et al.*, 2014). Although most polymorphisms are functionally neutral, some have effects on regulation of gene expression or on the function of the coded protein. These functional polymorphisms, despite being of low penetrance, could contribute to the differences between individuals in susceptibility to and severity of disease (Conde *et al*., 2013).

Many studies have examined the relationship between certain cytokine gene polymorphism, cytokine gene expression *in vitro*, and the susceptibility to and clinical severity of diseases (Hollegaard and Bidwell, 2006; Ulger *et al.*, 2014). Single nucleotide polymorphisms are an increasingly important tool for studying structure and history of genome, and one of their most common applications is in disease-association studies, which aim to explore statistically significant associations between SNP alleles and diseases, in order to pinpoint candidate causative genes (Ning *et al*., 2014). With respect to cytokine genes, SNPs are frequently encountered in the 5'and 3' regulatory regions or introns of a cytokine gene and/or its receptors, which have been shown to play a role in regulating the extent of gene expression through one or more of the following possible mechanisms: by altering the structure of transcription factor binding sites within promoters, manipulating the structure of enhancers or silencers within introns or at distant regions, and altering the binding sites within nuclear matrix for architectural transcription factors that modulate promoter activity (Longhi *et al*., 2013).

Inflammatory bowel disease has also been the subject of cytokine gene polymorphisms world wide, but unfortunately they have been not investigated in Iraqi patients, and therefore, this study came to shed light on 13 cytokine and cytokine receptor SNPs (*IL1A*, *IL1B*, *IL1R1*, *IL1RN*, *IL2*, *IL4*, *IL4R*, *IL6*, *IL10*, *IL12B*, *IFNG*, *TNF* and *TGFB1*).

2.2.1 Interleukin-1 Genes (*IL1A*, *IL1B*, *IL1R1* and *IL1RN*)

Three members of IL-1 have been recognized: IL-1α, IL-1β, IL-1 type I receptor (IL-1R) and IL-1 receptor antagonist (IL-1RA), which are controlled by four closely linked genes (*IL1A*, *IL1B*, *IL1R1* and *IL1RN*, respectively) that are mapped to the long arm of chromosome

2 (2q14.2) (Arend *et al*., 2008). The first two interleukins (IL-1α and IL-1β) are structurally related and recognize the same receptor (IL-1R), while IL-1RA binds IL-1R and blocks the activities of IL-1α and IL-1β (Sainz *et al*., 2008). Interleukin-1 is mainly produced by activated monocytes and macrophages, and acts systemically and locally, while IL-1RA is produced by hepatocytes during the inflammatory acute-phase response, probably to control IL-1 effects (van de Veerdonk and Netea, 2013). An imbalance between IL-1 and IL-1RA production has been observed in mucosal biopsies obtained from inflamed colonic tissue of IBD patients, and an IL-1RA administration to rabbit model of dextran-induced colitis prevented mucosal inflammation and necrosis (Ludwiczek *et al*., 2004).

Several SNPs have been described for *IL1A*, *IL1B*, *IL1R1*and *IL1RN* genes ($IL1A_{-889}$, $IL1B_{-511}$, $IL1B_{+3962}$, $IL1R1_{\text{pst1 1970}}$ and $IL1RN_{\text{mspa1 11100}}$), and studies in CD and UC patients of different ethnicities have reported different correlations with the two forms of IBD, but some of them suggested that these polymorphisms may play a role in etiopathogenesis of IBD (Craggs *et al*.,2001; Nohara *et al*.,2003; Ferreira *et al*., 2005; Celik *et al*., 2006; Corleto *et al*., 2009; Furusho *et al*., 2011; Ziaee *et al*., 2014; López-Hernández *et al*., 2015).

2.2.2 Interleukin-2 Gene (*IL2*)

Interleukin-2 was discovered in 1975 as a growth-promoting activity for bone marrow-derived T lymphocytes (Fallahzadeh *et al*., 2011). It is synthesized and released from activated T cells and has a key role in cell mediated immune response, and it was found that IL-2 increases lymphokine release from T, B and NK cells and has many immunological effects (Wrenshall *et al*., 2014). Additionally, it is a potent T cell growth factor that is assumed to amplify lymphocyte

responses *in vivo* (Fallahzadeh *et al.*, 2011).Functionally, it initiates a pro-apoptotic pathway through enhancing FasL expression on activated T cells, and since T cells also express Fas /CD95, this events leads to programmed cell death (apoptosis) of activated T lymphocytes. Also, it promotes production of NK-derived cytokines such as tumor necrosis factor alpha (TNF-α), IFN-γ and granulocyte monocyte-colony stimulating factor (GM-CSF), and can act synergistically with IL-12 to enhance NK cytotoxic activity (Oo *et al.*, 2012).

With respect to IBD, activated T lymphocytes have been suggested to be involved the mucosa inflammation of IBD patients, and therefore, IL-2 may play a stimulating role in the inflammation (Ito *et al.*, 2005). There is also emerging evidence that serum IL-2 show an increased level in CD patients with active disease; an observation that suggests that CD is Th1-driven disease, but may not be involved in UC (Sventoraityte *et al.*, 2008).

Interleukin-2 is encoded by a gene located on the long arm of chromosome 4 (4q26). Two polymorphisms in *IL2* gene (-330 T/G and +166 G/T) have been shown to influence IL-2 levels (Gao *et al.*, 2009). The profile of *IL2* SNPs in IBD has been the subject of two investigations. In the first, $IL2_{-330}$ SNP was investigated in Chinese UC patients and a positive association was reported (Shi *et al.*, 2011).In a further study, $IL2_{+166}$ SNP was investigated in Turkish CD and UC patients, and significant variations in genotypes and alleles were found between patients and controls (Gok *et al.*, 2014).

2.2.3 Interleukin-4 Gene (*IL4*)

Interleukin-4 was discovered in 1982, and secreted by activated T cells and basophils as a mature 129 amino acid glycoprotein. It is a pleiotropic cytokine that acts on T and B lymphocytes, monocytes,

polymorphonuclear cells, fibroblasts and endothelial cells (Maes *et al.*, 2012). As mentioned, it executes pleiotropic functions, including induction of Th2 differentiation, immunoglobulin class switching, B cell proliferation, and suppression of Th1 differentiation and macrophage activation (Gilmour and Lavender, 2008). It acts at various stages of cell differentiation and its effects are also dependent on the cytokine environment (Apte *et al.*, 2008). In particular, IL-4 blocks some of effects of IL-2 whereas IFN-γ blocks some of the effects of IL-4 (Olver *et al.*, 2007). Interleukin-4 is also termed anti-inflammatory because of its ability to suppress TNF-α, IL-1, IL-6, and prostaglandin E2 (PGE2) production by activated monocytes (Rai *et al*., 2011; Karimi *et al*., 2013). Interleukin-4 activates two types of receptors; the type 1receptor is comprised of the JAK1-bound IL4Rα and JAK3-bound common gamma chain(γc) (Sharma *et al*., 2008).

Interleukin-4 is encoded by a gene located on the long arm of chromosome 5 (5q31.1). Three polymorphisms in *IL4* gene ($IL4_{-1089}$ T/G, $IL4_{-590}$ T/C and $IL4_{+33}$ T/C) have been described, while, its receptor gene (*IL4R*) is mapped to 16p11.2-12.1, and the mostly studied SNP is $IL4R_{+1902}$ (Karimi *et al.*, 2013). Several studies have examined the IL$ *SNPs* in IBD patients, and although different associations were reported, the investigations agreed that *IL4* polymorphisms are involved in the etiopathogenesis of CD and UC (Aithal *et al*., 2001; Peng *et al*., 2002; Hong *et al*., 2008; Ahirwar *et al*., 2012; Connelly *et al*., 2014). The protective effect of these SNPs has also been highlighted; for instance, Gao *et al*. (2014) reported that $IL4_{-590}$ *C* allele is a protective allele against CD and UC in Turkish patients. Similarly, *IL4R* SNPs have been further investigated in IBD patients, and homozygosity in these SNPs have been suggested to have a

role in IBD pathogenesis and progression (Tachdjian *et al*., 2009; Gao *et al*., 2014; Karatzas *et al*., 2014).

2.2.4 Interleukin-6 Gene (*IL6*)

Interleukin-6 is produced primarily by mononuclear phagocytes, fibroblasts, and vascular endothelial cells, and some activated T cells can also secrete IL-6. It is multi-functional cytokine, and plays roles in adaptive immune response, inflammation, hematopoiesis and endocrine system (Balschun *et al*., 2004). However, studies of IL-6 deficient mice have shown that, while IL-6 contributes to numerous cellular events, it is crucial for the acute phase response, mucosal production of IgA, and fever response during inflammation. In particular, IL-6 induces the synthesis of acute phase proteins (particularly fibrinogen) by hepatocytes and stimulates pathogen clearance functions of neutrophils (Fisman *et al*., 2006). It also appears to have a major influence on the end stages of B cell differentiation, as well as supports the differentiation and/or maturation of B cells, T cells, macrophages, megakaryocytes, certain neurons and osteoclasts (Dienz and Rincon, 2009).

Although IL-6 was originally considered to be a pro-inflammatory cytokine, but several discoveries prompted a revision of its characteristics and indicated that IL-6 also has anti-inflammatory properties. For instance, IL-6 inhibited neutrophil accumulation after lipopolysaccharide (LPS) injection and antagonized the actions of IL-1β and TNF-α via induction of the soluble IL-1Ra and the soluble TNF-α receptor (Petersen and Pedersen, 2006). Furthermore, IL-6 was required to control the levels of pro-inflammatory cytokines, such as TNF-α, *in vivo* after endotoxic insults, both locally and systemically, and under certain conditions, IL-6 obtained

anti-inflammatory characteristics in macrophages (Neurath and Finotto, 2011).

There is emerging evidence that IL-6 plays a crucial part in intestinal inflammation, which is a main phenomenon of IBD, and it has been reported that intestinal macrophages and CD4+ T-cells produce elevated levels of IL-6 in IBD patients (Atreya and Neurath, 2005). A further observation documented that the formation of IL-6-sIL-6R complexes is increased in the patients, and such complexes can interact with CD4+T cell membrane and lead to an increased STAT3 expression and nuclear translocation, which leads to the induction of anti-apoptotic genes (for instance *Bcl-xl*) (Mudter and Neurath, 2007). Consequently, a resistance of lamina propria T-cells to apoptosis is augmented, and T-cell expansion therefore contributes to the persistence of chronic intestinal inflammation in IBD patients (Scheller *et al*., 2011). These findings depict the predominant pathogenic role of IL-6 in pathogenesis of CD and UC, and thus IL-6 has been a target for therapeutic strategies in the treatment of IBD (Allocca *et al*., 2013).

Interleukin-6 is encoded by a gene on the long arm of chromosome 7 (7q14.21). Two SNPs located in the promoter region of *IL6* gene ($IL6_{-174}$ G/C and $IL6_{+565}$ G/A) are the most frequently studied genetic variations of the gene, and earlier studies demonstrated that the first SNP is effective in regulating IL-6 production, and $IL6_{-174}$ *G* allele was associated with high levels of IL-6 (Rivera-Chavez, 2003). The mostly investigated SNP in IBD patients was $IL6_{-174}$ and the investigations concluded that such SNP might be associated with the etiology of IBD; although the observations have been inconsistent (Balding *et al*., 2004; Cantor *et al*., 2005; Sawczenko *et al*., 2005; Guerreiro *et al*., 2009; Sagiv-Friedgut *et*

al., 2010). The second SNP ($IL6_{+565}$) has not been investigated in IBD patients.

2.2.5 Interleukin-10 Gene (*IL10*)

Interleukin-10 was discovered in 1980 as a cytokine with inhibitory factors, and exhibits various immunomodulatory functions, and it is mainly secreted by Th2 type T cells, but also macrophages/monocytes, dendritic cells, B cells, and even Th1 cells, Th2 cells, lung mast cells, B cell derived tumor cell lines, and keratinocytes are also able to secrete IL-10, but recently, it is considered as T-regulatory (Treg) cell cytokine (Saraiva and O-Garra, 2010). The immunodulatory functions of IL-10 are various, ranging from supporting B cell differentiation and immunoglobulin secretion to inducing a strong anti-inflammatory response (Chaudhry *et al*., 2011). It is also an anti-inflammatory cytokine, and during infection, it inhibits the activity of Th1 cells, NK cells, and macrophages, all of which are required for optimal pathogen clearance but also contribute to tissue damage (Cope *et al*., 2011). Furthermore, IL-10 inhibits MHC-class II and co-stimulatory molecule B7-1/B7-2 expression on monocytes and macrophages and limits the production of pro-inflammatory cytokines (including IL-1α and β, IL-6, IL-12, IL-18, and TNF-α) and chemokines (Das *et al*., 2012). In this regard, it has been found that IL-10 inhibits the protective immune response to pathogens by blocking the production of pro-inflammatory cytokines, such as TNF-α and the Th1-polarizing cytokine IL-12, by directly acting on macrophages and dendritic cells (Pils *et al*., 2010).

With respect to IBD, it has been reported that IL-10 plays a pivotal anti-inflammatory role in CD patients, and an inactivation of IL-10 in mice was associated with an increased production of IL-12 and

IFN-γ. In humans, the inflamed mucosa and granulomas of CD demonstrated low level of IL-10 expression (Shih and Targan, 2008). However, it was most important to observe that endogenous IL-10 can constrain Th17 cell development through the control IL-1 production, which confirms the crucial anti-inflammatory role of IL-10 in patients with CD, while in UC the IL-10 effects have been not well-understood (Wilke *et al*., 2011).

Interleukin-10 is encoded by a gene located on the long arm of chromosome 1 (1q31-32). Three SNP has been found on the promoter region of *IL10* gene ($IL10_{-1082}$ G/A, $IL10_{-819}$ C/T and $IL10_{-592}$ C/A) and exhibited a strong effect on the transcription of *IL10* gene (D'Alfonso *et al*., 2000). In one comprehensive study, a meta-analysis of 17 published studies about three *IL10* SNPs in IBD ($IL10_{-1082}$, $IL10_{-819}$ and $IL10_{-592}$) was carried out by Zou *et al*. (2014). Their results demonstrated that $IL10_{-819}$ and $IL10_{-592}$ SNPs were associated positively with UC, while $IL10_{-1082}$ SNP was associated with CD or UC. A further meta-analysis also reported positive associations but they were different from those reported by the former meta-analysis (Lv *et al*., 2014).

2.2.6 Interleukin-12B Gene (*IL12B*)

Interleukin-12 is a pro-inflammatory cytokine that play a central role in immune response, because it links the activation of macrophages by microbial invaders to activation of both NK (innate response) and Th1 (adaptive response) effector functions. In particular, IL-12 is crucial for the generation of IFN-γ required for host defense against a broad range of intracellular pathogens (Trinchieri, 2003). Interleukin-12 is a heterodimer and composed of disulfide-linked p35 and p40 subunits that are unrelated in

structure. The p35 subunit is homologous to regions of IL-6 and G-CSF, while p40 subunit has the structure of a hematopoietin receptor molecule (Watford *et al*., 2004). It is produced primarily by activated macrophages and with some contribution by neutrophils, dendritic cells, monocytes, and B cells (Kang *et al*., 2005).

It has been demonstrated that of IL-12 expression is up-regulated in both active UC and CD biopsies, and it was positively correlated with the disease activity score (Nielsen *et al*., 2003). In addition, the Levels of IL12p40 were found to be higher in early rather than in late CD; an observation that suggests that IL-12-mediated modulation is strongly dependent on stage of disease (Kugathasan *et al*., 2007).

The two chains (p35 and p40) that form IL-12 complex are encoded by two genes; *IL12A* and *IL12B*, which are mapped to 3p12-13.2 and 5q31.1-q33.1, respectively, and SNPs for both genes have been described (Chen *et al*., 2009). The latter gene has been mostly encountered and in IBD, and several groups of investigators (Barrett *et al*., 2008; Franke *et al*., 2010; Anderson *et al*., 2011) presented evidence that *IL12B* is a gene that is associated with susceptibility for IBD. Disease-SNP studies also implicated the role *IL12B* SNPs in etiopathogenesis of IBD, although inconsistent observations have been made (Marquez *et al*., 2008; Yamazaki *et al*., 2009; Glas *et al*., 2012; Moon *et al*., 2013; Dubinsky *et al*., 2013).

2.2.7 Interferon Gamma Gene (*IFNG*)

Interferon-γ (IFN-γ) is a type II IFN that was believed that CD4+Th1 lymphocytes, CD8+ cytotoxic lymphocytes, and NK cells are its exclusive producer IFN-γ; however, it has also been evident that

B cells, NKT cells and professional APCs produce IFN-γ. Such production is controlled most notably by IL-12 and IL-18 (Schroder *et al*., 2004; Chan *et al*., 2006). The primary role of IFN-γ is macrophage activation to enhance phagocytosis, tumoricidal properties and intracellular killing of pathogens. In addition, IFN-γ induces macrophage to produce a variety of inflammatory mediators and reactive oxygen and nitrogen intermediates, and increases expression of high affinity immunoglobulin Fc receptors on phagocytes (Gattoni *et al*., 2006). Interferon-γ also upregulates the expression of MHC antigens by macrophages and this facilitates antigen presentation to T cells. It has also been shown that IFN-γ acts on CD4+ T cells to promote Th1 differentiation while inhibiting the generation of Th2 cells. It also promotes the maturation of CD8+ T cells to cytotoxic cells. These effects are mediated through specific IFN-γ receptors on cell surface (Schoenborn and Wilson, 2007).

As reviewed earlier in this chapter, progression of CD is mainly driven by CD4+ Th1, and IFN-γ has been considered as a signature cytokine of the disease (Strober and Fuss, 2011). However, further recent evidence has demonstrated that IFN-γ produced mainly by CD4+ T cells is required to upregulate MHC class-II expression on intestinal epithelial cells of mice that were experimentally induced to develop colitis (Thelemann *et al*., 2014). Their results suggested that, in addition to its pro-inflammatory roles, IFN-γ exerts a critical anti-inflammatory function in the intestine, which may protect against colitis by inducing MHC class-II expression on intestinal epithelial cells.

The gene encoding IFN-γ is mapped to the long arm of chromosome 12 (12q14), and several SNPs have been reported for this gene, and one

of them is $IFNG_{+874}$ A/T polymorphism (Bouzgarrou *et al.*, 2009). However, such polymorphism has shown no association with CD or UC (Cantor *et al*., 2005), but other SNP of *IFNG* have been investigated and their role in etiopathogenesis of IBD has been suggested (Gonsky *et al*., 2014).

2.2.8 Tumor Necrosis Factor-Alpha Gene (*TNF*)

Tumor necrosis factor-alpha (TNF-α) is a cytokine involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reactions. It is produced chiefly by activating macrophages, although it can be produced by many other cell types such as CD4+ lymphocytes, NK cells, neutrophils, mast cells, eosinophils, and neurons during acute inflammation and is responsible for a diverse range of signaling events within cells, leading to necrosis or apoptosis (Swardfager *et al.*, 2010). It also stimulates the migration of immune cells to the infection site; contributing to granuloma formation, and is capable of controlling the disease progression (Mootoo *et al*., 2009). It has demonstrated further that IFN-γ produced by Th1 lymphocytes is capable to induce apoptosis in enterocyte and triggers TNF-α release from activated mucosal macrophages. Th1 lymphocytes are also an important producer of TNF-α, which is the central cytokine that induces stromal cell differentiation into myofibroblasts and enhances their production of matrix metalloproteinases (MMPs), which is a tissue-degrading enzyme that also induces apoptosis of enterocyte by digesting the basement membrane (Biancheri *et al*., 2013). Therefore, TNF-α links the immune response of both type; innate and the adaptive immune responses, and has been suggested to play a crucial role in IBD pathogenesis, as demonstrated by the results of clinical trials with biological therapies targeting TNF-α (Macdonald *et al*., 2012). It has

also been found that the intestinal mucosa of CD patients showed an increased expression of TNF-α, and high levels were also observed in serum, intestinal mucosa and stool samples of patients (Hong *et al*., 2008). Similarly, UC has been suggested to be related to a dysregulated production of several cytokines, and TNF-α is one of them, whose expression in inflamed mucosa has been associated with disease severity (Rodri´guez-Pera´lvarez *et al*., 2012).

The *TNF* gene is located within the human MHC (HLA region) in the HLA-class III sub-region between HLA-class I and -class II sub-regions on the short arm of chromosome 6 (6p21.3). Two SNP have been determined on the promoter region of *TNF* gene; TNF_{-238} G/G and TNF_{-308} G/G (Zhan *et al.*, 2011). These SNPs have been considered to substantially influence the production capacity of TNF-α. The *TNF* gene promoter has been shown to contain numerous binding sites for transcriptional factors, suggesting that the presence of promoter polymorphism might directly influence transcriptional regulation of *TNF* gene (Basturk *et al.*, 2008). As TNF-α has been suggested to have a role in CD and UC pathogenesis, its SNPs have also been regarded to have positive and negative associations with IBD (Cao *et al*., 2006; Sýkora *et al*., 2006 Cucchiara *et al*., 2007), although some investigators reported no association between *TNF* SNPs and IBD (Zipperlen *et al*., 2005; Mittal *et al*., 2007; Naderi *et al*., 2014).

2.2.9 Transforming Growth Factor Beta Gene (*TGFB1*)

Transforming growth factor beta (TGF-β) belongs to a family of multi-functional cytokines that are produced by a wide variety of immune cells, and five isoforms are recognized, three of which exhibit expression in mammals (TGF-β1, TGF-β2 and TGF-β3). They are synthesized as precursor proteins that are cleaved inside the cell before

secretion to yield two products as dimers; LAP (latency-associated peptide) and mature TGF-β, which is termed latent TGF-β. The latter is converted to the biologically active form of TGF-β extracellularly after undergoing some conformational changes (Gorelik and Flavell, 2002). The three isoforms play an important role in the regulation of immune cell functions, and a disruption of TGF-β1 gene in mouse was associated with severe, multi-focal inflammatory response and even early death, while mutations in TGF-β2 or TGF-β3 resulted in 100% embryonic lethality; the three TGF-β isoforms have been reported to have similar immunological effects *in vitro* (Del Zotto *et al*., 2003). Such cytokine can act in autocrine and paracrine fashions to control differentiation, proliferation and activation of immune cells. Particularly, TGF-β1 can control the production of and response to cytokines that are associated with Th1 and Th2 T cells (Becker *et al*., 2006). In animal models, TGF-β production was associated with the pathogenesis of experimental colitis, and it has been demonstrated that protection from colitis progression was strictly influenced by up-regulation of cells that produce TGF-β1 (Rani *et al*., 2011). In humans, it has been observed that the unresponsiveness of mucosal T lymphocytes to luminal antigens is controlled by production of IL-10 and TGF-β from Treg cells (Ebert *et al*., 2009). In normal gut, Treg cells and via these two cytokines are involved in controlling inflammation, and in IBD patients, this physiological control of inflammation is disrupted because of defects in adequate production of IL-10 and TGF-β, which both have been regarded as important cytokines in pathogenesis of CD and UC (Feagins, 2010).

The *TGFB1* gene is mapped to the long arm of chromosome 19 (19q13.1), and two SNPs ($TGFB1_{\text{codon 10}}$ and $TGFB1_{\text{codon 25}}$) have been recognized in the translated region of the gene, which have been found to be associated with serum level of TGF-β1, in addition to further six

SNPs in the promoter region (Wang *et al*., 2007). Some of these SNPs have been reported to be positively or negatively associated with CD or UC; for instance, *TGFB1*$_{-509}$ and *TGFB1*$_{915}$ SNPs in CD (Schulte *et al*., 2001; Hume *et al*., 2006) and *TGFB1*$_{-800}$ in UC patients and controls (Tamizifar *et al*., 2008), while other showed no significant association (Liberek *et al*., 2008). However, Almeida *et al*. (2013) enhanced the concept that *TGFB1* SNPs might be associated with susceptibility to CD development.

Chapter Three
Subjects, Materials and Methods

Chapter Three
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3.1 Subjects

One hundred Iraqi Arab patients with inflammatory bowel disease (IBD) were enrolled in this study. The patients attended the Gastrointestinal Tract Units at Al-Kindy Teaching Hospital, Al-Yarmouk Teaching Hospital and Al-Zuafrania General Hospital in Baghdad for diagnosis and treatment during the period August 2013 - October 2014. The disease was clinically diagnosed by the consultant medical staff at the hospital, which was based on clinical, radiological, endoscopic, and histopathological findings according to the criteria of Lennard - Jones (1989). Under the supervision of this staff, information sheet (Appendix I) was filled for each patients, in which results of some laboratory tests were recorded (Hemoglobin, total leukocyte count and erythrocyte sedimentation rate).

According to the point view of consultants, the patients were distributed into two clinical groups:

A. Crohn's disease (CD) patients**:** They were 34 cases (12 males and 22 females), and their age mean ± S.D. was 40.65 ± 3.89 years.
B. Ulcerative colitis (UC) patients**:** They were 66 cases (24 males and 42 females), and their age mean ± S.E. was 40.19 ± 8.05 years.

For the purpose of a comparison, 43 apparently healthy controls of blood donors (12 males and 31 females) matched patients for age (38.67 ± 4.93 years) and ethnicity (Iraqi Arabs) were also enrolled in the study.

3.2 Materials

General laboratory equipment, chemicals and kits and their manufacturers are listed in tables 3-1, 3-2 and 3-3, respectively.

Table 3-1: General laboratory equipment.

Equipment	Company	Country
Camera	Sony	China
Centrifuge	Pioneer	Korea
Conical flask	Qiao Shang	England
Cool box	Hougi	China
Deepfreeze	Biko	Turkey
EDTA Tubes	FLmedical	Italy
Electrophoresis unit	Consort	Germany
Eppendorf tubes	Gilson	China
Incubator	Pioneer	Korea
Micropipettes	NNE	Germany
Microwave oven	LG	Korea
Nano Drop	Pioneer	Korea
Refrigerator	Vestal	Turkey
Spectrophotometer	Cecil E1021	England
Syringe 5ml	LLG.Abu Dhabi	UAE
Thermocycler	Pioneer	Korea
Tips	Gilson	China
UV Light Trans illuminator	GmbH	Germany
Vortex	Pioneer	Korea
Water bath	Pioneer	Korea

Table 3-2: Chemicals.

Chemical	Company	Country
Agarose	Bio Basic	Canada
Injection distal water	Bhud-Baddi	India
Ethanol	Promega	USA
Ethidium Bromide	Promega	USA
Isopropanol	Promega	USA
Master mix	Clinic Heidelberg	Germany
TAE Buffer	Promega	USA
Tag DNA polymerase	Promega	USA

Table 3-3: Kits.

Kit	Company	Country
CTS-PCR-SSP Tray Kit	Clinic Heidelberg	Germany
Wizard Genomic DNA Purification Kit	Promega	USA

3.3 Parameters of Study

Single nucleotide polymorphisms (SNPs) of 13 cytokine and cytokine receptor genes (*IL1A*, *IL1B*, *IL1R1*, *IL1RN*, *IL2*, *IL4*, *IL4R*, *IL6*, *IL10*, *IL12B*, *IFNG*, *TNF* and *TGFB1*) were detected by PCR-SSP (polymerase chain reaction-sequence specific priming).

3.4 Blood Sample Collection

From each participant, 2 – 3 ml of venous blood was collected in EDTA tubes using 5ml disposable syringe. The blood was kept frozen (a maximum of 8 weeks) at - 20°C until DNA isolation.

3.5 Cytokine Gene Polymorphisms

3.5.1 Isolation of Genomic DNA

The genomic DNA was extracted from EDTA blood using Wizard® Genomic DNA Purification Kit (Promega Corporation, USA), which is designed for DNA isolation from whole blood.

A. Kit Contents

- Cell lysis solution
- Nuclei lysis solution
- Protein precipitation solution
- DNA rehydration solution
- RNase Solution

B. Extraction Procedure:

The instructions of manufacturer were followed to isolate genomic DNA from whole blood, with some modification. They are summarized in the following steps:

i. To 500 µl of blood sample, 900µl of cell lysis solution were added in a sterile 1.5ml microcentrifuge tube, which was inverted 5 - 10 times before incubating for 10 minutes at room temperature. The tube was then centrifuge at 14,000 × *g* for 60 seconds at room temperature. The supernatant was discarded without disturbing the visible white pellet.
ii. The white pellet was suspended gently in 300 µl of nuclei lysis solution to lyse white blood cells, and incubated with mixing at 37°C until the clumps are disrupted. This was followed by adding 1.5µl of RNase solution to the nuclear lysate, and the tube inverted 2–5 times. Then it was incubated at 37°C for 15 minutes, and cooled to room temperature.

iii. Protein precipitation solution (150 µl) was added to the cooled nuclear lysate, and vortexed vigorously for 10 - 20 seconds. The tube was centrifuged at 14,000 × *g* for 3 minutes at room temperature, and the supernatant was transferred to a clean 1.5ml microcentrifuge tube containing 300 µl of room-temperatured isopropanol. The solution was gently mixed by inversion until white thread-like strands of DNA form a visible mass.

iv. The solution was centrifuged at 14,000 × *g* for 60 seconds at room temperature, and at this step, the DNA was visible as a small white pellet. The supernatant was decanted, and 200 µl of room temperatured 70% ethanol was added. The tube was gently inverted several times to wash the DNA pellet and the sides of the microcentrifuge tube, and centrifugation was repeated.

v. The ethanol was carefully aspirated, the tube was invert on clean absorbent paper and the pellet was air-dried for 10–15 minutes; to which, 100µl DNA rehydration solution was added and incubated at 65°C for 1 hour with mixing. Then, it was stored at 2–8°C.

C. Assessment of DNA Yield

The DNA yield was spectrophotometrically assessed using Cecil E1021 spectrophotometer (England), in which the sample was read at two optical densities that were 260 and 280 nm. The second reading was divided by the first reading, and if the outcome was 1.8 - 2.0, the sample was considered as free of contamination and having a sufficient amount of DNA for a further analysis. In some case Nano Drop was used for these assessments. The DNA concentration was calculated using the following formula as given by Sambrook *et al*. (1989):

DNA Concentration (µg /µl) = OD at 260 nm× Dilution Factor × µg/ml

3.5.2 Genotyping of Cytokines

The Cytokine CTS-PCR-SSP Tray Kit was used to detect cytokine gene polymorphisms. The kit was developed in the laboratories of Department of Transplantation Immunology, University Clinic Heidelberg (Heidelberg, Germany), and it is employed as the official reagent set for the Cytokine Component of the 13th International Histocompatibility Workshop. The kit enables the detection some of the single nucleotide polymorphisms (SNPs) described in the promoter regions of *IL1A*, *IL1B*, *IL1R1*, *IL1RN*, *IL2*, *IL4*, *IL6*, *IL10*, *IL12B*, *TNF* and *IFNG* genes, as well as some polymorphisms in the translated regions of *TGFB1*and *IL4R* genes. For all the polymorphisms mentioned above, there have been reports that they have a functional correlation and that they are associated with high or low production of the corresponding cytokine. The method of detection is a PCR-SSP (polymerase chain reaction-sequence specific priming) assay, which allowed the definition of the polymorphic variants of the genes that are present in the individual under test. Details of these polymorphisms are given in Figure 3-1.

Cytokine	Chromosome	db SNP	position	SNP
IL1 α	2q14	rs1800587	-889	T/C
IL1β	2q14	rs16944 rs1143634	-511 +3962	T/C T/C
γ IFN	12q14	rs2430561	+874	A/T
IL12	5p31-33.2	rs3212227	-1188	C/A
TNF α	6p21.3	rs1800629 rs361525	-308 -238	G/A A/G

Cytokine	Chromosome	db SNP	position	SNP
IL1 R	2q14.2	rs2234650	pstI 1970	C/T
IL1RA	2q14.2	rs315952	Mspa1 11100	T/C
IL 2	4q26-27	rs2069762 rs2069763	-330 +166	T/G G/T
IL 4Rα	16p11.2-12.1	rs1801275	+1902	G/A

Cytokine	Chromosome	db SNP	position	SNP
TGFβ1	19q13.1	rs1800470 rs1800471	+896 (cdn10) +915 (cdn 25)	C/T Leu/Pro C/G Arg/Pro
IL4	5q31.1	rs2243248 rs2243250 rs2070874	-1098 -590 -33	T/G C/T C/T
IL-6	7p14-21	rs1800795 rs1800797	-174 +565	G/C G/A
IL10	1q31-32	rs1800896 rs1800871 rs1800872	-1082 -819 -592	G/A C/T C/A
IL 13	5q31-33	rs7719175 rs1800925	-7402 -1055 (-1112)	T/G C/T

Figure 3-1: Human cytokine and cytokine gene receptors and their single nucleotide polymorphisms**.** The chromosomal localization, db SNP names, positions of functional polymorphisms in relation to gene start site and the nucleotide substitutions are given (Kaur and Mehra, 2012).

A. Kit contents

- **PCR Trays**: Each tray of the Cytokine CTS-PCR-SSP Tray Kit contains PCR primer mixes prepipetted and lyophilized in thin-walled, plastic, 96-well PCR trays for cytokine genotyping of two individuals (48 PCR primer mixes for each individual). Each kit included 10 trays for 20

typings in total. The PCR mixes were named numerically for each cytokine, and the mix position, allele specificities and PCR fragment size of each PCR-SSP primer mix of the Cytokine CTS-PCR-SSP Tray Kit are given in table 3-4.

- A separate master mix (CYT) was also supplied with the kit and contained the reaction buffer, but no Taq DNA polymerase was supplied. The latter item was purchased from Promega (USA) or Fermentas (Germany).

Table 3-4: Mix position, allele specificities and PCR fragment size of each PCR-SSP primer mix of the Cytokine CTS-PCR-SSP Tray Kit.

Position on tray (Figure 1)		Mix name	Allelic specificity (based on SNP nomenclature commonly used in literature)	Corresponding genotype/haplotype§	Size of the allele-specific amplicon (base pairs)	Size of the amplification control (bp)	Cytokine Official Full Name†	Cytokine Official Symbol†	dbSNP-ID†
H1	H7	IL-1α Mix No. 1	*T at pos -889*	**T**	220 bp	440 bp	*Interleukin 1, alpha*	*IL1A*	*rs1800587*
G1	G7	IL-1α Mix No. 2	*C at pos -889*	**C**	220 bp	440 bp			
F1	F7	IL-1β Mix No. 1	*C at pos -511*	**C**	215 bp	440 bp	*Interleukin 1, beta*	*IL1B*	*C/T at -511: rs16944*
E1	E7	IL-1β Mix No. 2	*T at pos -511*	**T**	215 bp	440 bp			
D1	D7	IL-1β Mix No. 3	*T at pos +3962*	**T**	340 bp	440 bp			*T/C at +3962: rs1143634*
C1	C7	IL-1β Mix No. 4	*C at pos +3962*	**C**	340 bp	440 bp			
B1	B7	IL-1R Mix No. 1	*C at pos pst1 1970*	**C**	290 bp	440 bp	*Interleukin 1 receptor type 1*	*IL1R1*	*rs2234650*
A1	A7	IL-1R Mix No. 2	*T at pos pst1 1970*	**T**	290 bp	440 bp			
H2	H8	IL-1RA Mix No.1	*T at pos mspa1 11100*	**T**	300 bp	440 bp	*Interleukin 1 receptor ant agonist*	*IL1RN*	*rs315952*
G2	G8	IL-1RA Mix No. 2	*C at pos mspa1 11100*	**C**	300 bp	440 bp			
F2	F8	IL-4Rα Mix No. 1	*G at pos +1902*	**G**	140 bp	440 bp	*Interleukin 4 receptor*	*IL4R*	*rs1801275*
E2	E8	IL-4Rα Mix No. 2	*A at pos +1902*	**A**	140 bp	440 bp			
D2	D8	IL-12 Mix No. 1	*C at pos -1188*	**C**	800 bp	440 bp	*Interleukin 12B*	*IL12B*	*rs3212227*
C2	C8	IL-12 Mix No. 2	*A at pos -1188*	**A**	800 bp	440 bp			
B2	B8	γ-IFN Mix No. 1	*A at pos +874*	**A**	180 bp	440 bp	*Interferon, gamma*	*IFNG*	*rs2430561*
A2	A8	γ-IFN Mix No. 2	*T at pos +874*	**T**	180 bp	440 bp			
H3	H9	TGF-β Mix No. 1	*C at Codon 10 ; G at Codon 25*	**CG**	80 bp	440 bp	*Transforming growth factor, beta 1*	*TGFB1*	*SNP in codon 10: rs1800470* *SNP in codon 25: rs1800471*
G3	G9	TGF-β Mix No. 2	*C at Codon 10 ; C at Codon 25*	**CC**	80 bp	440 bp			
F3	F9	TGF-β Mix No. 3	*T at Codon 10 ; G at Codon 25*	**TG**	80 bp	440 bp			
E3	E9	TGF-β Mix No. 4	*T at Codon 10 ; C at Codon 25*	**TC**	80 bp	440 bp			
D3	D9	TGF-β Mix No. 5	*C at Codon 10*	**CG** or **CC**	195 bp	440 bp			*SNP in codon 10: rs1800470*
C3	C9	TGF-β Mix No. 6	*T at Codon 10*	**TG** or TC	195 bp	440 bp			

B3	**B9**	TNF-α Mix No. 1	*G at pos –308 ; G at pos -238*	**GG**	110 bp	440 bp	*Tumor necrosis factor (TNF superfamily, member 2)*	*TNF*	*G/A at -308: rs1800629* *G/A at -238: rs361525*
A3	**A9**	TNF-α Mix No. 2	*A at pos –308 ; G at pos -238*	**AG**	110 bp	440 bp			
H4	**H10**	TNF-α Mix No. 3	*G at pos –308 ; A at pos -238*	**GA**	110 bp	440 bp			
G4	**G10**	TNF-α Mix No. 4	*A at pos –308 ; A at pos -238*	**AA**	110 bp	440 bp			
F4	**F10**	IL-2 Mix No. 1	*T at pos –330 ; G at pos +166*	**TG**	560 bp	90 bp	*Interleukin 2*	*IL2*	*T/G at -330: rs2069762* *G/T at +166: rs2069763*
E4	**E10**	IL-2 Mix No. 2	*G at pos –330 ; G at pos +166*	**GG**	560 bp	90 bp			
D4	**D10**	IL-2 Mix No. 3	*G at pos –330 ; T at pos +166*	**GT**	570 bp	90 bp			
C4	**C10**	IL-2 Mix No. 4	*T at pos –330 ; T at pos +166*	**TT**	570 bp	90 bp			

B. Assay procedure

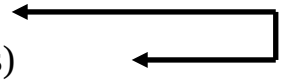
The first step in the assay included a preparation of PCR Reaction Mix, which consisted of 138 µl master mix (CYT), 3 µl Taq DNA polymerase (5 Unit/µl), 329 µl deionized distilled water and 50 µl DNA sample (0.10-0.15 µg/µl). The mix was vortexed thoroughly and kept on ice. This was followed by taking the PCR tray and placing it inside a sample holder, and the adhesive seal was carefully removed from the Tray. To each well of the first 48 wells, 10 µl of the PCR Reaction Mix was added in the sequence given in figure 3-2.

	1	2	3	4	5	6	7	8	9	10	11	12
A	**Lane 1** (Pos.1-8)	**Lane 1** (Pos.9-16)	**Lane 1** (Pos.17-24)	**Lane 2** (Pos.25-32)	**Lane 2** (Pos.33-40)	**Lane 2** (Pos.41-48)	**Lane 3** (Pos.49-56)	**Lane 3** (Pos.57-64)	**Lane 3** (Pos.65-72)	**Lane 4** (Pos.73-80)	**Lane 4** (Pos.81-88)	**Lane 4** (Pos.89-96)
B	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲
C												
D												
E												
F												
G												
H												

Figure 3-2: Cytokine genotyping PCR tray showing the sequence of PCR Reaction Mix adding. The word "Lane" refers to a well's corresponding gel lane.

The tray was placed in the thermal cycler and the thermal cycling profile was optimized and validated for the use with the CTS-PCR-SSP TRAY KITS, and as the following:

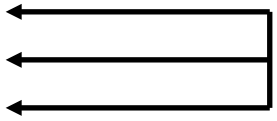
- Initial denaturation: 94°C (2 minutes)
- Denaturation: 94°C (15 seconds)
- Annealing+Extension: 65°C (1 minutes)



10 cycles

Followed by:

- Denaturation: 94°C (15 seconds)
- Annealing: 61°C 50 (seconds)
- Extension: 72°C (30 seconds)
- Hold: 4°C (15 minutes)



20 cycles

C. Agarose gel electrophoresis

After thermocycling, absence or presence of PCR products was visualized by submarine agarose gel electrophoresis.

1. **Preparation of agarose gel**: The agarose gel was prepared at a concentration of 2% agarose, by mixing 7 grams of agarose, 7 ml of 50x TAE (Tris-acetate-EDTA) buffer and 350 ml of deionized distilled water in a 500ml conical flask. The mixture was boiled to dissolve the agarose, using a magnetic stirring hotplate or a microwave oven. After that, it was cooled to 60°C, and by then 17 µl of ethidium bromide (10 mg/ml) was added and well-mixed. The gel was poured on an electrophoresis plate (25 x 25 cm) fixed on an even surface, and then four combs (each with 24 teeth) were placed and the gel was allowed to set for one hour at room temperature.

2. **Running Electrophoresis**: When the thermocycling was finished, the PCR tray was handled outside the thermocyler carefully and the strip caps that covered the wells were removed from the tray. This was

followed by removing the combs from the gel, which was covered with the running TAE buffer (approximately 2 - 3 mm above the gel surface). Then, 10 µl of each PCR product was loaded onto the respective gel wells in a sequence that was given in figure 3-2. The electrophoresis was run for 25 minutes at 170 volts (0.4 volt / cm^2).

3. **Interpretation of electrophoresis patterns**: After the end of electrophoresis, the power was turned off, electrodes were disconnected and the gel was removed and place on UV light transilluminator (312 nm) to visualize the bands. Finally a picture was taken, and the genotype of each cytokine was recorded according the chart provided by the supplier of the genotyping kit (Figure 3-3).

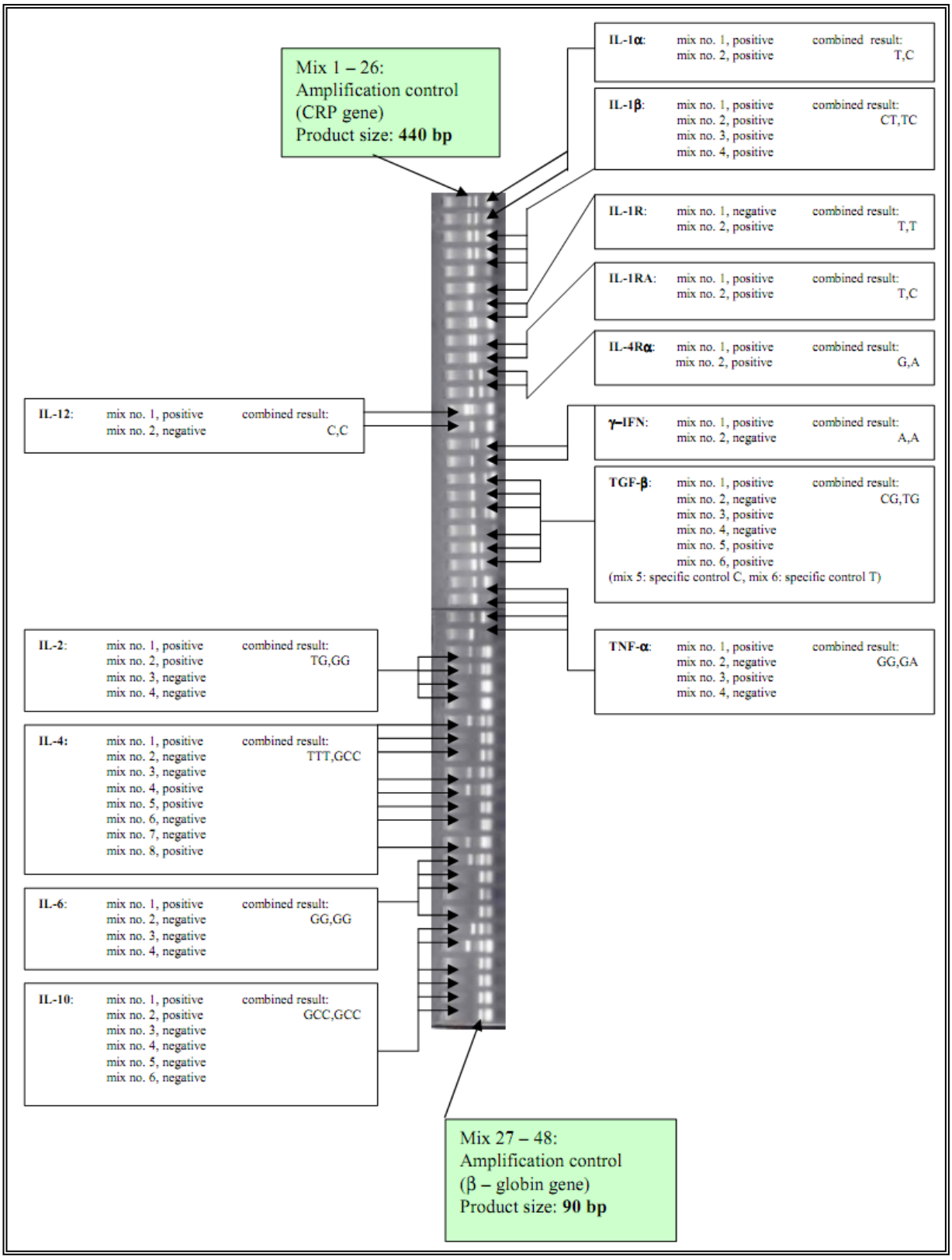


Figure 3-3: Visualized pattern bands of cytokine SNPs after agarose gel electrophoresis**.** Two internal positive controls were included: a fragment of either the human β-globin gene (90 bp) for *IL2*, *IL4*, *IL6*, and *IL10* genes, or the human C-reactive protein gene (440 bp) for *IL1A*, *IL1B*, *IL1R1*, *IL1RN*, *IL4R*, *IL12B*, *IFNG*, *TGFB1* and *TNF*.

3.6 Statistical Analysis

Alleles and genotypes of cytokines were presented as percentage frequencies, and significant differences between their distributions in IBD patients and controls were assessed by two-tailed Fisher's exact probability (P). In addition, relative risk (RR), etiological fraction (EF) and preventive fraction (PF) were also estimated to define the association between an allele or a genotype with the disease. The RR value can range from less than one (negative association) to more than one (positive association). If the association was positive, the EF was calculated, while if it was negative, the PF was given. (Ad'hiah,1990). These estimations were calculated by using the WINPEPI computer programs for epidemiologists. The latest version of the WINPEPI package (including the programs and their manuals) is available free online at http://www.brixtonhealth.com.

Allele frequencies of cytokine genes were calculated by direct gene counting methods, while a significant departure from Hardy-Weinberg equilibrium (HWE) was estimated using H-W calculator for two alleles, which is available free online at http://www.had2know.com/ academics/ hardy-weinberg-equilibrium-calculator-3-alleles.html. Hardy-Weinberg equilibrium is the expected frequencies of genotypes if mating is non-assortative and there are no mutations from one allele to another. When there are two alleles for a particular gene; A and B, and their respective population frequencies are p and q, then the expected frequencies of the genotypes AA, AB and BB are p^2, 2pq and q^2, respectively. Significant differences between the observed and expected frequencies are assessed by Pearson's Chi-square test (Ad'hiah, 1990).

Chapter Four
Results and Discussion

Chapter Four
Results and Discussion

4.1 Presentation of Subjects

The investigated subjects were distributed according to age, hemoglobin (Hb), total leukocyte count (TLC) and erythrocyte sedimentation rate (ESR).

4.1.1 Age and Gender

Females and males had approximated age means in CD patients (40.0 ± 4.6 and 40.3 ± 2.1 years, respectively) and controls (39.7 ± 3.5 and 37.3 ± 5.9 years, respectively), while female UC patients showed a significant ($P \leq 0.01$) increased age mean compared to male UC patients (47.0 ± 3.2 *vs*. 32.0 ± 3.6 years) (Figure 4-1).

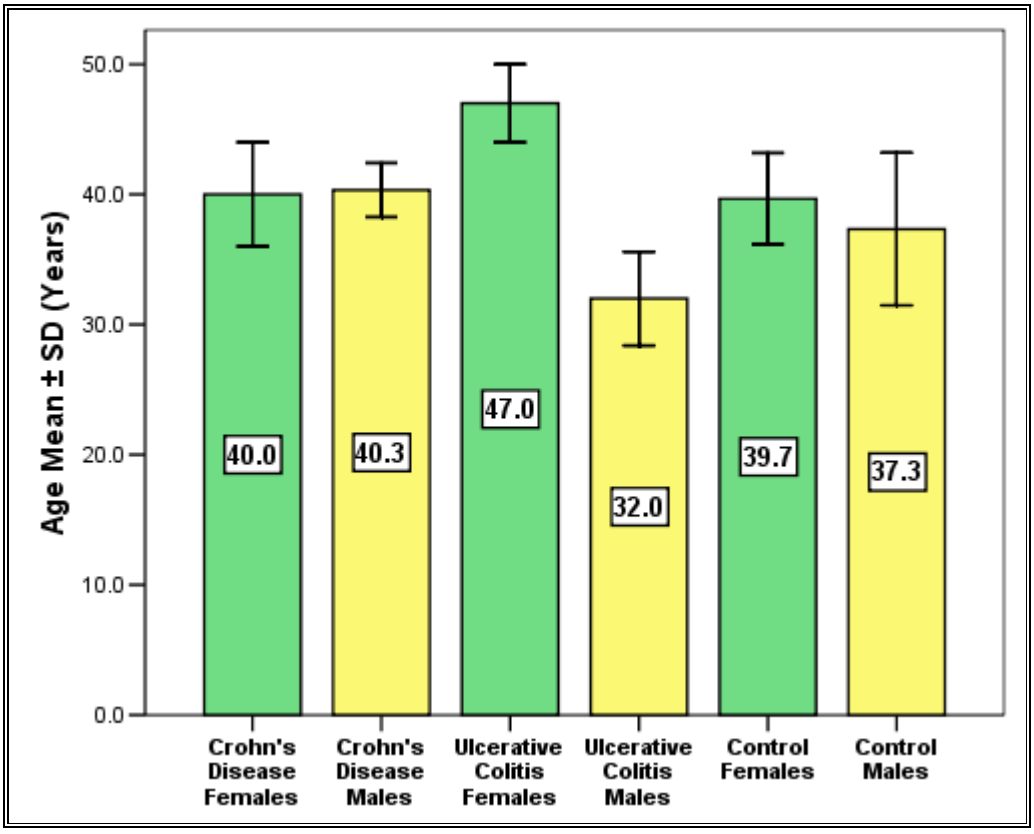


Figure 4-1: Crohn's disease and ulcerative colitis patients and controls distributed by gender and age.

4.1.2 Hemoglobin (Hb)

As a general observation, the Hb mean was lower in females than in males, but a significant difference was reached in CD patients (7.3 ± 2.1 *vs*. 12.0 ± 2.2 mg/dL) and controls (11.3 ± 1.2 *vs*. 14.7 ± 1.5 mg/dL) (Figure 4-2). However, the Hb mean was within the normal range, with the exception of female CD patients, which might be considered as anemic.

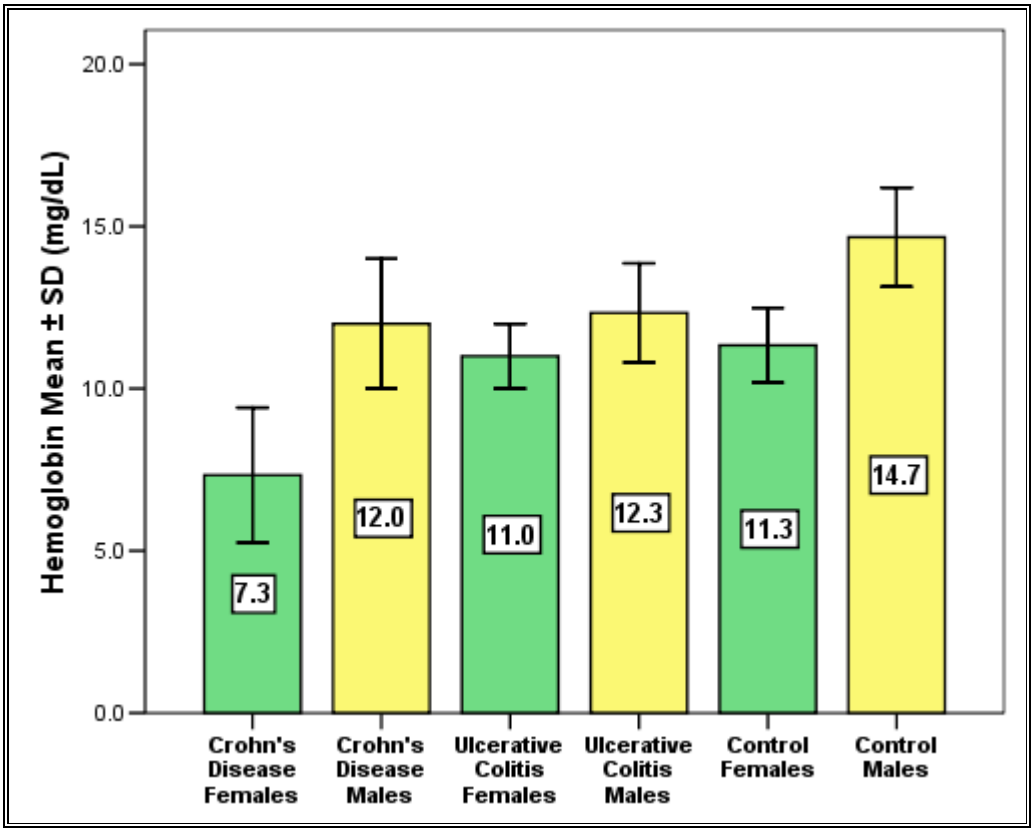


Figure 4-2: Hemoglobin mean in female and male Crohn's disease and ulcerative colitis patients and controls.

4.1.3 Total Leukocyte Count (TLC)

Females showed an increased mean of TLC compared to males in CD (11.7 ± 2.5 *vs*. 11.3 ± 1.5 × 10^3 cells/cu.mm.blood) and UC (12.7 ± 2.4 *vs*. 11.7 ± 2.6 × 10^3 cells/cu.mm.blood) patients and controls (9.0 ± 1.9 *vs*. 8.0 ± 2.1 × 10^3 cells/cu.mm.blood), but no significant gender-related variation was observed between females and males of patients and controls (Figure 4-3).

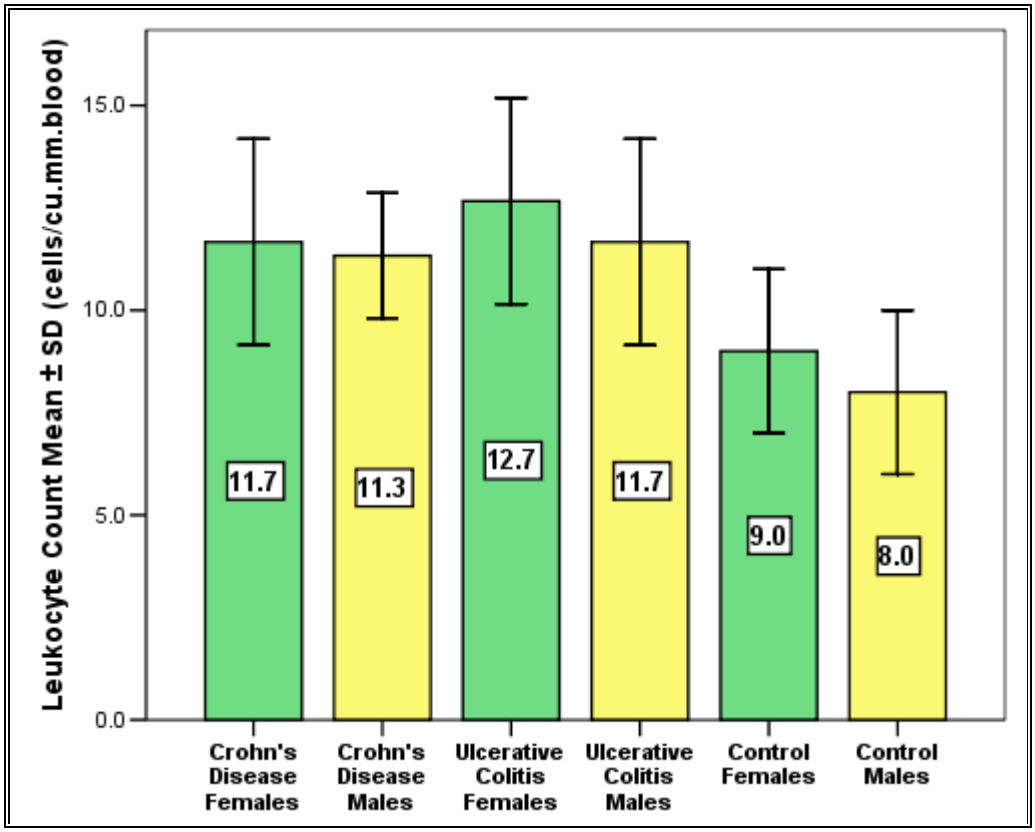


Figure 4-3: Total leukocyte count mean in female and male Crohn's disease and ulcerative colitis patients and controls.

4.1.4 Erythrocyte Sedimentation Rate (ESR)

The ESR showed significant variations between patients (CD and UC) and controls, as well as between female and male patients. Among CD patients, females recorded a significant increased ESR mean compared to male patients (67.0 ± 6.2 *vs*. 50.7 ± 5.1 mm.). A similar observation was also made in UC patients (65.3 ± 4.9 *vs*. 55.7 ± 5.1 mm.). However, these means were also significantly higher than the corresponding means of female and male controls (8.7 ± 3.2 and 9.0 ± 1.5 mm., respectively) (Figure 4-4). These results may highlight that ESR is important laboratory marker for IBD of both type (CD and UC).

It is the rate at which red blood corpuscles migrate through the plasma, and it depends on the concentration of plasma and on the number and size of these corpuscles. Several factors contribute to its increase in patients; for instance, clinical conditions like anemia and

thalassemia, as well as infectious and inflammatory conditions (Thomas *et al*., 1993). However, compared to C-reactive protein, ESR peaks much less rapidly and may also takes several days to decrease, even if the clinical condition of the patient or the inflammation is ameliorated. Therefore, ESR is an important indirect measurement of plasma acute phase protein concentration (Vermeire *et al*., 2006).

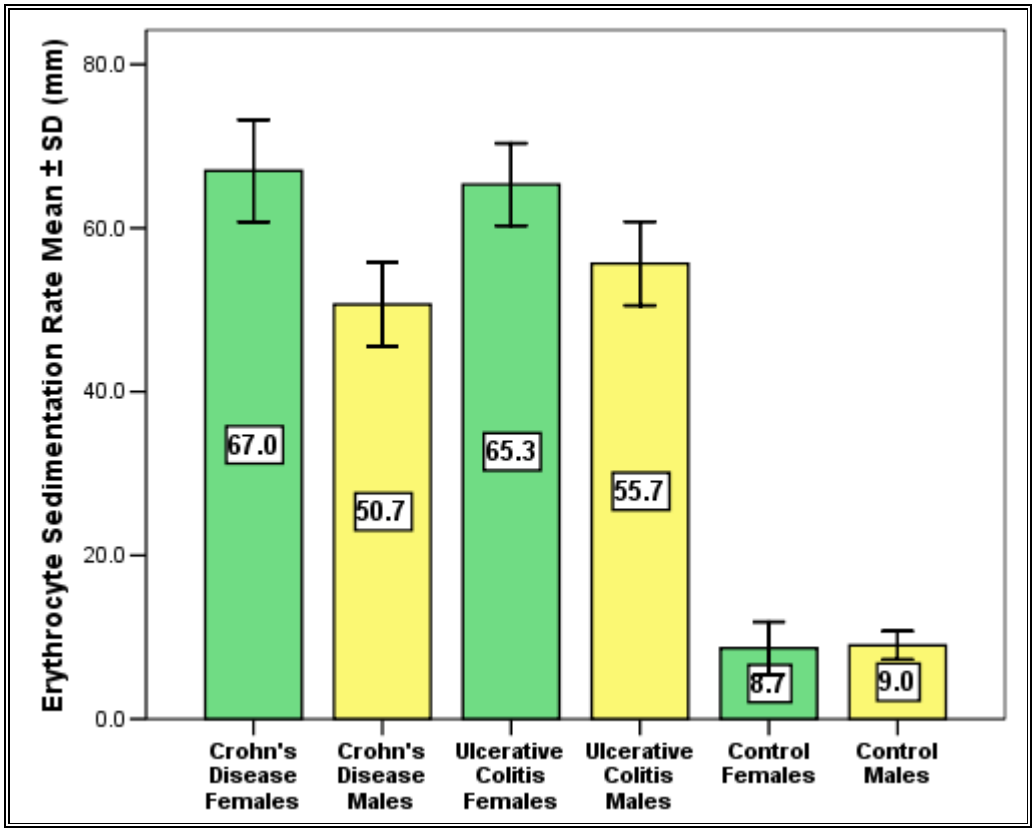


Figure 4-4: Erythrocyte sedimentation rate mean in female and male Crohn's disease and ulcerative colitis patients and controls.

4.2 Cytokine Gene Polymorphisms

Genetic polymorphisms of 13 cytokine and cytokine receptor genes (*IL1A*, *IL1B*, *IL1R1*, *IL1RN*, *IL2*, *IL4*, *IL4R*, *IL6*, *IL10*, *IL12B*, *IFNG*, *TNF* and *TGFB1*) were determined in 34 Crohn's disease (CD) and 66 ulcerative colitis (UC) patients, as well as 43 controls. The method of determination was PCR-SSP (polymerase chain reaction-specific sequence primer) assay, followed by electrophoresis on 2% agarose gel (Patterns of band migration is given Figure 4-5).

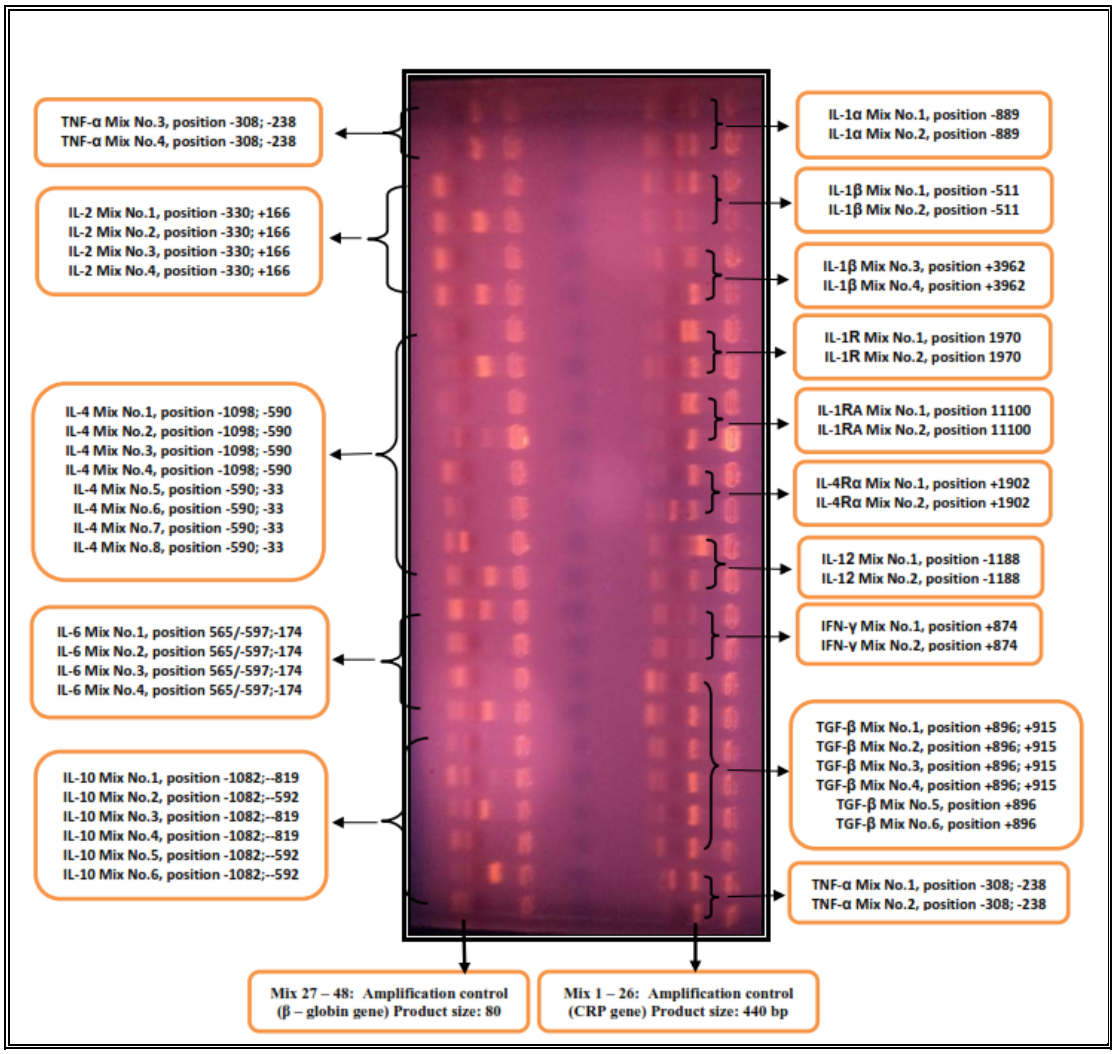


Figure 4-5: Agarose gel electrophoresis patterns of cytokine amplified gene regions (*IL1A*, *IL1B*, *IL1R1*, *IL1RN*, *IL2*, *IL4*, *IL4R*, *IL6*, *IL10*, *IL12B*, *IFNG*, *TNF* and *TGFB1* genes and internal controls are pointed with arrows) visualized by UV trans-illumination.

4.2.1 Interleukin-1 Alpha Gene (*IL1A*)

Genetic polymorphism of *IL1A* gene was determined in the promoter region at position -889 ($IL1A_{-889}$), which was presented with three genotypes (TT, TC and CC) that corresponded to two alleles (*T* and *C*). Among CD and UC patients, no significant difference was observed between the observed and expected frequencies of the three genotypes (a good agreement with Hardy-Weinberg equilibrium; HWE), while in controls, a departure from HWE was observed (i.e. a significant difference between the observed and expected genotype frequencies);

however comparing both groups of patients to controls revealed some significant differences (Table 4-1A).

Table 4-1A: Observed numbers and percentage frequencies and Hardy-Weinberg equilibrium (HWE) of *IL1A*$_{-889}$ genotypes and alleles in inflammatory bowel disease (Crohn's disease and ulcerative colitis) patients and controls.

Groups			*IL1A*$_{-889}$ Genotype or Allele					HWE P ≤
			CC	CT	TT	*C*	*T*	
Crohn's Disease (No. = 34)	Observed	No.	20	10	4	50	18	Not significant
		%	58.8	29.4	11.8	73.5	26.5	
	Expected	No.	18.4	13.2	2.4	Not Estimated		
		%	54.1	38.2	7.0			
Ulcerative Colitis (No. = 66)	Observed	No.	36	22	8	94	38	Not significant
		%	54.6	33.3	12.1	71.2	28.8	
	Expected	No.	33.5	27.1	5.5	Not Estimated		
		%	50.7	41.0	8.3			
Controls (No. = 43)	Observed	No.	11	13	19	35	51	**0.01**
		%	25.5	30.2	44.2	40.7	59.3	
	Expected	No.	7.1	20.8	15.1	Not Estimated		
		%	16.6	48.3	35.2			

The frequencies of CC genotype and *C* allele were significantly increased in CD (58.8 and 73.5%, respectively) and UC (54.6 and 71.2%, respectively) patients compared to controls (25.5 and 40.7%, respectively). The relative risks (RRs) of such positive associations were 4.16 and 4.55, respectively in CD, and 3.49 and 3.60, respectively in UC. In contrast, TT genotype and *T* allele frequencies were significantly decreased in CD (11.8 and 26.5%, respectively) and UC (12.1 and 28.8%, respectively) patients compared to controls (44.2 and 59.3%, respectively). The preventive fractions (PFs) of such negative associations were 0.37 and 0.45, respectively in CD, and 0.37 and 0.43, respectively in UC. However, comparing CD to UC patients revealed no significant variations between *IL1A*$_{-889}$ genotype or allele frequencies (Table 4-1B).

Table 4-1B: Statistical analysis of associations between *IL1A*$_{-889}$ genotypes or alleles and inflammatory bowel disease (Crohn's disease and ulcerative colitis).

Type of Comparison	*IL1A*$_{-889}$ Genotype or Allele	Statistical Evaluation			
		Relative Risk	Etiological or Preventive Fraction	Fisher's Exact Probability	95% Confidence Intervals
Crohn's Disease *Versus* Controls	CC	4.16	0.45	**0.005**	0.60-10.79
	CT	0.96	0.01	1.000	0.36-2.54
	TT	0.17	0.37	**0.002**	0.05-0.55
	C	4.55	0.59	**$1.7*10^{-5}$**	2.25-9.20
	T	0.25	0.45	**$1.7*10^{-5}$**	0.12-0.49
Ulcerative Colitis *Versus* Controls	CC	3.49	0.39	**0.003**	1.52-8.01
	CT	1.15	0.04	0.835	0.51-2.62
	TT	0.17	0.37	**$2.3*10^{-4}$**	0.07-0.45
	C	3.60	0.52	**$1.0*10^{-5}$**	2.04-6.37
	T	0.28	0.43	**$1.0*10^{-5}$**	0.16-0.49
Crohn's Disease *Versus* Ulcerative Colitis	CC	0.84	0.09	0.832	0.37-1.92
	CT	1.20	0.06	0.822	0.49-2.91
	TT	1.03	0.004	1.000	0.29-3.66
	C	0.89	0.08	0.868	0.46-1.71
	T	1.12	0.03	0.868	0.58-2.16

These results may point out for the role of *IL1A*$_{-889}$ genotypes and alleles in conferring predisposition (increased frequencies of CC genotype and *C* allele in patients) to develop inflammatory bowel disease (IBD) of both types (CD and UC) in the sample Iraqi population, and in addition, a protection role (decreased frequencies of TT genotype and *T* allele in patients) against IBD can also be highlighted. Reviewing the literature revealed that there was one published study (López-Hernández *et al*., 2015), in which *IL1A*$_{-889}$ gene polymorphism was investigated in Spanish IBD patients (57 CD and 27 UC) and no significant difference was reported between patients and controls in the distribution of genotype and allele frequencies, but they were comparable to the corresponding frequencies in present study CD (for instance, *C* allele frequency: 73.5 *vs*. 78.1%) and UC (for instance, *C* allele frequency: 71.2 *vs*. 72.2%) patients. Whereas, a big difference between the two controls was observed; the

frequency of *C* allele in the present controls was much lower than that of López-Hernández's study (40.7 *vs*. 75.2%). A variation in the distribution of polymorphic systems is well-documented across populations of different ethnicities including Iraqi Arabs, and in one study, the well-known polymorphic system; HLA, showed significant variations between Iraqi Arabs and other world populations (Ad'hiah, 2009). However, the present study control might be associated with some defect due to its small sample size (43 subjects), which might contributed to its significant departure from HWE. The deviation from HWE has also been identified as a principal source of divergent results that are observed in different ethnicities, because the factors that influence the equilibrium are ethnicity-based (Koushik *et al*., 2004).

4.2.2 Interleukin-1 Beta Gene (*IL1B*)

Genetic polymorphism of *IL1B* gene was determined in the promoter region at position -511 ($IL1B_{-511}$), which was presented with three genotypes (TT, TC and CC) that corresponded to two alleles (*T* and *C*). Genotype frequencies CD patients and controls were in a good agreement with HWE, while in UC patients, a significant departure ($P \leq 0.01$) was observed (Table 4-2A). The latter patients also showed significant variations compared to controls. The TC genotype frequency was significantly increased in UC patients compared to controls (63.6 *vs*. 39.5%; P = 0.018), and the associated RR was 2.68. In contrast, frequency of CC genotype was significantly decreased (6.1 *vs*. 32.2%; P = 0.061), and such negative association scored PF value of 0.18. When the comparison was made between CD patients and controls or UC patients, no significant differences were observed (Table 4-2B).

Table 4-2A: Observed numbers and percentage frequencies and Hardy-Weinberg equilibrium (HWE) of *IL1B*$_{-511}$ genotypes and alleles in inflammatory bowel disease (Crohn's disease and ulcerative colitis) patients and controls.

Groups			*IL1B*$_{-511}$ Genotype or Allele					HWE P ≤
			TT	TC	CC	*T*	*C*	
Crohn's Disease (No. = 34)	**Observed**	**No.**	12	18	4	42	26	N.S.
		%	35.3	52.9	11.8	61.8	38.2	
	Expected	**No.**	12.9	16.1	4.9	Not Estimated		
		%	38.2	4.2	14.6			
Ulcerative Colitis (No. = 66)	**Observed**	**No.**	20	42	4	82	50	**0.01**
		%	30.0	63.6	6.1	62.1	37.9	
	Expected	**No.**	25.5	31.1	9.5	Not Estimated		
		%	38.6	47.1	14.4			
Controls (No. = 43)	**Observed**	**No.**	16	17	10	49	37	N.S.
		%	37.2	39.5	32.2	56.9	43.0	
	Expected	**No.**	13.9	21.1	7.9	Not Estimated		
		%	32.5	49.0	18.5			

Table 4-2B: Statistical analysis of associations between *IL1B*$_{-511}$ genotypes or alleles and inflammatory bowel disease (Crohn's disease and ulcerative colitis).

Type of Comparison	*IL1B*$_{-511}$ Genotype or Allele	Statistical Evaluation			
		Relative Risk	Etiological or Preventive Fraction	Fisher's Exact Probability	95% Confidence Intervals
Crohn's Disease *Versus* Controls	**TT**	0.92	0.03	1.000	0.37-2.32
	TC	1.72	0.22	0.259	0.70-4.22
	CC	0.44	0.13	0.243	0.13-1.53
	T	1.22	0.11	0.621	0.64-2.32
	C	0.82	0.08	0.621	0.43-1.56
Ulcerative Colitis *Versus* Controls	**TT**	0.73	0.09	0.533	0.33-1.64
	TC	2.68	0.39	**0.018**	1.22-5.85
	CC	0.21	0.18	**0.016**	0.06-0.72
	T	1.24	0.12	0.481	0.71-2.15
	C	0.81	0.08	0.481	0.47-1.40
Crohn's Disease *Versus* Ulcerative Colitis	**TT**	0.80	0.07	0.655	0.33-1.90
	TC	1.56	0.23	0.389	0.68-3.57
	CC	0.48	0.06	0.439	0.12-2.03
	T	1.02	0.01	1.000	0.56-1.85
	C	0.98	0.01	1.000	0.54-179

Based on these findings, it is possible to highlight that *IL1B*$_{-511}$ TC is a susceptibility genotype in UC patients, and etiologically may contribute to 39% of the disease (EF = 0.39). In contrast, CC may be considered a protective genotype against the development of UC;

although PF did not exceeded 0.20. Whereas, CD failed to show such association, and possibly ILB_{-511} polymorphism is not related to the disease in Iraqi patients. Other investigated populations have shown contradictory results regarding ILB_{-511} polymorphism in IBD of both types (CD and UC). Two earlier studies were carried out in north-east English and Turkish patients, and no significant differences in the distributions of *IL1B* genotypes were observed between IBD patients and controls (Craggs *et al*., 2001; Celik *et al*., 2006). A further study from China reported that ILB_{-511} *T* allele was significantly increased in UC patients, but the author suggested that combination polymorphisms between MCP-1 (monocyte chemoattractant protein-1) and ILB_{-511} can increase UC risk significantly (Li *et al*., 2009). A mutation in ILB_{-511} has also be associated with complex disease behavior in CD of Italian patients (Corleto *et al*., 2009). A contradictory result has also been found in Mexican UC patients, in whom the frequency of ILB_{-511} TC genotype was significantly decreased compared to healthy controls (Yamamoto-Furusho *et al*., 2011), while in the present study such genotype was significantly increased in UC patients. These inconsistencies could be related to race differences, and inter-population discrepancies in cytokine polymorphisms are often observed, and differences in allelic frequencies among ethnic groups have been reported (Trajkov *et al*., 2009).

4.2.3 Interleukin 1 Receptor Type 1 Gene (*IL1R1*)

Genotype frequencies of $IL1R1_{\text{pstl } 1970}$ (TT, TC and CC) were in agreement with HWE in both groups of patients (CD and UC), while among controls, there was a significant difference ($P \leq 0.01$) between the observed and expected genotype frequencies (i.e. a departure from HWE) (Table 4-3A).

Table 4-3A: Observed numbers and percentage frequencies and Hardy-Weinberg equilibrium (HWE) of *IL1R1*$_{\text{pstl 1970}}$ genotypes and alleles in inflammatory bowel disease (Crohn's disease and ulcerative colitis) patients and controls.

Groups			*IL1R1*$_{\text{pstl 1970}}$ Genotype or Allele					H-W P ≤
			TT	TC	CC	*T*	*C*	
Crohn's Disease (No. = 34)	Observed	No.	16	14	4	46	22	N.S.
		%	47.1	41.2	11.8	67.7	32.4	
	Expected	No.	15.6	14.9	3.6	Not Estimated		
		%	45.8	43.8	10.5			
Ulcerative Colitis (No. = 66)	Observed	No.	26	32	8	84	48	N.S.
		%	39.4	48.5	12.1	63.6	36.4	
	Expected	No.	26.7	30.6	8.7	Not Estimated		
		%	40.5	46.3	13.2			
Controls (No. = 43)	Observed	No.	9	12	22	30	56	**0.01**
		%	20.9	27.9	51.2	34.9	65.1	
	Expected	No.	5.2	19.5	18.2	Not Estimated		
		%	12.2	45.4	42.4			

However, these genotypes showed significant variations between patients and controls. Frequencies of TT genotype and *T* allele (47.1 and 67.7%, respectively) were significantly increased (P = 0.026 and $8.6*10^{-5}$, respectively) in CD patients compared to controls (20.9 and 34.97%, respectively). The estimated RR values of such positive associations were 3.36 and 3.90, respectively. In contrast, CC genotype (11.8 *vs*. 51.2%) and allele *C* (32.4 *vs*. 65.1%) frequencies were significantly decreased (P = $2.8*10^{-4}$ and $8.6*10^{-5}$, respectively) in CD patients compared to controls. The PF values of such negative associations were 0.45 and 0.48, respectively. In the case of UC, the patients demonstrated significant increased frequencies of TC genotype (48.5 *vs*. 27.9%; P = 0.045) and T allele (63.6 *vs*. 34.9%; P = $5.1*10^{-5}$) compared to controls. The RR values of such differences were 2.43 and 3.27, respectively. As in CD, UC patients also demonstrated significant decreased frequencies of CC genotype (12.1 *vs*. 51.2%; P = $1.7*10^{-5}$) and *C* allele (36.4 *vs*. 65.1%; P = $8.6*10^{-5}$) compared to controls, and both negative associations scored PF of 0.44. However, comparing CD to UC patients

revealed no significant variations in the distribution of *IL1R1*$_{\text{pstl 1970}}$ genotypes or alleles (Table 4-3B).

Table 4-3B: Statistical analysis of associations between *IL1R1*$_{\text{pstl 1970}}$ genotypes or alleles and inflammatory bowel disease (Crohn's disease and ulcerative colitis).

Type of Comparison	*IL1R1*$_{\text{pstl 1970}}$ Genotype or Allele	Statistical Evaluation			
		Relative Risk	Etiological or Preventive Fraction	Fisher's Exact Probability	95% Confidence Intervals
Crohn's Disease *Versus* Controls	TT	3.36	0.33	**0.026**	1.26 - 8.68
	TC	1.81	0.18	0.237	0.71 - 4.64
	CC	0.13	0.45	**$2.8*10^{-4}$**	0.04 - 0.42
	T	3.90	0.50	**$8.6*10^{-5}$**	2.00 - 7.63
	C	0.26	0.48	**$8.6*10^{-5}$**	0.13 - 0.50
Ulcerative Colitis *Versus* Controls	TT	2.46	0.23	0.059	1.02 - 5.90
	TC	2.43	0.29	**0.045**	1.08 - 5.49
	CC	0.13	0.44	**$1.7*10^{-5}$**	0.05 - 0.34
	T	3.27	0.44	**$5.1*10^{-5}$**	1.86 - 5.75
	C	0.31	0.45	**$5.1*10^{-5}$**	0.17 - 0.54
Crohn's Disease *Versus* Ulcerative Colitis	TT	1.37	0.126	0.524	0.60 - 3.12
	TC	0.74	0.124	0.531	0.33 - 1.70
	CC	0.97	0.004	1.000	0.27 - 3.42
	T	1.19	0.11	0.640	0.65 - 2.21
	C	0.84	0.059	0.640	0.45 - 1.55

The protein encoded by *IL1R1* gene is a cytokine receptor belongs to IL-1 receptor family, and it is an important mediator involved in many cytokine induced immune and inflammatory responses, and recently, it has been presented that a dysregulation in its expression may lead to the activation of aberrant immune cells that may contribute to auto-inflammatory responses (Yang, 2015). The obtained results favor such presentation in the ground of its genetic effects on both types of IBD, and the genotypes or alleles of *IL1R1*$_{\text{pstl 1970}}$ polymorphism showed both types of associations (positive and negative) with CD and UC in the samples of Iraqi patients. Therefore, the functional role of such receptor might have been altered due to the deviations of some genotype and allele

frequencies. Unfortunately, there has been no further investigation that can confirm these findings in IBD patients, but such polymorphism has been investigated in systemic lupus erythematosus (SLE) of Iranian patients, and authors concluded that particular *IL1* gene variants could affect individual susceptibility to develop the disease (Ziaee *et al*., 2014).To validate the present results, further investigations are certainly required.

4.2.4 Interleukin 1 Receptor Antagonist Gene (*IL1RN*)

Neither CD patients nor controls showed a deviation from HWE, while among UC patients, a significant difference ($P \leq 0.05$) between the observed and expected genotype frequencies of $IL1RN_{\text{maspl 11100}}$ gene polymorphism (TT, TC and CC) was observed (i.e. a significant deviation from HWE). In addition, UC patients showed some variations in the distribution of $IL1RN_{\text{maspl 11100}}$ genotypes and alleles compared to controls or CD patients, but the differences felt short of significant (i.e. the P was slightly higher than 0.05), while no such differences were observed between CD patients and controls (Tables 4-4A and 4-4B).

Table 4-4A: Observed numbers and percentage frequencies and Hardy-Weinberg equilibrium (HWE) of $IL1RN_{\text{maspl 11100}}$ genotypes and alleles in inflammatory bowel disease (Crohn's disease and ulcerative colitis) patients and controls.

Groups			***IL1RN*maspl 11100 Genotype or Allele**					**H-W P ≤**
			TT	**TC**	**CC**	***T***	***C***	
Crohn's Disease (No. = 34)	**Observed**	**No.**	12	16	6	40	28	N.S.
		%	35.3	47.1	17.7	58.8	41.2	
	Expected	**No.**	11.8	16.5	5.8	Not Estimated		
		%	34.6	48.4	16.9			
Ulcerative Colitis (No. = 66)	**Observed**	**No.**	38	20	8	96	36	**0.05**
		%	57.6	30.3	12.1	72.7	27.7	
	Expected	**No.**	34.9	26.2	4.9	Not Estimated		
		%	52.9	39.7	7.4			
Controls (No. = 43)	**Observed**	**No.**	18	16	9	52	34	N.S.
		%	41.9	37.2	20.9	60.5	39.5	
	Expected	**No.**	15.7	20.6	6.7	Not Estimated		
		%	36.6	47.8	15.6			

Table 4-4B: Statistical analysis of associations between *IL1RN*$_{\text{maspl 11100}}$ genotypes or alleles and inflammatory bowel disease (Crohn's disease and ulcerative colitis).

Type of Comparison	*IL1RN*$_{\text{maspl 11100}}$ Genotype or Allele	Statistical Evaluation			
		Relative Risk	Etiological or Preventive Fraction	Fisher's Exact Probability	95% Confidence Intervals
Crohn's Disease *Versus* Controls	TT	0.76	0.10	0.641	0.30 - 1.89
	TC	1.50	0.16	0.486	0.61 - 3.70
	CC	0.81	0.04	0.779	0.26 - 2.51
	T	0.93	0.04	0.870	0.49 - 1.78
	C	1.07	0.03	0.870	0.56 - 2.04
Ulcerative Colitis *Versus* Controls	TT	1.88	0.27	0.121	0.87 - 4.07
	TC	0.73	0.09	0.533	0.33 - 1.64
	CC	0.52	0.10	0.281	0.19 - 1.46
	T	1.74	0.31	**0.075**	0.98 - 3.10
	C	0.57	0.17	**0.075**	0.32 - 1.02
Crohn's Disease *Versus* Ulcerative Colitis	TT	0.40	0.34	**0.057**	0.17 - 0.94
	TC	1.02	0.01	1.000	0.42 - 2.49
	CC	1.55	0.06	0.545	0.50 - 4.84
	T	0.54	0.34	**0.055**	0.29 - 0.99
	C	1.87	0.19	**0.055**	1.01 - 3.44

These results were consistent with the findings of other studies, which showed no significant differences between IBD (CD and UC) patients and controls in the distribution of *IL1RN* genotypes or alleles at different positions (Nohara *et al*., 2003; Ferreira *et al*., 2005; Corleto *et al*., 2009); therefore, it may be possible to exclude the role of such locus in pathogenesis of IBD.

4.2.5 Interleukin 2 Gene (*IL2*)

The SNP of *IL2*$_{+166}$ was presented with three genotypes (TT, TG and GG) that corresponded to two alleles (*T* and *G*). These genotypes were in a good agreement with HWE in both groups of patients, but they were significantly deviated in controls ($P \leq 0.01$). In addition, comparing IBD patients to controls also revealed significant differences in the distribution of *IL2*$_{+166}$ genotypes and alleles. Among CD patients, frequencies of TT genotype (41.2 *vs.* 6.9%; RR = 9.33)

and *T* allele (64.7 *vs.* 43.0%; RR = 2.43) were significantly increased in patients compared to controls (P = 0.001 and 0.009, respectively). In contrast, TG genotype (47.1 *vs*. 72.1%; P = 0.035; PF = 0.47) and *G* allele (35.3 *vs.* 56.9%; P = 0.009; PF = 0.34) frequencies were significantly decreased in CD patients. Almost, similar observations were made in UC patients, and frequencies of TT genotype (48.5 *vs.* 6.9%; RR = 12.55) and *T* allele (66.7 *vs.* 43.0%; RR = 2.62) were significantly increased in patients compared to controls ($P = 2.6*10^{-6}$ and 0.001, respectively); while frequencies of TG genotype (36.4 72.1%; $P = 3.8*10^{-4}$; PF = 0.56) and *G* allele (33.3 *vs*. 56.9%; P = 0.001; PF = 0.36) were significantly decreased in UC patients. However, no significant variation was observed between CD and UC patients in the distribution of $IL2_{+166}$ genotypes and alleles (Tables 4-5A and 4-5B).

The presented results strongly suggest that $IL2_{+166}$ polymorphism is involved in both groups of IBD in terms of susceptibility (positive association) and protection (negative association); especially in UC patients, in whom the RR of TT genotype reached 12.55, and the protective effect of TG genotype was 0.56. Therefore, *IL2* allelic changes at position +166 might be associated with increased and decreased risk of IBD in Iraqi population, and this may also contribute to a better clinic diagnosis of CD and UC. Such conclusion has also been favored by Gok and co-workers (2014) who investigated the prevalence of $IL2_{+166}$ polymorphism in 69 Turkish IBD patients (18 CD and 51 UC) and 100 controls, and reported similar findings. No further investigation that can validate these results, but Shi *et al*. (2011) have investigated a further SNP in this region ($IL2_{-330}$) in Chinese UC patients and reported that such SNP was positively associated with the disease.

Table 4-5A: Observed numbers and percentage frequencies and Hardy-Weinberg equilibrium (HWE) of $IL2_{+166}$ genotypes and alleles in inflammatory bowel disease (Crohn's disease and ulcerative colitis) patients and controls.

Groups			*IL2*$_{+166}$ Genotype or Allele					H-W P ≤
			TT	TG	GG	*T*	*G*	
Crohn's Disease (No. = 34)	**Observed**	**No.**	14	16	4	44	24	N.S.
		%	41.2	47.1	11.8	64.7	35.3	
	Expected	**No.**	14.2	15.5	4.2	Not Estimated		
		%	41.9	45.7	12.5			
Ulcerative Colitis (No. = 66)	**Observed**	**No.**	32	24	10	88	44	N.S.
		%	48.5	36.4	15.2	66.7	33.3	
	Expected	**No.**	29.3	29.3	7.3	Not Estimated		
		%	44.4	44.4	11.1			
Controls (No. = 43)	**Observed**	**No.**	3	31	9	37	49	**0.01**
		%	6.9	72.1	20.9	43.0	56.9	
	Expected	**No.**	7.9	21.1	13.9	Not Estimated		
		%	18.5	49.0	32.5			

Table 4-5B: Statistical analysis of associations between $IL2_{+166}$ genotypes or alleles and inflammatory bowel disease (Crohn's disease and ulcerative colitis).

Type of Comparison	*IL2*$_{+166}$ Genotype or Allele	Statistical Evaluation			
		Relative Risk	Etiological or Preventive Fraction	Fisher's Exact Probability	95% Confidence Intervals
Crohn's Disease *Versus* Controls	**TT**	9.33	0.37	**0.001**	2.44 -35.67
	TG	0.34	0.47	**0.035**	0.14 - 0.88
	GG	0.50	0.10	0.366	0.14 - 1.77
	T	2.43	0.38	**0.009**	1.27- 4.66
	G	0.41	0.34	**0.009**	0.21-0.79
Ulcerative Colitis *Versus* Controls	**TT**	12.55	0.45	**$2.6*10^{-6}$**	3.58 -44.00
	TG	0.22	0.56	**$3.8*10^{-4}$**	0.10 - 0.50
	GG	0.67	0.07	0.450	0.25 - 1.81
	T	2.62	0.42	**0.001**	1.52 - 4.62
	G	0.38	0.36	**0.001**	0.22 - 0.66
Crohn's Disease *Versus* Ulcerative Colitis	**TT**	0.74	0.12	0.531	0.33 - 1.70
	TG	1.56	0.17	0.389	0.68 - 3.57
	GG	0.75	0.04	0.767	0.22 - 2.54
	T	0.92	0.06	0.875	0.50 - 1.69
	G	1.09	0.03	0.875	0.59 - 2.01

4.2.6 Interleukin 4 Gene (*IL4*)

Genetic polymorphism of *IL4* gene was determined at position -590 of the promoter region ($IL4_{-590}$ SNP), which was presented with three genotypes (TT, TC and CC). These genotypes were significantly

departed from HWE in UC patients and controls ($P \leq 0.001$ and 0.05, respectively), while CD patients were in a good agreement with such equilibrium (Table 4-6A). Comparing *IL4*$_{-590}$ genotypes and alleles between IBD patients (CD and UC) and controls also revealed some significant variations. Among CD patients, it was observed that frequencies of TT genotype (52.9 *vs*. 11.6%; $P = 1.2*10^{-4}$) and *T* allele (70.6 *vs*. 24.4%; $P = 1.6*10^{-8}$) were significantly increased in patients compared to controls, and the associated RR values were 8.55 and 7.43, respectively. In contrast, CC genotype (11.8 *vs*. 62.8%, $P = 5.6*10^{-6}$) and *C* allele (29.4 *vs*. 75.6%; $P = 1.6*10^{-8}$) frequencies were significantly decreased in CD patients, and the associated PF values were 0.58 and 0.65, respectively. In the case of UC, frequencies of TC genotype (69.7 *vs*. 25.6%; RR = 6.69; $P = 1.1*10^{-5}$) and *T* allele (62.1 *vs*. 24.4%; RR = 5.08; $P = 4.6*10^{-8}$) were significantly increased in patients compared to controls, while CC genotype (3.0 *vs*. 62.8%; PF = 0.62; $P = 2.5*10^{-12}$) and *C* allele (37.9 vs. 75.6%; PF = 0.61; $P = 1.6*10^{-8}$) frequencies were significantly decreased. However, no such variation was observed between CD and UC patients (Table 4-6B).

Table 4-6A: Observed numbers and percentage frequencies and Hardy-Weinberg equilibrium (HWE) of *IL4*$_{-590}$ genotypes and alleles in inflammatory bowel disease (Crohn's disease and ulcerative colitis) patients and controls.

Groups			***IL4*$_{-590}$ Genotype or Allele**					**H-W P ≤**
			TT	**TC**	**CC**	***T***	***C***	
Crohn's Disease (No. = 34)	**Observed**	**No.**	18	12	4	48	20	N.S.
		%	52.9	35.3	11.8	70.6	29.4	
	Expected	**No.**	16.9	14.1	2.9	Not Estimated		
		%	49.8	41.5	8.7			
Ulcerative Colitis (No. = 66)	**Observed**	**No.**	18	46	2	82	50	**0.001**
		%	27.3	69.7	3.0	62.1	37.9	
	Expected	**No.**	25.5	31.1	9.5	Not Estimated		
		%	38.6	47.1	14.4			
Controls (No. = 43)	**Observed**	**No.**	5	11	27	21	65	**0.05**
		%	11.6	25.6	62.8	24.4	75.6	
	Expected	**No.**	2.6	15.9	24.4	Not Estimated		
		%	5.9	36.9	57.1			

Table 4-6B: Statistical analysis of associations between *IL4*$_{-590}$ genotypes or alleles and inflammatory bowel disease (Crohn's disease and ulcerative colitis).

Type of Comparison	*IL4*$_{-590}$ Genotype or Allele	Statistical Evaluation			
		Relative Risk	Etiological or Preventive Fraction	Fisher's Exact Probability	95% Confidence Intervals
Crohn's Disease *Versus* Controls	TT	8.55	0.47	**$1.2*10^{-4}$**	2.75 - 26.61
	TC	1.59	0.13	0.453	0.60 - 4.18
	CC	0.08	0.58	**$5.6*10^{-6}$**	0.02 - 0.26
	T	7.43	0.61	**$1.6*10^{-8}$**	3.64 - 15.14
	C	0.13	0.65	**$1.6*10^{-8}$**	0.07 - 0.27
Ulcerative Colitis *Versus* Controls	TT	2.85	0.18	0.058	0.98 - 8.28
	TC	6.69	0.59	**$1.1*10^{-5}$**	2.85 - 15.72
	CC	0.02	0.62	**$2.5*10^{-12}$**	0.00 - 0.09
	T	5.08	0.50	**$4.6*10^{-8}$**	2.78 - 9.26
	C	0.20	0.61	**$4.6*10^{-8}$**	0.11 - 0.36
Crohn's Disease *Versus* Ulcerative Colitis	TT	3.0	0.35	0.016	1.28 - 7.05
	TC	0.24	0.53	0.001	0.10 - 0.56
	CC	4.27	0.01	0.176	0.75 - 24.17
	T	1.46	0.22	0.274	0.78 - 2.73
	C	0.68	0.12	0.274	0.37 - 1.28

According to the presented results, *IL4*$_{-590}$ SNP can be highlighted as an important genetic marker in the pathogenesis of CD and UC; especially if we the RR values of 8.55 and 7.43 for TT genotype and *T* allele, respectively in CD, and 6.69 and 5.08 for TC and *T* allele, respectively in UC. Therefore, the genetic predisposition conferred by these genotypes cannot be ignored, and the estimated EF values are in favor of such generalization (EF range: 0.47 – 0.61). The protective effects, as estimated by PF values (PF range: 0.58 – 0.62), of CC genotype and *C* allele are also a further feature of *IL4*$_{-590}$ SNP. However, only one study reported the protective effect of *IL4*$_{-590}$ *C* allele against IBD of both types (CD and UC) in Turkish patients (Gao *et al*., 2014), and no further investigation has reported such SNP in IBD patients. However, other studies investigated other polymorphisms in intron and promoter regions of *IL4* gene and the results were almost

conflicting due to ethnic variations, but they agreed that that IL-4 is an important cytokine involved in mucosal immunity and its polymorphisms play a critical role in IBD development (Aithal *et al*., 2001; Peng *et al*., 2002; Hong *et al*., 2008; Ahirwar *et al*., 2012; Connelly *et al*., 2014).

4.2.7 Interleukin 4 Receptor Gene (*IL4R*)

Three genotypes (GG, GA and AA) were recognized at $IL4R_{+1902}$ gene. These genotypes were in a good agreement with HWE in UC patients and controls, but a significant departure ($P \leq 0.01$) from the equilibrium was noticed in CD patients (Table 4-7A).

Table 4-7A: Observed numbers and percentage frequencies and Hardy-Weinberg equilibrium (HWE) of $IL4R_{+1902}$ genotypes and alleles in inflammatory bowel disease (Crohn's disease and ulcerative colitis) patients and controls.

Groups			$IL4R_{+1902}$ Genotype or Allele					H-W P ≤
			GG	GA	AA	*G*	*A*	
Crohn's Disease (No. = 34)	Observed	No.	16	6	12	38	30	0.01
		%	47.1	17.7	35.3	55.9	44.1	
	Expected	No.	10.6	16.8	6.6	Not Estimated		
		%	31.2	49.3	19.5			
Ulcerative Colitis (No. = 66)	Observed	No.	38	24	4	100	32	N.S.
		%	57.6	36.4	6.1	75.8	24.2	
	Expected	No.	37.9	24.2	3.9	Not Estimated		
		%	57.4	36.7	5.9			
Controls (No. = 43)	Observed	No.	5	22	16	32	54	N.S.
		%	11.6	51.1	37.2	37.2	62.8	
	Expected	No.	5.9	20.1	16.9	Not Estimated		
		%	13.9	46.7	39.4			

In addition, comparing IBD patients to controls also revealed significant differences in the distribution of $IL4R_{+1902}$ genotypes and alleles. Among CD patients, frequencies of GG genotype (47.1 *vs.* 11.6%; RR = 6.76) and *G* allele (55.9 *vs.* 37.2%; RR = 2.14) were significantly increased in patients compared to controls (P = 0.001 and 0.023, respectively). In contrast, GA genotype (17.7 *vs*. 51.1%; P = 0.004; PF = 0.41) and *A* allele (44.1 *vs.* 62.8%; P = 0.023; PF =

0.33) frequencies were significantly decreased in CD patients. In UC patients, frequencies of GG genotype (57.6 *vs.* 11.6%; RR = 10.31) and *G* allele (75.8 *vs.* 37.2%; RR = 5.27) were significantly increased in patients compared to controls ($P = 1.0*10^{-6}$ and $1.9*10^{-8}$, respectively); while frequencies of AA genotype (6.1 *vs.* 37.2%; $P = 7.0*10^{-5}$; PF = 0.33) and *A* allele (24.2 *vs.* 62.8%; $P = 1.9*10^{-8}$; PF = 0.51) were significantly decreased in UC patients. However, comparing CD to UC patients revealed that frequencies of $IL2_{+166}$ AA genotype and *A* allele were significantly increased in CD patients (Tables 4-7B).

Table 4-7B: Statistical analysis of associations between $IL4R_{+1902}$ genotypes or alleles and inflammatory bowel disease (Crohn's disease and ulcerative colitis).

Type of Comparison	$IL4R_{+1902}$ Genotype or Allele	Statistical Evaluation			
		Relative Risk	Etiological or Preventive Fraction	Fisher's Exact Probability	95% Confidence Intervals
Crohn's Disease *Versus* Controls	**GG**	6.76	0.40	**0.001**	2.17 - 21.3
	GA	0.20	0.41	**0.004**	0.07 - 0.58
	AA	0.92	0.03	1.000	0.37 - 2.32
	G	2.14	0.30	**0.023**	1.12 - 4.07
	A	0.47	0.33	**0.023**	0.25 - 0.89
Ulcerative Colitis *Versus* Controls	**GG**	10.31	0.52	$\mathbf{1.0*10^{-6}}$	3.64 - 29.21
	GA	0.55	0.23	0.165	0.25 - 1.18
	AA	0.11	0.33	$\mathbf{7.0*10^{-5}}$	0.03 - 0.35
	G	5.27	0.61	$\mathbf{1.9*10^{-8}}$	2.93 - 9.50
	A	0.19	0.51	$\mathbf{1.9*10^{-8}}$	0.11 - 0.34
Crohn's Disease *Versus* Ulcerative Colitis	**GG**	0.65	0.20	0.398	0.29 - 1.49
	GA	0.38	0.23	0.067	0.14 - 1.02
	AA	8.45	0.31	$3.3*10^{-4}$	2.50 - 28.62
	G	0.41	0.45	0.006	0.22 - 0.75
	A	2.47	0.26	0.006	1.33 - 4.58

As in $IL4_{-590}$ SNP, $IL4R_{+1902}$ genotypes and alleles seem to have a role in IBD pathogenesis, and the SNP may have a functional effect on IL-4Rα in the patients. Such conclusion was addressed by Olavesen *et al*. (2000), who screened *IL4R* gene for SNPs by fluorescent chemical cleavage analysis, and tested allelic association in families of CD and UC patients, and no association

was found. Paavola *et al*. (2001) carried out a similar analysis, but their results indicated that homozygosity for $IL4R_{+1902}$ *G* allele was less frequent in UC patients only, while CD patients did not show such variation. Further conflicting results were given by the present study, in which AA homozygosity was the less frequent, while GG homozygosity dominated CD and UC patients, and the associated RRs were 6.76 and 10.31, respectively, and Gao *et al*. (2014) supported some these findings. However, $IL4R_{+1902}$ SNP has been best presented as adenine replacement for guanine at nucleotide 1902 (1902 A > G); leading to a change from a glutamine to arginine residue at position 576 (Q576R), which has been considered as a 'loss of function' SNP, and might be associated with an increased risk develop inflammatory diseases (Tachdjian *et al*., 2009). More recently, the promoter methylation status of genes involved in inflammation and autoimmunity, including *IL4R* gene, has been examined in CD but no hypermethylation was observed in the patients, while in UC, the hypermethylation was noticed (Karatzas *et al*., 2014). The authors suggested that the observed epigenetic changes might indicate that CD and UC patients exhibit specific DNA methylation signatures with potential clinical applications. Therefore, the investigated SNP of *IL4R* gene may potentiate for both types of IBD via these mechanisms or other pathways that have not been well-defined and require further intense investigations.

4.2.8 Interleukin 6 Gene (*IL6*)

Analysis of HWE revealed that both groups of IBD patients were in a good agreement with the equilibrium, while controls showed a significant departure ($P \leq 0.001$) (Table 4-8A). However, comparing patients to controls revealed that $IL6_{+565}$ GG genotype frequency was significantly increased in CD (70.6 *vs*. 13.9%; RR =14.80; $P = 1.5*10^{-8}$)

and UC (69.7 *vs*. 13.9%; RR = 14.18; P = $4.4*10^{-7}$) patients, and a similar increased frequency of *G* allele was observed in CD and UC patients. In contrast, the GC genotype frequency was significantly decreased in CD (23.5 *vs*. 76.7%; PF = 0.70; P = $3.7*10^{-6}$) and UC (24.2 *vs*. 76.7%; PF = 0.69; P = $7.0*10^{-6}$) patients, and the *C* allele frequency behaved in a similar manner. No such variation was observed between CD and UC patients (Table 4-8B).

The significant differences of $IL6_{+565}$ polymorphism between IBD patients (CD and UC) and controls that were observed in present study may point to a potential susceptibility (GG genotype and *G* allele) role in the progression of the disease, and may also affect serum level of IL-6; thus contributing to the pathophysiology of CD and UC. A protective effect of GC genotype or *C* allele also worths some consideration. Unfortunately, such polymorphism has not been investigated in CD and UC patients, but most investigations have been concerned with a further position of *IL6* gene ($IL6_{-174}$) and concluded that $IL6_{-174}$ SNP could be involved in etiopathogenesis of IBD (Balding *et al*., 2004; Cantor *et al*., 2005; Sawczenko *et al*., 2005; Guerreiro *et al*., 2009; Sagiv-Friedgut *et al*., 2010). Therefore, further investigations are certainly required to validate the present findings. This reasoned by the fact that IL-6 has been considered as one of the key roles in signaling pathway in IBD development. The clinical relevance of serum IL-6 in CD and UC patients has been addressed by Takac *et al*. (2014) in cross-sectional, case-control study that involved 100 IBD (CD and UC) patients and 71 blood donors. Their results indicated that IL-6 serum level is a clinically relevant parameter for both groups of IBD disease, and it was strongly correlated with inflammatory activity of CD and UC. However, the molecular mechanism of such relevance has not been understood; but *IL6* SNPs may have their effect as suggested by present results.

Table 4-8A: Observed numbers and percentage frequencies and Hardy-Weinberg equilibrium (HWE) of $IL6_{+565}$ genotypes and alleles in inflammatory bowel disease (Crohn's disease and ulcerative colitis) patients and controls.

Groups			$IL6_{+565}$ Genotype or Allele					H-W P ≤
			GG	GC	CC	*G*	*C*	
Crohn's Disease (No. = 34)	**Observed**	**No.**	24	8	2	56	12	N.S.
		%	70.6	23.5	5.9	82.4	17.7	
	Expected	**No.**	23.1	9.9	1.1	Not Estimated		
		%	67.8	29.1	3.1			
Ulcerative Colitis (No. = 66)	**Observed**	**No.**	46	16	4	108	24	N.S.
		%	69.7	24.2	6.1	81.8	18.2	
	Expected	**No.**	44.2	19.6	2.2	Not Estimated		
		%	66.9	29.8	3.3			
Controls (No. = 43)	**Observed**	**No.**	6	33	4	45	41	**0.001**
		%	13.9	76.7	9.3	52.3	47.7	
	Expected	**No.**	11.8	21.5	9.8	Not Estimated		
		%	27.4	49.9	22.7			

Table 4-8B: Statistical analysis of associations between $IL6_{+565}$ genotypes or alleles and inflammatory bowel disease (Crohn's disease and ulcerative colitis).

Type of Comparison	$IL6_{+565}$ Genotype or Allele	Statistical Evaluation			
		Relative Risk	Etiological or Preventive Fraction	Fisher's Exact Probability	95% Confidence Intervals
Crohn's Disease *Versus* Controls	**GG**	14.80	0.66	**$1.5*10^{-8}$**	4.83 - 45.37
	GA	0.09	0.70	**$3.7*10^{-6}$**	0.03 - 0.27
	AA	0.61	0.04	0.689	0.11 - 3.46
	G	4.25	0.63	**$1.4*10^{-4}$**	2.01 - 8.99
	A	0.24	0.37	**$1.4*10^{-4}$**	0.11 - 0.50
Ulcerative Colitis *Versus* Controls	**GG**	14.18	0.65	**$4.4*10^{-7}$**	5.22 - 38.54
	GA	0.10	0.69	**$7.0*10^{-6}$**	0.04 - 0.24
	AA	0.63	0.04	0.710	0.15 - 2.63
	G	4.10	0.62	**$4.6*10^{-6}$**	2.23 - 7.54
	A	0.24	0.36	**$4.6*10^{-6}$**	0.13 - 0.45
Crohn's Disease *Versus* Ulcerative Colitis	**GG**	0.83	0.12	0.811	0.33 - 2.09
	GA	0.96	0.01	1.000	0.37 - 2.51
	AA	0.97	0.02	1.000	0.17 - 5.46
	G	1.25	0.36	0.401	2.01 - 8.99
	A	0.24	0.37	0.401	0.11 - 0.50

4.2.9 Interleukin 10 Gene (*IL10*)

Single nucleotide polymorphism of *IL10* gene at position -1082 showed a significant departure from HWE in CD patients, while UC patients and controls were in a good agreement with the equilibrium (Table 4-9A). Comparing both groups of patients to controls also

revealed no significant variations in the distribution of *IL10*$_{-1082}$ genotypes and alleles (Table 4-9B).

Table 4-9A: Observed numbers and percentage frequencies and Hardy-Weinberg equilibrium (HWE) of *IL10*-$_{1082}$ genotypes and alleles in inflammatory bowel disease (Crohn's disease and ulcerative colitis) patients and controls.

Groups			*IL10*-$_{1082}$ Genotype or Allele					H-W P ≤
			GG	GA	AA	*G*	*A*	
Crohn's Disease (No. = 34)	Observed	No.	10	22	2	42	26	**0.05**
		%	29.4	64.7	5.9	61.8	38.2	
	Expected	No.	12.9	16.1	4.9	Not Estimated		
		%	38.2	47.2	14.6			
Ulcerative Colitis (No. = 66)	Observed	No.	26	30	10	82	50	N.S.
		%	39.4	45.5	15.2	62.1	37.9	
	Expected	No.	25.5	31.1	9.5	Not Estimated		
		%	38.6	47.1	14.4			
Controls (No. = 43)	Observed	No.	13	20	10	46	40	N.S.
		%	30.2	46.5	23.3	53.5	46.5	
	Expected	No.	12.3	21.4	2.3	Not Estimated		
		%	28.6	49.8	21.6			

Table 4-9B: Statistical analysis of associations between *IL10*-$_{1082}$ genotypes or alleles and inflammatory bowel disease (Crohn's disease and ulcerative colitis).

Type of Comparison	*IL10*-$_{1082}$ Genotype or Allele	Statistical Evaluation			
		Relative Risk	Etiological or Preventive Fraction	Fisher's Exact Probability	95% Confidence Intervals
Crohn's Disease *Versus* Controls	GG	0.96	0.01	1.000	0.36 - 2.54
	GA	2.11	0.34	0.166	0.85 - 5.25
	AA	1.38	0.08	0.606	0.50 - 3.77
	G	1.40	0.18	0.329	0.74 - 2.67
	A	0.71	0.13	0.329	0.37 - 1.35
Ulcerative Colitis *Versus* Controls	GG	1.50	0.31	0.414	0.67 - 3.37
	GA	0.96	0.09	1.000	0.45 - 2.06
	AA	0.59	0.09	0.318	0.22 - 1.55
	G	1.43	0.18	0.210	0.82 - 2.47
	A	0.70	0.13	0.210	0.41 - 1.21
Crohn's Disease *Versus* Ulcerative Colitis	GG	0.64	0.14	0.383	0.27 - 1.54
	GA	2.20	0.35	0.091	0.95 - 5.11
	AA	0.35	0.09	0.213	0.07 - 1.66
	G	0.98	0.09	1.000	0.54 - 1.79
	A	1.02	0.06	1.000	0.56 - 1.85

These results may suggest that *IL10*$_{-1082}$ SNP is not involved in the etiopathogenesis of IBD in Iraqi patients. In contrast, Almeida *et al*. (2013) reported that *IL10*$_{-1082}$ *G* allele was significantly linked with

age at diagnosis of CD in a mixed-race Brazilian population. However, Zou *et al*. (2014) carried out a meta-analysis of 17 studies that included 4132 IBD patients and 5109 controls and found no significant association between *IL10*$_{-1082}$ genotypes or alleles and the two types of disease (CD and UC). Accordingly, it would be possible to confirm the results of present study. Whereas, a further meta-analysis in Caucasian patients reported a different observation, and such SNP showed significant association with CD, with RRs for the GG + GA genotypes and GG genotype versus AA genotype of 1.28 and 1.24 (Lv *et al*., 2014).

4.2.10 Interleukin 12B Gene (*IL12B*)

A significant departure from HWE was observed in UC patients ($P \leq 0.001$) and controls ($P \leq 0.01$), while the observed and expected genotype frequencies of *IL12B*$_{-1188}$ were in a good agreement with equilibrium in CD patients (Table 4-10A).

Table 4-10A: Observed numbers and percentage frequencies and Hardy-Weinberg equilibrium (HWE) of *IL12B*$_{-1188}$ genotypes and alleles in inflammatory bowel disease (Crohn's disease and ulcerative colitis) patients and controls.

Groups			***IL12B*$_{-1188}$ Genotype or Allele**					**H-W P ≤**
			AA	**AC**	**CC**	***A***	***C***	
Crohn's Disease (No. = 34)	**Observed**	**No.**	14	18	2	46	22	N.S.
		%	41.2	52.9	3.0	67.7	32.4	
	Expected	**No.**	15.6	14.9	3.6	Not Estimated		
		%	45.8	43.8	10.5			
Ulcerative Colitis (No. = 66)	**Observed**	**No.**	18	44	4	80	52	**0.001**
		%	27.3	66.7	6.1	60.6	39.4	
	Expected	**No.**	24.2	31.5	10.2	Not Estimated		
		%	36.7	47.8	15.5			
Controls (No. = 43)	**Observed**	**No.**	14	13	16	41	45	**0.01**
		%	32.5	30.2	37.2	47.7	52.3	
	Expected	**No.**	9,8	21.5	11.8	Not Estimated		
		%	22.7	49.9	27.4			

When comparisons were made between patients and controls significant variations were observed in the distributions of *IL12B*$_{-1188}$

genotype and allele frequencies. Among CD patients, it was noticed that the frequency of *A* allele was significantly increased in patients compared to controls (67.7 *vs*. 47.7%; RR = 2.29; P = 0.015), while *C* allele was significantly decreased (32.4 *vs*. 52.3%; PF = 0.29; P = 0.015). In the case of UC patients, the heterozygous genotype AC was observed with a significant increased frequency in patients compared to controls (66.7 *vs*. 30.2%; RR = 4.62; P = $3.6*10^{-4}$), while CC genotype frequency was significantly decreased (6.1 *vs*. 37.2%; PF = 0.33; P = $7.0*10^{-5}$). However, no such variations were observed between CD and UC patients (Table 4-10B).

Table 4-10B: Statistical analysis of associations between *IL12B*$_{-1188}$ genotypes or alleles and inflammatory bowel disease (Crohn's disease and ulcerative colitis).

Type of Comparison	*IL12B*$_{-1188}$ Genotype or Allele	Statistical Evaluation			
		Relative Risk	Etiological or Preventive Fraction	Fisher's Exact Probability	95% Confidence Intervals
Crohn's Disease *Versus* Controls	AA	1.45	0.12	0.481	0.58 - 3.65
	AC	2.60	0.32	0.061	0.03 - 6.54
	CC	0.11	0.33	**0.001**	0.02 - 0.49
	A	2.29	0.38	**0.015**	1.19 - 4.43
	C	0.44	0.29	**0.015**	0.23 - 0.84
Ulcerative Colitis *Versus* Controls	AA	0.80	0.06	0.671	0.35 - 1.84
	AC	4.62	0.52	**$3.6*10^{-4}$**	2.03 - 10.47
	CC	0.11	0.33	**$7.0*10^{-5}$**	0.03 - 0.35
	A	1.69	0.24	0.070	0.98 - 2.91
	C	0.59	0.21	0.070	0.34 - 1.02
Crohn's Disease *Versus* Ulcerative Colitis	AA	1.87	0.19	0.179	0.79 - 4.42
	AC	0.65	0.29	0.198	0.24 - 1.30
	CC	0.97	0.02	1.000	0.17 - 5.46
	A	1.36	0.17	0.357	0.74 - 2.51
	C	0.74	0.10	0.357	0.40 - 1.36

These results point out for the important role of *IL12B*$_{-1188}$ genotype and alleles in etiopathogenesis of IBD, especially the genotype AC in UC patients, which scored EF value of 0.52, as well as the allele *A* in CD patients (EF = 0.38). In addition to these predisposing genotypes and alleles, *C* allele and CC genotype might

be associated with a protection against CD and UC, respectively. These results are in general agreement with the meta-analyses carried out by three groups of investigators (Barrett *et al*., 2008; Franke *et al*., 2010; Anderson *et al*., 2011) who demonstrated that *IL12B* as a susceptibility gene for IBD. Further cohort studies in Spanish (Marquez *et al*., 2008), Japanese (Yamazaki *et al*., 2009) and German (Glas *et al*., 2012) IBD patients established an association of *IL12B* SNPs with IBD susceptibility; although some differences in the results were observed. However, the role of *IL12B* SNPs for IBD susceptibility in Iraqi IBD patients has not been investigated; therefore these results came to contribute to the general knowledge of IBD SNPs in CD and UC, and highlight the importance of $IL12B_{-1188}$ SNP in Iraqi CD and UC patients, as well as confirming other recent studies in this regard (Moon *et al*., 2013; Dubinsky *et al*., 2013). However, it remains that the phenotypic effects of *IL12B* SNPS and potential gene-gene interactions that contribute to CD and UC susceptibility are largely not well-defined.

4.2.11 Interferon Gamma Gene (*IFNG*)

Both groups of patients (CD and UC) were in a good agreement with HWE with respect to the distribution of $IFNG_{+874}$ genotypes (AA, AT and TT), while the controls demonstrated a significant departure ($P \leq 0.01$) from the equilibrium (Table 4-11A). In addition, significant variations in genotype and allele frequencies of $IFNG_{+874}$ between patients and controls were observed. The AT genotype showed a significant increased frequency in CD patients compared to controls (58.8 *vs*. 30.2; RR = 3.30; P = 0.020), while TT genotype frequency was significantly decreased (11.8 *vs*. 39.5%; PF = 0.31; P = 0.009). Among UC patients, AA genotype (54.6 *vs*. 30.2%; RR = 2.77; P = 0.018) and *A* allele (74.2 *vs*. 45.4; RR = 3.47; $P = 2.7*10^{-5}$) frequencies were

Significantly increased in patients compared to controls . In contrast, TT genotype (6.1 *vs*. 39.5%; PF = 0.35; P = $2.8*10^{-5}$) and *T* allele (25.8 *vs*. 54.7%; PF = 0.38; P = $2.7*10^{-5}$) frequencies were significantly decreased. A significant decreased frequency of *A* allele (58.8 *vs*. 74.2%) and increased frequency of *T* allele (41.2 *vs*. 25.8%) was further observed in CD patients compared to UC patients (Table 4-11B).

Table 4-11A: Observed numbers and percentage frequencies and Hardy-Weinberg equilibrium (HWE) of $IFNG_{+874}$ genotypes and alleles in inflammatory bowel disease (Crohn's disease and ulcerative colitis) patients and controls.

Groups			$IFNG_{+874}$ Genotype or Allele					H-W P ≤
			AA	AT	TT	*A*	*T*	
Crohn's Disease (No. = 34)	**Observed**	**No.**	10	20	4	40	28	N.S.
		%	29.4	58.8	11.8	58.8	41.2	
	Expected	**No.**	11.	16.5	5.8	Not Estimated		
		%	34.6	48.4	16.9			
Ulcerative Colitis (No. = 66)	**Observed**	**No.**	36	26	4	98	34	N.S.
		%	54.6	39,4	6.1	74.2	25.8	
	Expected	**No.**	36.4	25.2	4.4	Not Estimated		
		%	55.1	38.3	6.6			
Controls (No. = 43)	**Observed**	**No.**	13	13	17	39	47	**0.01**
		%	30.2	30.2	39.5	45.4	54.7	
	Expected	**No.**	8.8	21.3	12.8	Not Estimated		
		%	20.6	49.6	29.9			

Table 4-11B: Statistical analysis of associations between $IFNG_{+874}$ genotypes or alleles and inflammatory bowel disease (Crohn's disease and ulcerative colitis).

Type of Comparison	$IFNG_{+874}$ Genotype or Allele	Statistical Evaluation			
		Relative Risk	Etiological or Preventive Fraction	Fisher's Exact Probability	95% Confidence Intervals
Crohn's Disease *Versus* Controls	**AA**	0.96	0.12	1.000	0.36 - 2.54
	AT	3.30	0.41	**0.020**	1.30 - 8.36
	TT	0.20	0.31	**0.009**	0.06 -0.67
	A	1.72	0.24	0.107	0.91 - 3.26
	T	0.58	0.22	0.107	0.31 - 1.10
Ulcerative Colitis *Versus* Controls	**AA**	2.77	0.34	**0.018**	1.24 - 6.18
	AT	1.50	0.13	0.414	0.67 - 3.37
	TT	0.10	0.35	**$2.8*10^{-5}$**	0.03 - 0.32
	A	3.47	0.52	**$2.7*10^{-5}$**	1.96 - 6.17
	T	0.29	0.38	**$2.7*10^{-5}$**	0.16 - 0.51
Crohn's Disease *Versus*	**AA**	0.35	0.35	**0.021**	0.15 - 0.83
	AT	2.20	0.32	0.090	0.96 - 5.05
	TT	2.07	0.06	0.439	0.49 - 8.69

Ulcerative Colitis	***A***	0.50	0.37	**0.035**	0.27 - 0.92
	T	2.02	0.20	**0.035**	1.09 - 3.74

As the results suggested, the *IFNG*$_{+874}$ SNP seem to play susceptibility and protective roles in the etiology of CD and UC; however, Cantor *et al*. (2005) were not in favor of such findings, and instead they cited that no association between UC or CD susceptibility and *IFNG*$_{+874}$ SNP could be established. A more recent study investigated a further polymorphism in a conserved region of *IFNG* gene and the authors reported for the first time that *IFNG* (*rs1861494*) *T* allele with enhanced IFN-γ production in IBD patients, and the polymorphism might be indicative of more aggressive disease (Gonsky *et al*., 2014). Therefore, SNPs of *IFNG* have to be further inspected in IBD to determine their susceptibility or protective effects.

4.2.12 Tumor Necrosis Factor Alpha Gene (*TNF*)

The SNP genotypes of *TNF* gene at position -308 showed a significant departure ($P \leq 0.05$) from HWE in CD patients and controls, while no departure was observed in UC patients (Table 4-12A).

Table 4-12A: Observed numbers and percentage frequencies and Hardy-Weinberg equilibrium (HWE) of *TNF*$_{-308}$ genotypes and alleles in inflammatory bowel disease (Crohn's disease and ulcerative colitis) patients and controls.

Groups			***TNF*$_{-308}$ Genotype or Allele**					**H-W P ≤**
			GG	**GA**	**AA**	***G***	***A***	
Crohn's Disease (No. = 34)	**Observed**	**No.**	10	22	2	42	26	**0.05**
		%	29.4	64.7	5.9	61.8	38.2	
	Expected	**No.**	12.9	16.1	4.9	Not Estimated		
		%	38.2	47.2	14.6			
Ulcerative Colitis (No. = 66)	**Observed**	**No.**	34	26	6	94	38	N.S.
		%	51.5	39.4	9.1	71.2	28.8	
	Expected	**No.**	33.5	27.1	5.5	Not Estimated		
		%	50.7	41.0	8.3			
Controls (No. = 43)	**Observed**	**No.**	8	29	6	45	41	**0.05**
		%	18.6	67.4	13.9	52.3	47.7	
	Expected	**No.**	11.8	21.5	9.8	Not Estimated		
		%	27.4	49.9	22.8			

Comparing patients to controls revealed that *TNF*$_{-308}$ genotypes and alleles were not significantly varied between CD patients and controls, while UC patients demonstrated a significantly (P = 0.006) increased frequency of *G* allele (71.2 *vs*. 52.3%; RR = 2.25) and a decreased frequency of *A* allele (28.8 *vs*. 47.7; PF = 0.26) compared to controls. The UC patients also showed a significant decreased frequency of GA genotype (39.4 *vs*. 64.7%; P = 0.021) compared to CD patients (Table 4-12A).

Table 4-12B: Statistical analysis of associations between *TNF*$_{-308}$ genotypes or alleles and inflammatory bowel disease (Crohn's disease and ulcerative colitis).

Type of Comparison	*TNF*$_{-308}$ Genotype or Allele	Statistical Evaluation			
		Relative Risk	Etiological or Preventive Fraction	Fisher's Exact Probability	95% Confidence Intervals
Crohn's Disease *Versus* Controls	GG	1.82	0.13	0.291	0.64 - 5.21
	GA	0.89	0.07	0.813	0.35 - 2.26
	AA	0.39	0.08	0.291	0.07 - 2.00
	G	1.47	0.19	0.256	0.77 - 2.80
	A	0.68	0.15	0.256	0.36 - 1.29
Ulcerative Colitis *Versus* Controls	GG	4.65	0.40	0.001	1.89 - 11.41
	GA	0.31	0.46	**0.006**	0.14 - 0.70
	AA	0.62	0.05	0.535	0.19 - 2.03
	G	2.25	0.39	**0.006**	1.28 - 3.96
	A	0.44	0.26	**0.006**	0.25 - 0.78
Crohn's Disease *Versus* Ulcerative Colitis	GG	0.39	0.31	0.055	0.16 - 0.94
	GA	2.82	0.41	**0.021**	1.21 - 6.59
	AA	0.63	0.03	0.713	0.12 - 3.21
	G	0.65	0.24	0.202	0.83 - 2.83
	A	1.53	0.13	0.202	0.35 - 1.21

The *TNF*$_{-308}$ might be more important in the susceptibility of UC than CD, and the two spotted alleles (*G* and *A*) have been suggested to have a role in predisposition and protection against this type of IBD. However, similar studies failed to find any significant variation in the frequency of the *TNF*$_{-308}$ alleles in IBD patients compared to controls (Zipperlen *et al*., 2005; Mittal *et al*., 2007; Naderi *et al*., 2014). Whereas on other studies and as in this study, the allele frequency of

such polymorphism showed a significant variation between UC patients and controls (Cao *et al*., 2006; Sýkora *et al*., 2006 Cucchiara *et al*., 2007). The variability of the results might be due to ethnic variations among different IBD populations, as suggested by Naderi *et al*. (2014).

4.2.13 Transforming Growth Factor Beta Gene (*TGFB1*)

Genotype frequencies of $TGFB1_{\text{codon } 25}$ demonstrated a significant departure from HWE in IBD patients (CD and UC) and controls, while comparing patients to controls or CD patients to UC patients revealed no significant variations in genotype (GG, GC and CC) or allele (*G* and *C*) frequencies between them (Tables 4-13A and 4-13B). Previous studies suggested a role for *TGFB1* SNPs in IBD clinical parameters. Of interest, $TGFB1_{-509}$ and $TGFB1_{915}$ SNPs were reported to be associated with CD (Schulte *et al*., 2001; Hume *et al*., 2006) and differences in allele frequencies and genotypes of $TGFB1_{-800}$ SNP were found between UC patients and controls (Tamizifar *et al*., 2008). In present study, a further SNP was investigated ($TGFB1_{\text{codon } 25}$) and it could not find any significant association between its genotypes or alleles and CD or UC. A similar conclusion has also been presented by Liberek *et al*. (2008), how demonstrated that four SNPs of *TGFB1* had no influence on susceptibility to IBD. In contrast, Almeida *et al*. (2013) defined that *TGFB1* SNPs may be associated with susceptibility to CD development. Therefore, it might be obvious that further studies, including larger and ethnically differentiated groups of IBD patients, are necessary to target the matter of differences between results of studies that correlate *TGFB1* polymorphisms and IBD. Accordingly, no firm conclusions could be proposed until the influence of any particular *TGFB1* SNPs on certain biochemical or

clinical effects are elucidated in the ground of molecular mechanism principles.

Table 4-13A: Observed numbers and percentage frequencies and Hardy-Weinberg equilibrium (HWE) of *TGFB1*$_{\text{codon 25}}$ genotypes and alleles in inflammatory bowel disease (Crohn's disease and ulcerative colitis) patients and controls.

Groups			*TGFB1*$_{\text{codon 25}}$ Genotype or Allele					H-W P ≤
			GG	GC	CC	*G*	*C*	
Crohn's Disease (No. = 34)	**Observed**	**No.**	10	22	2	42	26	**0.05**
		%	29.41	64.71	3.03	61.76	38.24	
	Expected	**No.**	12.97	16.06	4.97	Not Estimated		
		%	38.25	47.23	14.62			
Ulcerative Colitis (No. = 66)	**Observed**	**No.**	18	44	4	80	52	**0.001**
		%	27.27	66.67	6.06	60.61	39.39	
	Expected	**No.**	24.24	31.52	10.24	Not Estimated		
		%	36.73	47.8	15.52			
Controls (No. = 43)	**Observed**	**No.**	8	28	7	44	42	**0.05**
		%	18.6	65.1	16.2	51.16	48.84	
	Expected	**No.**	11.26	21.49	10.26	Not Estimated		
		%	26.18	49.97	23.85			

Table 4-13B: Statistical analysis of associations between *TGFB1*$_{\text{codon 25}}$ genotypes or alleles and inflammatory bowel disease (Crohn's disease and ulcerative colitis).

Type of Comparison	*TGFB1*$_{\text{codon 25}}$ Genotype or Allele	Statistical Evaluation			
		Relative Risk	Etiological or Preventive Fraction	Fisher's Exact Probability	95% Confidence Intervals
Crohn's Disease *Versus* Controls	**GG**	1.82	0.13	0.291	0.64 - 5.21
	GC	0.98	0.12	1.000	0.39 - 2.49
	CC	0.32	0.11	0.284	0.06 - 1.62
	G	1.54	0.21	0.196	0.81 - 2.93
	C	0.65	0.17	0.196	0.34 - 1.23
Ulcerative Colitis *Versus* Controls	**GG**	1.64	0.10	0.362	0.65 - 4.16
	GC	1.07	0.04	1.000	0.48 - 2.39
	CC	0.33	0.10	0.108	0.09 - 1.20
	G	1.47	0.19	0.208	0.85 - 2.53
	C	0.68	0.31	0.208	0.39 - 1.18
Crohn's Disease *Versus* Ulcerative Colitis	**GG**	1.11	0.02	0.818	0.45 - 2.74
	GC	0.92	0.05	1.000	0.39 - 2.16
	CC	0.97	0.02	1.000	0.17 - 5.46
	G	1.05	0.02	1.000	0.58 - 1.91
	C	0.95	0.01	1.000	0.52 - 1.73

4.3 Concluding Discussion

The presented results of the 13 cytokine SNPs in CD and UC patients are the first report in Iraqi patients, and their findings highlighted the role of these SNPs in etiopathogenesis of both groups of IBD, and paved the way for further investigations to determine the role cytokine gene polymorphisms in susceptibility to IBD or their protective effects. Such conclusion is favored by genome-wide association studies (GWAS), which identified many novel susceptibility loci for CD and UC, and SNPs in genes encoding cytokines and cytokine receptors have been suggested to affect the inflammatory cascade course and thereby may increase the risk of developing IBD (Magyari *et al*., 2014). In addition, the association between a single genetic SNP and IBD can be influenced by other genetic factors that any definite conclusions cannot be elucidated until the molecular mechanism of a putative genetic link is understood (Almeida *et al*., 2013).

Conclusions and Recommendations

Conclusions and Recommendations

I. Conclusions

Based on the findings of present study, it is possible to conclude that the profile of the 13 cytokine gene polymorphisms, as defined by single nucleotide polymorphism (SNP), in both forms of IBD (CD and UC) can be grouped as the following:

1. The SNPs of *IL1RN*$_{\text{maspl 11100,}}$ *IL10*$_{\text{-1082}}$ and *TGFB1*$_{\text{codon 25}}$ failed to show any association with CD or UC.
2. Significant variations in genotype and allele frequencies of *IL1B*$_{\text{-511}}$ and *TNF*$_{\text{-308}}$ SNPs were observed between UC patients and controls, but not between CD and controls.
3. Eight SNPs (*IL1A*$_{\text{-889}}$, *IL1R1*$_{\text{pstl 1970}}$, *IL2*$_{\text{+166}}$, *IL4*$_{\text{-590}}$, *IL4R*$_{\text{+1902}}$, *IL6*$_{\text{+565}}$, *IL12B*$_{\text{-1188}}$ and *IFNG*$_{\text{+874}}$) showed significant positive and negative association with CD and UC; therefore, their predisposing and protecting effects are suggested.
4. The patients (CD and UC) were significantly discriminated by genotypes or alleles of *IL4R*$_{\text{+1902,}}$ *IFNG*$_{\text{+874}}$ and *TNF*$_{\text{-308}}$ SNPs.

II. Recommendations

Based on results and review of literature, it is possibly to highlight the following recommendations:

1. Further SNPs of the 13 investigated cytokines have to be investigated to determine their predisposing or protecting effects against IBD.
2. It would be better to correlate the serum level of cytokines with their SNPs in IBD patients and controls.
3. For cytokines that showed no association, DNA sequencing of other promoter regions might be required.

References

References

Abraham, C. and Cho, J.H. (2009). Inflammatory bowel disease. *N. Engl. J. Med*., 361: 2066-2078.

Abreu, N.P.; Bergamaschi, C.T.; Marco, G.S.; Razvickas, c.v.and Schor , N.(2005).Effect of isotonic rehydration sports drink and exercise on urolithiasis. In rats .*Braz. J. Med. Biol.Res*.,38(4): 577-82.

Ad'hiah A. H. (1990). Immunogenetic Studies in Selected Human Diseases. Ph.D. Thesis, Department of Human Genetics, University of Newcastle upon Tyne, England.

Ad'hiah A. H. (2009).Distribution of HLA polymorphism in as sample of Iraqi Arabs in comparision with three Arab Galf populations .I.J.S.., 50: 120-125.

Ad'hiah, A. H.; Jasim H. A. and Fadhil, A. M. A. (2008) HLA antigens and inflammatory bowel disease in a sample of Iraqi patients. *Eastern Mediterranean Health Journal*, 14: 1155-1162.

Ahirwar, D. K.; Kesarwani, P.; Singh, R.; Ghoshal, U. C.; Mittal, R. D.(2012).Role of tumor necrosis factor-alpha (C-863A) Polymorphism in pathogenesis of inflammatory bowel disease in Northern India. *J. Gastrointest, Cancer*, 43(2): 196-204.

Ahmad, T.; Marshall, S. and Jewell, D. (2009). Genetics of inflammatory bowel disease: the role of the HLA complex. *World J. Gastroenterol*., 12**:** 3628-3635.

Aithal,G. P.; Daly, A. K.; Day, C.p. and Keteven, P. G.(2001).Association of polymorphisms in the cytochrome P450 CYP2 C9 with warfarin dose requirement and risk of bleeding complications. *Lancet*., 355:717-719.

Allison, L.; Bayer, A.Y. and Thomas, R. M.(2007). Function of the IL-2R for Thymic and Peripheral $CD4^{+}CD25^{+}$ $Foxp3^{+}$ T Regulatory Cells. *The Journal of Immunology*, 178 (7):*4062-4071*.

Allocca, M.; Jovani, M.; Fiorino, G.; Schreiber, S. and Danese, S.(2013). Anti-IL-6 treatment for inflammatory bowel disease: next cytokine. Next target. *Curr. Drug, Targets*, 14(12): 1508-21.

Ananthakrishnan, A.N. (2013). Environmental risk factors for inflammatory bowel disease. *Gastroenterol. Hepatol*., (N Y)., 9: 367-374.

Andersen, V.;Ernst, A.; Christensen, J.; Østergaard, M.; Jacobsen, B.A.; Tjønneland, A.; Krarup, H.B. and Vogel, U. (2010). The polymorphism rs3024505 proximal to IL-10 is associated with risk of ulcerative colitis and Crohns disease in a Danish case-control study. *B.M.C. Med. Genet.*, 11: 82.

Andoh, A.; Bamba, S.; Tsujiykawa, T.; Sasaki, M.and Fujiyama, Y.(2011). Immunomodulators and immunosuppressants for Japanese patients with ulcerative colitis. *ISRN Gastroenterol*., 36(2): 209-22.

Andoh, M.; Ogasawara, Y.; Sakata, A.; Ito, T.; Fujita, H.; Kawabata, H.; Ando, S.(2014). Isolation of the rickettsial agent genetically similar to Candidatus Rickettsia kotlanii, from Haemaphysalis megaspinosa in Japan. *Vector Borne Zoonotic, Dis*., 14(9):681-4.

Apte, S.; Baz, A.; Kelso, A. and Kienzle, N. (2008). Interferon-gamma and interleukin-4 reciprocally regulate CD8 expression in CD8+ T cells. *Proc Natl Acad Sci.,USA*., 45:17475-17480.

Arend,W.P.;Palmer, C. and Gabay, C.(2008).IL-1,IL-18, and IL-23 families of cytokines. *Immunol . Rve.*, 223:20-38.

Arreola, S. L.; Intanon, M.; Suljic, J.; Kittl, R.; Pham, N.H.; Kosma, P.;Haltrich,D. and Nguyen, T. H.(2014). Two B-Galactosidases from the Human Isolate Bifidobacterium breve DSM20213: Molecular cloning and expression, biochemical characterization and synthesis of galacto –oligosacchariges .*Pols. One*., 9(8):e104056.

Atreya, R. and Neurath, M.E. (2005). Involvement of IL-6 in the pathogenesis of inflammatory bowel disease and colon cancer. *Clin. Rev. Allergy Immunol*., 28(3):187-96.

Balding ,J.; Livingstone, W. J.; Conroy,J.; Mynett-Johnson,L.; Weir, D.G.; Mahmud,N. and Smith,O.P.(2004). Inflammatory bowel disease: the role of inflammatory cytokine gene polymorphisms. *Mediators of Inflammation*, 13(3), 181- 187.

Balschun, D.; Wetzel, W.; Del Rey, A.; Pitossi, F.; Schneider, H.; Zuschralter, W. and Besedovsky, H.O.(2004). Interleukin-6: a cytokine to forget. *FASEB, J.* ,18(14): 1788-90.

Barreiro- de Acosta,M. and Peña, A.S.(2007). Clinical applications of NOD2/CARD15 mutations in Crohn's disease. *Acta Gastroenterol Latinoam*;37(1):49-54.

Barrett, C. F.; Van Den Maagdenbrg, A.M.; Frants, R.R.;Ferrari, M. D.(2008). Familial hemiplegic migraine. Adv. Genet.63:57-83.

Barta, S.K.; Samuel, M. S.; Xue, X.; Wang, D.; Lee, J. Y.; Mounier, N.; Ribera, J. M.; Spina, M.; Tirelli,U.; Weiss, R.; Galicier, L.; Boue, F.; Little, R. E.; Dunleavy, K.; Wilson, W. H.; Wyen, C.; Remick,S. C.; Kaplan, L.D. ;Ratner, L. ;Noy, A. and Sparano, J.A.(2015).Changes in the influence of lymphoma –and HIV –specific factors on outcomes in AIDS- related non –Hodgkin lymphoma. *Ann. Oncol*., 26(5): 958-66.

Basturk, B. ; Karasu, Z. ; Kilic, M.; Ulukaya, S. ;Boyacloglu, S.; Oral, B.(2008). Association of TNF-alpha -308 polymorphism with the outcome of hepatitis B virus infection in Turkey. Infect Genet. Evol, 8(1): 20-5.

Becker, C.; Fantini, M. G.; Neurath, M. F.(2006). High resolution colony in live mice. Nat. Protoc., 1(6):2900-4.

Bengtson, M.B.; Aamodt, G.; Vatn, M.H. and Harris, J.R. (2010). Concordance for IBD among twins compared to ordinary siblings; a Norwegian population-based study. *J. Crohns Colitis*, 4: 312-318.

Birrenbach, T. and Bocker, U. (2004). Inflammatory bowel disease and smoking: a review of epidemiology, pathophysiology, and therapeutic implications. *Inflamm. Bowel Dis*., 10: 848-859.

Bitton, A.; Dobkin, P.L.; Edwardes, M. D.; Sewitch, M. J.; Meddings, J. B.;Rawal, S. ;Cohen, A.; Vermeire, S.; Dufresne, L.; Franchimont, D. and Wild, G.E.(2008). Predicting relapse in Crohn's disease: a biopsychosocial model. *Gut.*57(10):1386-92.

Bonen, D.K.; Cho, J. H. and Zheng. C.Q.(2003).The genetics of inflammatory bowel disease. *Gastroenterology,* 124(2):521-36.

Bouzgarrou,N.; Hassen, E.; Farhat, K.; Bahri, O.; Gabboui, S. ; Maamouri, N.; Ben Mami, N.; Saffar,H.; Trabelsi, A.; Triki,H. and Chouchane,L.(2009).Combined analysis of interferon-gamma and interleukin-10 gene polymorphisms and chronic hepatitis. *Hum. Immunol*.,70(4):230-6.

Campillo, J. A.; Hernandez, R. L.; Banaclocha, H. M.; Bolarin, J. M.; Gimeno, L.; Mrowiec,A.; Lopez,M.; Heras, B. L.; Minquela, A.; Moya-Quiles, M. R.; Legas, I.; Frias-Iniesta, J. F.; Garcia-Alonso,A. M.; Alvarez-Lopez, M. R.; Martnez-Escribano, J. A. and Muro, M. (2015). MHC class 1chain –related gene a diversity in patients with cutaneous malignant melanoma from southeastern Spain. *Dis. Markers*, 6 pages.

Cantor, M. J.; Nickerson, P.; Bernstein, C. N. (2005) The role of cytokine gene polymorphisms in determining disease susceptibility and phenotype in inflammatory bowel disease. *Am. J. Gastroenterol*,100(5):1134-42.

Cao, F.; Lane,S.; Raniga, P. A.; Lu, Y.; Zhou, Z.; Ramon, K.; Chen, J. and Liu, H.(2006).The Flo8 transcription factor is essential for hyphal development and virulence in Canadida albcans. *Mol. Biol. Cell*, 17(1):295-307.

Celik,Y. Ü. D. ; Kiliç, M. Y. ; Törüner, M. ; Özen, S. C. ; Özkan, M. ; Soykan, I.; Çetinkaya, H. ; Ülker, A. ; Özden, A. and Bozdayi, A. M. (2006). Cytokine gene polymorphisms in Turkish patients with

inflammatory bowel disease. *Scandinavian Journal of Gastroenterology*, 14(5): 559-565.

Chamouard, P.; Monneaux, F.; Richert, Z.; Voegeli, A.C.; Lavaux, T.;Gaub, M.P.; Baumann, R.; Oudet, P. and Muller, S. (2009). Diminution of circulating CD4 + CD25 high T cells in naïve Crohn's disease. *Dig. Dis. Sci.*, 54: 2084-2093.

Chan, C.W.; Crafton, E.;Fan, H.N.; Flook, J.;Yoshimura, K.; Skarica, M., Brockstedt, D., Dubensky, T.W., Stins, M.F., Lanier, L.L., Pardoll, D.M. and Housseau, F. (2006). Interferonproducing killer dendritic cells provide a link between innate and adaptive immunity. *Nat. Med.*, 12: 207-213.

Chang, M. (2003). Dynamics of hepatitis C virus replication in human liver. *Am. J. Pathol.*, 163:433-444.

Chaudhry, S.; Khan, A.A. ; Butt, A. K.; Idrees, M.; Izhar, M. and Iqbal,H. A.(2011). Helicobacter pylori in dental plaque ; is it related to brushing frequency , plaque load and oral health status.*J. Col. Physicians, Surg.Pak.*, 21(10): 589-92.

Cobrin,G. M. and Abreu M T. (2005).Defects in mucosal immunity leading to Crohn's disease. *Immunological Reviews*, 206 (10): 277–295.

Conde, S. R.; Feitosa, R. N.; Freitas, F. B.; Hermes, R. B.; Demachki, S.; Araujo, M. T.; Soares, M. C.; Ishak, R. and Vallinoto, A. C. (2013). Association of cytokine gene polymorphisms and serum concentrations with the outcome of chronic hepatitis B. *Cytokine*, 61:940-944.

Connelly,J.T.; Dalbay ,M.T. and Thorpe, S.D. (2015) . Adipogenic differentiation of hMSCs is mediated by recruitment of IGF-1r onto the primary cilium associated with cilia elongation**.**.*Stem Cells*, 33 (6): 1952-1961.

Cope, A., LeFriec, G., Cardone, J. and Kemper, C. (2011). The Th1 life cycle: molecular control of IFN-γ to IL-10 switching. *Trends Immunol.*, 32: 278-286.

Corleto, V. D.; Cotesta, D.; Petramala, L.; Panzuto, F.; Pagnini, C.; Masoni, L.; Verrienti, A.; Delle Fave, G.;Filette, S.and Letizia, C.(2009).Late recurrence after surgical resection of a pancreatic tumor in von Hippel –Lindau disease. *JOP*., 10(5):562-5.

Corona,E.; Chen, R.; Sikora, M.; Morgan, A.; Patel, C.; Ramesh, A.; Bustamante, C. and Butte, A.J.(2013). Analysis of the genetic basis of disease in the context of worldwide human relationships and migration. *Pols. Genet*., 9(5):e1003447.

Cosnes, J. (2008). What is the link between the use of tobacco and IBD? *Inflamm. Bowel Dis*., 14(2): S14-S15.

Craggs, A.; West, S.; Curtis, A.; Welfare, M.; Hudson, M.; Donaldson, P.and Mansfield, J.(2001). Absence of a genetic association between IL-1RN and IL-1B gene polymorphisms in ulcerative colitis and crohn disease in multiple populations from northeast England. *Scand. J. Gastroenterol*., 36(11): 1173-8.

D' A lfonso, S. Rampi, M.; Rolando, V.; Giordano, M.; Momigliano,-Richiardi, P.(2000). New polymorphisms in the IL-10 promoter region. *Genes Immun*., 1(3): 231-3.

Dai, C.; Chuang, W. and Lee, L. (2008). Association between transforming growth factor–beta 1 polymorphism and virologic characteristics of chronic hepatitis C. *Transplant Res.,* 152:151-156.

Das, A.; Ellis, G.; Pallant, C.; Lopes, A.R.; Khanna, P. and Peppa, D. (2012). IL-10-producing regulatory B cells in the pathogenesis of chronic hepatitis B virus infection. *J. Immunol.*, 189:3925-3935.

De Almeida, J. P.; Hajjar, L. A.; Fukushima, J. T.; Nakamura, R. E.; Albertini, R. and Galas, F. R.(2013). Bronchial injury and pneumothorax after reintubation using an airway exchange catheter. *Braz. J. Anesthesiol*., 63(1): 107-9.

Del Zotto, B.; Mumolo, G.; Pronio, A.M.; Montesani, C.; Tersigni,R. and Boirivant, M.(2003). TGF-beta1 production in inflammatory

bowel disease: differing production patients in crohn s disease and ulcerative colitis. *Clin. Exp. Immunol*., 134(1): 120-6.

Dennis, D. T. UNIT 6.29 Cytokine, Growth Factor, and Chemokine Ligand Database. *Current Protocols in Immunology*,76-85.

Di Sabatino, A.;Biancheri, P.; Rovedatti, L.; Macdonald, T.T. and Corazza, G.R. (2012). New pathogenic paradigms in inflammatory bowel disease. *Inflamm. Bowel Dis*., 18: 368-371.

Dienz, O. and Rincon, M.(2009). The effects of IL-6 on CD4 T cell responses. *Clin Immunol*., 130(1):27-33.

Doan, T.; Melvold, R.; Viselli, S. and Waltenbaugh, C. (2008). Lppincotts IIIustrated Review: *Immunology, Lippincott Williams and Wilkins,* pp 67.

Dubinsky, M. C.; Dassopoulos, T.; Bentsen, J. L.; Martin, C. F.; Galanko, J.A.; Seidman, E.G.; Sandler, R. S. and Hanauer, S.B.(2013). Iccreased effectiveness of early therapy with anti-tumor necrosis factor-a vs an immunomodular in children with crohn s disease. *Gastroenterology*, 146(2):383-91.

Duerr, R.H. (2007). Genome-wide association studies herald a new era of rapid discoveries in inflammatory bowel disease research. *Gastroenterology* , 132: 2045-2049.

Ek, C.; Kim, I.R.;Chang, O.; Kang, D.;Nam, S.J.; Lee, J.E.; Lee, S.K., Im, Y.H.; Park, Y.H.; Yang, J.H.and Cho, J.(2014). Impact of chemotherapy-induced alopecia distress on body image, psychosocial well-being, and depression in breast cancer patients. *Psychooncology*, 23(10):1103-10.

Elango, T.; Banaganapalli, B.; Shaik, N.A.; Al-Abbasi,F.A .(2015). In-Silico Analysis of Inflammatory Bowel Disease (IBD) GWAS Loci to Novel Connections. *PLoS One*., 10(3): e0119420.

Fallahzadeh, M. K.; Roozbeh, J.; Geramizadeh, B. and Namazi, M. R.(2011). Interleukin-2 serum levels are elevated in patients with uremic pruritus: a novel finding with practical implications. *Nephrol. Dial. Transplant*, 26(10): 3338-44.

Fang, F.; Yu, L.; Zhong, Y. and Yao, L. (2010). TGFB1 509 C/T polymorphism and colorectal cancer risk: a meta-analysis. *Medicine,* 27:1324-1328.

Fantini, M.C.; Becker, C.; Tubbe, I.; Nikolaev. A.; Lehr, H.A.; Galle, P. and Neurath, M.F. (2006). Transforming growth factor beta induced FoxP3+ regulatory T cells suppress Th1 mediated experimental colitis. *Gut.,* 55: 671-680.

Fareed, M. and Afzal, M. (2013). Single nucleotide polymorphism in genome-wide association of human population: A tool for broad spectrum service. *Egypt, J. Med. Hum. Genet.,* 14:123-134.

Feagan, B.G.**;** Greenberg, G.R.; Wild, G.; Fedorak, R.N.; Paré, P.; McDonald, J.W.; Cohen, A.; Bitton, A.; Baker, J.; Dubé, R.; Landau, S.B.; Vandervoort, M.K. and Parikh, A.(2008). Treatment of active Crohn's disease with MLN0002, a humanized antibody to the alpha4beta7 integrin. *Clin. Gastroenterol. Hepatol.,* 6 (12):1370-7.

Ferreira, T. B.; Ferreira, A. L.; Carrondo, M. J. and Alves, P. M.(2005).Antibody response of autogenous splenic tissue implanted in the abdominal cavity of mice. *J. Clin. Oncol*., 29(12):1623-9.

Festen, E. M.; Franke, L. and Franke, L.(2013).Genetic analysis of innate immunity in crohn s disease and ulcerative colitis identifies two susceptibility loci harboring CARD9 and IL18RAP. *Am. J. Hum. Genet.,* 82:1202-10.

Fisman, E.Z.; Benderly, M.; Esper, R.J.; Behar. S.; Boyko, V.; Adler, Y.; Tanne, D.; Matas, Z.; Tenenbaum, A.(2006). Interleukin-6 and the risk of future cardiovascular events in patients with angina pectoris and/or healed myocardial infarction. *Am. J. Cardiol*., 98:14-18.

Frank, B.; Hoffimeister, M.; Kloop, N.; Lllg, T.; Chang-Claude, J.; Brenner, H.(2010).Polymorphisms in inflammatory pathway genes and their association with colorectal cancer risk. *Int. J. Cancer*., 15;127(12):2822-30.

Fursho,N.; Kubota, H.; Sato, K.; Akiyama, H. and Kawamura, Y.(2011). Analytical method for natamycin in wine using high-performance liquid chromatography.*Kokuritsu Lyakuin Shokuhin Esei Kenkyusho Hokoku*,(129):100-4.

Fuss, I.J.; Heller, F.; Boirivant, M.; Leon, F.; Yoshida, M.; Fichtner-Feigl, S.; Yang, Z.; Exley, M.; Kitani, A.; Blumberg, R.S.; Mannon, P. and Strober, W. (2004). Nonclassical CD1d-restricted NK T cells that produce IL-13 characterize an atypical Th2 response in ulcerative colitis. *J. Clin. Invest*., 113: 1490-1497.

Gao, J.W.; Zhang, A.Q.; Pan, W.; Yue, C. L.; Zeng, L.; Gu,W. and Jiang, J.(2014).Association between IL-6-174 G/C polymorphism and the risk of sepsis and mortality: a systematic review and meta-analysis.*Plos. One*., 10(3): e0118843.

Gattoni, A.; Parlato, A.; Vangieri, B.; Bresciani. M. and Derna, R.(2006).Interferon- gamma: biologic functions and HIV therapy (type I/II) (1 of 2 parts). *Clin.Ter*., 157(4): 377-86.

Geremia, A.; Biancheri, P.; Allan, P.; Corazza, G.R. and Di Sabatino, A. (2014). Innate and adaptive immunity in inflammatory bowel disease. *Autoimmun. Rev.,* 13: 3-10.

Gilmour, J. and Lavender, P. (2008). Control of IL-4 expression in T helper 1 and 2 cells. *Immunology*, 124:437–444.

Glas, J.; Seiderer, J.; Wagner, J.; Olszak, T.; Fries, C.; Tillack, C.; Friedrich, M .; Beigel, F.; Stallhofer, J.; Steib, C.; Wetzke, M .; Göke, B .; Ochsenkühn, T.; Diegelmann,J.; Czamara,D. and Brand, S. (2012).Analysis of *IL12B* Gene Variants in Inflammatory Bowel Disease. *PLoS One*., 7(3): e34349.

Gok,Y.; Akkoc, S.; Albayrak, S.; Akkurt, M. and Tabir, M. N.(2014). Crystal structure of 1,3-bis (4-methylbenzyl)-1H-1,3-benzinmidazol-3-ium bromide monohydrate. *Appl. Organomet. Chem.*, 28: 244-251.

Gonsky, R.; Deem, R.L.; Landers, C. J.; Haritunians, T. ;Yang, S.; Targan, S. R.(2014).IFNGrs 1861494 polymorphism is associated

with IBD disease severity and functional changes in both IFNG methylation and protein secretion. *Inflamm. Bowel, dis*., 20(10): 1794-801.

Gorelik, L. and Flavell, R. A. (2002).Transforming growth factor –beta in T-cell biology. *Nat. Rev. Immunol*., 2(1): 46-53.

Guerreiro, C. S.; Ferreira, P.; Tavares, L.; Santos, P.M.; Neves, M.; Brito, M. and Cravo, M.(2009).Fatty acids,IL-6,and TNF alpha polymorphisms: an example of nutrigenetics in crohn s disease. *Am. J. Gastroenterol*.,104(9): 2241-9.

Guerreiro,C. S.; Ferreira, P.; Travaes, L.; Santos, P. M.; Neves, M.; Brito, M. and Cravo, M.(2009).Fatty acids, IL-6,and TNFalpha polymorphisms: an example of nutrigenetics in Crohn s disease. *Am. J. Gastroenterol*., 104(9): 2241-9.

Halme, L.; Paavola-Sakki, P.; Turunen, U.; Lappalainen, M.; Farkkila, M. and Kontula, K. (2006). Family and twin studies in inflammatory bowel disease. *World, J. Gastroenerol*., 12: 3668-3672.

Heller, F.;Florian, P.;Bojarski, C.; Richter, J.; Christ, M.; Hillenbrand, B.; Mankertz, J.; Gitter, A.H.; Bürgel, N.; Fromm, M.'; Zeitz, M.; Fuss, I.; Strober, W. and Schulzke, J.D. (2005). Interleukin-13 is the key effector Th2 cytokine in ulcerative colitis that affects epithelial tight junctions, apoptosis, and cell restitution. *Gastroenterology*, 129: 550-564.

Hollegaard, M. V. and Bidwell, J. L. (2006). Cytokine gene polymorphism in human disease: on-line databases, Supplement 3. *Genes Immun.*, 7:269-276.

Hong,K. S.; Park,S. Y.; Whang, S.; Seo, S. Y.; Lee, D. H.; Kim, H. J.; Cho, J. Y.; Cho, Y. J.; Jang, W.and Kim, C. Y.(2008). Intravenous recombinant tissue plasminogen activator thrombolysis in a patient with acute ischemic stroke secondary to aortic dissection.*J. Cli. Neurol*., 5(1): 49-52.

Huebner,c.; Petermann ,I.; Lam, W.J.; Shelling, A.N. and Ferguson, L.R.(2010). Characterization of single-nucleotide polymorphisms relevant to inflammatory bowel disease in commonly used gastrointestinal cell lines. *Inflamm .Bowel .Dis*.,16(2):282-95.

Hume, H. A.; Robitaille, N. and Delvin, E. E.(2006).Newborn screening for sickle cell disease:A1988-2003 Quebec experience. Paediatr. Child Health, 11(4): 223-7.

Inohara, N.;Ogura, Y.;Fontalba ,A.; Gutierrez, O.; Pons, F.; Crespo, J.;Fukase, K.;Inamura, S.; Kusumoto, S.;Hashimoto, M.; Foster, S.J.; Moran, A.P.; Fernandez-Luna, J.L.and Nuñez, G.(2003). Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. *J. Biol. Chem.*, 278(8):5509-12.

Ito, R.; Shin-Ya, M., Kishida, T., Urano, A., Takada, R., Sakagami, J., Imanishi, J. , Kita, M., Ueda, Y. , Iwakura, Y., Kataoka, K., Okanoue, T. and Mazda, O. (2005). Interferon-gamma is causatively involved in experimental inflammatory bowel disease in mice. *Clin Exp Immunol*., 146**:** 330-338.

Johansson, C.; Rytter,E.; Nygren, J.; Vessby, B.;Basu,S.and Moller, L.(2008).Down-regulation of oxidative DNA lesions in human mononuclear cells after antioxidant supplementation correlates to increase of gamma –tocopherol. *Int. J. Vitam. Nutr.Res.*, 78(5):183-92.

Joossens, M.; De Preter, V.; Ballet, V.; Rutgeerts, P. and Vemeire, S.(2011).Effect of oligofructose –enriched inulin (OF-1N) on bacterial composition and disease activity of patients with crohn s disease .results from a double –blinded randomized. Gut.,61(6):958-63.

Jostins, L.; Ripke, S.; Weersma, R.K.; Duerr, R.H. McGovern, D.P.; Hui ,K.Y.;Lee, J.C.; Schumm, L.P.; Sharma, Y.; Anderson, C.A.; Essers, J.; Mitrovic, M.; Ning, K.; Cleynen, I.; Theatre, E.; Spain, S.L.; Raychaudhuri, S.; Goyette, P.; Wei, Z.;Abraham, C.;Achkar, J.P.; Ahmad, T.; Amininejad, L.; Ananthakrishnan, A.N.; Andersen V, Andrews, J.M.; Baidoo, L.;Balschun, T.; Bampton, P.A.; Bitton,

A.; Boucher, G.;Brand, S.; Büning, C.;Cohain, A., Cichon, S.; D'Amato, M.; De Jong, D.; Devaney, K.L.;Dubinsky, M.;Edwards, C.;Ellinghaus, D.; Ferguson, L.R.; Franchimont, D. ; Fransen, K.; Gearry, R.; Georges, M.;Gieger, C.; Glas, J.; Haritunians, T.; Hart, A.; Hawkey, C.; Hedl, M., Hu, X., Karlsen, T.H.; Kupcinskas, L.; Kugathasan, S.; Latiano, A.; Laukens, D.; Lawrance, I.C.; Lees, C.W.;Louis, E.; Mahy, G.;Mansfield, J.and Morgan, A.R. (2012).Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature*, 491(7422):119-24.

Kang, B.Y., Kim, E. and Kim, T.S. (2005). Regulatory mechanisms and their therapeutic implications of interleukin-12 production in immune cells. *Cell Signal*, 17: 665-673.

Karatzas, P.S.; Mantzaris, G. J.; Saeioleas,M. and Gazouil, M.(2014).DNA methylation profile of genes involved in inflammatory and autoimmunity in inflammatory bowel disease. *Medicine, (Baltimore)*, 93(28):e309.

Karimi, H.;Ramin, Y.; Khadijeh, A.; Bita, G.; Saman, N. and Seyed, A. (2013). Cytokine Gene Polymorphisms and Viral Hepatitis Infections in Kidney Transplant Recipients. *Spring.*, 44:114-120.

Kaur, G. and Mehra,N.(2012).Cytokine gene polymorphisms: methods of detection and biological significance. *Methods Mol. Biol*.882:549-68.

Khor, S.M.; Ng, S.L.; Lim, P.E. and Seng, C.E.(2011). The effects of nickel(II) and chromium(VI) on oxygen demand, nitrogen and metal removal in a sequencing batch reactor. *Environ Technol*. 33(16):1903-14.

Kim, S.W.; Kim, E.S.; Moon, C.M.; Park, J.J.; Kim, T.I. and Cheon, J. H. (2011). Genetic polymorphisms of IL-23R and IL-17A and novel insights into their associations with inflammatory bowel disease. *Gut*., 60: 1527-1536.

Koushik, K.;Bandi, N. ;Sundaram, S. and Kompella, U. B. (2004).Evidence for LHRH-receptor expression in human airway

epithelial (Calu-3) cells and its role in the transport of an LHRH agonist. *Pharm. Res*., 21(6):1034-46.

Kuballa, P.; Huett, A.; Rioux, J. D.; Daly, M. J. and Xavier, R. J.(2008). Impaired Autophagy of an intracellular pathogen induced by a crohn s disease associated ATG16L1variant. *Pols. One*.3(10): e3391.

Kugathasan, S. ;Saubermann, I. J. ;Smith, L. ;Kou, D.; Itoh, J.; Binion, D. G.; Levine, A. D.; Blumberg, R.S. and Fiocchi, C. (2007). Mucosal T-cell immunoregulation varies in early and late inflammatory bowel disease. *Gut.*, 56(12): 1696-705.

Kugathasan, S.; Sauberman, L.J.; Smith, L.; Kou, D.; Itoh, j.; Binion, D.G. ; Levine, A. D.; Blumberg, R. S.and Fiocchi, C.(2007).Mucosal T-cell immunoregulation varses in early and late inflammatory bowel disease. *Gut.*, 56(12):1696-705.

Lees, C.W. and Satsangi, J. (2009). Genetics of inflammatory bowel disease: implications for disease pathogenesis and natural history. *Expert. Rev. Gastroenterol. Hepatol*., 3: 513-534.

Lennard-Jones, J.E.(1989).Classification of inflammatory bowel disease.*Gut*.,1702: 2-6;discussion 16-9.

Leslie, K.; Myles, P.S.; Chan, M.T.; Paech, M.J.; Peyton, P.; Forbes, A. and McKenzie, D. E.(2008). Trial Group. Risk factors for severe postoperative nausea and vomiting in a randomized trial of nitrous oxide-based vs nitrous oxide-free anaesthesia. *Br. J. Anaesth*, 101(4):498-505.

Li, X. L.; Li, X. F.; Ning, B. A.; Wu, D. C.; Wang, H. Y.; Chen, X.; Ma, X. H.; Ou,G. R. and Gau, Z. X.(2009).Preparation of clenbuterol monoclonal antibody with subtractive immunization method. *Zhongguo Ying Yong Sheng Li Xue Za Zhi,Chinese*, 25(4): 573-6.

Liberek, K.; Ratajczak, E.; Zietkiewicz, S.(2008).Distinct activities of Escherichia coli small heat shock proteins IbpA and IbpB promote efficient protein disaggregation, J. Mol. Biol., 386(1): 178-89.

Loftus, E.V. (2004). Clinical epidemiology of inflammatory bowel disease: incidence, prevalence, and environmental influences. *Gastroenterology*, 126: 1504-1517.

Longhi, R.; Zembrzuski, V.; Basta, P. and Croda, J. (2013). Genetic polymorphism and immune response to tuberculosis in indigenous populations: a brief review. *Braz J Infect Dis.*, 17:363-368.

Lopez-Hernandez,R.; Campillo, J. A.; Martínez-Banaclocha, H.; Bolarín, J.M.; Gimeno, L.; Mrowiec, A.; López, M.; Las Heras, B.; Minguela, A.; Moya-Quiles, M. R. ; Legáz, I.; Frías-Iniesta, J.F. ; García-Alonso, A. M. ; Álvarez-López, M. R. ; Martínez-Escribano, J. A. and Muro, M. (2015). MHC Class I Chain-Related Gene A Diversity in Patients with Cutaneous Malignant Melanoma from Southeastern Spain. *Disease Markers*, Article, ID: 831864, 6 pages.

Ludwiczek,O.;Vannler, E.;Borqqraefe, I.;Kaser, A.;Simqmund, B.;Dinarello, C.A. and Tilq,H. (2004). Imbalance between interleukin-1 agonists; relationship to severity of inflammatory bowel disease. *Clin.Eux.Immunol*.,138(2):323-9.

Lv,Y.; Liang, R.; Hu, X.; Liu, Z.; Liao, X.; Lin, Y.; Yuan, C.; Liao, S.; Li, Q.; Zhang, J. and Li, Y.(2014).Combination of oxaliplatin and S-1 versus sorafenib alone in patients with advanced hepatocellular carcinoma.*Pharmazie.* 69(10): 759-63.

Macdonald, I. K.; Murray, A.; Healey, G.F.; Parsy-Kowalska, C. B.; Allen, G.; McElveen, J.; Robertsom, C.; Sewell, H.F.; Chapman, C. J.and Robertson, J. F.(2012): Application of a high throughput method of biomarker discovery to improvement of the early CDT-lung test. *Pols. One.* 7(12): e51002.

Maerten, P.; Shen, C.; Colpaert, S.; Liu, Z.; Bullens, DA.; van Assche, G.; Penninckx, F.; Geboes, K.; Vanham, G.; Rutgeerts, P. and Ceuppens, J. L. (2004). Involvement of interleukin 18 in Crohn's disease: evidence from in vitro analysis of human gut inflammatory cells and from experimental colitis models. *Clin. Exp. Immunol*., 135: 310-317.

Maes, T., Joos, G. and Brusselle, G. (2012). Targeting interleukin-4 in asthma: lost in translation. *Am .J. Respir. Cell Mol. Biol*., 47:261-270.

Magyari,L.; Kovesdi, E.; Sarios, P.; Javorhazy, A.;Sumegi,K. and Melegh, B.(2014). Interleukin and interleukin receptor gene polymorphisms in inflammatory bowel diseases susceptibility. *World, J. Gastroenterol.*, 20(12): 3208-22.

Mansour-Ghanaei, F.; Joukar,F.; Naghipour,M. R.; Forouhari,A. and Seyed Saadat,SM. (2015). Seven-day quintuple regimen as a rescue therapy for *Helicobacter pylori* eradication. *World J. Gastroenterol*., 14; 21(2): 661–666.

Marks, D.J.; Harbord, M.W.; MacAllister, R.; Rahman, F.Z.;Young, J.; Al-Lazikani, B.; Lees, W.; Novelli, M.; Bloom, S. and Segal, A.W. (2006). Defective acute inflammation in Crohn's disease: a clinical investigation. *Lancet*, 367: 668-678.

Marquez, C.; Clark, T.; Hare, C. B.; Johon, M.D. and Klsusner, J. D.(2008).Methamphetamine use, transmission risk behavior and internet use among HIV-infected patients in medical care, san Francisco. AIDS. Behav, 16(2): 396-403.

Martinez, A.N.; Mehra, S.; Kaushal D.(2008). Role of interleukin 6 in innate immunity to Mycobacterium tuberculosis infection. J. Infect Dis., 207(8):1253-61.

McCarroll, S.A.; Kuruvilla, F.G.; Korn ,J.M.; Cawley, S. ;Nemesh. J.; Wysoker, A.; Shapero, M.H.; de Bakker, P.I.; Maller, J.B.; Kirby, A.; Elliott, A.L.; Parkin, M.; Hubbell, E.; Webster, T.; Mei, R.; Veitch ,J.;Collins, P.J.; Handsaker, R.; Lincoln, S.; Nizzari, M.; Blume, J.; Jones, K.W. Rava ,R. Daly, M .J.;Gabriel, S.B.and Altshuler, D.(2008). Integrated detection and population-genetic analysis of SNPs and copy number variation. *Nat. Genet.*, 40(10):1166-74.

Meconi, S.; Vercellone, A.; Levillain, F.; Payre, B.; Al Saati,T.; Capilla, F.; Desreumaux,P.; Darfeuille-Michaud, A. and Altare, F.(2007).

Adherent-invasive Esccherichia coli isolated from crohn s disease patients induce granulomas in vitro.*Cell Microbiol*., 9(5): 1252-61.

Mehta,T.(2006).Promoting ALA as a source of omega-3.*Can. Fam. Physician,* 10; 52(10): 1205-1206.

Mittal, D.; Hudson, T. J.; Fortney, J. M.; Pyne, J. M. and Lu, L.(2007). Reduction of patiet-reported antidepressant side effects,by type of collaborative care. *Psychiatr. Serv*., 66(3):272-8.

Monteleone, G.; Kumberova, A.; Croft, N. M.; McKenzie, C.; Steer, H.W. and McDonald, T. T.(2001). Blocking Smad7 restores TGF-B1 signaling in chronic inflammatory bowel disease. *J. Clin. Invest*, 108(4):601-609.

Moon,J. I.; Kwon.C. H.; Joh,J. W.; Choi, G.S.; Jung,G.O.; Kim, J.M.; Shin, M.; Choi, S. J.; Kim,S. J. and Lee, S. K.(2012). Primary versus living donor liver transplantation forpatients with hepatocellular carcinoma: impact of microvascular invasion on survival.*Transplant Proc*., 44(2): 487-93.

Mootoo, A.; Stylianou, E.; Arias, M.A. and Reljic, R. (2009). TNF-alpha in tuberculosis: a cytokine with a split personality. *Inflamm Allergy Drug Targets.* 8:53-62.

Műzes, G.;Molnár, B.; Tulassay, Z. and Sipos, F. (2012). Changes of the cytokine profile in inflammatory bowel diseases. *World J. Gastroenterol*., 18: 5848-5861.

Nadeem,O. K.**;** Hazel, M. M.; Steven, L. N; Leach, T.; Daniel, A. L.; Munday, J. S. ; Huinao, K. ; Day, A. S. (2015) .Inflammatory Bowel Disease Therapies and Gut Function in a Colitis Mouse Model. *science. report*, 14: (585-585).

Naderi, N.; Farnood, A.; Dadaei, T.; Habibi, M.; Balaii, H.;Firouzi F.;Mahban A.; Soltani, M.;Zali, M.(2014).Association of tumor necrosis factor alpha gene polymorphisms with inflammatory bowel disease in Iran .*Iran J. Public Health*, 43(5):630-6.

Neurath, M. F.and Finotto, S.(2011).IL-6 Signaling in autoimmuity ,chronic inflammation –associated cancer.*Cytokine Growth Factor Rev.*, 22(2):83-9.

Ng, S.C.; Woodrow, S.; Patel, N.; Subhani, J. and Harbord, M. (2012). Role of genetic and environmental factors in British twins with inflammatory bowel disease. *Inflamm. Bowel Dis*., 18: 725-736.

Ning, S.; Zhao, Z.;Ye, J.; Wang, P.; Zhi, H.; Li, R.; Wang, T. and Li, X. (2014). Link SNP: a database of linking disease-associated SNPs to human large intergenic non-coding RNAs. BMC. *Bioinformatics.*, 15:152.

Nohara, Y.;Mizuno, M. Kamei, H.;Yamada, M.; Fuji,K.;Satoh, N.; Yasui,H.; Muramatsu, S.; Noda, Y.and Funuta, K.(2003). Prepartion of a manual treatment of pressure ulcers which draws attention to moisture of the affected part. *Gan. To Kaqaku Ryoho*., (1): 95-7.

O'Garra, A. and Vieira, P. (2004). Regulatory T cells and mechanisms of immune system control. *Nat. Med*., 10: 801-805.

Ogura,Y.; Bonen ,D.K.;Inohara, N.; Nicolae, D.L.; Chen, F.F.; Ramos R.; Britton, H.;Moran, T.; Karaliuskas, R.;Duerr, R.H., Achkar, J.P.; Brant, S.R.; Bayless, T.M.; Kirschner, B.S., Hanauer, S.B.;Nuñez, G.and Cho, J.H.(2001). A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature.*, 411(6837):603-6.

Olavesen, M. G.; Hampe, J.; Miza, M. M.; Saiz, R.; Lewis, C. M.; Bridger, S.; Teare, D.; Easton, D. E.; Hermann, T.; Scolt, G.; Hirst, J.; Sanderson, J.; Hodgson, S.; Lee, J.; Macpherson, A.; Schrreiber, S. Lennard-Jone, J. E.; Curran, M. E. and Mathew, C.G.(2000). Analysis of single-nucleotide polymorphisms in the interleukin-4 receptor gene for association with inflammatory bowel disease. *Immunogenetics*, 51(1): 1-7.

Olver, S.; Apte, S. ; Baz, A. and Kienzle, N. (2007). The duplicitous effects of interleukin-4 on tumour immunity: how can the same cytokine improve or impair control of tumour growth. *Tissue Antigens*, 69:293-298.

Oo, Y.;Mutimer, D. and Adams, D. (2012). Low-dose interleukin-2 and HCV-induced vasculitis. *Engl. J. Med*., 366:1353-1354.

Paavola, P.; Helio, T.; Kiuru, M.; Halme, L.; Turunen, U.; Terwilliger, J.;Karvonen, A. L.; Julkunen, R.; Niemela, S.; Nurmi, H.; Farkkilia, M. and Kontula, K.(2001).Genetic analysis in finnish families with inflammatory bowel disease supports linkage to chromosome 3p21.*Eur. J. Hum. Genet*., 9(5):328-34.

Palmieri, O.; Latiano, A.; Salvatori, E.; Valvano, M.R.; Bossa, F.; Latiano, T.; Corritore, G.; di Mauro, L.; Andriulli, A. and Annesec, V. (2010). The A2518G polymorphism of monocyte chemoattractant protein-1 is associated with Crohn's disease. *Am. J. Gastroentero*., 105: 1586-1594.

Peng, Q.; Li, H.; Lao, X.; Deng, Y.; Chen, Z.; Qin, X. and Li, S. (2014). Association of IL-2 polymorphisms and IL-2 serum levels with susceptibility to HBV-related hepatocellular carcinoma in a Chinese Zhuang population. *Infect. Genet. Evol.*, 27:375-381.

Peng, Y.; Lu, J.X.; Ye, S.Y.; Huang, Q. H.(2002).Studies of DNA D7S21 locus in Hebel Han population.*Yi. Chuan*., 24(2):223-6.

Petersen, A. M.;Petersen, B. K.(2006).The role of IL-6 in mediating the anti-inflammatory effects of exercise.J. Physiol. Pharmacol.,57 (10):43-51.

Pils, M. (2010). Monocytes /macrophages and/or neutrophils are the target of IL-10 in the LPS endotoxemia model. *Eur. J. Immunol.*, 40:443-448.

Pingzhao, H.; Aleixo, M.; Muise, X. X.; John, H.; Brumell, Mark S. Silverberg, H. and Wei X.(2013). Association Between a Multi-Locus Genetic Risk Score and Inflammatory Bowel Disease. *Bioinformatics and Biology Insights*, 7: 143-152.

Qin, Z.; Clements, T.; Wang, L.; Khatri, M.; Pilai, S. P.; Zhang, Y.; Lejeune, J. T. and Lee, C. W.(2010).Detection of influenza viral

gene in European starling and experimental infection. *Influenza Other Respir. Viruses.*, 5(4): 268-75.

Rai, M.; Graeve, T.; Twardziok, S. and Schmidt, M. (2011). Evidence for regulated interleukin-4 expression in chondrocyte-scaffolds under in vitro inflammatory conditions. *PLoS One*., 6:e25749.

Riha, R.; Yang, A.; Rabnott, G.; Tunnicliffe, A.; Fong, K. and Zimmerman, P. (2004). Cytokine gene polymorphisms in idiopathic pulmonary fibrosis. *J. Intern Med.,* 34:126-129.

Rivera-Chave,F. A.;Peters- Hybki,D.L.; Barber, R. C.and Okeefe, G. E. (2003). Interleukin-6 promoter haplotypes and interleukin-6 cytokine resposes, Shock, 20(3): 218-23.

Rodrigues-Peralvarez, M. L.;Garcia-Sanchez, V.; Villar,-Pastor, C.M.; Gonzales, R.; Iglesias-Flores, E.;Muntane, J. and Gomez-Camacho, F. (2012).Role of serum cytokine profile in ulcerative colitis assessment. *Inflamm. Bowel Dis.*, 18(10): 1864-71.

Rodriquez-Peralvarez, M.; Luong, T.V.; Andreana, L.; Meyer, T.; Dhillon, A. P. and Burroughs, A. K.(2013).Asystematic review of microvascular invasion in hepatocellular carcinoma: diagnostic and prognostic variability.*Ann. Surg.Oncol*., 20(1): 325-39.

Ryan, M.D.; Wattchow, D.;Walker, M. and Hakendorf,P.(2004). Adhesional small bowelobstruction after colorectal surgery. *ANZ. J. Surq.*, 74(11): 1010-2.

Saake, M.; Breuer, L.; Gölitz, P.; Köhrmann, M.; Schwab, S.; Dörfler, A.; Kloska, S.(2014). Clinical/perfusion CT CBV mismatch as prognostic factor in intraarterial thrombectomy in acute anterior circulation stroke. *Clin. Neurol. Neurosurg., 121:39-45.*

Sabbah, A. ; Chang, T.H.; Harnack, R.; Frohlich, V.; Tominaga, K.; Dube, P.H.; Xiang, Y.and Bose, S.(2009). Activation of innate immune antiviral responses by Nod2. *Nat. Immunol.*, 10(10): 1073-80.

Sagiv-Friedgut,K.; Karban, A.; Weiss, B.; Shamir, R.; Shaoul, R.; Bujanover, Y.; Reif, S.; Boaz, M.; Shani, I.; Levine, A.;Levine, A. and Leshinsky-Silver,E.(2010). Early- onset crohn disease is associated with male sex and a polymorphism in the IL-6promoter. *J. Pediatr. Gastroenterol. Nutr.* , 50(1): 22-6.

Sainz, J.; Perez, E.; Gomez-Lopera, S. and Jurado, M.(2008). IL1gene cluster polymorphisms and its haplotypes may predict the risk to develop invasive pulmonary aspergillosis and modulate C-reactive protein level. *J. Clin. Immunol*., 28(5): 473-85.

Salonen,A.; de Vos, W. M. and Palva, A.(2010). Gastrointestinal microbiota in irritable bowel syndrome: present state and perspectives. *Microbiology, 156(11):* 3205-15.

Sambrook, P. N.; Day, R. O. ;Graham, G.G.; Bieri, D. ;Brown, M.; Cairns, D. ; Harris, G.; Hounshell, J.; Plat-Hepwoth, S. and Reeve, R.(1989).Concentration- response relationships for salicylate induced ototoxicity in normal volunteers. *Br. J. Clin. Pharmacol*., 28(6): 695-702.

Sanchez-Munoz, F.; Dominguez-Lopez, A. and Yamamoto-Furusho, J.K. (2008).Role of cytokines in inflammatory bowel disease. *World, J. Gastroenterol*., 14: 4280-4288.

Saraiva, M. and O'Garra, A. (2010). The regulation of IL-10 production by immune cells. *Nat. Rev. Immunol*., 10: 170–181.

Sawczenko, A.; Azooz, O.; Paraszczuk, J.; Idestrom, M.; Croft, N. M. ;Savage, M.O.; Ballinger, A. B. and Sanderson, I. R.(2005). Intestinal inflammation –induced growth retardation acts through IL-6 in rats and depends on the -174 IL-6 G/C polymorphism in chilsren. *Proc. Nat. Acad. Sci. U S A.*, 102(37): 13260-5.

Scheller,H.; Lehmann, K.M.; Devigus, A.;Igiel, C.; Wentaschek, S.; Azar, M. S.(2011).Rapeatability of color-measuring devices. *Eur. J. Esthet. Dent.*, 6(4): 428-35.

Schmidt, C.; Giese, T.; Ludwig, B.; Mueller-Molaian, I.; Marth, T.; Zeuzem, S.; Meuer, S.C. and Stallmach, A. (2005). Expression of

interleukin-12-related cytokine transcripts in inflammatory bowel disease: elevated interleukin-23p19 and interleukin-27p28 in Crohn's disease but not in ulcerative colitis. *Inflamm. Bowel Dis*., 11:16-23.

Schoenborn, J. and Wilson, C. (2007). Regulation of interferon-gamma during innate and adaptive immune responses. *Adv Immunol.*, 96:41-101.

Schroder, K.; Hertzog, P.; Ravasi, T. and Hume, D. (2004). Interferon-gamma: an overview of signals, mechanisms and functions. *J. Leukoc. Biol.*, 75:163-189.

Sharma, P.; Chakraborty, R.; Wang, L.; Min, B.; Tremblay, M. L.; Kawahara, T.; Lambeth, J. D. and Haque, S. J. (2008). Redox regulation of Interleukin-4 signaling. *Immunity*, 29: 551-564.

Shaw P. J.; Lamkanfi, M. and Kanneganti, T. D. (2011). NOD-like receptor (NLR) signaling beyond the inflammasome. *Eur. J. Immunol.* 40, 624–627.

Shi, G. H.; Zong, L.; Chen, P. and Wang, L.(2011).Gastric cavemous hemangioma: Arare case with upper gastrointestinal bleeding. *Oncol. Lett*. 2(6):1073-1075.

Singh, R .; Bollina, V.; Higgins, E. E.; Clarke, W. E.; Eynck, Ch. C.; Gugel, R. S .; Snowdon, R.; Isobel A. ; Parkin, P. P. (2015). Single-nucleotide polymorphism identification and genotyping in *Camelina sativa. Molecular, Breeding*, 35:35.

Strober, W. and Fuss, I. J. (2011). Proinflammatory cytokines in the pathogenesis of inflammatory bowel diseases. *Gastroenterology*, 140: 1756-1767.

Sventoraityte, J.; Zvirbliene, A.; Kudelis, G.; Zalinkevicius, R. ; Zvirbliene A.; Praskevicius, A.; Kupcinskas, L. and Tamosiunas, V.(2008).Immune system alterations in patients with inflammatory bowel disease during remission. *Medicina. (Kaunas*), 44(1): 27-33.

Swardfager, W.; Lanctôt, K.; Rothenburg, L. ;Wong, A.; Cappell, J. and Herrmann, N. (2010). A meta-analysis of cytokines in Alzheimer's disease. *Biol. Psychiatry.,* 68:930-941.

Sykora, J.; Subrt, I.; Didek, P.; Siala, K.; Schwarz, J.; Machalova, V.;Varvarovska, J.; Pazdiora, P.; Pozler, O. and Stozicky, F.(2006). Cytokine tumor necrosis factor – alpha apromoter gene polymorphism at position -208 G→A and pediatric inflammatory bowel disease: implication in ulcerative colitis and crohn s disease. *J. Pediatr. Gastrsoentero. Nut*., 42(5): 479-87.

Tachdjian, R.; Mathias, C.; Al Khatib, S.; Bryce, P. J.; Kim, H.S.; Blaeser, F.; O Connor, B.D.; Rzmkiewicz, D.; Chen, A.; Holtzman, M. J.; Hershey, G. K.;Garm, H.; Harb, H.;Renz, H.; Oettgen, H.C. and Chatila, T.A.(2009). Pathogenicity of a disease- associated human IL-4 receptor allele in experimental asthma. *J. Exp. Med*., 206(10): 2191-204.

Takac, B.; Mihalijevic, S.; Stefanic, M.; Glavas-Obrovac, L.; Kibel, A. and Samardzija,M.(2014).Importance interleukin 6 in pathogenesis of inflammatory bowel disease. *Coll. Antropol*., 38(2): 659-64.

Takatori, H.; Kanno, Y.; Watford, W.T.; Tato, C.M.; Weiss, G.; Ivanov I.I.; Littman, D.R. and O'Shea, J. J.(2009). Lymphoid tissue inducer-like cells are an innate source of IL-17 and IL-22. *J. Exp. Med.*, 206(1):35-41.

Tamizifar, B.; Lankarani, K. B.; Naeimi, S.; Rismankar-Zadeh, M.;Taghavi, A.;Ghaderi, A.(2008).Promoter polymorphism of transforming growth factor –beta 1 gene and ulcerative colitis. *World, J. Gastroenterol.*, 14(2): 243-7.

Targan, S.R.; Landers, C.J.; Yang, H.; Lodes, M.J.; Cong, Y.; Papadakis, K.A.; Vasiliauskas, E.; Elson, C.O. and Hershberg RM. (2005). Antibodies to CBir1 flagellin define a unique response that is associated independently with complicated Crohnäs disease. *Gastroenterology*, 128: 2020-2028.

Thelemann, C.; Eren, R. O.; Coutaz, M.; Brasseit, J.; Bouzourene, H.; Rosa, M.; Duval, A.; Lavanchy, C.; Mack, V.; Mueller, C.; Reith,

W. and Acha-Orbea, H.(2014). Interferon-Y induced expression of MHC class II on intestinal epithelial cells and protects mice from colitis. *Polo. One.,* 9(1): e86844.

Thia, K.T.; Loftus, E.V.; Sandborn, W.J. and Yang, S.K.(2008). An update on the epidemiology of inflammatory bowel disease in Asia. *Am. J. Gastroenterol.,* 103(12): 3167-82.

Thomas, M. J. ;Brennan, V. M. ; Chapel, H. H.(1993).Rapid subcutaneous immunoglobulin infusions in children. Lancet., 342(8884):1432-3.

Torkvist, L.; Noble, C. L.; Lördal, M.; Sjöqvist, U.; Lindforss, U.; Nimmo, E.R.; Russell, R. K.; Löfberg, R. and Satsangi, J.(2006). Contribution of CARD15 variants in determining susceptibility to Crohn's disease in Sweden. *Scand . J. Gastroenterol*., 41(6):700-5.

Trajkov, D.; Trajchevska, M.; Arsov,T.; Petlichkovska, A.; Strezova, A.; Efinska-Madenovska,O.; Sandevski, A. and Spirosk, M.(2009). Association between 22 cytokin gene polymorphisms and dilated cardiomyopathy in macedonian patients. Kardiol. *Pol.,* 67(11):1237-47.

Travassos, L. H.; Cameiro, L. A.; Ramieet, M.; Hussey, S.; Kim, Y.G.; Magalhaes, J. G.; Yuan, L.; Soares, F.; Chea, E.; Le Bourhis, L.; Boneca, I. G.; Allaoui, A. ; Jones, N. L.; Nunes, G.; Girardin S. E.and Philott, D. J.(2010).NOD1 and NOD2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. *Nat. Immunol*., 11(1): 55-62.

Tremelling, M.; Cummings, F. ;Fisher, S. A. ;Mansfleld, J.; Gwilliam, R. ; Keniry, A.; Nimmo, E. R.; Drummond, H.; Onnie, C. M. ; Prescott, N. J. ; Sanserson, J.; Bredin, C. ; Forbes, A. ; Lewis, C. M. ; Cardon, L. ; Deloukas, P.; Jewell, D. ;Mathew, C. G. ; Parkes, M. and Satsangi, J.(2007). IL-23Rvariation determines susceptibility but not disease phenotype in inflammatory bowel disease. *Gastoenterology,* 132(5): 1657-64.

Trifunovic, N **.;** Milutinovic**,** V.**;** Salom**,** J. and Kos**,** A.(2015). Paradigm Shift in Big Data Super Computing: Data Flow vs. Control Flow . *Journal of Big Data,* 2:4.

Trinchieri, G. (2003). Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat. Rev. Immunol.,* 3: 133-146.

Turgut , T.; Adel, Shalata, and Robert J. Desnick.(2004). Crohn Disease: Frequency and Nature of CARD15 Mutations in Ashkenazi and Sephardi/Oriental Jewish Families. *American Journal of Human Genetics.*,74(4):623-636.

Ulger, M.; Emekdas, G.; Aslan, G.; Tas, D.; Ilvan, A.; Tezcan, S.; Calıkoglu, M.; Erdal, M. and Kartaloglu, Z. (2014). Determination of the cytokine gene polymorphism and genetic susceptibility in tuberculosis patients. *Mikrobiyol , Bul.,* 47: 250-264.

Van de Veerdonk,F. L. and Netea, M. G.(2013).New insights in the Immunobiology of IL-1 family members. *Front. Immunol*. 8(4):167.

Vermeirs, S.; Van Assche, G. and Rutgeerts, P.(2006).Focus on mechanisms of inflammation in flammatory bowel disease sites of inhibition: current and therapies. *Gastroenterol. Clin. North. Am*., 35(4):743-56.

Wang, Z. W. ; Feng, J. I. ; Teng, W. J. ; Yuan, X. G. and Ye, X. M. (2011) Risk factors and gene polymorphisms of inflammatory bowel disease in population of Zhejiang, China. *World, J. Gastroenterol. 17(1): 118-122.*

Warle, M.C.; Farhan, A.; Metselaar, H.J.; Hop, W.C.; Perrey, C.; Zondervan, P.E. ; Zondervan, P.E.; Kap, M.; Kwekkeboom, J.; Ijzermans, J.N.; Tilanus. H.W.; Pravica, V.; Hutchinson, I.V. and Bouma, G. J. (2008). Are cytokine gene polymorphisms related to in vitro cytokine production profiles? *Liver. Transpl.*, 9: 170-181.

Watford, W.T., Hissong, B.D., Bream, J.H., Kanno, Y., Muul, L. and O'Shea, J. J. (2004). Signaling by IL-12 and IL-23 and the immunoregulatory roles of STAT4. *Immunol. Rev.,* 202: 139-156.

Wehkamp J.; Harder, J.; Weichenthal, M.; Schwab, M.; Schäffeler, E.; Schlee, M.; Herrlinger, K.R.; Stallmach, A.; Noack, F.; Fritz, P.; Schröder, J.M.; Bevins, C.L.; Fellermann, K.and Stange, E.F. (2004). NOD2 (CARD15) mutations in Crohn's disease are associated with diminished mucosal alpha-defensin expression. *Gut.* 53(11):1658-64.

Wilke,R. A.; Ramsey, L. B.and Johnson, S. G.(2011).The clinical pharmacogenomics implementation consortium: CPIC guideline for SLCO1B1 and simvastatin- induced myopathy. *Clin. Pharm. Therapeutics,* 92: 112-117.

Wrenshall, L. E.; Clabaugh, S. E.; Cool, D. R.; Arumgan, P.; Grunwald, W. C.; Smith, D. R.; Liu, G.C. and Miller, J. D.(2014).Identification of a cytotoxic from of dimeric interleukin -2 in murine tissues. *Pols. One*., 9(7): e 102191.

Yamamoto-Furusho,J. K.;Picazo-Ferrera, K.; Santiago-Hermandez, J. and Bustamante-Quan, Y.(2011).The role of appendectomy in the ulcerative colitis in Mexico. Rev. Gastroenterol. Mex., 76(4):316-21.

Yamazaki, K.; Watanabe, A.; Kinugasa, Y.; Tsukamoto, S.; Yamaguchi, T.; Shiomi, A.; Tsushima, T.; Yokota, A.; Machida, N.; Fukutomi, A.;Onozawa, Y. and Yasui, H.(2009).Influence of primary tumor resection onsurvival in asymptomatic patients with incurable stage Iv colorectal cancer. *Int. J. Clin. Oncol*., 19(6):1037-42.

Yang, C.; Dou, B.; Chai, Y.; Yuan, R. and Xiang, Y.(2015).Target-induced reconfiguration of DNA probes for recycling amplification and signal-on electrochemical detection of hereditary tyrosinemia type 1 gene. *Analyst*., 4(1): 63-66.

Zhan, P., Wang, J. and Wei, S. (2011). TNF-308 gene polymorphism is associated with COPD risk among Asians: meta-analysis of data for 6.118 subjects. *Mol. Biol. Rep.,* 38: 219-227.

Zhang, Y.Z. and Li, Y.Y. (2014). Inflammatory bowel disease: Pathogenesis. *World, J. Gastroenterol*., 20: 91-99.

Ziaee, N. ;Moradi, J.; Abbasipour, F.; Zaringhalam, J.; Maleki, B.; Khodadoustan, A.(2014).Anethole a medicinal plant compound decreases the production of pro-inflammatory TNF-a and IL-1B in a Rat model of LPS- induced periodontitis. *Iran. J. Pharm. Res.*13(4):1319-25.

Zipperlen, P.; Nairz, K.; Rimann, I.; Basler, K.; Hafen, E.; Hengartner, M. and Hajnal, A.(2005).Auniversal method for automated gene mapping. *Genome, Biology*, 6(2):R19.

Zou, S.; Zhang, A.; Zhou, X.;Zhao, H.; Guan, Y.; Yan.G. L.; Ma, C. W.; Liu. Q. and Wang, X.(2014). Correction Rapidly improved determination of metabolites from biological data sets using the high-efficient Transomics tool. *Mol. Biosyst*. 11(1):317.

Patient Code:

Appendix

Patient's Information Sheet and Laboratory Investigations

- **Personal Information**

  Name:

  Gender:

  Age:

  Tests:

  ESR:

  RBCs:

  WBCs:

  Hb:

  PCV:

  General Urine Examination:

  General Stool Examination:

  Others:

- **Final Diagnosis**: **Crohn’s Disease** ( )

  Ulcerative Colitis ( )

الخلاصـــــــة

يعد مرض المعي الالتهابي مشكلة صحية عالمية , والتي تنطوي على شكلين وهما: رئيسين هما: داء كرون والتهاب القولون التقرحي. ويعتبر الاستعداد الوراثي المناعي هو احد عوامل الخطر للمرض .الحر كيات الخلوية هي من بين هذه العوامل , لذلك تم التحري في هذه الدراسة عن تعدد الأشكال للنيوكليوتيدة المفردة لثلاثة عشر من جينات الحر كيات الخلوية مع مستقبلاتها وهي (البين ابيضاض 1- ألفا و البين ابيضاض 1- بيتا و البين ابيضاض 1ومستقبلاته و البين ابيضاض 2 والبين ابيضاض 4 و البين ابيضاض 4 ومستقبله و البين ابيضاض 6 و البين ابيضاض 10 و البين ابيضاض 12- بيتا وانترفيرون كاما وعامل التنخر الورمي وعامل تحول النمو – بيتا), في عينات من المرضى العرب العراقيين, (34مريض بداء الكرون و66مريض بالتهاب القولون التقرحي , وتم التحري عن المرضى في مستشفى الكندي التعليمي ومستشفى اليرموك التعليمي ومستشفى الزعفرانية العام في بغداد للتشخيص والعلاج خلال الفترة (أب 2013 إلى تشرين الأول 2014) مقارنة بعينات السيطرة (43شخص).

وتم التوصل الى النتائج التالية من الدراسة الحالية :

1. جين البين ابيضاض-1 الفا في الموقع (-889): ازداد تردد النمط الجيني CC والاليل C بشكل كبير لدى مرضى داء الكرون (58,8 و73,5% على التوالي), وفي مرض التهاب القولون التقرحي (54,6 و71,2% على التوالي), مقارنة بالسيطرة (25,5 و40,7% على التوالي) ,في المقابل النمط الجيني TT والاليل T الترددات انخفضت بشكل ملحوظ لدى مرضى داء الكرون (11,8 و26,5 % على التوالي), ومرضى التهاب القولون التقرحي (12,1 و28,8 % على التوالي) مقارنة بمجموعة السيطرة (44,2 و59,3 % على التوالي).

2. جين البين ابيضاض-1 بيتا في الموقع (-511): لوحظ زيادة تردد النمط الجيني TC بشكل ملحوظ لدى المرضى الذين يعانون من التهاب القولون التقرحي مقارنة بمجموعة السيطرة (63,6 و39,5 %) تحت احتمالية تساوي (0,018) , بينما انخفض تردد النمط الجيني CC (6,1 مقابل 32,2 %), عند احتمالية تساوي (0,061). وقد لوحظ عدم وجود اختلافات معنوية لدى المرضى الذين يعانون من داء كرون .

3. جين البين ابيضاض-1 ومستقبله- الاول في الموقع (-1970): ازداد تردد النمط الجيني TT والاليل T (47,1 و67,7 % على التوالي) بشكل ملحوظ تحت احتمالية تساوي (0,026

و8,6 ×10^{-5} على التوالي) في المرضى الذين يعانون من داء كرون مقارنة بمجموعة السيطرة (20,9 و34,97 % على التوالي). في المقابل , النمط الجيني CC (11,8 مقابل 51,2 %) , والاليل C (32,4 مقابل 65,1 %) كانت الترددات منخفضة بشكل ملحوظ وتحت احتمالية تساوي (2,8 ×10^{-4} و 8,6 ×10^{-5} على التوالي), لدى مرضى التهاب القولون التقرحي اظهر زيادة كبيرة في ترددات النمط الجيني TC (48,5 ومقابل 27,9 %) وتحت احتمالية تساوي (0,045) والاليل T (63,6 مقابل 34,9 %) وتحت احتمالية تساوي (5,1 ×10^{-5}) مقارنة بمجموعة السيطرة , كما هو الحال في داء الكرون. إما لمرضى التهاب القولون التقرحي اظهر التردد انخفاضا ملحوظا للنمط الجيني CC (12,1 مقابل 51,2 %) وتحت احتمالية تساوي (1,7 × 10^{-5}) , والاليل C (36,4 مقابل 65,1 %) وتحت احتمالية تساوي (8,6 ×10^{-5}).

4. جين البين ابيضاض 2 في الموقع (+166): من بين المرضى لداء الكرون كانت ترددات النمط الجيني TT (41,2 مقابل 6,9 %), والاليل T (64,7 مقابل 34,0 %) مرتفعة بشكل كبير مقارنة مع مجموعة السيطرة تحت احتمالية تساوي (0,001 و0,009 على التوالي) في المقابل النمط الجيني TG (47,1 مقابل 72,1 %) وتحت احتمالية تساوي (0,009) , كانت الترددات منخفضة بشكل ملحوظ تقريبا .

5. جين البين ابيضاض 4 في الموقع (-590): لوحظ أن ترددات النمط الجيني TT (52,9 مقابل 11,6 %) , وتحت احتمالية تساوي (1,2 ×10^{-4}) والاليل T (70,6 مقابل 24,4 %) وتحت احنمالية تساوي (1,6 ×10^{-8}) سجل زيادة كبيرة في المرضى الذين يعانون من داء كرون مقارنة بمجموعة السيطرة بالمقابل النمط الجيني CC (11,8 مقابل 62,8 %) وتحت احتمالية (5,6× 10 $^{-6}$) والاليل C (29,4 مقابل 75,6 %) وتحت احتمالية (1,6 ×10^{-8}) كانت الترددات منخفضة بشكل ملحوظ في حالة التهاب القولون التقرحي. إما ترددات النمط الجيني TC (69,7 مقابل 52,6 %) وتحت احتمالية تساوي (1,1 ×10^{-5}) سجل زيادة معنوية لدى المرضى. في حين الاليل T (62,1 مقابل 24,4 %) , وتحت احتمالية تساوي (4,6 ×10^{-8}) مقابل النمط الجيني CC (3,5 مقابل 62,8 %) وتحت احتمالية تساوي (2,5 ×10^{-12}) والاليل C (37,9 مقابل 75,6 %) وتحت احتمالية وتساوي (1,6 ×10^{-8}) هذه الترددات سجلت انخفاضا ملحوظا لدى المرضى.

6. جين البين ابيضاض 4 ومستقبله في الموقع (+ 1902): لوحظ زيادة في ترددات النمط الجيني GG (41,1 مقابل 11,6 %) , والاليل G (55,9 مقابل 37,2 %) بشكل ملحوظ لدى المرضى الذين يعانون من داء الكرون مقارنة مع مجموعة السيطرة وتحت احتمالية

تساوي (0,001 و0,023). في المقابل النمط الجيني GA (17,7 مقابل 51,1 %) وتحت احتمالية تساوي (0,004), والاليل A (44,1 مقابل 62,8 %) وتحت احتمالية تساوي (0.023) سجل انخفاضا معنويا لدى المرضى الذين يعانون من التهاب القولون التقرحي . النمط الجيني GG (57,6 مقابل 11,6 %) وكذلك الاليل G (75,8 مقابل 37,2 %) كانت هناك زيادة معنوية وتحت احتمالية تساوي (1,0 ×10^{-6} و1,9 ×10^{-8} على التوالي) .بينما النمط الجيني AA (6,1 مقابل 37,2 %) وتحت احتمالية تساوي (7,0 ×10^{-5}) والاليل A (24,2 مقابل 62,8 %) وتحت احتمالية تساوي (1,9 ×10^{-8}) سجل انخفاضا معنويا.

7. جين البين ابيضاض 6 في الموقع (+565): عند مقارنة المرضى مع مجموعة السيطرة وجد بأن النمط الجيني GG سجل زيادة معنوية كبيرة لدى مرضى داء الكرون (70,6 مقابل 13,9 %) وتحت احتمالية تساوي (1,5 ×10^{-8}) كما في مرضى التهاب القولون التقرحي (69,7 مقابل 13,9 %) عند احتمالية تساوي 4,4 ×10^{-7}), وأيضا لوحظت في الاليل G بالمقابل انخفضت ترددات النمط الجيني GC بشكل كبير لدى مرضى داء الكرون (23,5 مقابل 76,7 %) وتحت احتمالية تساوي (3,7 ×10^{-6}) , ولدى مرضى التهاب القولون التقرحي (24,2 مقابل 76,7 %) وتحت احتمالية تساوي (7,0 ×10^{-6}).

8. جين البين ابيضاض-12بيتا في الموقع (-1188): سجل الاليل A زيادة معنوية كبيرة لدى مرضى الكرون مقارنة مع مجموعة السيطرة (67,7 مقابل 47,7 %) وتحت احتمالية تساوي (0,015). بينما انخفض تردد الاليل C بشكل ملحوظ (32,4 مقابل 52,3 %) وتحت احتمالية (0,015) بينما لدى التهاب القولون التقرحي المتباين النمط الجيني AC لوحظ زيادة معنوية كبيرة مقارنة مع مجموعة السيطرة (66,7 مقابل 30,2 %) تحت احتمالية تساوي (3,6 ×10^{-4}). بينما سجل النمط الجيني CC انخفاضا معنويا كبيرا (6,1 مقابل 37,2 %) وتحت احتمالية تساوي (7,0 ×10^{-5}).

9. جين الانترفيرون كاما في الموقع (+874): اظهر النمط الجيني AT زيادة معنوية ملحوظة في التردد لدى مرض داء الكرون مقارنة مع مجموعة السيطرة (58,8 مقابل 30.2 %) وتحت احتمالية تساوي (0,020). بينما النمط الجيني TT سجل انخفاضا معنويا (11,8 مقابل 39,5 %) وتحت احتمالية (0,009). لدى مرضى التهاب القولون التقرحي . أما النمط الجيني AA (54,6 مقابل 30,2 %) تحت احتمالية تساوي (0,018) والاليل A (74,2 مقابل 45,4 %) وتحت احتمالية تساوي (2,7 ×10^{-5}) سجلا انخفاضا معنويا ملحوظا.

10. جين عامل التنخر الورمي –الفا في الموقع(-308): عند المقارنة لمرضى داء كرون مع مجموعة السيطرة لم تكشف عن تفاوت معنوي , في حين اظهر مرض التهاب القولون

التقرحي فرقا معنويا تحت احتمالية (0,006), وزيادة في تردد الاليل G (71,2 مقابل 52,3 %) وانخفاضا في تردد الاليل A (28,8 مقابل 47,7 %).

11. جين البين ابيضاض -1 ومستقبله الاول في الموقع (- 11100) , و جين البين ابيضاض- 10 في الموقع (-1082) وعامل تحول النمو- بيتا (الشفرة 25) عند المقارنة بين المرضى ومجموعة السيطرة لم تسجل فروقا معنوية في الأنماط االجينيه أو الاليلات.

أن النتائج المعروضة لتعدد الأشكال الوراثي لثلاثة عشر جين من الحركيات الخلوية هي الأولى في مرضى عراقيين، وتشير هذه النتائج الى دورهذه الأشكال الوراثية في إمراضية داء كرون والتهاب القولون التقرحي، والتي مهدت الطريق لمزيد من الدراسات في هذا المجال.

جامعــة بغـــداد

تعدد الأشكال للنيوكليوتيدة المفردة لبعض الحركيات الخلوية في مرض المعي الالتهابي لدى مرضى عراقيين

أطروحة تقدمت بها

إبتســـام بـــــداي حســـــان الكـــناني

ماجستير علوم الحياة /كلية التربية (ابن الهيثم), جامعة بغداد,(2005)م.

إلى

مجلس كلية العلوم للبنات/جامعة بغداد كجزء من متطلبات نيل درجة الدكتوراه فلسفة في علوم الحياة / الوراثة

بأشـــــراف

أ.م .د. بتــول علـــي شـــهاب

أ. د. علـــي حسين ادحيـــة

تموز 2015م

شوال 1436هـ