POLYMORPHIC ELEMENTS OF THE HUMAN IL-6 GENE IN GASTRIC CANCER

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NIOVE E. JORDANIDES BSc (Hons)

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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SCOTLAND

This thesis is dedicated
to my family
for their encouragement and
unwavering belief in me

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DECLARATION

The work on this thesis is based upon my own independent work except where acknowledged.

NIOVE E. JORDANIDES BSc (HONS)

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ABBREVIATIONS

AA	Amino acid
BO	Barrett's Oesophagus
EDTA	Ethylenedianinetetra-acetic acid disodium salt
GC	Gastric Carcinoma
GORD	Gastroesophagial reflux disease
HGF	Hepatocyte grpwth factor
IFN-β	Interferon-β
Ig	Immunoglobulin
IL-	Interleukin
MIN	Microsatellite instability
mRNA	Messenger RNA
NF-κB	Nuclear factor κB
PCR	Polymerase chain reaction
RA	Rheumatoid arthritis
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
SLE	Systemic lupus erythematosus
TBE	Tris/borate, EDTA buffer
TEMED	N, N, N, N-tetramethylethylenediamine
TNF-α	Tumour necrosis factor-α
VNTR	Variable number tandem repeat
LPS	Lipopolysaccharide

SUMMARY

There has been much interest focusing on the contribution of cytokines to the genetic variation known to occur between the immune responses of different individuals. Interleukin-6 (IL-6), originally defined as a B cell growth factor, was rapidly identified as under-pinning the induction of the acute-phase response and being heavily involved in inflammatory responses generally. More recently, the role of IL-6 in human malignancy has become a topic of interest. The aim of this study was to examine four polymorphic elements, three in the promoter 5'region and one VNTR in the 3'region and define the relationships of these alleles in a West of Scotland control population. The relationships were then observed in two malignant cohorts; patients diagnosed with Gastric carcinoma and patients diagnosed with Barrett's Oesophagus, to determine possible disease related associations. Investigation of an African American cohort and subsequent rheumatoid individuals also provided a comparison in a different ethnic group and the associations of the polymorphic elements in an autoimmune disease. Digestion of amplified DNA determined the polymorphisms in the 5' promoter region and PAGE separation resolved the minisatellite alleles at the 3' VNTR. The data revealed that strong significant associations between the alleles form three prominent 5' promoter haplotype families and four dominant extended haplotypes, which include the 3'VNTR in the West-of-Scotland population. In the gastric carcinoma cohort the 4 polymorphisms were similarly distributed to the control population and did not prove to be genetic markers relating to this disease. Similarly no statistical significance was detected with the Barrett's Oesophagus cohort. However, the presence of *H.pylori* in the malignant cohorts proved to be of significant importance in the distribution the 5' haplotype families. The haplotype IL6.01 was significantly more prominent in *H.Pylori* positive patients of the gastric cohort, where as the haplotype IL6.02 was more prominent in the Barrett's Oesophagus patients. In the African American population, no statistical difference was detected between the control cohort and the Rheumatoid arthritis patients. The results reported here demonstrate that markers cannot necessarily be considered independent of one another across human cytokine genes. Additionally, where markers fall in, or close to, functional elements such as transcription factor binding sites, it should be remembered that other polymorphic elements might be contributing to the observed effect.

INTRODUCTION

CHAPTER 1

Cytokines are small soluble proteins made by many cell types that are produced to regulate immune and inflammatory responses and mediate cell growth, differentiation and repair. In general, cytokines are synthesised in response to inflammatory or antigenic stimuli and their action is initiated by binding to specific receptors on the target cells altering their RNA and protein synthesis. Cytokine secretion is usually a self-limiting event due to production regulated by the AU rich sequences in the 3' untranslated regions, which render most cytokine mRNAs unstable. Cytokines have multiple different effects on a variety of cell types, which results in considerable overlapping and redundancy between them.

According to their principle actions, cytokines can be organised into three groups. The first mediates and regulate specific immunity and are produced by T-lymphocytes in response to specific antigen recognition. The second are collectively called colony-stimulating factors and stimulate the growth of bone-marrow progenitors. The final group mediates innate immune mechanisms and includes antiviral, pro-inflammatory and regulatory cytokines [Abbas et al, Roitt,].

Recently there has been much interest focusing on the contribution of cytokines to the genetic variation known to occur between the immune responses of different individuals. While the majority of work has been carried out on the TNF locus, other cytokines have featured such as IL-1 and IL-10. More recently Interleukin-6 and its role in human disease has been studied.

1. INTERLEUKIN-6

Interleukin-6 is a multifunctional cytokine with a wide spectrum of biological activities. This diversity created considerable confusion during identification and thus this protein was previously known by terms that reflected the characteristics identified in the studies. There were three main lines of investigation running in parallel, which eventually led to the conclusion that they were all the same molecule.

The original discovery of interleukin- 6 was in 1980 by Weissenbach et al who isolated two cDNA clones from human fibroblasts stimulated for Interferon-β (IFN-β) expression. One clone coded for IFN-β and the second for a protein of molecular weight 23-26 kilodaltons. This clone was termed "IFN-β_2". However, Content and co-workers [Content et al, 1982] renamed this protein as "26kD factor" or "26kD protein" after observing that it lacked antiviral activity. Further investigation detected different transcriptional regulation [Poupart et al, 1984] and sequencing in 1986 [Haegermann et al, 1986] confirmed lack of structural homology with IFN-β. Although investigations suggested that this protein played an intermediary role in inflammation or immunoregulatory processes by detecting its induction by a leukocyte-derived factor belonging to the IL-1 family [Content et al, 1985], the physiological functions remained ambiguous at this point.

Meanwhile a "B-cell differentiating factor" (initially called "B-BCDF") was isolated and purified from a human B-lymphoblastoid cell line [Yoshizaki et al, 1984], which was later renamed "B-cell stimulating factor 2" (BSF-2) when it was observed to induce the final maturation of B-cells into immunoglobulin-secreting cells. Purification to homogeneity [Hirano et al, 1985] and cloning from a human T-cell leukaemia virus [Shimizu et al, 1985] led to sequencing [Hirano et al, 1986] that later showed identity to IFN-β_2 [Sehgal et al, 1987].

The final area of investigation concentrated on growth factors. "Interleukin hybridoma plasmacytoma 1" (IL-HP1) was purified from the supernatant of a murine helper T cell (Van Snick et al, 1987) and a similar molecule from a macrophage supernatant termed "plasmacytoma growth factor" (PCT-GF) [Nordan and Potter, 1986]. This led to the purification of a human "hybridoma/plasmacytoma growth factor" (HPGF) from an osteosarcoma cell line treated with IL-1 [Van Damme et al, 1987], which showed identical amino acid terminal sequence to that predicted from the 26Kd cDNA clone. Additionally, a "fibrinogen stimulating factor" detected by Ritchie and co workers, which was later renamed "Hepatocyte stimulating factor" (HSF), was observed to share immunological and functional identity to IFN-β_2 [Gauldie et al, 1987].

The realisation that all these molecules were the same cytokine led to the proposal that it should be renamed "Interleukin-6" to avoid further confusion over nomenclature [Poupart et al, 1987, Gauldie et al, 1987]. The various nomenclatures relating to IL-6 are summarised in Table 1.

IL-6 production is secreted by a large number of cell types, including monocytes and macrophages [Aarden et al, 1987], fibroblasts [Weissenbach et al, 1980], endothelial cells [Jirik et al, 1989], keratinocytes [Grossman et al, 1989], T cells [Garman et al, 1987], synoviocytes [Guerne et al, 1989], and pancreatic cells [Campbell et al, 1989]. These cells do not constitutively produce IL-6, but instead it is induced and regulated by a number of stimuli, the most common of which are LPS, IL-1 and TNF-α. Yet IL-6 has also been observed to inhibit the production of both TNF-α and IL-1 in order to attenuate the inflammatory response caused by the primary stimulus. Therefore these substances can be seen to create a cytokine cascade but with a regulatory feedback loop.

1.2. IL-6 STRUCTURE

The human IL-6 gene has been localised to chromosome 7p21 [Bowcock et al, 1988] and consists of 5 exons and 4 introns. [Yasukawa et al, 1987]. Its organisation is homologous to that of granulocyte colony stimulating factor (G-CSF), suggesting that these genes have evolved from a common ancestral gene [Hirano et al, 1986]. Additionally, a high degree of similarity has been described between human IL-6 cDNA and murine IL-6 at both nucleotide and amino acid sequences, with 65% and 42% homology respectively [Tanabe et al, 1988]. IL-6 codes for a protein of 184 amino acids, which includes 2 potential N-glycosylation sites and 4 cysteine residues and is formed by the cleavage of a 28aa signal peptide from a 212aa precursor protein [Hirano et al, 1986].

TABLE 1

Alternative synonyms for Interleukin-6 (IL-6)

Name	Author
IFN-β_2	Weissenbach et al, 1980
FSF	Ritchie et al, 1983
26k	Content et al, 1982
HSF	Ritchie et al, 1983
B-BCDF/BCDF	Yoshizaki et al, 1984
BSF-2	Hirano et al, 1985
IL-HP1	Van Snick et al, 1986
PCT-GF	Nordan and Potter, 1986
HPGF	Van Damme et al, 1987

Due to extensive post-translational modifications, natural human IL-6 exists in a number of different forms with molecular weights ranging form 21K-30K [Hirano et al, 1986, May et al, 1988]. Both monocytes and fibroblasts have shown to secrete at least 5 different isoforms although the higher forms are absent when the cells are inhibited by tunicamycin, suggesting that these forms are N-glycosylated [Bauer et al, 1988, May et al, 1988]. Yet a preferential shift in the secretion of these 29KDa triplets can be obtained by partially inhibiting the cellular protein synthesis, observed by the inclusion of cyclohexamide [May et al, 1988]. This suggests that the multiple forms of IL-6 are actively regulated. Additional post-translation modifications have been observed, such as sulphation and phosphorylation at serine residues in specific tissues. However these appear to have no significant effect on the biological activity of IL-6 [Simpson et al, 1997].

1.3. IL-6 RECEPTOR AND SIGNAL TRANSDUCTION

The human IL-6 receptor is an 80KDa glycoprotein consisting of 468 amino acids including a 19aa signal peptide, a 28aa transmembrane domain and six potential glycosylation sites [Yamasaki et al, 1988]. The first domain in the extracellular region consists of 90aa which is homologous to the immunoglobulin (Ig) superfamily [Yamasaki et al, 1988] followed by a cytokine binding domain (CBD) consisting of two fibronectin type III- like domains (FN-III) (Simpson et al, 1997). The CBD is characteristic of the class I cytokine receptor family which includes interleukins-2 to -9, IL-11, IL-13, Leukaemia Inhibitory Factor (LIF), Erythropoietin (Epo), Ciliary neurotrophic factor (CNTF), growth hormone (GH) and prolactin [Thoreau et al, 1991, Kishimoto et al, 1994].

There is also a short cytoplasmic segment of 82aa that, unlike other growth factor receptors, lacks tyrosine kinase domains [Yamasaki et al, 1988]. This region however is not essential for signal transduction and neither is the transmembrane domain [Taga et al, 1989].

The IL-6 receptor can also exist in soluble form (sIL-6R) and has been detected in normal human urine and serum [Novick et al, 1989, Narazaki et al, 1993]. Its physiological role however is not yet well understood.

For IL-6 transduction, a signal transducer (termed gp130) is required to bind to the IL-6-IL-6R binary complex to form the high affinity ternary complex IL-6--IL-6R--gp130 [Taga et al, 1989]. The cDNA of the human gp130 encodes for a 918aa protein, including a 22aa signal peptide, an extracellular region of 527aa, a transmembrane domain of 22aa and a cytoplasmic domain of 277aa [Hibi et al, 1990]. This protein of mature molecular weight 101KDa also has 14 potential glycosylation sites. The cytoplasmic domain of gp130 has no intrinsic catalytic activity [Hibi et al, 1990]. A segment of ~200aa in the extracellular region possesses typical features of this cytokine family. Additionally this region comprises of six units of fibronectin type III modules and shows closest similarity to the G-CSF receptor than to any of the other family members [Hibi et al, 1990].

Transcriptional activation studies have demonstrated that there are two signaling pathways mediating the IL-6 effects. The binding of IL-6 to IL-6R results in the homodimerisation of gp130 and activation of the tyrosine kinases of the JAK family (Janus Kinase), which then phosphorylate the STAT family transcription factors (signal transducers and activators of transcription) [Darnell et al, 1994]. The second pathway involves the G-protein Ras and Raf, which activate the MAPK's (mitogen activating protein kinases) [Gadient 1997].

1.4. BIOLOGICAL ACTIVITIES

IL-6 has a wide range of biological activities, which, as previously discussed, led to the long list of synonyms. These activities include pro-inflammatory responses such as T-cell activation, B-cell differentiation, acute phase response, as well as some anti-inflammatory activities.

B-cell differentiation

One of the earliest descriptions of IL-6 activity was as a role in B-cell differentiation. IL-6 was shown to induce immunoglobulin (Ig) production in stimulated *Staphylococcus aureus* Cowan strain I and Epstein Barr virus (EBV) transformed cell lines without inducing B-cell growth [Hirano et al, 1985]. Further study confirmed the importance of IL-6 to immunoglobulin production by observing the strong inhibition of Ig production in mononuclear cells stimulated by pokeweed mitogen when treated with

anti-IL-6 antibody [Muraguchi et al, 1988]. The strong effect of IL-6 was observed even when added on the fourth day of an eight-day culture suggesting that IL-6 acted late in the response. IL-6 activity was also demonstrated when it was identified in the supernatant of LPS-activated monocytes [Tosato et al, 1988]. The addition of these fluids to EBV-infected human B-cells enhanced its production 30-50 fold.

IL-6 has also been shown to augment antigen specific antibody synthesis in primary and anmnestic responses both *in vitro* and *in vivo* [Takatsuki et al, 1988]. The inhibition of IL-6 by anti-IL-6 antibody confirmed this cytokine as the enhancing agent. Furthermore, IL-6 was enhanced the primary and secondary antibody responses, in mice with a stronger effect observed in the secondary response.

B-cell hybridoma and plasmacytoma activity

An activity related to B-cell differentiation is the ability of IL-6 to promote hybridoma and plasmacytoma growth [Aarden et al, 1987, Nordan and Potter, 1986, Van Damme et al, 1987]. IL-6 was shown to be required for the proliferation of newly formed B-cell hybridomas in pristane-treated mice [Bazin and Lemieux, 1987], whereby IL-6 sensitive cells induced the formation of ascetic tumours. Furthermore, IL-6 was required for the survival of newly formed B-cell hybridomas as its absence eventually led to tumour cell death. The role of IL-6 in plasmacytoma growth was observed by Suematsu et al [1989] in IL-6 transgenic mice. All the mice showed plasmacytoses expressing high IL-6 concentrations and polyclonal IgG1. The plasma cells were shown to have infiltrated into the lungs, liver and kidney, yet upon transplantation could not induce plasmacytomas. This study therefore indicated that IL-6 could induce polyclonal plasmacytosis, but not plasmacytoma.

T-cell

The involvement of IL-6 with T-cells was first detected whilst searching for new factors involved in T-cell activation. IL-6 was induced proliferation of purified peripheral murine T-lymphocytes in the presence of lectins [Garman et al, 1987] and thus act as a second signal for production of IL-2. IL-6 has since been shown to be also involved in the differentiation of cytolytic T-cells. Takai et al [1988] observed that three cytokines were involved in differentiation of $Ly2^{+}$ CTL differentiation, one being IL-6.

Further studies on cytotoxic T-cell (CTL) differentiation have used recombinant IL-6 [Okada et al, 1988]. Recombinant IL-6 (rIL-6) alone was unable to induce CTL in cocultures of human T-cells stimulated by allogeneic leukocytes. It was observed that rIL-2 was required as well during the early phase of induction. Yet the activity of IL-6 was not mediated by the induction of IL-2 production, as anti-IL-2 antibody had no effect on IL-6. Furthermore the ability of rIL-6 to generate CTL when added 72 hours after culture initiation suggested a role as a late acting differentiation factor.

Haematopoiesis

IL-6 has three main roles in haematopoiesis; in progenitor cell expression, in megakaryocyte differentiation and in myelopoiesis.

Studies in IL-3 dependent haematopoietic progenitor cells have shown that combination with IL-6 decreases the time that dormant cells reside in G0 phase thus increasing the speed of colony appearance [Ikebuchi et al, 1987]. IL-6 was also able to synergise with GM-CSF and M-CSF [Hoang et al, 1988, Bot et al, 1989] although the synergy with M-CSF was only observed in highly purified progenitor cells [Bot et al, 1989]. Furthermore, early addition of IL-6 was required to produce an enhancing effect.

Studies have also suggested that IL-6 has a significant effect on megakaryocyte development. In the presence of IL-3, the addition of IL-6 directly augmented the maturation of murine megakaryocytes defined by size increments, Acetylcholinesterase activity and ploidy. The absence of IL-3 produced no detectable effect. *In vivo*, increased numbers of megakaryocytes in bone marrow were observed in transgenic mice [Suematsu et al, 1989].

Early studies implicating the ability of IL-6 to influence myelopoiesis was observed by Chen et al [1988]. IL-6 induced the terminal differentiation of the myeloleukemic M1 cells into growth arrested macrophage-like cells and partial differentiation of histocytic lymphoma U937cells. Further studies indicating IL-6 stimulating myelopoiesis were confirmed *in vivo* where by myeloid hyperplasia was observed in rats 12 hours after administration of rIL-6 [Ulich et al, 1989].

Acute phase response

The acute phase response is the change in the transcription of certain hepatic proteins in response to inflammation. When this response was induced by LPS, IL-1 or TNFα, the cytokine IL-6 was shown to act as the mediator [Geiger et al, 1988]. The importance of IL-6 in acute phase response was further confirmed *in vivo* [Marikovic et al, 1989, Geiger et al, 1988b]. Recombinant IL-6 combined with glucocorticoids injected into rats induced the mRNA for the major rat acute phase proteins α_2-macroglobulin, fibrinogen thiostatin and hemopexin. Hattori et al [1990] observed the effect to be due to the formation of a complex between the IL-6 response element of the genes and the hepatic nuclear proteins. Furthermore IL-6 has been observed to stimulate the full spectrum of acute phase proteins in adult human hepatocytes, i.e. C-reactive protein, serum amyloid A, fibrinogen, α_1-antichymotrypsin and haptoglobin [Castell et al, 1989]. The association between serum IL-6 levels in severely burned patients and C-reactive protein further supports a role for IL-6 in the acute phase response.

Nervous system

There has been much evidence indicating that IL-6 has an essential effect on the neurons in the peripheral and central nervous system. IL-6 was first described to have neuroprotective actions on CNS neuronal subpopulations by Hama et al [1989]. The survival of cholinergic neurons and catecholaminergic neurons in the basal forebrain and mesencephalon respectively was supported by IL-6. Additionally, IL-6 can induce neuronal differentiation of PC12 cells (a rat adrenal pheochromocytoma) into neuronal cells, an activity similar to the differentiation process observed by the nerve growth factor [Satoh et al, 1988].

In addition to neuronal effects, IL-6 has been observed to induce astrocyte proliferation *in vitro* [Selmaj et al, 1990], suggesting that increased levels of IL-6 may have an effect in astrogliosis. Additionally, IL-6 can stimulate nerve growth factor synthesis in cultured astrocytes [Frei et al, 1989].

Bone metabolism

IL-6 also plays an important role in bone metabolism through induction of osteoclast activity [Tamura et al, 1993, Kurihara et al, 1990]. Multinucleated cells (MNC)

expressing the osteoclast phenotype, adapted from long term human marrow cultures, can be stimulated in the presence of mouse IL-6 and sIL-6. Studies have shown that in response to the combination of recombinant IL-1 and TNF, IL-6 secretion in osteoblast-like cells and murine bone-marrow stromal cells increases up to 10,000 fold, yet is inhibited by 17β-oestradiol [Girasole et al, 1992]. Furthermore, inhibition of osteoclast precursors was detected in oestrogen-depleted mice when treated with anti-IL-6 monoclonal antibodies. These suggest that the inhibition of IL-6 gene expression by oestrogen may partly inhibit osteoclast activation [Jilka et al, 1992]. This was further supported in IL-6 deficient mice, which were observed to be protected from bone loss associated with ovariectomy [Poli et al, 1994].

Anti-inflammatory activities

IL-6 has a number of properties that lead to inflammation and is essentially a pro-inflammatory cytokine. IL-6 can suppress the inflammatory functions mediated by T-cells by inhibiting delayed type hypersensitivity and adjuvant arthritis [Mihara et al, 1991]. Furthermore IL-6 can induce the synthesis of metalloproteinases-1, an inhibitory enzyme to the matrix metalloproteases [Lotz and Guerne, 1991]. This suggests that IL-6 may also have protective mechanisms that balance the catabolic effects of other cytokines such as TNFα and IL-1β.

1.5. THE IL-6 PROMOTER

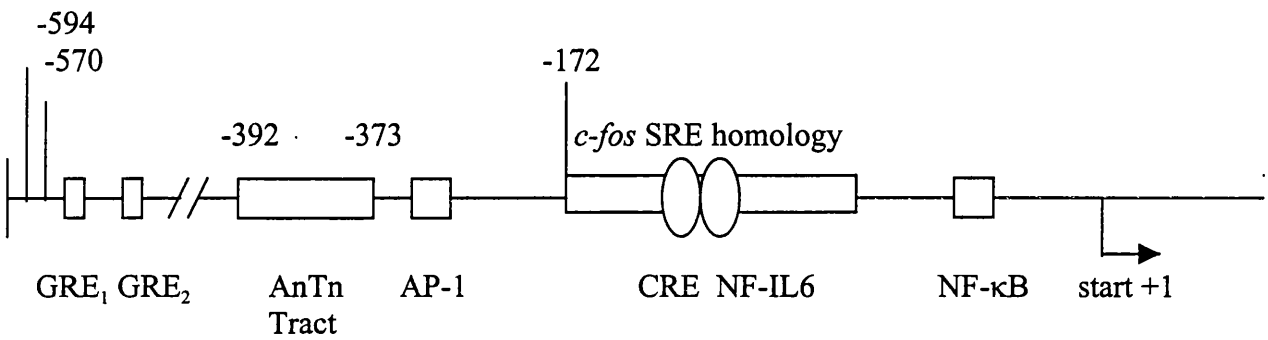
The 5' flanking region of the human IL-6 gene is a very complex region whose strong homology in the first 300bp to murine IL-6 underlines its importance [Tanabe et al, 1988]. The regulatory elements can be observed in Figure.1. The 5' flanking region of the IL-6 gene contains a number of putative cis-acting response elements, which mediate the activation of the promoter, including those for AP-1, NF-κB, and the serum response element (SRE), which is composed of the cAMP response element (CRE) and nuclear factor IL-6 (NF-IL-6). Brach et al [1992] observed NF-κB and NF-IL-6 activity during the synthesis of IL-6 in human blood monocytes when induced by LTB_4. These have since been detected to act as bacterial and mycobacterial response elements when stimulated by LPS or Lipoarabinomannan (LAM). This lipid glycoprotein of the mycobacterial cell wall was able to induce mononuclear phagocytes to release IL-6 (Zhang et al, 1994). Transcriptional activation involving NF-κB to inducing IL-6 gene

expression in mononuclear phagocytes was again observed with recombinant human Leukaemia Inhibitory Factor (LIF) [Gruss et al, 1992].

Simultaneous activity of multiple regulatory elements has also been shown to mediate IL-6 gene expression. The transcription factors MRE, AP-1, NF-κB and NF-IL6 were required for stimulation with cAMP and postglandins as mutations within these elements significantly reduced inducibility of IL-6 in monocytic cells [Dendorfer et al, 1994], suggesting that each of the sites contribute to the expression.

Combinations of trans and cis-acting elements can mediate repression of the IL-6 promoter, such as the regulatory factor *Fos*. Nuclear extracts from HeLa cells were inhibited by the *fos* E DNA fragment and *c-fos* oligonucleotide [Ray et al, 1989]. Additionally, wild type p53 and retinoblastoma susceptibility gene product have also been identified as transcriptional repressors in modulating IL-6 gene expression during cellular differentiation and oncogenesis [Santhanam et al, 1991].

Oestrogen has also been recognised to repress expression of IL-6. Using IL-6 promoter CAT constructs containing either 1.2kb fragment or a 225bp fragment of the promoter, Pottratz et al [1994] observed that 17β-oestradiol had no effect on the activity unless these were co-transfected with an hER plasmid. Furthermore, they observed that the latter IL-6 promoter plasmid was inhibited by 17β-oestradiol in MBA 13.2, cells which constitutively express oestrogen receptor. These results therefore suggested that 17β-oestradiol inhibits IL-6 gene transcriptional activation by an ER-dependent mechanism.



Transcription factor	Location
GRE_1	-557 to -552
GRE_2	-466 to -461
AP-1	-283 to -277
CRE	-163 to -158
NF-IL6 (C/EBPβ)	-158 to -145
NF-κB	-73 to -64

FIGURE 1. Schematic representation of the 5' flanking region of the IL-6 gene identifying the 3 SNPs and transcription factor binding sites

1.6. ALLELIC VARIANTS

There have been a number of polymorphic elements observed in the IL-6 gene. A polymorphism can be defined as the occurrence of two or more genetically determined forms in a frequency of greater than 1%, suggesting that this Mendelian trait could not be maintained by mutation alone. The majority of recognised DNA polymorphisms are single base-pair changes and is known as single nucleotide polymorphisms (SNPs). These are generally detected by an alteration of a restriction enzyme recognition site and are therefore also termed as restriction fragment length polymorphisms (RFLPs).

DNA sequence variation can also arise in DNA sequences known as variable number tandem repeats (VNTR). These are arrays of short nucleotide sequences dispersed throughout the genome that are repeated in a tandem row. This family of repetitive non-coding DNA sequences can be classified into 3 groups. Satellite sequences, located in the heterochromatin arrays of repeats ranging in size from 5 to 100bp and are organised in clusters of approximately 100Mb. Microsatellite sequences are approximately 100bp long made up of 2-6bp repeats, whilst Minisatellite sequences range from 0.5 to 30kb made up of 15-70bp repeats. Both these groups are found in the euchromatin region.

These polymorphic elements can affect the gene in a number of ways. Non-conservative mutation within the coding region can produce a change in the protein structure, resulting in a functional change of the expressed protein. Conservative mutations (silent), which do not alter the amino acid sequence, can influence the protein expression by altering the mRNA stability and level of gene transcription. Polymorphisms in the 5' promoter and 3' regulatory region of the gene may have the most significant effect on transcription. Polymorphisms in the promoter may alter the structure of transcription factor binding sites; where as polymorphisms in the 3' regulatory site may modulate enhancers and silencers.

There have been 4 single base pair polymorphisms detected in the promoter region of IL-6. In 1998, Olomolaiye et al identified a G/C substitution at –172 using the restriction enzyme Nla-III. It is postulated that the allele G is ancestral and that the allele C represents a relatively recent change in the 5' sequence. It has since been identified in many populations, including Caucasians, Gujarati Indians and Afro-

Caribbeans [Fishman et al, 1997]. The proximity of the SNP to a glucocorticoid receptor site suggested that a base pair substitution might potentially influence the binding and hence, its ability to repress transcriptional activation [Ray et al, 1990]. This instigated further analysis of the polymorphism and its effect in disease populations and identified an association with systemic-Onset Juvenile chronic arthritis [Fishman et al, 1997]. Since these unique findings, this SNP has also been investigated in Systemic Lupus Erythematosus (SLE) [Schotte et al, 2001], Alzheimer's [Bagli et al, 2000] and Inflammatory Bowel Disease [Kelin et al, 2001], all of which showed no association.

Bowcock et al [1987] also observed a base pair substitution in the 5' flanking region using the restriction enzyme BglI. This was observed in a Caucasian and an African population. Further analysis showed no disease association with Rheumatoid arthritis, SLE or pauci-articular JCA [Fugger et al, 1989]. Similarly, a G/A base pair substitution at -594 detected in Caucasians [Faulds et al, Accession number AF048962] has, to date, shown no disease association. A fourth base pair substitution has only been recently observed by Dr McNicholl and has yet to be published. This is a G/C substitution at – 570 detected in an African American population.

Polymorphisms have also been observed in the 3' region of the gene. The restriction enzyme MspI identified a base pair substitution within the fifth exon, and has been observed in Pygmies, Chinese and Melanesians.

A VNTR has also been detected in IL-6 by Bowcock [1988] Located within 1kb of the 3'flanking region, this AT rich region produces insertions of between 0.07 and 0.23 kb during DNA replication. Bowcock identified 4 alleles by using 10 different enzymes, although mentioned that more may be present due to unresolvable microheterogeneity caused by the low-resolution of agarose gel separation. Recently, Murray et al [1997] utilised the ABI machine to observe another two alleles in a Caucasian population and further demonstrated an association between genotype for the 3'region and peak bone mineral density in women.

CHAPTER 2

Dysregulation of the body's carefully regulated system often leads to diseases. These can be classified into three main types: Infectious, Neoplastic, and Autoimmune diseases. Neoplastic diseases are generally caused by over production of cells due to the dysregulation of replication, thus producing a tumour. Cells that remain localised produce 'Benign tumours', which pose little risk to the body. However abnormal cells that are able to spread into alien sites are classed as 'malignant tumours' and can potentially be life threatening. In autoimmune diseases, dysregulation of the immune system produced by defects in congenital or acquired mediators of immunity can result in immunodeficiencies, again possibly resulting in mortality. In the majority of diseases there is no exclusive causative factor, but rather a combination of both genetic and environmental factors contributing the observed effect.

There have been many studies that have indicated IL-6 to be involved in human malignancy and autoimmune diseases. IL-6 production has been noted in a number of tumour cell lines including multiple myeloma [Kawano et al, 1988], renal cell carcinoma [Miki et al, 1989] ovarian carcinoma (Watson et al, 1990), cervical caricinoma [Eustace et al, 1993], lung cancer [Jian et al, 1998], and Kaposi's sarcoma of AIDS [Miles et al, 1990], showing its ability to act as an autocrine growth stimulator during human melanoma progression. Significantly increased serum levels of IL-6 production have also been detected in Cholangiocarcinoma [Goydos et al, 1998], Crohn's disease and ulcerative colitis [Mahida et al 1991, Hyams et al, 1993, Holtkamp et al, 1995], and colorectal carcinoma [Kinoshita et al, 1999].

2.1 GASTRIC CANCER

Gastric cancer is characterised as a malignant neoplasm of the stomach mucosa [Wu et al, 1997]. Although recent advances have led to early diagnosis and treatment, it still remains to be the second most common malignant disease world wide [Everett et al, 1998] with poor prognosis [Morris et al, 1997]. There are two histological types of gastric cancer termed 'Intestinal' and 'Diffuse'. The intestinal tumours are associated with intestinal metaplasia of the stomach and often ulcerate while the diffuse generally lead to a thickening of the stomach lining. The intestinal type is more prevalent in older men while the diffuse is more common in women of blood group A. The observation that the diffuse type has a hereditary basis suggests that the two histological types

possess different genetic pathways [Wu et al, 1997]. According to the invasiveness of tumour cells, gastric cancer can also be divided into early and advanced stages. Adenocarcinoma in early gastric cancer is confined to the mucosa or sub-mucosa and detection of cancer in this stage provides patients a high survival rate [Everett et al, 1998].

The transformation of the gastric mucosa and progression of stomach cancer are presently incompletely understood. However dietary, environmental and genetic factors have a role. Ever since *Helicobacter pylori* were first discovered in 1982, it has been extensively studied in association with gastric cancer. Early supportive evidence linking *Helicobacter pylori* to gastric cancer was provided by Parsonnet et al [1991] who tested for the presence of IgG antibodies to this bacterium. In comparison to control subjects, patients seropositive for this bacterium were three times more likely to have gastric adenocarcinoma in the following 1-24 years, thus suggesting a strong association between *H. pylori* exposure and gastric malignancy. Furthermore *H. pylori* was linked with both histological variants present in either the body or antrum of stomach. Such compelling evidence therefore persuaded the IARC (International Agency for Research on Cancer) in 1994 [Nyrén et al, 1998] to classify this gram-negative bacterium as a group I carcinogen that presents a definitive contribution to the gastric cancer.

The direct link of gastric cancer and H. pylori has since been confirmed in further studies. Watanabe et al [1998] successfully demonstrated that long-term infection of *H. pylori* in Mongolian gerbils induced gastric adenocarcinoma. Tumours were present in 37% of animals in this study and all consisted of well-differentiated intestinal type epithelium in the pyloric region. This corresponds to the intestinal type adenocarcinoma observed in humans, suggesting a direct involvement between *H. pylori* infection and gastric carcinogenesis.

However H. pylori infection cannot explain all epidemiological features. It has been observed in several developing countries that although the infection occurs at an early age, the frequency of gastric cancer is very low [Holcombe et al, 1992]. Additionally, although the male to female ratio of cancer is 2:1, infection is the same in both sexes. [Forman et al, 1998].

There is also increasing evidence that some gastric cancers are caused by an inherited predisposition. Familial factors account for ~8% of all stomach cancers and first-degree relatives of gastric cancer patients have an increased relative risk of ~3 fold [La Vecchia et al, 1992]. The down- regulation of the adhesion molecule E-cadherin resulting in loss of cell-cell adhesion and an increase in invasiveness is one such genetic abnormality that is especially common in poorly differentiated diffuse type gastric cancer [Guilford et al, 1998].

Prominent genetic alterations also play a role in tumourigenesis. Microsatellite instability (MI), which is involved in various cancers, is observed in 39% of all gastric cancers [Han, 1993]. Nakashima et al [1995] however noted the incidence increased to 53.3% when including multiple gastric cancer cases and observed 78.5% of patients represented MI in at least one of their multiple cancers. This suggests that microsatellite instability has a more important role in the development of multiple cancers in the same host than in solitary gastric cancer. Microsatellite instability in hereditary nonpolyposis colon cancer (HNPCC) occurs due to germline mutations in the mismatch repair genes MSH2 and MLH1 and carriers of these mutations have a 4-fold risk of gastric cancer in addition to increased colorectal cancer [Lynch et al, 1996]. Yet these germline mutations are rarely observed in sporadic or familial non-HNPCC gastric cancer suggesting alternative genes to be involved in these cases.

Tumour progression of gastric cancer subtypes can also be related to genetic alterations. Overexpression of the p53 gene was shown to be related to tumour progression in diffuse type of gastric cancer as was the proto-oncogene c-met, which was also noted in advanced gastric cancer. Overexpression of the proto-oncogene c-erbB-2 however was significantly higher in the intestinal type and advanced stage. Loss of heterozygosity of APC (adenomatous polyposis coli) and DCC (deleted in colorectal cancer) was observed in 30% of intestinal type with the latter significantly detected in advanced stage [Wu et al, 1997]. Furthermore, the inactivation of APC gene is observed in ~20% of sporadic gastric cancers suggesting a key role in initiation in sporadic gastric cancer [Hsieh, 1995].

Certain cytokines also have a role in this disease. Constitutive interleukin-8 (IL-8) expression was observed in both normal and inflamed gastric mucosa, with increased

expression observed in the latter [Crabtree et al, 1994]. Similarly, interleukins 1α and 3 (IL-1α, IL-3), and granulocyte-macrophage colony stimulating factor (GMCF) were expressed in human gastric carcinoma cell lines [Ito et al, 1993, Dippold et al, 1991]. Furthermore, three polymorphisms in IL-1, which are believed to enhance production of IL-1β, have been associated with gastric cancer [El-Omar et al, 2000].

Other causative agents include smoking, which increases risk of disease to 1.8/2.2 (intestinal and diffuse type respectively). Additionally diet has been implicated showing an inverse relationship between intake of fresh fruit and vegetables and the risk of gastric cancer [Ye et al, 1999].

2.2. IL-6 AND GASTRIC CANCER

There have been several recent studies carried out supporting a relationship between IL-6 and gastric cancer. IL-6 has been shown to act as an autocrine growth factor in some gastric carcinoma cell lines. In this study IL-6 mRNA was expressed and high IL-6 levels secreted in 3 of 8 gastric carcinoma cell lines. This was further demonstrated by the addition of anti-IL-6 AB which suppressed cell growth [Ito et al, 1997].
Studies have also reported that serum IL-6 reflects disease status of gastric cancer and may be used as a serum marker to monitor disease progression [Wu et al, 1996]. Serum levels in patients with gastric cancer, benign gastric lesions, hepatitis-B patients and normal subjects were measured and the results showed that the mean serum levels in gastric cancer patients (mean ± Se 10.0 ± 9.5pg/ml) were significantly higher than in these control groups, (2.5 ± 0.3pg/ml, 2.6 ± 0.5pg/ml and 2.8 ± 0.9pg/ml respectively). Similar significantly higher results were also observed by comparing the median serum IL-6 levels. Further studies showed that of the six gastric cancer lines, five secreted IL-6 suggesting that gastric stromal cells secrete IL-6. In addition serum IL-6 levels correlated with disease progression and decreased after removal of tumour bearing tissues, thus resulting in a relationship between gastric cancer stages and IL-6. These results may thus indicate that the immune response directed against the tumour was inhibited by IL-6 [Wu et al, 1996].

2.3. BARRETT'S OESOPHAGUS

Barrett's Oesophagus is a pre-malignancy that has as yet eluded consensus upon definition, diagnosis and treatment. It is now generally accepted to be the replacement of squamous mucosa with metaplastic columnar epithelium and goblet cells in the distal oesophagus [Navaratnam and Winslet, 1998, Pereira et al, 1998]. The identification of columnar mucosa observed near the oesophageal junction however, causes uncertainty in ascertaining whether there is representation of Barrett's epithelium or gastric epithelium. This is due to difficulty in identifying the junction of oesophagus and stomach by the endoscopic techniques generally used to establish diagnosis. Furthermore the length of the segment has been in dispute as to its importance in the definition. In some cases BO is only represented if metaplasia is observed at a distance of at least 3cm. This therefore recognises the term 'short segment' Barrett's Oesophagus, indicated by columnar glandular mucosa within 2-3cm of the proximal edge of the gastric folds (Pereira et al, 1998). In contrast, by disregarding the length all metaplasia in this segment is regarded as Barrett's Oesophagus [Spechler et al, 1996, Spechler et al, 1996b].

BO is observed predominantly in Caucasians, with a male to female ratio of 2:1 [Navaratnam and Winslet, 1998]. This pre-malignant condition can develop into invasive cancer through a series of steps. These are characterised by low-grade dysplasia and high-grade dysplasia, which eventually develops to adenocarinoma [Hammeteman et al, 1989]. Patients with BO are at 3-5- fold more at risk in developing oesophageal adenocarcinoma [Hameeteman, 1989] and an estimated 75% of all adenocarcinomas of the oesophagus originate from BO.

Barrett's Oesophagus was first reported in 1906 by Tileston who observed a peptic ulcer in the oesophagus that showed close resemblance to ulcers detected in the stomach. However in 1950 Norman Barrett published a report that supporting the view of other investigators since and suggested that the organ was actually a tubular segment of stomach in patients with a congenitally short squamous lined oesophagus [Spechler et al, 1996].

The true prevalence of BO is difficult to determine although it is estimated that 1% of the population are affected. This is due to many patients being asymptomatic. For those patients that do experience symptoms such as heartburn, a significant proportion self-medicate with antacids when affected with mild disease severity [Cameron et al, 1990]. However, once cancer develops, the prognosis is poor unless there is early diagnosis. Survival rates after surgical resection are placed at <20% in the next 5 years unless treated early, when 5 year survival is >90%. Although all ages can be affected, the median age for development of BO is approximately 40 years, whilst mean age for cancer diagnosis is approximately 60 years [Cameron et al, 1992]. The increasing prevalence observed with increasing age suggests that Barrett's Oesophagus is an acquired abnormality.

Gastro-oesophageal reflux (GORD) has been recognised as the main aetiological factor and ~12% of patients experiencing GORD symptoms were diagnosed as having BO [Winters et al, 1987]. Acid and bile reflux occurs characteristically when there is failure of the lower oesophageal sphincter secondary to inappropriate transient relaxation of LOS, leading to retrograde oesophageal flow. [Navarantnam and Winslet, 1998]. It has been shown that acid reflux is directly related to development of BO, whilst bile reflux may have a synergistic role [Champion et al, 1994].

There is increasing evidence that molecular abnormalities are involved in BO and its progression to BO associated adenocarcinoma. The development of adenocarcinoma includes mobilisation of cells from G_0 to G_1 [Reid et al, 1993]. Loss of heterozygosity studies, detected in premalignant epithelium and oesophageal adenocarinoma have indicated allelic imbalance of 4q, 5q, 9p, 12q, 17p and 18q [Gleeson et al, 1998], suggesting involvement in BO progression. The observations of p53 gene mutations, which are located on chromosome 17p, in Barrett's adenocarcinomas further confirm association with malignant transformation [Neshat et al, 1994, Hamelin et al, 1994]. Genetic instability has also been related to BO. Microsatellite instability initially observed in diploid cells and secondary alterations in aneuploid cells of BO oesophageal adenocarcinomas suggests that MI may continue through the multiple stages of neoplastic progression as well as at onset. [Meltzer et al, 1994]. To date, there have been no studies in the relationship of IL-6 to Barrett's Oesophagus.

2.4. RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a common chronic inflammatory disorder involving the synovial membranes of multiple joints, which result in cartilage and bone invasion. Unsuccessful treatment eventually leads to joint destruction resulting in loss of function, disability and increased mortality [Pope, 1996]. RA is a relatively recent condition and first described in 1800 by Austin-Jacob and Landre-Beauvais, who considered it to be a form of gout. It was not until 1858 however that Garrod first termed this condition as 'Rheumatoid arthritis' [Landre-Beauvais, 2001].

RA affects about 1% of the population and about 5% of women over 65 years of age [Deighton, 1994]. The disease is more common in women at a rate of 3:1 over men and although it generally occurs later in life at approximately 40-50 years, all ages can be affected. This disease can eventually restrict the patients' ability to work thus having an important impact in economy.

Mortality in patients with RA often occurs at an earlier age than the general population. Evidence reports a decrease in survivorship of approximately 50% [Mitchell et al, 1986]. The two major causes of death however (i.e. cardiovascular disease and malignancy) remain the same in both rheumatic patients and the general population. Yet in RA patients, there is an increased mortality risk from infection or sepsis and from gastrointestinal causes. Additionally, increased mortality has been directly linked to complications of RA such as vasculitis, cervical subluxation, amyloidosis and rheumatoid lung (Mitchell et al, 1986).

The pathogenesis of RA has been described in five overlapping stages by Harris [1990]. The causes of RA are presently unknown although it is increasingly clear that both environmental and inherited factors have a role. Environmental triggers suggested include infectious agents such as viruses and bacteria. The genetic factors shown to be consistently involved in a predisposition to RA are in the HLA-DR locus on the MHC situated on the short arm of chromosome 6. Studies have shown that 93% of patients carry HLA-DR4, HLA-DR1 or both [Stastny, 1978].

HLA-DR4 can be further divided into 5 subtypes: Dw4, Dw10, Dw13, Dw14, and Dw15 [Gregersen et al, 1987]. It has been determined that only the subtypes Dw4, Dw14, and Dw15 were associated with RA whilst Dw10 and Dw13 were not [Stastny et al, 1978, Nepom et al, 1986]. The subtypes associated with RA shared a common sequence of 5 amino acids in the 3rd hypervariable region suggesting a critical area relating to RA susceptibility. A similar sequence at the 5aa stretch was also observed in HLA-DR1, which further supports this hypothesis.

Studies have also suggested that HLA-DR4 may also be related to disease severity. On the basis of radiological findings in the hand, RA patients with HLA-DR4 had a more destructive disease [Calin et al, 1989] than those who did not. Supporting evidence was observed when RA patients carrying the disease-associated alleles on both chromosomes were more likely to develop severe destructive disease.

Disease severity has also been observed with HLA-DRB1. Most severe rheumatoid arthritis was identified with patients homozygous to HLA-DRB1*0401 or 0404, and combinations of 0401 with 0404 or 0408. This suggests that genotyping may provide useful predictive biomarkers [Weyand et al, 1992].

Non-inherited factors relating to HLA antigens also influence susceptibility to RA. DR4 non-inherited maternal antigens (NIMA) were increased in DR4 negative patients compared to DR4 non-inherited paternal antigens (NIPA). This therefore suggests that patients who were in contact to DRB1*0401 and or *0404 positive HLA antigens during the foetal period have an increased risk of RA. It is suggested this may be due to positive mother's T cells, molecules or peptides influencing the T cell of the foetus [Van der Horst-Bruinsma et al, 1998].

Interleukin-1 (IL-1) is another factor that is associated with RA. Its biological activity was first observed in exudate synovial fluids [Wood et al, 1983]. Elevated IL-1 has since been observed in plasma of RA patients [Eastgate et al, 1988] with IL-1β showing correlation with disease activity. Joint damage has also been reported to correlate with IL-1 production, suggesting a pivotal role in this process and a possible target for therapy [Miyasaka et al, 1988]. A single nucleotide polymorphism at -889 in IL-1α has been shown to be genetically associated with juvenile rheumatoid arthritic patients

[McDowell et al, 1995] and chronic polyarthritis [Jouvenne et al, 1999], suggesting a contribution to the pathogenesis of these diseases and a possible prognostic marker.

High concentrations of TNF-α have also been reported in synovial fluids from RA patients [Saxne et al, 1988]. Additionally transgenic mice for a human 3' modified TNF-α developed inflammatory synovitis in the joints, suggesting deregulated production of TNF aids in the pathogenesis [Keffer et al, 1991].

In vitro studies have shown that IL-8 is released from rheumatoid mononuclear cells isolated from bone marrow, peripheral blood, and synovial fluid, with highest production observed from the last source [Seitz et al, 1991].

Specific polymorphisms and associated haplotypes in Interleukin-10 (IL-10) have also been directly associated with RA. A significant over-representation of the IL10.R2 allele, located upstream of the coding region, was detected in RA patients, suggesting that IL-10 may have an active role in the pathogenesis of this disease [Eskdale et al, 1998]. Further association was observed with the low IL-10 producing haplotype ATA and severity of juvenile rheumatoid arthritis [Crawley et al, 1999].

The other inherited factor that is associated with RA is the female gender. A possible role for exogenous female sex hormones was suggested in a study by the Royal College of General practitioners in 1978, showing that oral contraceptives protected against development of RA. Since then however, there have been extensive studies with various findings thus providing inconclusive results. Endogenous sex hormones have also been implicated in the aetiology of RA. Studies have observed consistently lower levels of the immunosuppressive hormone testosterone in men with RA [Spector et al, 1988]. Furthermore, an association was observed between the diurnal rhythm of prolactin levels and disease activity [Chikanza et al, 1990]. This study combined with the presence of prolactin receptors on T and B lymphocytes [Gagnerault et al, 1993] may suggest there is an interaction between increased prolactin levels and the immune system. These findings compliment the recent study on the effects of breast-feeding. Women who developed RA within 12 months after their first pregnancy were more likely to have breast-fed [Brennan and Silman, 1994]. The risk of RA development was observed highest for first-born (5.4) and greatest amongst women who delayed motherhood till after their 29th year (13.8). In breast-feeding mothers, there are low

levels of anti-inflammatory steroids and high prolactin levels. This therefore suggests that the prolactin may therefore act as a stimulus for the development of RA.

2.5. IL-6 AND RHEUMATOID ARTHRITIS

In RA, some of the abnormalities seem to be related to IL-6. Studies have shown that cells isolated from the synovial fluid of patients with RA constitutively expressed IL-6 mRNA [Hirano et al, 1988]. Additionally, significantly elevated levels of IL-6 were observed in the synovial fluid of RA patients in comparison to osteoarthritic patients [Housiau et al, 1988]. When IL-6 from serum and synovial fluid was detected from RA individuals, the latter source produced levels ~1000 fold higher than the former. This suggests that the source of IL-6 is from the rheumatic joint [Brozik 1992]. Similarly, significantly elevated serum IL-6 levels were also detected in patients with active systemic -onset juvenile rheumatoid arthritis. However these remained similar to controls during remission, suggesting that the high IL-6 levels relate to joint inflammation [De Benedetti et al, 1991] and contribute to local and systemic arthritic manifestations [Guerne et al, 1989].

Polymorphisms detected in IL-6 gene and identified with restriction enzymes MspI and BglII have been studied in relation to Rheumatoid arthritis, but have shown no association [Fugger et al, 1990, Blankenstein et al, 1989]. However, the polymorphism at –172 has shown significant association to this disease, as mentioned earlier. Studies in JRA patients have shown that the increased serum levels result from a difference in the control of IL-6 expression by the polymorphism at -172. When the constructs (-172G and -172C) were stimulated by LPS or IL-1 in a luciferase reporter system transiently transfected into HeLa cells, the transcriptional activity for -172G construct increased significantly in relation to the unstimulated level. In contrast, the -172C construct showed lower expression than the -172G construct when unstimulated and its activity did not increase after stimulation. Furthermore, plasma levels of IL-6 in healthy subjects were significantly lower when associated with the -172C allele than when the -172G allele was present. These results combined with the significantly less common CC genotype in S-JCA may indicate the reason for increased IL-6 levels in comparison to the control population [Fishman et al, 1998]. Similarly, elevated interleukin-6 plasma levels were regulated by -172 polymorphism of the IL6 gene in primary Sjogren's syndrome (pSS) and this correlated with the clinical manifestations of the disease

[Hulkkone et al, 2001]. However, they noted that the -172 allele frequencies were similar in patients and normal subjects and hence the G/C polymorphism does not predispose patients to pSS. In contrast, Collado-Escobar et al [2000] determined that the polymorphism at -172 played no role in susceptibility to Ankylosing Spondylitis. These studies therefore suggest that there are additional variations in close proximity, which are contributing to the overall effect.

Previous studies in cytokines such as TNF and IL-10 [Gallagher et al, 1997, Eskdale et al, 1999] have shown polymorphisms to associate to one another as haplotypes across the locus. The aim of this project was therefore to examine four polymorphic elements in the human IL-6 gene, three point mutations in the 5' promoter and one VNTR in the 3' region, and determine their relationship to one another. Furthermore, these polymorphic elements were then to be observed in malignant and autoimmune cohorts and determine the possibility of disease related associations.

MATERIALS AND METHODS

3.1. PATIENTS

Patient and control samples were obtained from a number of sources. DNA from 115 healthy unrelated individuals from the West of Scotland were obtained and provided by the tissue typing unit of Glasgow Royal Infirmary as the control group. At the time of sampling no individuals were known to be suffering from either a malignant or autoimmune disease. DNA from 69 patients classed with Gastric cancer and 98 patients with Barrett's Oesophagus were available in the laboratory, having been obtained from a previous project. However additional DNA was extracted to replenish stocks. The Barrett's Oesophagus patients were diagnosed with benign and malignant forms and separated accordingly to provide 51 samples of benign Oesophagus and 47 of malignant oesophagus.

In order to compare the distribution of IL-6 alleles in an ethnically distinct control population, 53 control African American DNA samples were obtained from Dr Janet McNicholl, Centres for Disease Control, Atlanta. A group of 109 rheumatoid arthritic patients from this source were also examined.

3.2. ISOLATION AND STANDARDISATION OF GENOMIC DNA

DNA was extracted from both frozen blood and tissue sections embedded in paraffin. Extraction from frozen blood was achieved by following the 'Genomix kit' protocol for 'Old Blood' exactly. DNA from tissue sections was achieved by first soaking the slides in xylene for 10 minutes to remove the paraffin, followed by 30 seconds in two more xylene baths, in order to remove remaining traces. The tissue sections were then rehydrated. This was achieved by dipping the slides for 30 seconds in a series of decreasing alcohol concentrations. The baths were at concentrations of 100%, another 100%, 90% and 70%, before soaking in tap water and distilled water. The tissue sections were then scraped off the slide and suspended in PCR buffer. Equal volumes of PCR buffer and Tween containing 1μg/μl proteinase K was added before incubating at 37°C for three days. This allowed digestion of the cells. After spinning down, the pellet was removed, leaving genomic DNA in solution.

The amount of DNA obtained was determined by spectrophotometric analysis. A 1:100 dilution of the DNA was placed in a cuvette and absorbencies at 260nm and 280nm obtained. The absorbance at 260nm provided the concentration of nucleic acid in the sample and the absorbance at 280nm provided the RNA concentration. The ratio between 260/280 allowed estimation of the purity of the nucleic acid. An O.D ratio value of 1.8 and above was taken to show that a pure DNA preparation had been obtained. Calculation of the 260nm reading multiplied by 50 (1 O.D = 50ng), multiplied by 100 (dilution factor), provided the amount of DNA in 1μl.

3.3. GENOTYPE DETERMINATION OF 5' SNP

The 3 point mutations studied in this thesis were at positions -594, -570, and -172. The position numbers correspond to the nucleotide number of the sequence in Accession number AF048962 [Faulds et al]. Genotyping at the three point mutations studied in this thesis was achieved by amplification of the region encompassing the three sites by PCR, followed by restriction enzyme digestion.

Amplification was carried out in two stages. Firstly an 861bp segment (primers 6PR-1 and 6PR-2) of the IL-6 promoter sequence (shown in the above accession number) was amplified. This product then became the template DNA for nested (primers Nla-F and Nla-R) and semi-nested PCR experiments (primers 6PR-1 and Fok/Fnu-R). This allowed further amplification thus increasing product concentration hence allowing clearer digestion detection to be obtained. Fig.2 contains the position and direction of the primers used for these analyses. Additionally, the three SNPs are indicated in capitals, with the alternative bases shown under the main sequence, the codon for the initiating methionine (ATG) and the transcription site (T).

FIGURE 2. Polymorphic elements in the human IL-6

Optimised amplification of 861bp region

In a 50µl final reaction volume 50-150ng DNA was amplified with 0.15µM each primer, 5µl of 10x Buffer IV (Advanced Biotechnologies), 200µM of each deoxyribonucleotide triphosphates (dATP, dTTP, dCTP, dGTP), 1.5mM $MgCl_2$ (AB) and 2U Taq DNA polymerase (AB). The primers were designed with consideration to maintaining a comparable length and similar Tm values. The primers deemed suitable were as follows:

5' GGA.GTC.ACA.CAC.TCC.ACC.TGG 3'	6PR-1
5' GTG.ACT.GAC.AGC.AGA.GCT.GG 3'	6PR-2

Replacing the template DNA with sterile water in order to confirm positive results was also set up as a mandatory negative control for every experiment.

The PCR conditions were carried out in a 40-well Biometra thermocycler and comprised an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles of 94°C, 56°C and 72°C each for 1 minute. A final extension step at 72°C for 1 minute completed the reaction. 5µl of PCR product was mixed with 5µl orange dye. A ϕX174 RF DNA/Hae III fragment ladder (Gibco) to confirm a correct size band and the negative control were added adjacent to the PCR product. These were loaded on a 2% 1x TAE agarose gel (Nusieve 3:1) stained with ethidium bromide at a concentration of 0.08% immersed in 1x TAE buffer and run at 50mA.

Optimised amplification of -594 and -570 SNP by semi-nested PCR

The 861bp product obtained from the original amplification was used as the template DNA. The reagents remained exactly the same as above except the reverse primer was replaced with the following:

5' GGA.GTC.ACA.CAC.TCC.ACC.TGG 3'	6PR-1
5' CCA.AGC.CTG.GAT.TAT.GA 3'	Fok/Fnu-R

The reaction conditions remained the same except that an annealing temperature of 49°C replaced the previous 56°C. The resulting 152bp product produced was checked as above for contamination.

Endonuclease digestion of Fok-I allele

The -594 A/G SNP first observed by Faulds et al [Accession number AF048962] was detected by the restriction enzyme Fok-I. A sequence C[A]TCC provides a cleavage site for this enzyme at the 5' end of the first C. The presence of G in the brackets prevents cleaving.

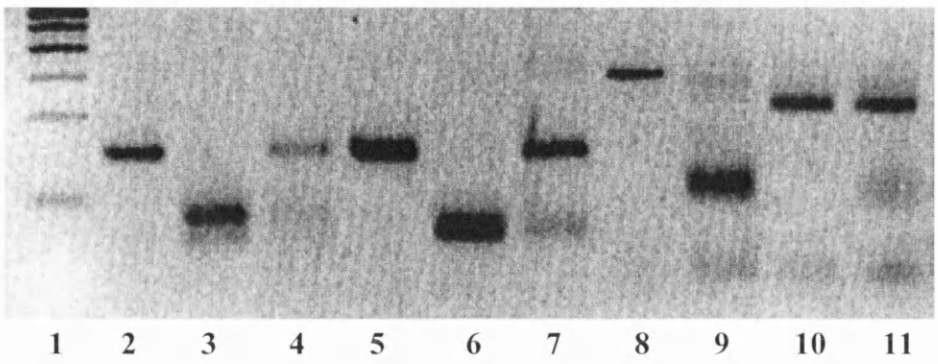
In a total volume of 25µl, 10µl of amplified DNA was digested by adding 2.5µl of 10x Buffer (New England Biolabs) and 2U Fok-I enzyme (New England Biolabs). After incubation at 37°C for 3 hours, 5µl orange dye was added to the digested product. This was run against a ϕX174 RF DNA/Hae III (Gibco) fragment ladder on an ethidium bromide stained 3% agarose gel in 1x TAE buffer at 50mA until clear separation was observed under the UV illuminator.

An adenine nucleotide provides a cleavage site in the 152bp band producing two bands of 66bp and 86bp where as a guanine nucleotide annuls the site and the thus band remains 152bp. A 1*1 homozygote (AA) was detected by the two bands of 66bp and 86bp while a 2*2 homozygote (GG) was detected by the single 152bp band. A 1*2 heterozygote (AG) was observed by the detection of bands 66bp, 86bp and 152bp. A representative gel picture is illustrated in fig. 3.

Endonuclease digestion of Fnu4H-I allele

The restriction endouclease Fnu4-HI was used to detect the -570 G/C SNP. A sequence of GCN[G]C where N represents any nucleotide provides a cleavage site at SNP site. Similar conditions were used to digest the amplified product as for Fok-I allele except 2.5U Fnu4HI enzyme was added.

A guanine nucleotide creates a cleavage site for this enzyme producing bands of sizes 80bp and 72bp. A cytosine nucleotide abolishes the site and the band remains at 152bp.



Lane 1: X174RF DNA/ HAE III ladder

Lane 2: Undigested 152bp fragment

Lane 3: Homozygote -594.A by Fok-I endonuclease digestion

Lane 4: Heterozygote -594.G/A by Fok-I endonuclease digestion

Lane 5: Homozygote -594.G by Fok-I endonuclease digestion

Lane 6: Homozygote -570.G by Fnu4H-I endonuclease digestion

Lane 7: Heterozygote -570.C/G by Fnu4H-I endonuclease digestion

Lane 8: Undigested 295bp fragment

Lane 9: Homozygote -172.G by Nla-III endonuclease digestion

Lane 10: Homozygote -172.C by Nla-III endonuclease digestion

Lane 11: Heterozygote -172.G/C by Nla-III endonuclease digestion

FIGURE 3 Agarose gel displaying endonuclease digestion of polymorphisms at positions -594, -570 and -172 within the IL-6 locus.

Again a 1*1 homozygote for C alleles was detected by the two small bands and a homozygote 2*2 GG by the larger single band. A heterozygote 1*2 CG was detected by all three bands. This can be observed in fig.3.

Optimised amplification of -172bp SNP by nested PCR

The original 861bp product was again used as the template DNA for this reaction. The reagents remained exactly the same except the primers were substituted with the following internal primers:

5' TTG.TCA.AGA.CAT.GCC.AAA.GTG.CT 3' Nla-F
5' CAT.CTC.CAG.TCC.TAT.ATT.TAT.TG 3' Nla-R

These primers were designed by O.O. Olomolaiye et al (1998). The PCR conditions remained the same except an annealing temperature of 52°C for 1 minute was used. This amplification protocol produced a 295bp product encompassing the -172 SNP.

Endonuclease digestion of Nla-III allele

The -172G/C SNP was first observed by O.O Olomolaiye et al [1998] and detected with the restriction endonuclease Nla-III. This enzyme recognises a [C]ATG sequence and cleaves at the 5' end of the C base.

10µl of amplified DNA was digested with 2.5µl of 10x Buffer, 5U Nla-III enzyme and 0.25µl of 100x BSA (all NEB) in a 25µl reaction volume. After 3 hours of incubation at 37°C the digested products were observed on an agarose gel as above.

In the 295bp band there are already two cleavage sites present producing digested products of 233bp, 53bp and 9bp. A cytosine at -172 produces an additional restriction site cleaving the 233bp into 122bp and 111bp. A guanine nucleotide does not allow this cleaving. Therefore a 1*1 homozygote for allele C was detected by bands at 122bp, 111bp and 53bp. The additional 9bp was too small to be observed on the 3% agarose gel. A 2*2 homozygote for G was detected by the presence of 233bp and 53bp (9bp too small to be observed). The heterozygote CG genotype was observed by bands of 233bp, 122bp, 111bp and 53bp (9bp too small to be detected), as shown in fig. 3.

3.4 GENOTYPE DETERMINATION OF 3' VNTR

The 3' variable number tandem repeat (VNTR) first observed by Bowcock et al [1989] are a group of repetitive non-coding DNA sequences. These examples of sequences are becoming increasingly important as markers in the genome.

Optimised amplification of 3' VNTR

In a 25µl reaction volume 100ng of DNA was added to 200µM of dTTP, 200µM of dCTP, 200µM of dATP, 20µM dCTP, 3mM $MgCl_2$, 2.5µl of 10x Buffer (Bioline), 0.5U Bioline Taq, 1µM of each primer and 0.03µl α^{32} P. The primers used were designed by Bowcock et al [1989] and were of the following sequence:

5' GCA.ACT.TTG.AGT.GTG.TCA.CG 3'
5' GAC.GTG.ATG.GAT.GCA.ACA.C 3'

The amplification conditions were in accordance to those described by Bowcock et al and comprised of an initial denaturation step at 94°C for 5 minutes, followed by 28 cycles of 94°C for 15 seconds, 55°C for 30 seconds and 72°C for 1 minute. Each PCR had both negative and positive controls to detect contamination and confirm consistency across all the PCR experiments.

Denaturing PAGE

The products were separated on a 3.5% denaturing polyacrylamide gel produced by 8.75ml of 19:1 acrylamide (DNA sequencing grade): N,N-methylenebisacrylamide gel mix, 1x TBE, 7M urea, 50µl TEMED and 750µl 10% w/v ammonium persulphate. Prior to loading on a preheated gel, 4µl formamide loading buffer (including 10mM EDTA) was added to the products, which were then denatured at 80°C for 10 minutes. These were then immediately placed on ice to prevent renaturation. 4µl of each sample was loaded on the gel and run on a Stratagene gel rig at 75W for 5 hours. The gel was then dried for 1 hour and autoradiographed with Kodak film.

The 3' VNTR alleles were compared and determined individually by two independent people. Control DNA's with a range of genotypes were run on every gel in order to assume consistency of allele calling.

3.5 STATISTICAL ANALYSIS

After obtaining individual allele and genotype frequencies at the 5'SNP's and 3'VNTR, the polymorphisms were compared with each other. This allowed detection of possible relationships amongst the polymorphisms across the promoter and gene. These comparisons were achieved by constructing 2x2 contingency tables and using χ^2 analysis on Minitab for Windows software. A probability of less than 0.05 was deemed significant. Due to the number of allelic combinations possible with 3'VNTR and a 5'SNP, these multiple comparisons were Bonferroni corrected (pc). A probability of less than 0.05 after Bonferroni correction was deemed to be very significant. Power analysis was also carried out to determine the power of detecting a significant difference with the cohort size by calculating against an odds ratio (OR) of 2. This test was carried out using the 'PS Power and Sample Size Calculations' program.

These analyses were tested in all the populations. Furthermore comparisons between the patient groups and the controls were carried out in order to show possible significant variations in diseases. In addition comparisons between the different disease groups of the same ethnicity were analysed. Finally, a comparison with the two ethnic control groups was also tested in order to highlight any conceivable inconsistencies. The comparisons at each stage examined possible differences in allele and genotype numbers, allele and genotype associations, and haplotype observations.

RESULTS

4.1. Allelotype and genotype data of West of Scotland control population

The genotypes of the West of Scotland normal control population are shown in Table 2. The allele frequencies and their genotype counterparts for the three single nucleotide polymorphism (SNP) sites and 3' variable number tandem repeats (VNTR) are shown in Table 3. The results show that at the -594 polymorphic site, allele A was present at 41.7% and allele G at 58.3%. Similarly, at the -172, alleles C and G were present 44.2% and 55.8% respectively. At -570, allele C was rarely observed (4.5%) whilst allele G was common at 95.5%.

Chi-square analysis showed strong significant associations between the point mutations in the 5' promoter region. Alleles A and C at positions -594 and -172 respectively were seen in strong linkage disequilibrium (χ^2 =88.268, $p<0.00001$), as were the two G alleles at these sites (i.e. $p<0.00001$). Additionally, -594.A and -570.G were significantly associated as were -594.G and -570.C (χ^2=5.0960, p=0.024). No significant association was observed between either alleles at -172 and -570.

Based upon the observed associations and the allele distribution in the 5' promoter region, the following 3 haplotype families were considered to be prominent:

-594.A	-570.G	-172.C
-594.G	-570.G	-172.G
-594.G	-570.C	-172.G

accounting for 98.9% of the total possible haplotypes. These groups can subsequently be proposed terms that both differentiate the different allele combinations and determine their respective frequencies. It is suggested they be termed IL6.01, IL6.02, and IL6.03 respectively. The two additional haplotype families detected were too infrequent to be suggested as prominent haplotype families in this population.

TABLE 2

The results of the normal control West of Scotland population

SAMPLE NO.	5' REGION			3' VNTR
	FOK-1	FNU4H-I	NLA-III	
	-594	-570	-172	
30001	AG	nd	CG	4*4
30002	AG	nd	CG	3*7
30003	AA	GG	CC	nd
30006	GG	GG	GG	4*4
30007	AG	GG	CG	3*7
30008	AG	GG	CG	nd
30009	GG	GC	GG	nd
30010	AG	GG	GG	nd
30011	AG	GC	CG	3*4
30012	AG	GG	CG	3*6
30013	AA	GG	CC	3*3
30014	AG	GG	CG	nd
30015	AG	GG	CG	nd
30016	GG	GG	GG	nd
30017	AG	GG	CC	nd
30018	AG	GG	CG	3*4
30019	GG	GG	GG	4*6
30020	GG	GG	GG	4*7
30021	AA	GG	CC	nd
30022	AG	GG	CG	3*4
30023	GG	GG	nd	3*13
30024	GG	nd	GG	nd
30025	GG	GC	GG	nd
30027	AG	GG	CG	3*7
30028	AG	GG	CG	3*9
30029	nd	nd	nd	3*7

SAMPLE NO.	5' REGION			3' VNTR
	FOK-1	FNU4H-I	NLA-III	
	-594	-570	-172	
30030	AG	GG	nd	3*7
30031	GG	GC	CG	7*7
30032	AG	GG	CG	3*4
30033	GG	GC	nd	7*7
30034	AG	GG	CG	3*4
30036	AG	GG	CG	3*7
30037	AG	GG	CG	3*7
30038	AG	GG	CG	3*4
30039	GG	GG	GG	6*7
30040	GG	GG	GG	7*7
30041	AA	GG	CC	3*3
30042	AG	GG	CC	3*3
30043	GG	GG	GG	4*4
30044	nd	nd	nd	3*4
30045	GG	GG	GG	4*4
30046	GG	nd	GG	4*4
30047	AG	GG	CG	nd
30049	AG	GG	CG	3*4
30050	AG	GG	CG	3*7
30051	AG	GG	CG	3*4
30052	AG	GC	CG	3*7
30053	AG	GG	CG	3*4
30054	AG	GG	CG	3*7
30056	AA	GG	CC	3*3
30057	AG	GG	CG	3*4
30058	nd	nd	CG	3*4
30059	AA	GG	CC	3*3
30060	GG	GC	GG	4*7
30061	GG	GG	GG	4*7

SAMPLE NO.	5' REGION			3' VNTR
	FOK-1	FNU4H-I	NLA-III	
	-594	-570	-172	
30062	GG	GG	GG	4*4
30063	AG	GG	CG	3*4
30064	AA	GG	CC	4*4
30065	GG	GG	GG	7*8
30066	AG	GG	CG	3*4
30067	AG	GC	CG	4*7
30068	AG	GG	CG	3*4
30069	AG	GG	CG	3*4
30070	AA	GG	CC	3*3
30072	AG	GG	CG	nd
30073	GG	GG	GG	nd
30074	GG	GG	GG	nd
30075	GG	GG	GG	nd
30076	AG	GG	CG	nd
30077	AG	GG	CG	nd
30078	AG	GG	CG	nd
30079	AG	GG	CG	3*7
30080	AA	GG	CC	3*3
30081	AG	GG	CG	3*8
30082	GG	GG	GG	4*8
30083	AA	GG	CC	3*3
30084	AA	GG	CC	3*3
30085	AA	GG	CC	3*3
30086	AA	GG	CC	3*3
30087	AA	GG	CC	3*3
30088	AG	GG	CG	nd
30089	GG	GG	GG	4*7
30090	GG	GG	GG	4*4
30091	nd	nd	CC	3*3

SAMPLE NO.	5' REGION			3' VNTR
	FOK-1 -594	FNU4H-I -570	NLA-III -172	
30092	AG	GG	CG	nd
30093	AG	GG	CG	3*4
30094	nd	nd	nd	4*7
30095	nd	GG	CG	3*4
30096	GG	GC	GG	6*7
30097	AG	GG	CG	3*7
30098	nd	nd	nd	4*7
30099	AG	GG	CG	3*4
30100	GG	GG	GG	4*4
30101	AG	GG	CG	nd
30102	AG	GG	CG	3*7
30103	GG	GG	GG	6*7
30104	nd	nd	nd	3*7
30105	AG	GG	CG	3*4
30106	GG	GG	GG	7*8
30107	AA	GG	CC	3*3
30108	AG	GG	CG	3*4
30109	AG	nd	CG	3*7
30110	GG	GG	GG	4*4
30112	AA	GG	CC	nd
30113	AA	GG	CC	3*3
30114	GG	GG	GG	4*4
30115	AG	GG	CG	3*4
30136	GG	GG	GG	3*7
30137	GG	GG	GG	3*7
30138	GG	GG	GG	3*7
30158	nd	nd	CG	4*4
30172	AG	GG	CG	3*4

nd = not defined

TABLE 3

Allele distribution at four polymorphic loci in the normal control West of Scotland population

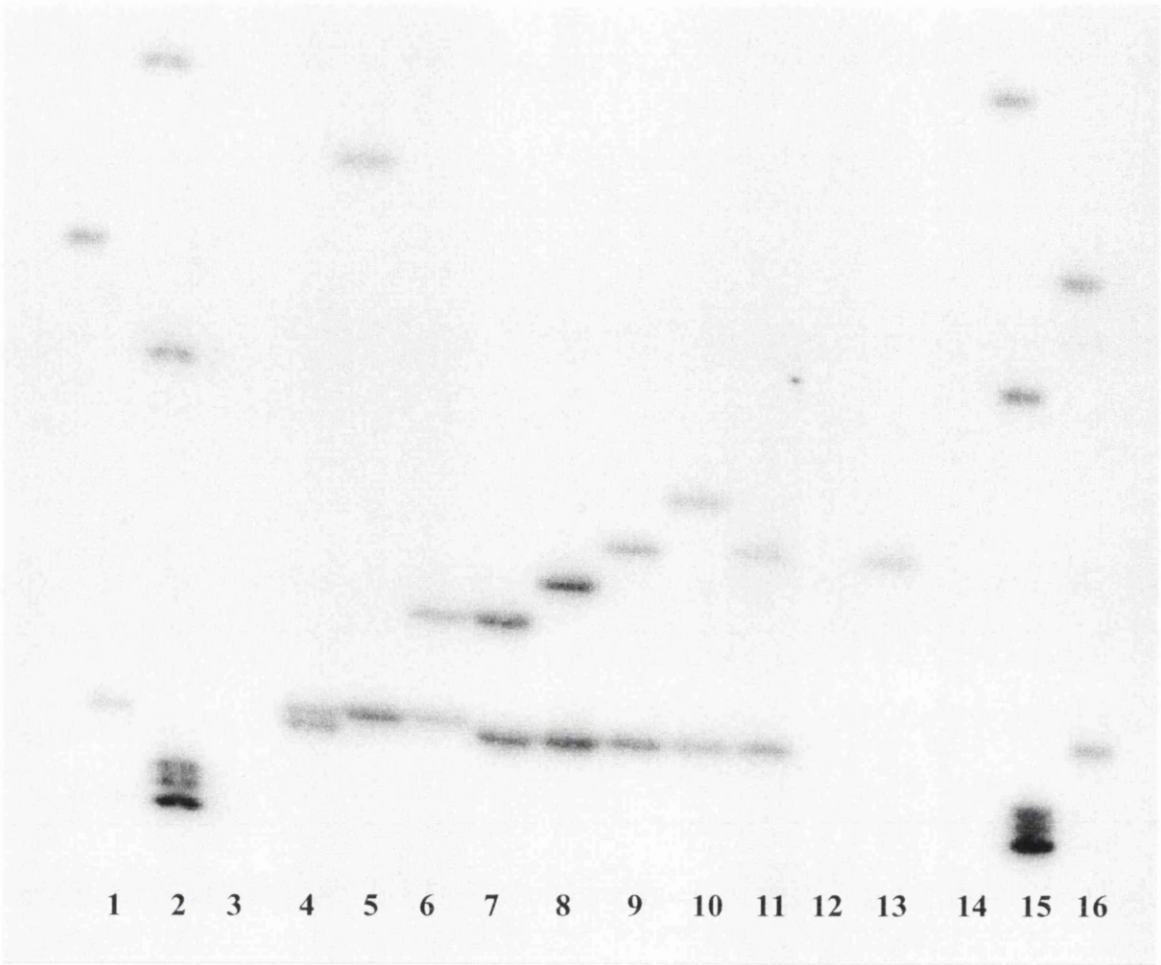
Locus	Allele	Number Observed (%)	Genotype	Number Observed (%)
-594	A	86 (41.7)	A*A	17 (16.5)
	G	120 (58.3)	A*G	52 (50.5)
			G*G	34 (33)
-570	C	9 (4.5)	G*G	90 (91.8)
	G	189 (95.5)	G*C	9 (8.2)
			C*C	0 (0)
-172	C	92 (44.2)	C*C	20 (19.2)
	G	116 (55.8)	C*G	52 (50)
			G*G	32 (30.8)
3'VNTR	3 (610bp)	75 (42.1)	3*3	15 (16.9)
	4 (616bp)	56 (31.5)	3*4	23 (25.8)
	6 (639bp)	5 (2.8)	3*6	1 (1.1)
	7 (648bp)	36 (20.2)	3*7	18 (20.2)
	8 (658bp)	4 (2.2)	3*8	1 (1.1)
	9 (672bp)	1 (0.6)	3*9	1 (1.1)
	13 (773bp)	1 (0.6)	3*13	1 (1.1)
			4*4	12 (13.5)
			4*6	1 (1.1)
			4*7	7 (7.9)
			4*8	1 (1.1)
			6*7	3 (3.4)
			7*7	3 (3.4)
			7*8	2 (2.2)

These were:

-594.G	-570.G	-172.C
-594.A	-570.G	-172.G

Analysis of the 3' VNTR displayed 7 irregularly sized alleles in the West of Scotland control population, although an additional 6 alleles were observed in the various disease groups studied from the West of Scotland area. The individual genotypes can be observed in Table 2. The allele sizes in the West of Scotland control population ranged from 610bp to 773bp and the proportions of the respective alleles for the control population are shown in Table 2 and illustrated in fig.4. The data show that allele 3 is most frequent followed by alleles 4 and 7 (42.1%, 31.5%, and 20.2% respectively). The four prominent genotypes, 3*4 (25.8%), 3*7 (20.2%), 3*3 (16.9%) and 4*4 (13.5%) accounted for 76.2% of the total.

Associations between the multi-allelic 3' VNTR and point mutations in the 5' region were seen with alleles 3, 4, 6, and 7. Allele 3 was strongly associated with both -594.A and
-172.C (χ^2=57.388, pc<0.00013, χ^2=58.434, pc<0.00013). A weak association between -570.G and 3' VNTR allele 3 was also observed but which diminished when corrected for multiple comparisons (χ^2 =5.095, p=0.024, pc=0.312). 3' VNTR allele 4 was associated with -594.G and -172.G (χ^2 =11.908, pc=0.0078, χ^2 =15.919, pc=0.00013 respectively), but not with either of the -570 alleles. Again this was seen with allele 6, where weak associations with -594.G and -172.G were observed which became insignificant when Bonferroni corrected (χ^2 =4.3090, p=0.0379, pc=0.4927, χ^2 =5.1240, p=0.0236, pc=0.3068). Finally there was a strong association between IL-6 3' VNTR allele 7 and -594.G, -172.G and -570.G (χ^2 =13.147, pc=0.0039, χ^2 =10.52, pc=0.0156, χ^2 =13.263, pc=0.0039 respectively).



Lane 1: 123bp ladder
Lane 2: 100bp ladder
Lane 3: water blank
Lane 4: 3/4
Lane 5: 4/13
Lane 6: 4/6
Lane 7: 3/6
Lane 8: 3/7
Lane 9: 3/8
Lane10: 3/9
Lane 11: 3/8
Lane 12: blank
Lane 13: 8/8
Lane 14: blank
Lane 15: 100bp ladder
Lane 16: 123bp ladder

Gel by Mrs. J. Eskdale

FIGURE 4. Demonstration of alleles sizes at the IL-6 3'VNTR locus

Based upon the associations observed and the allele distribution it is postulated that there are four potential extended relationships between 5' SNP and 3' VNTR. Furthermore it is proposed that these associations spanning the IL-6 gene functioned as haplotypes:

i.	-594.A	-570.G	-172.C	3' VNTR.3
ii.	-594.G	-570.G	-172.G	3' VNTR.4
iii.	-594.G	-570.G	-172.G	3' VNTR.7
iv.	-594.G	-570.C	-172.G	3' VNTR.7

The 2nd and 3rd extended associations contain the –570.G allele by default. It is suggested that the haplotypes be termed IL6.0103, IL6.0204, IL6.0207 and IL6.0307 respectively. The former part of the terminology relates to the 5' promoter haplotype families as shown earlier, and the latter part of the terminology further differentiates one haplotype from another by making use of the 3' allele.

In support of these proposed haplotypes, 13 individuals were homozygous for IL6.0103, 8 were homozygous for IL6.0204 and one for IL6.0207. Furthermore, genotyping data were consistent with a further 18 being heterozygous for IL6.0103/ IL6.0204 and 9 more for IL6.0103/ IL6.0207. Subtraction of these haplotypes from the population revealed a number of supposed haplotypes and their distribution can be observed in Table 4. It can be seen that these four haplotypes mentioned potentially account for 87% of the total genotypes observed. The remaining were seen too infrequently to be suggested as prominent haplotypes in this population.

TABLE 4

Putative haplotypes observed in the West of Scotland Control population

5'			3'	Number	
-594	**-570**	**-172**	**VNTR**	**observed (%)**	
A	**G**	**C**	**3**	**60 (41.1)**	**IL6.0103**
A	G	C	4	3 (2.1)	IL6.0104
G	G	G	3	3 (2.1)	IL6.0203
G	**G**	**G**	**4**	**41 (28.1)**	**IL6.0204**
G	G	G	6	5 (3.4)	IL6.0206
G	**G**	**G**	**7**	**21 (14.4)**	**IL6.0207**
G	G	G	8	4 (2.7)	IL6.0208
G	G	G	9	1 (0.7)	IL6.0209
G	C	G	4	1 (0.7)	IL6.0304
G	**C**	**G**	**7**	**5 (3.4)**	**IL6.0307**
G	G	C	3	1 (0.7)	IL6.0403
G	G	C	7	1 (0.7)	IL6.0407

4.2. Allelotype and genotype of West of Scotland Gastric cancer patients

The genotype data observed from the West of Scotland gastric cancer patient population including *H. Pylori* status (where known) is shown in Table 5. This data is missing in some patients as these were diagnosed before it became standard routine practice to look for *H. Pylori* in the pathology slides. Table 6 contains the frequencies of the alleles and genotypes at the SNPs and 3' VNTR. Allele A at -594 was observed at 44.8% and G at 55.2%. Similarly at -172, the alleles were distributed at 48.5% and 50.5% (C and G respectively). At -570, allele C was very rare and seen only at 1.5% in comparison to 98.5% for -570G.

Chi-squared analysis showed strong linkage disequilibrium between -594.A and -172.C, and -594.G and -172.G, (χ^2=68.9830, p<0.00001). No significant associations were observed at -570 and either of the alleles at -594 or -172.

In this population 4 haplotype families were observed, although the two prominent were IL6.01 and IL6.02, accounting for 95.5% of the total in this population. Tumour location bore no significance in the distribution. However, there was a significant difference when segregated regarding *H.pylori* status. The haplotype termed IL6.02 was observed more frequently in *H.Pylori* negative patients in comparison to positive patients where as haplotype IL6.01 was more common in positive patients, (χ^2=7.435, p=0.0243).

At the 3' region additional alleles were observed in comparison to the normal population and these can be observed in Table 6. Fig.5 demonstrates the overall range of alleles observed in the various West of Scotland cohorts. The results show that allele 3 was most frequently observed (48.1%) followed by allele 4 (25.0%) and allele 7 at 17.3%. Five prominent genotypes constituted 82.6% of the total observed. These were 3*3 (26.9%), 3*7 and 3*4 (both at 19.2%), 4*4 (9.6%) and 4*7 (7.7%).

TABLE 5

The results of the West of Scotland Gastric population

PATH. NO.	5' REGION			3' REGION	H.P. STATUS
	FOK-I	FNU4H-I	NLA-III		
	-594	-570	-172		
40001	GG	GC	GG	nd	nd
40003	GG	GG	CG	nd	nd
40005	GG	nd	CG	nd	nd
40006	AA	GG	CC	3*3	nd
40007	AG	GG	CG	3*7	nd
40008	nd	GG	nd	nd	nd
40009	nd	nd	nd	7*8	nd
40010	AA	GG	CC	3*3	positive
40011	GG	GG	GG	7*13	nd
40014	GG	GG	GG	3*7	nd
40016	AA	GG	CC	nd	nd
40018	AG	GG	CG	nd	positive
40020	AG	GG	CG	3*4	nd
40021	AG	GG	CC	3*7	nd
40022	AG	GG	CG	3*4	nd
40023	AG	GG	CG	3*6	nd
40024	GG	GG	GG	6*7	nd
40026	AG	GG	CG	3*3	nd
40027	GG	GG	GG	nd	negative
40028	GG	GG	GG	nd	nd
40029	AG	GG	CG	3*3	nd
40031	AG	GG	CC	3*7	negative
40032	AG	GG	CG	3*3	nd
40033	AA	GG	CC	3*3	positive
40035	AG	GC	CG	3*7	nd
40036	AG	GG	CG	3*7	nd

PATH. NO.		5' REGION		3' REGION	H.P. STATUS
	FOK-I	FNU4H-I	NLA-III		
	-594	-570	-172		
40038	AA	GG	CC	3*3	nd
40041	GG	GG	GG	4*11	nd
40043	AG	GG	CG	3*4	negative
40044	AG	GG	CG	3*4	nd
40045	GG	GG	GG	4*7	nd
40056	GG	GG	GG	nd	nd
40058	AG	GG	CG	3*4	positive
40069	AA	GG	CC	3*3	nd
40071	GG	GG	GG	nd	nd
40073	AA	GG	CC	3*3	negative
40075	AG	GG	CG	3*7	negative
40079	AA	GG	CC	nd	nd
40085	AG	GG	CG	nd	positive
40086	GG	GG	GG	nd	positive
40091	GG	GG	GG	7*8	negative
40095	GG	GG	GG	4*4	nd
40096	AA	GG	CC	3*3	positive
40097	AG	GG	CG	3*4	positive
40100	AG	GG	CG	4*4	nd
40102	AA	GG	CC	3*7	positive
40123	GG	GG	GG	nd	negative
40140	AG	GG	CG	3*7	negative
40147	AG	GG	CG	nd	positive
40151	AG	GG	CG	3*4	positive
40153	AG	GG	CG	nd	positive
40156	GG	GG	GG	4*6	negative
40161	GG	GG	GG	4*4	negative
40165	AA	GG	CC	4*4	negative
40170	AG	GG	CG	3*4	nd

PATH. NO.	5' REGION			3' REGION	H.P. STATUS
	FOK-I	FNU4H-I	NLA-III		
	-594	-570	-172		
40173	GG	GG	GG	4*7	positive
40174	AA	GG	CC	3*3	positive
40175	AA	GG	CC	3*3	positive
40178	AA	GG	CC	3*3	positive
40189	GG	GG	CG	4*7	negative
40196	AG	GG	CG	3*13	positive
40206	AG	GG	CG	1*8	positive
40208	AA	GG	CC	3*3	positive
40209	AG	GG	CG	3*4	nd
40214	AG	GG	CG	3*4	negative
40215	GG	GG	GG	4*4	negative
40217	GG	GG	GG	4*7	positive
40219	AG	GG	CG	nd	positive
40221	AG	GG	CG	3*7	negative

nd = not defined

TABLE 6

Allele distribution at four polymorphic loci in the Gastric West of Scotland population

Locus	Allele	Number Observed (%)	Genotype	Number Observed (%)
-594	A	60 (44.7)	A*A	15 (22.4)
	G	74 (55.2)	A*G	30 (44.8)
			G*G	22 (32.8)
-570	C	2 (1.5)	G*G	65 (97)
	G	132 (98.5)	G*C	2 (3)
			C*C	0 (0)
-172	C	65 (48.5)	C*G	17 (25.3)
	G	69 (51.5)	C*G	31 (46.3)
			G*G	19 (28.4)
3'VNTR	1	1 (0.9)	1*8	1 (1.9)
	3	50 (48.1)	3*3	14 (26.9)
	4	26 (25)	3*4	10 (19.2)
	6	3 (2.9)	3*6	1 (1.9)
	7	18 (17.3)	3*7	10 (19.2)
	8	3 (2.9)	3*13	1 (1.9)
	11	1 (0.9)	4*4	5 (9.6)
	13	2 (1.9)	4*6	1 (1.9)
			4*7	4 (7.7)
			4*11	1 (1.9)
			6*7	1 (1.9)
			7*8	2 (3.8)
			7*13	1 (1.9)

Lane1: 3/12
Lane 2: 4/8
Lane 3: 2/4
Lane 4: 5/8
Lane 5: 1/8
Lane 6: 7/10
Lane 7: 5/11
Lane 8: 4/12
Lane 9: 7/13
Lane10: 6/13
Lane 11: 4/13
Lane 12: 5/8

FIGURE 5. Overall range of 3'alleles in a variety of West of Scotland cohorts

Five prominent genotypes constituted 82.6% of the total observed. These were 3*3 (26.9%), 3*7 and 3*4 (both at 19.2%), 4*4 (9.6%) and 4*7 (7.7%).

Calculations showed associations across the promoter SNPs and 3' VNTR. 3' VNTR allele 3 was significantly associated with -594.A and -172.C (χ^2=18.748, pc<0.000015, χ^2=34.9160, pc<0.00015). Similarly a strong association was also observed with -594.G and -172.G with 3' allele 4 (-594.G: 3' allele 4, χ^2=10.324, pc=0.0169, -172.G: 3' allele 4, χ^2=11.486, pc=0.0105). Additionally -594.G was also seen to be weakly associated with 3' allele 7 (χ^2=6.9180, p=0.0085) but which became insignificant when Bonferroni corrected (p_C=0.1105). No significant association was observed with either of the -570 alleles and the 3' VNTR.

As in the control population, the observed associations postulated that there were three potential relationships spanning the 5' SNP and 3' VNTR that showed haplotypic behaviour. These were IL6.0103, IL6.0204, and IL6.0207. The results verified these haplotypes. Of the 51 individuals tested, 11 were homozygous for IL6.0103 and 3 were homozygous IL6.0204. Genotyping further presented that 10 individuals were heterozygous IL6.0103/ IL6.0204, 5 individuals for IL6.0103/ IL6.0207 and 3 for IL6.0204/ IL6.0207. The haplotype IL6.0307 previously prominent in the West of Scotland control population occurred only once in this test population (0.9%). This is unlikely to be significant due to the small Gastric cohort and rarity of the C allele at -570. However, additional study is required to clarify this. Subtraction of these haplotypes revealed the remaining haplotypes, which can be observed with their frequencies in Table 7. It was observed that the three prominent haplotypes potentially accounted for 79.4% of the postulated haplotypes observed. There was no significant difference when these extended haplotypes were separated according to *H.pylori* status or tumour location.

TABLE 7

Putative haplotypes observed in the West of Scotland Gastric population

5'			3'	Number	
-594	-570	-172	VNTR	observed (%)	
A	G	C	1	1 (1)	IL6.0101
A	**G**	**C**	**3**	**46 (45.1)**	**IL6.0103**
A	G	C	4	3 (2.9)	IL6.0104
A	G	C	7	1 (1)	IL6.0107
G	**G**	**G**	**4**	**23 (22.5)**	**IL6.0204**
G	G	G	3	4 (3.9)	IL6.0203
G	G	G	6	3 (2.9)	IL6.0206
G	**G**	**G**	**7**	**12 (11.8)**	**IL6.0207**
G	G	G	8	2 (2)	IL6.0208
G	G	G	11	1 (1)	IL6.0211
G	G	G	9	2 (2)	IL6.0213
G	C	G	7	1 (1)	IL6.0307
G	G	C	7	3 (2.9)	IL6.0407

4.3. Barrett's Oesophagus patients

Analysis on patients diagnosed with Barrett's Oesophagus was carried out in two ways. Patients diagnosed with either the Benign or Malignant form of the disease were collectively grouped to produce a disease cohort for analysis against the West of Scotland control population and gastric cancer patients. The data were then categorised into the two sections to compare possible associations of the locus to malignancy in this disease.

Allelotype and genotype of West of Scotland Barrett's oesophagus patients

The combined data for the Barrett's Oesophagus patients with *H. Pylori* status are shown in Table 8, with their allele frequencies and their genotype counterparts in Table 9. The results show that both -594.A and -172.C were less common than their counterpart -594.G and -172.G, at 39.8% and 40.1%. Allele C at -570 was very rare and only seen 5.9%. Chi-squared analysis showed that -594.A and -172.C were in strong linkage disequilibrium as were the corresponding -594.G and -172.G (χ^2=116.368, p<0.00001). No other associations were observed amongst the 5' SNP.

Six putative haplotype families were represented in this population, five of which were identical to the control population. The sixth haplotype family absent in the control population and only detected once in this population was:

-594.A -570.C -172.C

Following the haplotype family nomenclature, this may be termed IL6.06. Consistent with the control population, the two prominent haplotype families in this group were IL6.01 and IL6.02, which accounted for 91.2%.

Separation of these haplotype families into *H.pylori* status revealed that there was a significant difference in distribution of the two prominent haplotypes (χ^2=12.111, p=0.0332). Haplotype family IL6.01 was more frequent in *H.pylori* negative patients, whilst IL6.02 was more frequent in positive patients.

TABLE 8

The results of West of Scotland Barrett's Oesophagus population

BENIGN BARRETT'S	5' REGION			3' VNTR	H.P. STATUS
	FOK-I	FNU4-HI	NLA-III		
Path No.	-594	-570	-172		
7031/95	AG	GG	CG	3*4	nd
7630/95	GG	GG	GG	4*13	negative
7895/95	AG	GC	CG	3*7	negative
8398/95	AA	GG	CC	3*3	nd
8540/95	AA	GG	CC	3*7	negative
8662/95	AG	GG	CG	3*7	negative
9492/95	AA	GC	CC	3*3	negative
9801/95	GG	GG	GG	7*8	positive
10074/95	AG	GG	CG	4*4	nd
10075/95	GG	GG	GG	7*8	negative
10409/95	GG	GG	GG	4*8	negative
10658/95	AA	GG	CG	nd	negative
10660/95	AA	GG	CC	3*3	negative
11492/95	GG	GG	GG	4*7	nd
11509/95	GG	GG	GG	4*4	nd
12045/95	AG	GC	CG	3*7	nd
12661/95	AG	GG	CG	4*4	nd
12662/95	AG	GG	CG	3*7	nd
12664/95	GG	GG	GG	6*7	positive
13005/95	AG	GG	CG	5*7	negative
13049/95	AA	GG	CC	3*3	nd
13331/95	AG	GG	CG	3*4	positive
1213/96	AG	GG	GG	nd	negative
1786/96	GG	GG	CC	3*3	positive
3039/96	AG	GC	CG	3*7	negative
3929/96	AA	GG	CC	nd	nd

BENIGN BARRETT'S		5' REGION		3' VNTR	H.P. STATUS
	FOK-I	FNU4-HI	NLA-III		
Path No.	-594	-570	-172		
5559/96	GG	GG	GG	4*7	positive
5596/96	GG	GG	GG	4*7	positive
6309/96	AG	GG	CG	3*3	negative
7942/96	AG	GG	nd	3*4	negative
7984/96	GG	GG	GG	4*4	positive
8486/96	AG	GG	CG	3*3	nd
10665/96	AG	GG	CG	3*4	negative
10686/96	AG	GC	CG	4*7	negative
10989/96	AA	GG	CC	3*3	negative
11552/96	AA	GG	CC	3*3	negative
14325/96	GG	GG	GG	3*4	negative
14600/96	AG	GG	CG	4*4	negative
14601/96	GG	GG	GG	4*7	negative
14645/96	AG	GG	CG	3*6	negative
14928/96	AG	GG	CG	3*4	negative
15274/96	AG	GG	CG	3*7	negative
1466/97	AA	GG	CC	nd	positive
1790/96	AA	GG	CC	nd	negative
2121/97	GG	GG	GG	6*7	negative
3126/97	AA	GG	CC	nd	negative
3485/97	GG	GC	GG	7*7	positive

MALIGNANT BARRETT'S	5' REGION			3' VNTR	H.P. STATUS
	FOK-I -594	FNU4-HI -570	NLA-III -172		
40004	AG	GG	CG	3*7	nd
40012	AG	GG	CG	nd	nd
40013	AG	GG	CC	3*7	nd
40015	GG	GG	GG	nd	nd
40017	GG	GG	GG	4*7	nd
40019	AG	GG	CG	nd	nd
40030	GG	GG	GG	4*7	nd
40037	GG	GG	GG	4*4	nd
40050	AA	GG	CC	3*3	positive
40051	AG	GG	CG	3*4	negative
40053	AG	GG	CG	nd	nd
40059	GG	GC	nd	nd	positive
40060	GG	GG	GG	nd	nd
40063	GG	GG	GG	nd	nd
40072	GG	GC	GG	7*8	negative
40074	AA	GG	CC	3*13	positive
40076	GG	GG	GG	nd	nd
40080	AA	GG	CC	4*4	positive
40094	nd	nd	GG	nd	nd
40098	GG	GG	GG	6*6	nd
40101	GG	GG	GG	7*13	nd
40105	GG	GG	GG	nd	nd
40106	nd	nd	CG	nd	positive
40112	AG	GG	CG	nd	negative
40118	AA	GG	CC	3*3	nd
40121	GG	GG	GG	nd	negative
40125	AG	GG	CG	nd	nd
40129	AG	GG	CG	nd	positive
40130	AG	GG	CG	3*4	positive

MALIGNANT BARRETT'S	5' REGION			3' VNTR	H.P. STATUS
	FOK-I -594	FNU4-HI -570	NLA-III -172		
40132	nd	nd	CG	nd	positive
40133	nd	nd	CG	nd	positive
40135	AG	GG	CG	3*7	nd
40142	AG	GG	GG	nd	nd
40144	nd	nd	CG	nd	positive
40146	GG	GG	GG	nd	positive
40158	GG	GG	GG	5*8	positive
40162	GG	GG	GG	4*8	negative
40163	GG	GG	GG	2*4	positive
40166	AG	GC	CG	3*7	negative
40180	GG	GG	GG	4*7	positive
40184	AG	GG	CG	3*6	negative
40187	AA	GG	CC	nd	nd
40193	AA	GG	CC	3*3	negative
40195	GG	GG	GG	4*7	negative
40199	AG	GG	CG	3*7	negative
40200	GG	GG	GG	4*7	positive
40201	GG	GC	GG	4*7	nd
40204	GG	GG	GG	4*4	positive
40205	GG	GC	GG	7*7	positive
40210	AG	GG	CG	3*8	nd
40211	AA	GG	CC	3*3	positive

nd = not defined

TABLE 9

Allele distribution at four polymorphic loci in the Barrett's Oesophagus population

Locus	Allele	Number Observed (%)	Genotype	Number Observed (%)
-594	A	74 (39.8)	A*A	19 (20.4)
	G	112 (60.2)	A*G	36 (38.7)
			G*G	38 (40.8)
-570	G	175 (94.1)	G*G	82 (88.2)
	C	11 (5.9)	G*C	11 (11.8)
			C*C	0 (0)
-172	C	77 (40.1)	C*C	20 (20.8)
	G	115 (59.9)	C*G	37 (38.5)
			G*G	39 (40.6)
3'VNTR	2	1 (0.7)	2*4	1 (1.4)
	3	50 (35.2)	3*3	13 (18.3)
	4	39 (27.5)	3*4	8 (11.3)
	5	2 (1.4)	3*6	2 (2.8)
	6	6 (4.2)	3*7	12 (16.9)
	7	34 (23.9)	3*8	1 (1.4)
	8	7 (4.9)	3*13	1 (1.4)
	13	3 (2.1)	4*4	8 (11.3)
			4*7	11 (15.5)
			4*8	2 (2.8)
			4*13	1 (1.4)

Genotype	Number Observed (%)
5*7	1 (1.4)
5*8	1 (1.4)
6*6	1 (1.4)
6*7	2 (2.8)
7*7	2 (2.8)
7*8	3 (4.2)
7*13	1 (1.4)

At the 3' region 3 prominent alleles were observed. These were allele 3 at 35.2%, 4 at 27.5% and 7 at 23.9%. The most common genotypes observed were 3*3 (18.3%), followed by 3*7 and 4*7 (16.9% and 15.5% respectively). Analysis by Chi-square test revealed associations between 3' VNTR alleles and 5' SNP. Allele 3 was strongly associated with -594.A and -172.C (χ^2=66.202, pc<0.00014, χ^2= 73.030, pc<0.00014). Alleles 4 and 7 were seen to be strongly associated with -594.G (χ^2= 9.33, pc=0.0322, χ^2= 8.906, pc=0.0392) and 172.G (χ^2=11.558, pc=0.0098, χ^2= 8.576, pc=0.0476). Additionally -570.C was also strongly associated to 3' VNTR 7 (χ^2= 11.395, pc=0.0084). Weak associations at allele 8 were observed with -594.G and -172.G χ^2= 5.734, p=0.0166, pc=0.2324, χ^2= 6.144, p=0.0132, pc=0.1848. A weak association at allele 13 with -172.G (χ^2= 3.85, p=0.0497, pc=0.6958) completed all the observed associations.

A number of putative haplotypes were seen in the Barrett's Oesophagus patients although, as in the control population, the four prominent were IL6.0103, IL6.0204, IL6.0207, and IL6.0307. From the population 8 were homozygous for IL6.0103 and 3 showed IL6.0204 homozygosity. Additionally, genotyping revealed heterozygosity in 3 individuals for the haplotypes IL6.0103/ IL6.0207, 5 for IL6.0204/ IL6.0207 and 2 for IL6.0103/ IL6.0204. In total these 4 prominent haplotypes represented 76.4% of the total population and the remaining can be observed in Table 10.

In comparison to the haplotype families, segregation of extended haplotypes according to *H. pylori* infection detected no significant difference in the distribution. This was further confirmed by Monte Carlo simulation.

Table 10

Putative haplotypes observed in the West of Scotland Barrett's Oesophagus population

5'			3'	Number	
-594	-570	-172	VNTR	observed (%)	
A	**G**	**C**	**3**	**43 (30.7)**	**IL6.0103**
A	G	C	4	6 (4.3)	IL6.0104
A	G	C	5	1 (0.7)	IL6.0105
A	G	C	7	1 (0.7)	IL6.0107
A	G	C	13	1 (0.7)	IL6.0113
G	G	G	2	1 (0.7)	IL6.0202
G	G	G	3	3 (2.1)	IL6.0203
G	**G**	**G**	**4**	**32 (22.9)**	**IL6.0204**
G	G	G	5	1 (0.7)	IL6.0205
G	G	G	6	6 (4.3)	IL6.0206
G	**G**	**G**	**7**	**23 (16.4)**	**IL6.0207**
G	G	G	8	7 (5)	IL6.0208
G	G	G	13	2 (1.4)	IL6.0213
G	**C**	**G**	**7**	**9 (6.4)**	**IL6.0307**
G	G	C	3	3 (2.1)	IL6.0403
G	G	C	7	1 (0.7)	IL6.0407
A	C	C	3	1 (0.7)	IL6.0603

Allelotype and genotype of West of Scotland Benign Barrett's patients

The 5' SNP allelotype and counterpart genotypes can be observed in Table 11. At position -594, allele G and -172.G were observed at 53.2% and 53.3% compared to the less common alleles at 46.8% and 46.7%. In contrast allele G at -570 was most common at 93.6%.

Probability analysis showed significant association between -594.A and -172.C (χ^2 =49.695, p<0.00001) as there was for -594.G and -172.G. Chi-squared analysis showed no further significant association with either alleles at -570 and those at -594 or -172.

Six putative haplotype families were represented in this population. However the two haplotype families, which also showed significant association, were IL6.01 and IL6.02 accounting for 89.1%. When these haplotypes families were classed in relation to *H.pylori* infection, a significant difference in the distribution was detected. Haplotype family IL6.01 was more frequent in *H.pylori* negative patients compared to infected individuals, who had a higher distribution of IL6.02.

Of the 7 alleles present at the 3' region, alleles 3, 4 and 7 were the most common (39%, 28% and 23.2% respectively) making up 90.2% of the total. The most common genotypes were 3*3 (21.6%), 3*7 (17.1%) and 3*4 (14.6%). Analysis by chi-square showed that the 5' SNP were significantly associated with alleles in the 3' region. -594.A and -172.A were significantly associated with allele 3 (χ^2=28.117, pc<0.00012, χ^2 =32.993, pc<0.00012 respectively). Additionally, allele G at -594 was weakly associated with 3' VNTR alleles 4, 7 and 8 (allele 4, χ^2=5.328, p=0.021, pc=0.252, allele 7, χ^2= 3.990, p=0.0458, pc=0.5496, allele 8, χ^2=4.194, p=0.0416, pc=0.4992 respectively) as was allele G at -172 (allele 4, χ^2=6.613, p=0.0101, pc=0.1212, allele 7, χ^2= 4.586, p=0.0322, pc=0.3864, allele 8, χ^2=4.377, p=0.0354, pc=0.4248 respectively).

The associations and data obtained showed that there were five potential relationships across the polymorphic region, which behaved as putative haplotypes. These were similar

TABLE 11

Allele distribution at four polymorphic loci in the Benign Barrett's population

Locus	Allele	Number Observed (%)	Genotype	Number Observed (%)
-594	A	44 (46.8)	A*A	12 (25.5)
	G	50 (53.2)	A*G	20 (42.6)
			G*G	15 (31.9)
-570	G	88 (93.6)	G*G	41 (87.2)
	C	6 (6.4)	G*C	6 (12.8)
			C*C	0 (0)
-172	C	43 (46.7)	C*C	12 (26)
	G	49 (53.3)	C*G	19 (41.3)
			G*G	15 (32.6)
3'VNTR	3	32 (39)	3*3	9 (21.6)
	4	23 (28)	3*4	6 (14.6)
	5	1 (1.2)	3*6	1 (2.4)
	6	3 (3.7)	3*7	7 (17.1)
	7	19 (23.2)	4*4	5 (12.2)
	8	3 (3.7)	4*7	5 (12.2)
	13	1 (1.2)	4*8	1 (2.4)
			4*13	1 (2.4)
			5*7	1 (2.4)
			6*7	2 (4.9)
			7*7	1 (2.4)
			7*8	2 (4.9)

to the prominent haplotypes observed in the West of Scotland control population, i.e. IL6.0103, IL6.0204, IL6.0207, and IL6.0307. In this population however, IL6.0208 was detected to be a prominent putative haplotype. Data from the population indicated that 5 individuals were homozygous for IL6.0103, 1 for homozygous IL6.0204 and 4 for heterozygous IL6.0103/ IL6.0204. Additionally, 3 were heterozygous for IL6.0103/ IL6.0307, 3 for heterozygous IL6.0103/ IL6.0307 and 3 for heterozygous IL6.0204/ IL6.0207. The four prominent haplotypes were seen to potentially account for 76.3% of the postulated haplotypes. Extraction of these haplotypes revealed the rest, which can be observed in Table 12.

TABLE 12

Putative haplotypes observed in the West of Scotland Benign Barrett's population

5'			3'	Number	
-594	-570	-172	VNTR	observed (%)	
A	**G**	**C**	**3**	**25 (31.3)**	**IL6.0103**
A	G	C	4	4 (5)	IL6.0104
A	G	C	5	1 (1.3)	IL6.0105
A	G	C	7	1 (1.3)	IL6.0107
G	G	G	3	3 (3.8)	IL6.0203
G	**G**	**G**	**4**	**18 (22.5)**	**IL6.0204**
G	G	G	6	3 (3.8)	IL6.0206
G	**G**	**G**	**7**	**13 (16.3)**	**IL6.0207**
G	**G**	**G**	**8**	**3 (3.8)**	**IL6.0208**
G	G	G	13	1 (1.3)	IL6.0213
G	**C**	**G**	**7**	**5 (6.3)**	**IL6.0307**
G	G	C	3	2 (2.5)	IL6.0403
A	C	C	3	1 (1.3)	IL6.0603

Allelotype and genotype of West of Scotland Barrett's malignant patients

The data observed for malignant Barrett's patients can be observed in Table 13. A smaller proportion of the allele A at -594 and C at -172 was observed (32.6% and 34%). At -570, allele G was observed 94.6%. Analysis detected strong linkage disequilibrium between the two G alleles and alleles A and C, both at -594 and -172 respectively (χ^2=64.8, p<0.00001), but no association with alleles at -570 and either of the alleles at -594 or -172.

The accumulation of obtained results and analysed data determined five haplotype families, although the two prominent were IL6.01 and IL6.02 accounting for 93.3%. In this study the distribution of the haplotypes according to *H.pylori* infection showed no significance. This data was also analysed against previous data obtained by H. Kasem whom showed the haplotype a2d4 at the TNF was significantly associated to this disease. There was no significant distribution of any observed haplotype family to the microsatellite alleles TNF a2 or d4, or the genotype a2d4. However there was a significant difference of the two prominent haplotype families IL6.01 and IL6.02, and alleles TNF a6 (χ^2=6.414, p=0.0113, χ^2=6.520, p=0.0107 respectively) and d3(χ^2=24.528, p<0.00001, χ^2=21.179, p<0.00001). Furthermore this distribution difference was detected against the genotype TNF a6d3 (χ^2=3.861, p=0.0494, χ^2=5.642, p=0.0175).

At the 3' region 3 alleles were prominent; allele 3 (30%), allele 4 (26.7%) and allele 7 (25%). The most common genotypes observed were 4*7 (20%), 3*7 (16.6%) and 3*3 (13.3%). Chi-squared analysis showed associations between the 5' SNP and the three common allele at the 3' VNTR. A strong association was observed with -594.A and -172.C with allele 3 (χ^2 =40.179, pc<0.00013, χ^2 =41.368, pc<0.00013 respectively). At allele 4 and 7 only weak associations which became insignificant when Bonferroni corrected were observed with both -594.G and -172.G (at allele 4: -594.G, χ^2 =4.176, p=0.041, pc=0.533, -172.G, χ^2 =65.065, p=0.0244, pc=0.3172, at allele 7: -594.G: χ^2 =5,

TABLE 13

Allele distribution at four polymorphic loci in the Malignant Barrett's population

Locus	Allele	Number Observed (%)	Genotype	Number Observed (%)
-594	A	30 (32.6)	A*A	7 (15.2)
	G	62 (67.4)	A*G	16 (34.8)
			G*G	23 (50)
-570	G	87 (94.6)	G*G	41 (89.1)
	C	5 (5.4)	G*C	5 (10.8)
			C*C	0 (0)
-172	C	34 (34)	C*C	8 (16)
	G	66 (66)	C*G	18 (38)
			G*G	24 (48)
3'VNTR	2	1 (1.6)	2*4	1 (3.3)
	3	18 (30)	3*3	4 (13.3)
	4	16 (26.7)	3*4	2 (6.6)
	5	1 (1.6)	3*6	1 (3.3)
	6	3 (5)	3*7	5 (16.6)
	7	15 (25)	3*8	1 (3.3)
	8	4 (6.7)	3*13	1 (3.3)
	13	2 (3.3)	4*4	3 (10)
			4*7	6 (20)
			4*8	1 (3.3)
			5*8	1 (3.3)
			6*6	1 (3.3)
			7*7	1 (3.3)
			7*8	1 (3.3)
			7*13	1 (3.3)

p=0.0253, pc=0.3289, -172.G: χ^2 =3.956, p=0.0467, pc=0.6071). Additionally -570.C was also weakly associated with allele 7 (χ^2 =6.4291, p=0.0112, pc=0.1344).

As in the West of Scotland control population, these associations and supported data suggested that there were four prominent extended haplotypes in this cohort. These were IL6.0103, IL6.0204, IL6.0207 and IL6.0307. From the population, 4 individuals were homozygous for IL6.0103 and 2 for IL6.0204. Additionally, 2 individuals were heterozygous for IL6.0103/ IL6.0207, 5 for IL6.0204/ IL6.0207, 1 individual for IL6.0103/ IL6.0307 and 1 for IL6.0204/ IL6.0307. Extraction of the putative haplotypes revealed the remaining in this population and is represented in Table 14. Separation of the haplotypes in relation to *H.pylori* status or tumour location presented no significance in the haplotype distribution.

TABLE 14

Putative haplotypes observed in the West of Scotland Malignant Barrett's population

5'			3'	Number	
-594	-570	-172	VNTR	observed (%)	
A	**G**	**C**	**3**	**18 (30)**	**IL6.0103**
A	G	C	4	2 (3.3)	IL6.0104
A	G	C	13	1 (1.7)	IL6.0113
G	G	G	2	1 (1.7)	IL6.0202
G	**G**	**G**	**4**	**14 (23.3)**	**IL6.0204**
G	G	G	5	1 (1.7)	IL6.0205
G	G	G	6	3 (5)	IL6.0206
G	**G**	**G**	**7**	**10 (16.7)**	**IL6.0207**
G	G	G	8	4 (6.7)	IL6.0208
G	G	G	13	1 (1.7)	IL6.0213
G	**C**	**G**	**7**	**4 (6.7)**	**IL6.0307**
G	G	C	7	1 (1.7)	IL6.0403

4.4. Allelotype and genotype of an African American control population

The data for the African American control population and the Rheumatoid arthritis patients at the 5'SNPs were obtained by Osiri et al and have since been published (1999). The data is also presented in Table 15, which also include the 3' VNTR. The allelotypes and genotypes at each locus are in Table 16. Osiri et co observed unequal distribution of alleles. At -594, allele G was most common and present 94.3%, as was allele G at -172 and allele G at -570 (91.5% and 95.3% respectively).

Two by two contingency tables and χ^2 analysis showed that -594.A was significantly associated to -172.C (χ^2=11.643, p=0.0006). No association was observed with either of the -570 alleles and the other SNP's.

They reported 5 haplotype families from their data, which they termed one to five according to their observed frequency. These were:

-594.G	-570.G	-172.G
-594.G	-570.C	-172.G
-594.A	-570.G	-172.C
-594.A	-570.G	-172.G
-594.G	-570.G	-172.C

These were identical to those detected in the West of Scotland control population, although the distribution differed. In this population, IL6.02 was most common, followed by IL6.03, IL6.01, Il6.05, and Il6.04. This thesis further developed on the 5' SNP data and analysed the 3' VNTR and their corresponding associations.

Analysis at the 3' region showed that additional alleles were present in comparison to those observed in the West of Scotland population. A nomenclature was thus designed to maintain the original numbers allocated in the Caucasian cohort, but distinguish the new alleles by adding a suffix 'S' or 'L', characterising a 'shorter' or 'longer' allele, to the allele number located closest. For example, an allele positioned slightly above allele 6 on the gel was identified as 6L identifying this allele to be longer than 6. Six new alleles

TABLE 15

The results of the African American control population

SAMPLE NO.	5' REGION			3' VNTR
	FOK-1	FNU4H-I	NLA-III	
	-594	-570	-172	
118	GG	GG	GG	nd
119	GG	GG	GG	3*6
120	GG	GG	GG	7*8
121	GG	GG	GG	6*7
122	GG	GG	GG	7*7
123	GG	GG	GG	nd
124	GG	GG	GG	4*13
125	GG	CG	GG	7*7
126	GG	CC	GG	nd
127	GG	GG	GG	nd
128	GG	GG	GG	4*13
129	GG	CC	GG	6*6
130	GG	GG	GG	6*6
131	GG	GG	GG	nd
132	GG	GG	GG	4*7
133	GG	GG	GG	7*11
134	GG	GG	GG	6*6
135	GG	GG	GG	6*6
136	GG	GG	GG	4*6
137	GG	GG	GG	7*7
138	GG	GG	GG	7*13
139	GG	CG	GG	7*7
140	GG	GG	GG	3*7

SAMPLE NO.	5' REGION			3' VNTR
	FOK-1	FNU4H-I	NLA-III	
	-594	-570	-172	
141	GG	GG	GG	4*10
142	GG	GG	GG	7*7
143	AG	GG	GC	3*3
144	GG	GG	GG	3*6
145	AG	GG	GC	3*7
146	GG	GG	GG	6*7
147	GG	GG	GG	nd
149	GG	GG	GC	3*3
150	GG	GG	GG	3*3
151	GG	GG	GG	6*13
152	GG	GG	GG	7*10L
153	GG	GG	GC	3*6
154	GG	GG	GG	6*13
155	GG	GG	GG	6*7
156	GG	GG	GG	6*13
157	GG	CG	GG	6L*7L
158	AG	GG	GG	10*13
159	GG	GG	GG	3*9
160	GG	GG	GG	4*7
161	AG	GG	GG	3*6
162	GG	CG	GG	7*7
163	GG	GG	GG	6*7
164	AG	GG	GG	3*4
165	GG	GG	GG	nd
166	GG	GG	GG	2*7
167	GG	GG	GG	4*7
168	AG	CG	GC	3*7

SAMPLE NO.	5' REGION			3' VNTR
	FOK-1 -594	FNU4H-I -570	NLA-III -172	
169	GG	GG	GG	3*7
170	GG	GG	GG	7*8
171	GG	GG	GG	10*13

TABLE 16

Allele distribution at four polymorphic loci in the African American control population

Locus	Allele	Number Observed (%)	Genotype	Number Observed (%)
-594	A	6 (5.7)	A*A	0 (0)
	G	100 (94.3)	A*G	6 (8.5)
			G*G	47 (88.7)
-570	G	97 (91.5)	G*G	46 (86.8)
	C	9 (8.5)	G*C	5 (9.4)
			C*C	2 (3.8)
-172	C	5 (4.7)	C*C	0 (0)
	G	101 (95.3)	C*G	5 (9.4)
			G*G	48 (90.6)
3'VNTR	2	1 (1)	2*7	1
	3	16 (17)	3*3	3
	4	8 (8.5)	3*4	1
	6	20 (21.3)	3*6	4
	6L	1 (1)	3*7	4
	7	29 (29)	3*9	1
	7L	1 (1)	4*6	1
	8	2 (2.1)	4*7	3
	9	1 (1)	4*10	1
	10	3 (3.2)	4*13	2
	10L	1 (1)	6*6	4

Allele	Number Observed (%)	Genotype	Number Observed (%)
11	1 (1)	6*7	4
13	8 (8.5)	6* 13	3
		6L*7L	1
		7*7	6
		7*8	2
		7*10L	1
		7*11	1
		7*13	1
		10*13	2

were detected in the individuals of African American background in relation to Caucasians from the West of Scotland, three of which were presented in the African American control population. These represented (3.2%) of the total observed alleles. These are presented in fig.6. Allele 7 was seen most frequent in this population (33%), followed by allele 6 (21.3%) and allele 3 (17%). No genotype was seen to prevail above any other. Analysis of the 3' VNTR only showed two weak associations to the 5' SNPs. This was with allele 3 where by a weak association was observed with -594.A and -172.C (χ^2=8.392, p=0.038, pc=0.722, χ^2=6.434, p=0.0112, pc=0.1792.

The detected associations postulated that there was one potential extended relationship between 5' SNP and 3' VNTR:

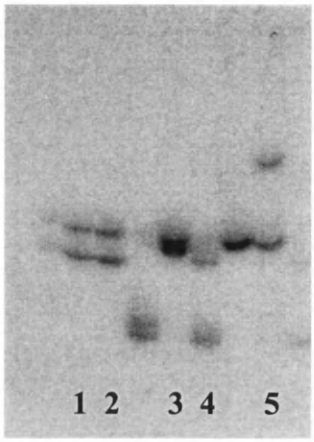
-594.A	-570.G	-172.C	3' VNTR.3

The data however suggested another two putative haplotypes prominent in this cohort:

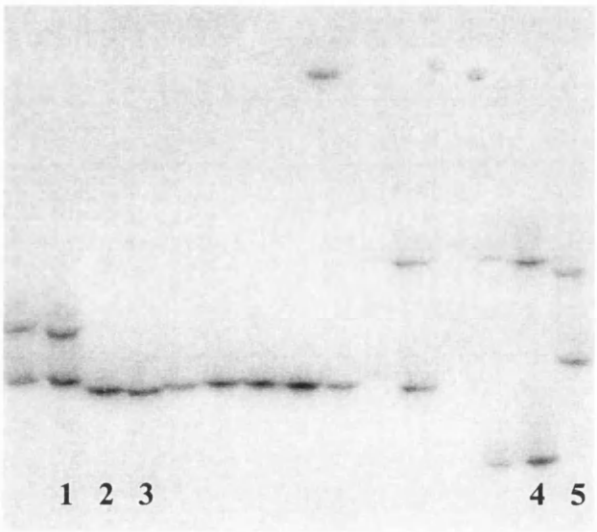
-594.G	-570.G	-172.G	3' VNTR.7
-594.G	-570.G	-172.G	3' VNTR.6

Consistent with the proposed nomenclature for the haplotypes in the West of Scotland control population, these can therefore be termed IL6.0103, IL6.0207, and IL6.0206 respectively. From the data, 3 individuals were homozygous for the haplotypes IL6.0207, 3 for IL6.0206 and 1 individual for IL6.0103. Additionally, 4 were heterozygous for IL6.0206/ IL6.0207, 2 for IL6.0103/ IL6.0206, and 1 individual for IL6.0103/ IL6.0207.

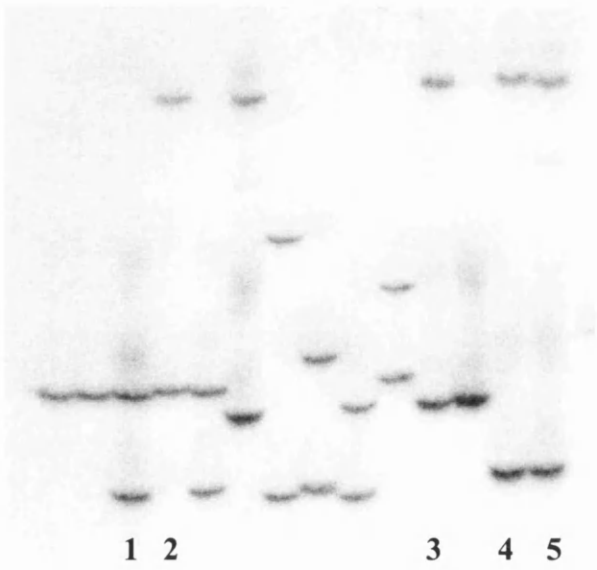
These putative haplotypes potentially accounted for 54.4% of the total haplotypes. Subtraction of these haplotypes revealed the additional haplotypes. However, there were a few indeterminate data that did not contain one of the prominent extended haplotypes. These data could not be conclusively categorised as there were potentially two different haplotype combinations. For this thesis, it was suggested that the probable haplotype combination more frequently observed in the African American population were most probable and therefore added to the total of extended haplotypes.



Lane 1: 6S / 7
Lane 2: 6S / 7
Lane 3: 7S / 7
Lane 4: 3 / 6L
Lane 5: 7 / 10



Lane 1: 6 / 8
Lane 2: 6S / 6S
Lane 3: 6S / 6S
Lane 4: 4 / 10L
Lane 5: 7 / 10



Lane 1: 3 / 7
Lane 2: 7L / 13
Lane 3: 6 / 13
Lane 4: 4 / 13L
Lane 5: 4 / 13

FIGURE 6. A demonstration of additional alleles in the African-American Population

Table 17 shows the total of both the concluded putative haplotypes, which disregard the few data, and the suggested potential haplotypes for these data. Further analysis of the haplotype distribution with other cohorts was examined with both sets of results. However, the presence or absence of the suggested haplotypes did not alter the outcome of the comparative analysis with other cohorts.

Table 17

Putative haplotypes observed in the African American control population

5'			3'	Number	
-594	-570	-172	VNTR	observed (%)	
A	**G**	**C**	**3**	**3 (3.7)**	**IL6.0103**
G	G	G	2	1 (1.2)	IL6.0202
G	G	G	3	9 (11)	IL6.0203
G	G	G	4	7 (8.5)	IL6.0204
G	**G**	**G**	**6**	**16 (19.5)**	**IL6.0206**
G	**G**	**G**	**7**	**26 (31.2)**	**IL6.0207**
G	G	G	7L	0 (0)	IL6.027L
G	G	G	8	2 (2.4)	IL6.0208
G	G	G	9	1 (1.2)	IL6.0209
G	G	G	10	2 (2.4)	IL6.0210
G	G	G	10L	1 (1.2)	IL6.0210L
G	G	G	11	1 (1.2)	IL6.0211
G	G	G	13	7 (8.5)	IL6.0213
G	C	G	6	2 (2.4)	IL6.0306
G	C	G	6L	0 (0)	IL6.0306L
G	C	G	7	3 (3.7)	IL6.0307
G	G	C	3	1 (1.2)	IL6.0403
A	G	G	3	0 (0)	IL6.0603
G	G	C	10	0 (0)	IL6.0610

4.5. Allelotype and genotype of Rheumatoid arthritis patients in African Americans

The data observed for the rheumatoid arthritis patients can be observed in Table 18 and the allelotypes and genotypes at each locus are in Table 19. The data for the 5' promoter SNPs were obtained by Osiri and co workers. They observed that the alleles of the 5' SNP's were unequally distributed. At -594, -570 and -172 the G alleles were the most common and present 94.9, 90.2% and 93.5% respectively. Two by two contingency tables and χ^2 analysis showed that -594.A and –172.C were in linkage disequilibrium to each other, as were -594.G and -172.G (χ^2=105.847, p<0.00001). No association was observed with either of the -570 alleles and the other SNP's.

From this data, they identified 4 haplotype families that were present in the control population. The fifth haplotype family, -594.G, -570.G, -172.C, present in the controls was absent in this population.

The data of the 3' VNTR revealed allele 7 was most frequent in this population (28.4%), followed by allele 6 (23.6%) and allele 3 (11.5%). No genotype was prominent. Analysis of the 3' allele 3 showed strong associations to allele A at -594 and allele C at -172 (χ^2=75.834, pc<0.00023, χ^2=55.331, pc=0.00025). Weak associations were also observed with allele 6 and allele 7 at -594.G and -172.G (χ^2=5.318, p=0.0211, pc=0.4853, χ^2=7.344, p=0.0067, pc=0.1675). No associations were observed with either of the allele at -570.

Based upon the associations observed and the allele distribution it was postulated that the four putative haplotypes detected were the same as those in the African American population. The data showed 9 individuals were homozygous for IL6.0207, 6 for IL6.0206, 1 individual for IL6.0103 and one for IL6.0203. Additionally, 10 were heterozygous for IL6.0203/ IL6.0207, 2 for IL6.0203/ IL6.0206, and 1 for IL6.0206/ IL6.0207. These putative haplotypes potentially accounted for 61.8% of the total haplotypes. Subtraction of these haplotypes revealed additional haplotypes and remaining data was calculated as before.

TABLE 18

The results of the African American Rheumatoid arthritis population

SAMPLE NO.	5' REGION			3' REGION
	FOK-I	FNU4-HI	NLA-III	
	-594	-570	-172	
1	GG	CG	GG	7*7
2	GG	GG	GG	3*7
3	GG	GG	GG	7L*13
4	GG	GG	GG	4*7
5	GG	GG	GG	6*13
6	GG	GG	GG	6*6
7	GG	GG	GG	4*13L
8	GG	CG	GG	4*13
9	GG	GG	GG	6*6
10	GG	CG	GG	8*8
11	AG	GG	GC	4*7
12	GG	CG	GG	4*7
13	GG	CC	GG	7*13
14	GG	GG	GG	13*13
15	GG	GG	GG	7*10
16	GG	GG	GG	4*7
17	GG	GG	GG	3*3
18	GG	CG	GG	6*9
19	GG	GG	GG	13*13
20	GG	GG	GG	4*7
21	GG	GG	GG	6*7
22	AG	GG	GC	3*4
23	GG	GG	GG	7S*7
24	GG	CG	GG	3*6L
26	GG	GG	GG	7*7

SAMPLE NO.	5' REGION			3' REGION
	FOK-I	FNU4-HI	NLA-III	
	-594	-570	-172	
28	GG	CG	GG	6S*7
29	GG	GG	GG	6*13
30	GG	GG	GG	6S*8
31	GG	GG	GG	6*10
32	GG	GG	GG	7*7
33	GG	GG	GG	6*7
35	GG	GG	GG	6*13
36	GG	GG	GG	4*7S
37	GG	GG	GG	4*7
38	AG	GG	GC	3*13
40	GG	GG	GG	6*6
41	GG	GG	GG	6S*13
42	GG	CG	GG	nd
43	GG	GG	GG	6S*6
44	GG	CG	GG	3*7
45	GG	GG	GG	4*6
47	GG	GG	GG	7*10L
48	GG	GG	GG	6*7
49	GG	GG	GG	6*10L
50	GG	GG	GG	1*6
51	GG	GG	GG	6*6
52	AG	GG	GC	4*9
53	GG	CG	GG	6*6
54	GG	GG	GG	5*9
55	GG	GG	GG	6*7
56	GG	GG	GG	6*7
57	GG	GG	GG	4*6
58	AG	GG	GC	3*3
59	GG	CG	GG	nd

SAMPLE NO.	5' REGION			3' REGION
	FOK-I	FNU4-HI	NLA-III	
	-594	-570	-172	
60	GG	GG	GG	7*7
61	AG	GG	GC	3*3
62	AG	GG	GC	3*6
63	GG	GG	GC	3*6
65	GG	GG	GG	3*4
67	GG	GG	GG	7*13
69	AG	GG	GC	3*3
70	GG	GG	GG	3*10
71	GG	GG	GG	3*6
72	AA	GG	CC	3*3
73	GG	GG	GG	4*10L
74	GG	GG	GG	4*6
75	GG	GG	GG	6*13
76	GG	CG	GG	6*10
77	GG	GG	GG	2*7
78	GG	GG	GG	7*7
79	GG	GG	GG	7*7
80	GG	GG	GG	nd
81	GG	GG	GG	nd
82	GG	GG	GG	6*7
83	GG	GG	GG	6*7
84	GG	GG	GG	7*7
85	GG	GG	GG	6S*13
86	GG	GG	GG	4*7
87	GG	CG	GG	6*7
88	GG	GG	GG	6*6
89	GG	GG	GG	7*13
90	AG	GG	GC	3*13
91	GG	GG	GG	6*8

SAMPLE NO.	5' REGION			3' REGION
	FOK-I	FNU4-HI	NLA-III	
	-594	-570	-172	
92	GG	GG	GG	6*8
93	GG	CG	GG	4*7
94	GG	CG	GG	7*7
95	GG	GG	GG	7*10
96	GG	GG	GG	6*6
97	GG	GG	GG	6*10L
98	GG	GG	GG	6*7
99	GG	CG	GG	6*13
100	GG	GG	GG	3*7
101	GG	GG	GG	6*7
102	GG	GG	GG	nd
103	GG	GG	GG	7*7
104	GG	GG	GC	4*10L
105	GG	GG	GG	6*7
106	GG	GG	GG	7*13
107	GG	CC	GG	6*7
108	GG	GG	GC	7*13
109	GG	GG	GG	6*8
110	GG	GG	GG	6S*6S
111	GG	GG	GG	7*7
112	GG	GG	GG	7*7
113	GG	GG	GG	3*6
114	GG	CG	GG	7*7
115	GG	GG	GG	6*7
116	GG	GG	GG	6S*13
117	GG	GG	GG	3*4

TABLE 19

Allele distribution at four polymorphic loci in the African American Rheumatoid arthritis patients

Locus	Allele	Number Observed (%)	Genotype	Number Observed (%)
-594	A	11 (5.1)	A*A	1 (0.9)
	G	205 (94.9)	A*G	9 (8.3)
			G*G	98 (90.7)
-570	G	195 (90.3)	G*G	89 (82.4)
	C	21 (9.7)	G*C	17 (15.7)
			C*C	2 (1.9)
-172	C	14 (6.5)	C*C	1 (0.9)
	G	202 (93.5)	C*G	12 (11.1)
			G*G	95 (88)
			Genotype	Number Observed(%)
3'VNTR	1	1 (0.5)	1*6	1
	2	1 (0.5)	2*7	1
	3	24 (11.5)	3*3	5
	4	20 (9.6)	3*4	3
	5	1 (0.5)	3*6	4
	6S	8 (3.8)	3*6L	1
	6	49 (23.6)	3*7	3
	6L	1 (0.5)	3*10	1
	7S	2 (1)	3*13	2
	7	59 (28.4)	4*6	3

		Genotype	Number Observed (%)
7L	1 (0.5)	4*7S	1
8	6 (2.9)	4*7	8
9	3 (1.4)	4*9	1
10	5 (2.4)	4*10L	2
10L	5 (2.4)	4*13	1
13	21 (10.1)	4*13L	1
13L	1 (0.5)	5*9	1
		6S*6S	1
		6S*6	1
		6S*7	1
		6S*8	1
		6S*13	3
		6*6	7
		6*7	13
		6*8	3
		6*9	1
		6*10	2
		6*10L	2
		6*13	5
		7S*7	1
		7*7	12
		7*10	2
		7*10L	1
		7*13	5
		7L*13	1
		8*8	1
		13*13	2

The total of extended haplotypes and those including the suggested haplotypes can be observed in table 20. The addition or extraction of the predicted putative haplotypes did not alter the overall analysis. Neither did their presence or absence affect further comparative analysis.

TABLE 20

Putative haplotypes observed in the African American Rheumatoid arthritis population

5'			3'	Number	
-594	-570	-172	VNTR	observed (%)	
A	**G**	**C**	**3**	**9 (5.1)**	**IL6.0103**
A	G	C	4	0 (0)	IL6.0104
A	G	C	9	0 (0)	IL6.0109
G	G	G	1	1 (0.001)	IL6.0201
G	G	G	2	1 (0.001)	IL6.0202
G	G	G	3	12 (6.7)	IL6.0203
G	G	G	4	14 (7.9)	IL6.0204
G	G	G	5	1 (0.001)	IL6.0205
G	G	G	6S	7 (3.4)	IL6.0206S
G	**G**	**G**	**6**	**42 (23.6)**	**IL6.0206**
G	G	G	7S	2 (0.01)	IL6.0207S
G	**G**	**G**	**7**	**47 (26.4)**	**IL6.0207**
G	G	G	7L	1 (0.001)	IL6.027L
G	G	G	8	5 (2.8)	IL6.0208
G	G	G	9	1 (0.001)	IL6.0209
G	G	G	10	4 (2.2)	IL6.0210
G	G	G	10L	4 (2.2)	IL6.0210L
G	G	G	13	17 (9.6)	IL6.0213
G	G	G	13L	1 (0.001)	IL6.0213L
G	C	G	3	0 (0)	IL6.0303
G	C	G	4	0 (0)	IL6.0304
G	C	G	6S	0 (0)	IL6.0306S
G	C	G	6	2 (0.01)	IL6.0306
G	C	G	6L	0 (0)	IL6.0306L
G	C	G	7	5 (2.8)	IL6.0307
G	C	G	8	1 (0.001)	IL6.0308

5'			3'	Number	
-594	**-570**	**-172**	**VNTR**	**observed (%)**	
G	C	G	6	0 (0)	IL6.0309
G	C	G	10	0 (0)	IL6.0310
G	C	G	13	1 (0.01)	IL6.0313
G	G	C	3	0 (0)	IL6.0403
G	G	C	10L	0 (0)	IL6.0410L
G	G	C	13	0 (0)	IL6.0413

4.6. Comparison of populations

Gastric Carcinoma cohort versus Normal control West of Scotland population

Chi-squared analysis has shown that although there were slight differences between gastric allelotypes and genotypes at the 5' SNP region, no significant association was observed. Additionally this was also true at the 3' VNTR region where by although additional alleles were observed, there were too few to present significance or affect the rest of the distribution (table 6). For the sample size in this comparison, the power to detect an odds ratio (OR) of 2 was greater than 0.9 at both –174 and –594. However, at –570, the low frequency of the C allele reduced the power to 0.53.

The distribution of the haplotype families in gastric cancer patients presented a similar pattern to the controls and thus showed no significance by chi-square analysis. Power analysis was carried out on all haplotypes that were observed at a frequency of greater than 10%. In these, the power to detect an OR of 2 was at least 0.79.

The same strong associations spanning the 5' SNP and 3' VNTR in the normal population were seen in the gastric population at -594 and -172 and 3' VNTR alleles 3 and 4. However at allele 7 the alleles at -172 were not associated. Additionally, neither alleles at -570 were associated with any 3' VNTR alleles. This however bore no significance to the distribution of haplotypes. This was confirmed by the Monte carlo simulation analysis.

Barrett's Oesophagus cohort versus Normal control West of Scotland population

No statistical difference was observed between these two groups, neither with the allelotypes or genotypes at the 5' SNP region. Despite the presence of additional alleles at the 3' VNTR, there was no significance difference in the distribution. Consequently, haplotype families and extended haplotypes showed no significant distributionAs observed with the gastric cohort, the power to detect an OR of 2 was greater than 0.9 for –172 and –594 and 0.66 for –570. Haplotype analysis also identified a power of greater than 0.79 to detect an OR of 2.

When the Barrett's Oesophagus cohort was split according to malignancy diagnosis, i.e. Benign and Malignant Barrett's, there was no significant difference at the 5' SNPs and thus also in the haplotype family distribution. However, comparison at the 3' VNTR revealed that the genotype 3*4 was slightly less frequent in the Malignant Barrett's cohort in comparison to the West of Scotland cohort (χ^2=4.972, p=0.0258, pc=0.7482). Yet this bore no significance when comparing the extended haplotypes of the malignant patients in comparison to the control individuals. Power analysis on all haplotypes with a frequency of greater than 10% showed a power of at least 0.89 at an OR of 2.

Gastric Carcinoma cohort versus Barrett's Oesophagus cohort

Analysis on allelotype distribution showed a significant difference between Barrett's Oesophagus patients and Gastric Carcinoma patients at -570. The former group had a higher frequency of the rare allele C in comparison to the Gastric cohort (χ^2=3.907, p=0.0481). However, no other significant difference was observed at the 5' SNPs or 3' VNTR.

The absence of significant difference was further demonstrated in the distribution of the 5' haplotype families. A significant difference was observed when the 3'VNTR alleles were added to form the extended haplotypes and a higher frequency of IL6.0307 was detected in Barrett's Oesophagus patients (χ^2=4.084, p=0.0433, pc>0.9999) in comparison to the Gastric cohort. Furthermore, IL6.0103 was slightly more frequent in Barrett's Oesophagus (χ^2=5.251, p=0.0219, pc=0.5475)

Gastric Carcinoma cohort versus Malignant Barrett's cohort

A significant distribution was detected at –172 between the gastric carcinoma cohort and malignant Barrett's Oesophagus population. Allele G was significantly more frequent in Malignant Barrett's compared to Gastric carcinomas (χ^2=4.398, p=0.0263), where patients showed an almost equal distribution of the two alleles. Consistent with this observation, the haplotype families containing allele C at -172 were significantly less frequent in Malignant Barrett's compared to Gastric Carcinomas (χ^2=4.602, p=0.0319).

Additionally, at the 3' VNTR allele 3 was slightly less frequent in Malignant Barrett's (χ^2=5.123, p=0.0236), which disappeared with Bonferroni correction (pc= 0.3776). This insignificance was further demonstrated with no significant genotype distributions.

H.Pylori status in Gastric carcinoma cohort versus Barrett's Oesophagus cohort

H.Pylori segregation in Barrett's Oesophagus and Gastric carcinoma displayed a significant reversal in the distribution of haplotype families IL6.01 and IL6.02. In *H.Pylori* positive patients, the distribution of the haplotype family IL6.02 was significantly more frequent in Barrett's Oesophagus patients (χ^2=11.107, p=0.0112), whilst in Gastric patients, a higher frequency of IL6.01 was observed.

Similarly, in patients whom *H.pylori* was absent, the distribution was again significantly reversed with the haplotype family IL6.02 significantly lower in Barrett's Oesophagus patients in comparison to Gastric patients (χ^2=12.279, p=0.0311).

H.Pylori status in Gastric carcinoma cohort versus Malignant Barrett's cohort

H.Pylori status again presented a role in the distribution of haplotypes in the analysis of the gastric carcinoma cohort and Malignant Barrett's cohort. IL6.02 was significantly more frequent in the Malignant Barrett's Oesophagus patients whom were *H.Pylori* positive in comparison to the Gastric cohort (χ^2=4.635, p=0.0313).
Differences between the two cohorts was also demonstrated when combining the 3' VNTR, whereby the extended haplotype IL6.0307 was slightly more frequent in the Malignant Barrett's Oesophagus cohort (χ^2=4.421, p=0.0355) in comparison to the Gastric cohort. Bonferroni correction diminished this slight significance (pc>0.9999)

Malignant Barrett's cohort versus Benign Barrett's cohort

Comparison of the two Barrett's cohorts detected a significant decrease of allele A at -594 in Malignant Barrett's in comparison to Benign Barrett's, whom showed a similar distribution of the two alleles. This significance however was not extended to the genotypes.

There was no significant distribution of the haplotype families between the two cohorts or due to *H.pylori* status. This was further demonstrated in similar distribution of the extended haplotypes.

African American Rheumatoid arthritis cohort versus African American control population

Data analysis determined that there was no significant difference in the distribution of allelotypes and genotypes both at the 5' SNPs and 3' VNTR. This lack of significance was further observed in the distribution of haplotype families. Although additional associations spanning across the locus were detected in the rheumatoid arthritis patients, this bore no significance in the distribution of the extended haplotypes. Power analysis for alleotypes and haplotypes demonstrated a power of at least 0.98 for an OR of 2, except for allele at –570 where the rarity of allele C decreased the power to 0.65.

African American control populations versus West of Scotland control populations

When analysing the two ethnically different cohorts, significant differences were observed. The distribution of alleles at -594 and -172 were significantly different in these cohorts (χ^2=43.835, p<0.00001, χ^2=51.553, p<0.00001 respectively). These differences were further portrayed in the distribution of genotypes (-594, χ^2=44.071, p<0.00001, -172, χ^2=50.742, p<0.00001).

The significant differences were further demonstrated in the comparison of extended haplotypes. In the African Americans, a higher distribution of haplotypes IL6.0206, IL6.0207, and IL6.0213 were detected in relation to the West of Scotland population (IL6.0206 χ^2 =16.842, p<0.00001, pc<0.00031, IL6.0207 χ^2=5.921, p=0.015, pc=0.0465, IL6.0213 χ^2 =13.137, p=0.0003, pc=0.0093). Additionally IL6.0203 was slightly more frequent (χ^2=7.039, p=0.008, pc=0.248). In comparison, haplotype IL6.0103 was significantly increased in the West of Scotland population (χ^2=41.508, pc<0.00031). These significant distributions correlate with the observation that a higher frequency of the haplotype family IL6.01 was detected in the West of Scotland population and IL6.02 in the African American cohort respectively (χ^2=55.621, p<0.00001, pc<0.00004).

DISCUSSION

In this study, the distribution of four polymorphic elements spanning across the locus of IL-6 in the West of Scotland were investigated and their relationship analysed by the chi-square test. This provided data to determine putative haplotype families in the promoter region and extended haplotypes encompassing the 3' VNTR. Once these putative haplotypes were established in the control population, additional disease populations were examined in order to determine variations. Investigation of an African American cohort and subsequent rheumatoid individuals provided a comparison of the putative haplotypes in a different ethnic group and its association with an autoimmune disease.

5.1. Control population analysis

In order to show variations of polymorphisms in disease patients, control populations are needed as comparable standards. Therefore, the West of Scotland and the African American control populations were primarily investigated.

The distribution observed in the West of Scotland population at the -172 polymorphism is analogous to that recently described for Caucasians by Fishman et al [1997]. Allele C was observed at 44.2% in this study in comparison to the 40.3% in Fishman's population. Faulds et al [accession number AF048692] described a similar distribution of the -594 polymorphism, with allele A at 43.2% in relation to our 41.7%. The polymorphism at -570 has not previously been described in Caucasians. In our population, allele C at -570 was sparsely distributed at 4.5%, with the G allele being much the more common, at 95.5%.

In the African American population, the polymorphisms at the 5' SNPs have not previously been reported. However, these can be compared to the West of Scotland population to show any ethnic variations. A significantly higher frequency of the G alleles at both -594 and -172 were observed in the African American population (94.3% and 91.5% respectively). However at -570, allele C was similarly sparsely distributed in both populations (West of Scotland, 4.5% and African Americans, 8.5%). In these populations, the sparseness of allele C suggests that it is barely polymorphic enough to

be of interest in its own right in these populations, although as later shown, it was of importance in defining the haplotypes.

The frequency of these markers has also been detected in different ethnic origins with varying distribution frequencies. At -172, Fishman et al have shown allele G to be present at 86% and 95.5% in Gujarati Indians and Afro-Caribbean's respectively. The latter cohort shows a similar distribution to that of the African American population studied here. Additionally, the rare allele C at -570 in the West of Scotland and African American population has been observed to be prominent in a recent study in Japanese women (82%) [Nakajima et al, 1999]. The differences in ethnic variations such as these are both common and expected, thus highlighting that a significant observation in one ethnic population may not always be of relevance or detected in another. Furthermore it emphasises the importance of presenting results with correct control populations.

Associations within the 5' locus of the West of Scotland population illustrated that the -594.A allele was in strong linkage disequilibrium with -172.C and that -594.G allele was similarly linked to the corresponding -172.G allele. In general, both these combinations were found with the common -570.G allele. However, it was observed that the rare -570.C allele was only found with -594.G and -172.G allele, suggesting it has arisen against this background.

These associations proposed that there were three combinations of 5' SNPs in the promoter. Subtraction from the data revealed a further two rare combinations, suggesting there were 5 haplotype families in the West of Scotland population:

-594.A	-570.G	-172.C
-594.G	-570.G	-172.G
-594.G	-570.C	-172.G
-594.G	-570.G	-172.C
-594.A	-570.G	-172.G

I have proposed that these haplotype families be termed IL6.01, IL6.02, IL6.03, IL6.04 and IL6.05.

In the African American population, the G alleles at -594 and -172 were also seen in linkage disequilibrium, as were their counterparts. Additionally, as in the West of Scotland, the rare allele C at -570 was only found with -594 and -172 G alleles. The associations and data revealed the same haplotype families and these have recently been published [Osiri et al, 1999]. However in this population the distribution was altered. Haplotype family IL6.02 was the most common, as it was for the West of Scotland cohort, although at a higher incidence (African American population, 84.1%, West of Scotland, 51.4%) followed by IL6.03 and IL6.01. The marked difference in distribution therefore reflects the ethnic disparity between the two cohorts.

The 3' variable number tandem region (VNTR) of IL-6 was originally described by Bowcock et al [1988] who observed 4 alleles although mentioned that additional alleles may be present due to the likelihood of unresovable microheterogeneity resulting from the low-resolution the agarose gel separation. In this study a total 13 irregularly spaced alleles were observed in a variety of control and patient groups from the West of Scotland Caucasians. In the control West of Scotland cohort, 7 were represented although three dominated the population; alleles 3, 4 and 7 (42.1%, 31.5%, and 20.2% respectively). These observations contrast with those of Murray et al [1997], who used an ABI machine to detect only 2 dominant alleles from a total of 6 conserved in their Caucasian control population.

In the African American population, an additional 6 alleles were observed, four of which were detected in the control panel. In order to retain the nomenclature allocated in the West of Scotland population, the new alleles were allocated a suffix S or L to determine a 'shorter' or 'longer' allele than the original. In this population, alleles 6 and 7 were the most common (23.6% and 28.4%). The distribution differences of the two cohorts again reflect the varieties detected in ethnic cohorts.

Associations between the alleles at the 5' SNP's and 3' VNTR suggested there were four potential relationships which integrate the alleles within the IL-6 locus in the West of Scotland population. The associations within these groups were such that it was proposed they behave as haplotypes:

-594.A	-570.G	-172.C	3' VNTR 3	
-594.G	-570.G	-172.G	3' VNTR 4	(-570.G by default)
-594.G	-570.G	-172.G	3' VNTR 7	(-570.G by default)
-594.G	-570.C	-172.G	3' VNTR 7	

These haplotypes were thus termed IL-6.0103, IL-6.0204, IL-6.0207 and IL-6.0307 respectively. The first part of the terminology is from their respective frequencies observed in this population and the latter part of the terminology makes use of the 3' VNTR to further differentiate one haplotype from another. These haplotypes potentially accounted for 87% of the genotypes observed. Subtraction of these haplotypes from the data in table 2 further revealed the seven remaining proposed haplotypes shown in table 4. However these were too infrequent to be proposed as prominent.

The distribution of the remaining IL-6 3' VNTR against the 5' combinations in the other individuals was then examined. Only two individuals showed any variation on the putative IL6.0103 haplotype, having allele 4 on the IL6.01 background:

-594.A	-570.G	-172.C	3' VNTR 4

No other 3' alleles appeared on the IL6.01 background, suggesting that the proposed haplotype IL6.0103 is either recent, or contains undefined elements which inhibits or prevents change. It may also suggest that, like the strongly conserved B8-DR3 haplotype and its associated TNF locus alleles in Caucasians, it is under selection pressure to remain stable [Gallagher et al, 1997]. The stability of this haplotype is further emphasised by the recent study by Fishman et al [1998] on a polymorphic [A]n[T]m tract in the promoter region, lying between the -570.G/C and -172G/C site in the IL-6 promoter. Although considerable independent variation was observed in the 'A' and 'T' runs, they detected only one individual showing variation when -172.C allele was present and all the remaining variants were in association with the -172.G allele. This further emphasizes the stability of the IL6.0103 haplotype. However, genotyping this locus using a recently described method would conclusively resolve this question [Tschentscher et al, 2000].

Fishman et al [1998] also demonstrated that the -172.C allele was significantly less frequent in S-JCA patients and that promoter constructs containing this allele were less transcriptionally active following stimulation by LPS. This was reflected in normal individuals who were homozygous for this allele and had lower serum IL-6 levels, in comparison to those whom carried the -172.G. This project demonstrates that this marker is in strong linkage disequilibrium with two other promoter alleles and one 3' allele. Therefore, it can be postulated that one or more of these additional alleles can contribute to the association of -172 allele with IL-6 secretion. Similarly, Linker-Israeli et al [1999] have demonstrated an association between the 3'VNTR region of the IL-6 gene and SLE. However, there may be additional SLE-related alleles involved in the dysregulation of IL-6 secretion.

In the African American population, strong associations at VNTR allele 3 were detected with -594.A and -172.C. This therefore suggests that these polymorphic loci may behave as the haplotype:

-594.A	-570.G	-172.C	3' VNTR 3

This haplotype termed IL-6.0103, as in the West of Scotland population, showed no variation at the 3' VNTR against the IL6.01 background, thus again suggesting undefined elements inhibiting its modification. However it was also only observed in 3 individuals (3.7%) thus suggesting that it is a strongly conserved recessive haplotype. Alternatively, it may also suggest that this haplotype is of a Caucasian origin and its presence in the African American population may have arisen due to ethnic mixing.

The data revealed an additional 3 prominent relationships, which may suggest haplotypic behaviour:

-594.G	-570.G	-172.G	3' VNTR 7
-594.G	-570.G	-172.G	3' VNTR 6
-594.G	-570.G	-172.G	3' VNTR 3

These relationships do not show any statistical associations other than the linkage disequilibrium at the -594 and -172 sites. This may be because there are so many other

alleles at the 3' VNTR against the common haplotype background any statistical significance would be reduced. Yet their frequency observed may suggest that there are selection factors influencing the gene to incline towards these extended alleles.

5.2. Gastric carcinoma analysis

Analysis of the four polymorphic elements in the gastric carcinoma cohort revealed similar distributions to those of the normal cohort. At the 3' region, although 2 additional alleles were detected, neither the distribution nor further analysis was altered. It is unlikely that the appearance of these additional alleles in this cohort are gastric cancer related, but probably due to the increased chance of detecting the rarer alleles with increasing sample numbers analysed. The statistical data revealed the same haplotypic families and extended associations as those of the normal cohort. Previous published data has indicated that levels of serum IL-6 correlate markedly with disease status in gastric cancer [Wu et al, 1996] and that IL-6 is an autocrine growth factor for some gastric cancer cell-lines [Ito et al, 1997]. Therefore a genetic determinant in IL-6 is of importance in gastric carcinoma. Disappointingly however, a major conclusion from this study is that the 4 polymorphisms analysed in this study are not the genetic markers that relate to this disease.

When the gastric cohort was analysed with respect to *H.pylori* status, a significant difference was detected with the 5' alleles. The 5' haplotype IL6.02 was significantly more frequent in *H.pylori* negative patients in comparison to the prevalent IL6.01 in *H.pylori* positive patients. An early study has shown that infected *H.pylori* dyspeptic patients have increased IL-6 levels [Gionchetti et al, 1994]. Therefore, it can be postulated that the 5' haplotype IL-6.02 may control IL-6 levels, which in turn may provide a protective role against *H.pylori* associated gastritis. However, further study relating the plasma levels of IL-6 in *H.pylori* associated gastric cancer patients and the haplotypic phenotypes would be required for clarification. A recent study that may possibly challenge our data is that of Fishman et al, (1998) who have shown that increased plasma IL-6 levels correlate with the presence of -172G. This would imply that IL6-02, the haplotype containing the allele –172.G, would be expected to be significantly associated with *H.pylori* positive patients. Therefore, from our data, it could be assumed that high plasma IL-6 levels are not favourable to *H.pylori* in gastric

carcinoma, and may be limiting its growth. However, the levels of IL-6 correlating with −172.G have only been observed in rheumatoid arthritis patients. A similar study in patients with inflammatory bowel disease found no significant difference of IL-6 production in relation to −172 alleles [Koss et al, 1999]. Therefore, it could also be postulated that this is an event related only to autoimmune diseases. A similar study in a gastric cancer cohort, as suggested above, is required to resolve this question. Furthermore, our data is unfortunately based on a limited cohort, as *H.Pylori* status was not known in the entire patient cohort as explained earlier. A larger study would be beneficial to provide a clearer picture.

Extending the haplotypes to include the 3' minisatellites eliminated the significant result, suggesting the 3' allele has no genetic role in this gastric cohort. Additionally, analysing the cohort in relation to tumour location also showed no significant difference.

5.3 Barrett's Oesophagus analysis

The Barrett's Oesophagus cohort included patients diagnosed with the Benign or Malignant form. Analyses were carried out on the combined cohort and then when segregated with respect to their diagnosis.

In this cohort, a new 5' haplotype family was observed and hence termed IL6.06 following the nomenclature pattern. However, it was only detected once and bore no significance in further statistical analysis in comparison to the normal cohort. Similarly, additional 3' alleles were detected, but again these did not affect the distribution or further analysis. Therefore our data concluded that the 4 polymorphisms investigated singularly and as haplotypic families in a collective cohort of Barrett's Oesophagus patients were not statistically different to the normal population.

Segregation of the cohort according to *H.pylori* status revealed no significant differences. However, when the patients were categorised according to diagnosis and *H.pylori* status is again reviewed, a significant difference between the positive *H.pylori* Benign population and the negative *H.pylori* Benign population was detected. As in the gastric cancer cohort, a difference was detected in the distribution of the two prominent

haplotype families IL6.01 and IL6.02. The data revealed that haplotype family IL6.01 was more frequent in *H.pylori* negative patients, whilst IL6.02 was more frequent in positive patients. This is in contrast to the gastric cohort, whom showed IL6.01 prevalent in *H.pylori* positive patients. *H.pylori* does not play a significant role in Barrett's Oesophagus as the stomach bacterium is not found in the oesophageal region. Hence, it is can be assumed that the possible protective role of IL6.02 against *H.pylori* would not be of importance. Further investigation as to the relevance of the significant distribution to IL-6.02 would address the role of this haplotype family in *H.pylori* and Barrett's Oesophagus patients.

A recent study by H.Kasem [1999] has shown that the haplotype a2d4 in the TNF is significantly associated to Barrett's Oesophagus. However our data concluded that there were no IL-6 haplotype families associated with this genotype, therefore concluding that these polymorphisms are not associated with disease. Analysis of other TNF polymorphisms indicated that a significant difference between the two prominent haplotype families IL6.01 and IL6.02, and the two alleles TNF a6 and d3, and the genotype TNF a6d3 in the malignant cohort. The function of the TNF alleles is not known, but it is clear that there is a strong link between the TNF genotype and IL6 haplotypes. Therefore any future study on markers in one cytokine should be with consideration to the other as it is possible all the polymorphisms are contributing to the effect.

Comparison of the Barrett's cohort in relation to the gastric cohort revealed a slight difference in the frequency of IL6.0307 and IL6.0103. However these differences are likely to be due to sample numbers as in the gastric cohort allele C at -570 was only observed twice, and Bonferroni correction diminishes the significance. A larger study would be assist in clarifying this observation

5.4 Rheumatoid arthritis analysis

Data analysis determined that there was no significant difference in the distribution of allelotypes and genotypes both at the 5' SNPs and 3' VNTR between the two African American cohorts. In the rheumatoid arthritis patients additional associations spanning across the locus were detected, but these did not affect the overall distribution of haplotype families. The recent by Fishman et al (1997) showed that the C allele at –172

correlates with lower IL-6 levels and S-JCA patients have the CC genotype significantly less frequently. However, in this cohort the alleles at -172 were not significantly different in their distribution. This can be explained by the ethnic variations. As stated earlier, the allele G at -172 has been investigated in various ethnic populations providing different frequencies. It is very possible therefore, that different factors affect the production of IL-6, as previous studies suggest [Hulkkone et al, 2001, Collado-Escobar, 2000]. Furthermore, it is possible that additional genetic markers will be acting against the result of -172 allele in the normal cohort where as this effect is 'switched off' in arthritic patients.

5.5 Experimental analysis

The methods applied for this thesis were quick, easy and effective. Amplification followed by complete digestion for the 5' polymorphisms give indisputable results. The only consideration required was to digest for the appropriate length of time. Incomplete digestion was possible with a short time span, whereas overnight digestion could potentially cause false cleaving. This was especially true for digestion with Fok-I as this enzyme was able to construct a complimentary nucleotide to a sequence allowing potential random cleaving between any two nucleotides. Therefore preliminary studies deemed 3 hours to be appropriate. Known standards showing the three digestion results were also added for each run to ensure complete and accurate digestion. Analysis of 3' VNTR region by denaturation PAGE was a more superior method to that previously carried out by Bowcock et al [1988] whom showed 4 alleles. The PAGE gels provided higher resolution, thus overcoming the unresovable heterogeneity encountered by the agarose gel separation. This enabled the additional alleles that Bowcock referred to be observed with more clarity. Yet still even this separation system is not totally accurate and it is felt there may be additional alleles not seen. This is based upon the difficulty in calling samples that look slightly variable in density upon the gels. The variation may be due to slight differences in the gel consistency or alternatively may be due to single base pair additions/deletions. From the procedures available at the moment, additional sequencing of samples that are ambiguous would clarify this. Yet this is not practical due to the expense, and it would lead to an additional complication relating as to the number of base addition/deletion/substitutions that would warrant a new allele.

CONCLUSION AND FUTURE STUDIES

To conclude, this study investigated 4 polymorphic elements spanning across the locus of IL-6 and analysed their relationship with each other. In the West of Scotland control population, the 3 polymorphisms in the 5' region showed strong linkage disequilibrium and hence these associations indicated that there were three common combinations. Further analysis revealed two rare combinations concluding that there are the following 5' haplotype families in this cohort:

-594.A	-570.G	-172.C
-594.G	-570.G	-172.G
-594.G	-570.C	-172.G
-594.G	-570.G	-172.C
-594.A	-570.G	-172.G

By observing the common practice of naming haplotype families, these are termed IL6.01, IL6.02, IL6.03, IL6.04 and IL6.05.

Further analysis of the 3' region minisatellite using PAGE overcame the unresovable heterogeneity encountered by Bowcock et al (1989) and enabled us to observe a total of 13 irregularly spaced alleles in a variety of control and patient groups from the West of Scotland Caucasians, 7 of which were represented in the control cohort. Statistical analysis revealed further associations with the minisatellite alleles, thus suggesting 4 putative extended haplotypes, which integrate the alleles within the IL-6 locus:

-594.A	-570.G	-172.C	3' VNTR 3	
-594.G	-570.G	-172.G	3' VNTR 4	(-570-I 1 by default)
-594.G	-570.G	-172.G	3' VNTR 6	(-570-I 1 by default)
-594.G	-570.C	-172.G	3' VNTR 6	

These haplotypes were thus termed IL6.0103, IL6.0204, IL6.0207 and IL6.0307 where the latter part of the terminology makes use of the 3' VNTR and further differentiates one haplotype from another.

The results obtained therefore suggest that the genetic polymorphisms in the promoter region cannot be considered independent from one another, but instead be remembered that other markers may be contributing to the overall observed effect. It would have been interesting to develop this and study the expression of IL-6 by the different haplotype families. This could be then compared in different cohorts in order to determine the effect of haplotype families and resulting IL-6 expression, in relation to patient groups. However, since the completion of this study, Terry et al (2000) have indeed looked at functional effect of 4 polymorphisms in IL-6. Three of these were the 5' promoter SNP's that were studied here, and the fourth being the AnTn track at -373. They identified their natural occurring haplotypes and compared their effects on reporter gene expression. The data revealed that different transcriptional regulation occurred in the ECV304 cell in comparison to the HeLa cell, thus suggesting that the haplotypes have cell-type specific regulation. Additionally, the haplotypes showed different transcriptional rates in the ECV 304 cell line, thus correlating with our hypothesis that the polymorphisms do not act independently but that other markers influence the overall functional effect.

Analysis of these haplotypes in a gastric carcinoma cohort revealed no statistical difference in the haplotype families or extended haplotypes in comparison to the control population. Similarly, analysis in the Barrett's Oesophagus patients either segregated according to diagnosis or combined, also showed a similar distribution to the control cohort. However, the presence of *H.pylori* significantly affected the frequency of the two prominent haplotype families, IL6.01 and IL6.02 in both cohorts. The gastric cohort revealed that the 5' haplotype IL6.01 was significantly more frequent in *H.pylori* positive patients. Yet in contrast, *H.pylori* positive Barrett's Oesophagus patients had a significantly higher frequency of the haplotype IL6.02. The inclusion of the 3' VNTR in the statistical analysis however, diminished the significance suggesting that in the patient cohorts, the minisatellite alleles only play a minor role. Additionally tumour location in the gastric cohort showed no statistical difference in haplotype frequencies.

Gene expression of the haplotype families would be of great advantage in these cohorts as there are clearly haplotype family preferences when observed in relation to *H.pylori* status. It would be of interest then to note if a haplotype family which may have

increased Il-6 production, may play a specific role in *H.pylori* associated gastritis or Barrett's Oesophagus.

Analyses of an African American cohort revealed the same haplotype families, but were shown in different frequencies; IL6.02 was the most common, followed by IL6.03 and IL6.01. In this ethnic population, an additional 6 minisatellite alleles were observed, four of which were detected in the control panel. Comparison to a rheumatoid arthritic cohort indicated no significant difference in the distribution of allelotypes, genotypes and subsequent haplotypes suggesting that the polymorphisms do not have an important role in disease susceptibility.

Since completion of this study, a recent group has also observed that there is no difference of the polymorphisms -594 and -172 between rheumatoid arthritic patients and comparative control. However they stressed that the polymorphisms may contribute to the pathogenesis of the disease by influencing the age of onset of RA [Pascual et al, 2000].

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