Genetic variations in the Interleukin-6 (IL-6) gene: Implication in coronary heart disease (CHD)

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Declaration

The majority of the work presented in this thesis was carried out by Le Anh Luong, with assistant from Professor Steve Humphries on the statistical analysis. All statistical calculation was carried out by Ms Emma Hawe in the Cardiovascular Genetics group at UCL. The IL-6 in vivo functional studies was done in collaboration with Dr Rachel Jeffrey and Professor Pat Woo in Rheumatology at UCL.
Abbreviations

A
ACE = Angiotensin I converting enzyme
ACTH = Adrenocorticotropic hormone
AIDS = Acquired immunodeficiency syndrome
ANCOVA = Analysis of covariance
ANOVA = Analysis of variance
ApoAI = Apolipoprotein AI
ApoE = Apolipoprotein E
APR = Acute-phase response
AP = Acute phase protein
AP-1 = Activated protein-1
APC = Activated protein C
APRF = Acute phase responsive factor
ASOH = Allele-specific oligonucleotide hybridization
AT1 = Angiotensin II type 1 receptor

B
BMI = Body mass index
BSF-2 = B-cell stimulating factor 2

C
CABG = Coronary artery bypass graft surgery
CAT = Chloramphenicol acetyl transferase
CDF = Cytolytic T-cell differentiation factor
C/EBPβ = CCAAT/Enhancing binding protein beta
CHD = Coronary heart disease
CI = Confidence interval
CIS: Cytokine-inducible SH2 containing protein
CRE = cAMP responsive element
CRP = C-reactive protein
CsCl2 = Caesium chloride

D
ddNTP = Dideoxyribonucleotides
DEX = Dexamethasone
DGGE = Denaturing gradient gel electrophoresis
DMSO = Dimethyl Sulfoxide
DNA = Deoxyribonucleic acid
DOTMA = N-[1-(2,3-dioleoyloxy)propyl]-n,n,n-trimethylammonium chloride
DOPE = Dioleoyl phosphatidylethanolamine
dsDNA = double stranded DNA
ECG = Electrocardiogram
ECTIM = Etude Cas-Témoin de l'Infarctus du Myocarde
EDTA = Ethylenediaminetetra-acetic acid

FBS = Fetal bovine serum

GRs = Glucocorticoid receptor
GCs = Glucocorticoids
GRE or GR = Glucocorticoid responsive element
gp130 = 130kDa glycoprotein

HDL = High density lipoprotein
HMG-CoA = 3-hydroxy-3-methylglutaryl coenzyme A
HPGF = Hybridoma/plasmacytoma growth factor
HSF = Hepatocyte stimulating factor
HUVEC = Human umbilical vein endothelial cells

IFN-γ = Interferon gamma
IHD = Ischaemic heart disease
IL-1β = Interleukin-1 beta
IL-2 = Interleukin-2
IL-3 = Interleukin-3
IL-4 = Interleukin-4
IL-6 = Interleukin-6
IL-10 = Interleukin-10
IL-6R = IL-6 receptor
IL-6REs = IL-6 responsive elements
IPTG = Isopropyl-1-thio-β-D-galacto-pyranoside

JAK/STAT = Janus/signal transducer activator of transcription

LARII = Luciferase assay reagent II
LDL = Low density lipoprotein
LDLc = Low density lipoprotein cholesterol
Lp(a) = Lipoprotein (a)
LPS = Lipopolysaccharides

M
mAbs = Monoclonal antibodies
MADGE = Microtitre array diagonal gel electrophoresis
MAPK = Mitogen activated protein kinase
MCP-1 = Monocytes chemoattractant protein-1
MCS = Multiple cloning sites
MCSF = Macrophage colony stimulating factor
MEM = Minimum essential medium
MgCl₂ = Magnesium chloride
MGI-2 = Monocyte-granulocyte inducer type-2
MI = Myocardial infarction
MRE = Multi-response element
mRNA = Messenger RNA

N
Na₂EDTA = Sodium EDTA
NF-IL6 = Nuclear factor IL-6
NF-κB = Nuclear factor kappa B
NPHSII = Northwick Park Heart Study II

O
OR = Odds ratio

P
PAI-1 = Plaminogen activator inhibitor type-1
PBS = Phosphate buffer saline
PCR = Polymerase chain reaction
PDGF = Platelet derived growth factor
PIAS: Protein inhibitor of activated STAT
PLB = Passive lysis buffer
PPARα = Peroxisome proliferator activator receptor α

R
RFLP = Restriction fragment length polymorphism
RR = Relative risk
SD = Standard deviation
SDS = sodium dodecyl sulphate
SEM = Standard error of the mean
SH-2 = Src-homology 2
sIL-6R = Extracellular domain of IL-6 receptor
SNP = Single nucleotide polymorphism
SOCS = Suppressor of cytokine signalling
SRE = Serum response element
SSCP = Single stranded conformational polymorphism
ss DNA = Single stranded DNA
STAT3 = Signal transducers activator of transcription-3

T
TAE = Tris acetate EDTA
TAF = T-cell activating factor
TBE = Tris borate EDTA
TDI = Template directed dye terminator incorporation
TE = Tris EDTA
TF = Tissue factor
TGF-β = Tumor growth factor-β
TGGE = Temperature gradient gel electrophoresis
TIMP-1 = Tissue inhibitor of metalloproteinases-1
TME = Tris-MES-EDTA
TNFα = Tumor necrosis factor alpha
TPA = 12-O-tetradecanoylphorbol-13-acetate

W
WHO = World health organization
WOSCOPS = West of Scotland Coronary Prevention Study
Wt = Wild-type

X
X-gal = 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
Abstract

Interleukin-6 (IL-6) is an inflammatory cytokine that plays an important role in the pathogenesis of coronary heart disease (CHD) such as atherosclerosis. Single stranded conformational polymorphism (SSCP) was used to screen for genetic variations in the 5'-untranslated and coding regions of the human IL-6 gene. Three novel variants were identified, a wobble position in codon F201 (TTT>TTC), -627C>A, and -572G>C (frequency of rare allele=0.04, 0.01 and 0.05 respectively), plus also the reported -597G>A, the AnTn tract (-392 to -373) and the -174G>C promoter variants. The -174G>C and -572G>A variants were in strong allelic association with each other (δ=0.94).

Functional properties of the IL-6 -174G>C and -572G>C variants were examined in hepatocyte cell lines (HepG2 and HuH7) using a dual luciferase reporter assay system. The -174C variant was associated with higher relative promoter strength than the -174G variant over a 24 hours time course period after dexamethasone (DEX) repression or IL-1β plus DEX induction in both HepG2 and HuH7 cells. The -572C variant also gave higher promoter activity than the -572G variant in response to the same stimulus and time periods in these cells. When the glucocorticoid response element (GRE) at position -557 to -552 was destroyed by mutation, the -572G promoter construct in HuH7 cells no longer showed repression by DEX and had higher induction from IL-1β plus DEX compared to the wild-type -572G variant. No promoter activity difference was found for the -572C GRE knockout compared to the -572C wild type variant. This suggests that the impact of the -572G>C variant is not acting via GRE-related mechanisms.

The IL-6 -174G>C and -572G>C promoter polymorphisms were examined in the second Northwick Park Heart Study (NPSHII) of ~3000 prospective healthy middle-aged men from the UK (frequency of rare allele 0.43 95% CI=0.42-0.44 and 0.05 95% CI=0.04-0.06 respectively). The -174C allele was not associated with fibrinogen levels, but was significantly associated with higher systolic blood pressure in smokers and non-smokers, an effect which was greater in men in the top two tertiles for body mass index (>24.68kg/m²). Compared to those with the GG genotype, men carrying the -174C allele had a relative risk of CHD of 1.54 (95% CI=1.0-2.23, p=0.048) and this effect was
greatest in smokers (RR=2.66, 95% CI=1.64-4.32). These effects remained statistically significant even after adjusting for classical risk factors including systolic blood pressure (p=0.04). The -572C allele was not associated with a significant effect on blood pressure, fibrinogen or relative risk for CHD. To confirm the finding of the NPHSII study, the same two polymorphisms were examined in MI survivors and healthy aged-matched controls from four European centres (HIFMECH study). A significant gradient of declining allele frequency was seen in the controls (p=<0.005) and the cases (p=0.032) for the -174C allele, but not for the -572C allele. There were no significant differences in genotype distribution or allele frequency between cases and controls in the North or the South. Significantly higher plasma concentrations for IL-6, fibrinogen and CRP were seen in MI survivors compared to the healthy controls in the North and South of Europe (p=<0.00005). Smoking was associated with greater risk in the South of Europe (OR=3.60, 95% CI=2.34-5.52) compared to the North (OR=2.30, 95% CI=1.48-3.55), but the North-South interaction was not significant. The -174G>C genotype was not significantly associated with levels of IL-6, fibrinogen, CRP or blood pressure in MI survivors or healthy control men from the North or South of Europe. Carriers for the -572C allele who were smokers had significantly higher systolic (5% higher, p=0.004) and diastolic blood pressure (3.4% higher, p=0.03) compared to -572GG genotype, an effect not seen in the nonsmokers. The lack of confirmation of the CHD risk associated with the functional -174C allele in the HIFMECH study and the novel associations seen with the -572C allele may be a result of the case-control design, but demonstrates that care must be taken in extrapolating from single studies, and that further work to demonstrate functionality in vitro and in vivo is required.
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CHAPTER 1

INTRODUCTION
Chapter 1: Introduction

1.1 Atherosclerosis – An inflammatory disease

There are growing evidence to support the role of inflammation in the pathogenesis of coronary heart disease (CHD) such as atherosclerosis, the principle cause of myocardial infarction (MI), stroke, and peripheral vascular disease in western industrialized countries (Sturk et al 1992, Ross et al 1993, de Maat et al 1996; Biasucci et al 1996). The principle risk factors for atherosclerosis have been considered for many years by investigators to consist largely of the accumulation of lipids such as triglyceride and cholesterol, particularly low density lipoprotein cholesterol (LDLc) within the arterial wall. Atherosclerosis is now universally recognized as an inflammatory disease whereby inflammation within the arterial walls contributes to the initiation and progression of atherosclerotic plaque formation (Ross et al 1993, 1999). Histopathological and immunocytochemical evidence suggests that active inflammatory processes may destabilize the fibrous cap tissue of the atherosclerotic plaque, thus triggering plaque rupture and enhancing the risk of coronary thrombosis (Van der Wal et al 1994). Prospective epidemiological studies have also shown a strong and consistent association between clinical manifestations of atherothrombotic disease and systemic markers of inflammation, including white blood cell count and various acute phase reactants such as fibrinogen, plasminogen activator inhibitor type-1 (PAI-1), C-reactive protein (CRP), interleukin-6 (IL-6) and von Willebrand factor (Ernst et al 1987; Juhan-Vague et al 1996; Koenig et al 1999; Ridker et al 2000a; Ridker et al 2000b; Thompson et al 1995). The key role of inflammation in the onset of acute ischemic syndromes is indicated by neutrophil activation (Dinerman et al 1990) and elevated levels of various acute phase proteins in unstable angina as well as the notable temporal relationship between acute or chronic infections and coronary events (Kruskal et al 1987; Berk et al 1990; Liuzzo et al 1994).
1.2 Pathogenesis of Atherosclerosis

During the 19th century, two theories were proposed for the pathogenesis of atherosclerosis. The ‘incrustation theory’ proposed by the pathologist von Rokitansky, suggested that arterial thickening was due to the deposition of fibrin from arterial blood (von Rokitansky 1844) and the ‘lipid infiltration’ theory by Virchow (Virchow 1856), which proposed that lipid infiltration in the arterial wall followed by complex formation with mucopolysaccharides was the initial event in atherosclerosis. Numerous pathophysiological observations in humans and animals have led to the formulation of the ‘response-to-injury’ hypothesis (Ross and Glomset 1973), where endothelial denudation, was cited as the first step in atherosclerosis. The most recent version of this hypothesis emphasizes endothelial dysfunction rather than denudation, where injury to the arterial wall leads to the initiation of compensatory processes by the endothelium, which then alters the normal vascular homeostatic properties of the artery wall. Endothelial dysfunction resulting from injury can then lead to increased adhesiveness of the endothelium to leukocytes and platelets as well as its permeability. Injury also induces the endothelium to have procoagulant instead of anticoagulant properties and increased production of endothelium derived vasoactive molecules, cytokines and growth factors. If left unabated, this can lead to the migration and proliferation of smooth muscle cells to the area contributing to advanced, complicated atherosclerotic lesions (Ross 1999). The erosion or uneven thinning that precedes plaque rupture, principally occurring at the shoulders of the fibrous cap where the mechanical stress exceeds that which the tissue can withstand (Richardson et al 1989; Loree et al 1992; Lee and Libby 1997). The atherosclerotic plaque is weakest here due to low collagen composition resulting from increased collagen degradation by collagenases, elastases and stromelysins, and the infiltration of macrophages and T-cells across the endothelium which increases and promotes plaque instability (Galis et al 1994; Schonbeck et al 1997). In addition, the lipid core is a source of prothrombotic materials in which tissue factor and coagulation factors have been found both in the necrotic core and cellular areas of the plaque in humans, further increasing the possibility of thrombosis (Fernandez-Ortiz et al 1994; Nemerson 1995; Marmur et al 1996; Mach et al 1997; Thompson et al 1995).
1.3 Factors that induce and promote inflammation or atherosclerosis

1.3.1 Modified lipoproteins

The degree to which low density lipoprotein (LDL) particles can be modified varies greatly (Steinberg 1997; Navab et al 1996; Diaz et al 1997). Modifications of LDL particles can occur through many processes, including oxidation, glycation (in diabetes), aggregation and incorporation with immune complexes. Modified LDL particles are considered the major cause of injury to the endothelium and underlying smooth muscle of the arterial wall (Steinberg 1997; Khoo et al 1998; 1992; Navab et al 1996; Morel et al 1983; Griendling and Alexander 1997). Oxidized LDL is cytotoxic to endothelial cells (Hessler et al 1983), inhibits the vasodilation that is normally induced by nitric oxide (Kugiyama et al 1990) and is mitogenic for macrophages and smooth muscle cells (Yui et al 1993; Chatterjee and Ghosh et al 1996). Oxidized LDL has proatherogenic properties through chemoattraction for monocytes and T-lymphocytes (Jonasson et al 1986; Quinn et al 1987; van der Wal et al 1989), the activation of which leads to the secretion of proteolytic enzymes, cytokines, chemokines and growth factors. LDL oxidation stimulates the release of macrophage colony stimulating factor (MCSF) and monocytes chemoattractant protein-1 (MCP-1) from endothelial cells (Quinn et al 1987; Rajavashisth et al 1990; Leonard and Yoshimura 1990), thus fueling the inflammatory response by allowing entry of new monocytes into the lesions which cause further injury and necrosis of the artery. Therefore, the removal and sequestration of these atherogenic modified LDL particles (by the LDL receptor on surfaces of endothelial cells and by scavenger receptor on macrophages) are an important part of the initial protective role in damping down the inflammatory process (Han et al 1997; Diaz et al 1997). The internalization of these modified LDL particles is principally carried out by macrophages via scavenger receptors and leads to the accumulation of cholesterol lipid-laden macrophages and the formation of foam cells in the arterial wall (Steinberg 1997; Khoo et al 1992; Navab et al 1996; Griendling and Alexander 1997; Han et al 1997). The accumulation of foam cells results in the thickening of the artery wall and reduced lumen diameter, which is then compensated for by gradual dilation, a phenomenon known as ‘remodeling’ (Glagov et al 1987). However, specific inflammatory mediators such as tumor necrosis factor alpha
(TNFα), interleukin-1 (IL-1), MCSF and MCP-1 secreted from monocyte-derived macrophages and T-lymphocytes can increase LDL binding to the endothelium and promote the migration and proliferation of smooth muscle cells around the atherosclerotic lesion core of lipid and necrotic tissues (Stopec et al 1993; Hajjar and Haberland 1997; Geng and Libby 1995). The build-up of lipid core and necrotic tissue within the fibrous cap, if large enough, can restrict blood flow and reduce the supply of nutrients to tissues, resulting in ischaemia. To compensate for this, blood pressure rises to increase flow rate, which results in erosion and uneven thinning of the fibrous cap. As a consequence of this, plaque instability occurs around the shoulders of the lesion where macrophages enter, accumulate and are activated into foam cells. It is here that the expression of cytokines, chemokines, growth factors and apoptosis may occur (Davies 1990; Fuster 1994; Lee and Libby 1997; Ross 1999), thus fueling a vicious cycle of inflammation within the arterial wall.

Oxidized LDL is expressed in the atherosclerotic lesions in both humans and animals (Yla-Herttuala et al 1989). Antioxidants can reduce the size of lesions and fatty streaks in animals with hypercholesterolemia and in nonhuman primates (Navab et al 1996; Carew et al 1987; Kita et al 1987; Sasahara et al 1994; Chang et al 1995). The antioxidant vitamin E has been shown to increase the resistance of human LDL to oxidation (Reaven et al 1993). Vitamin E intake is inversely correlated with the incidence of MI, and vitamin E supplements reduce coronary events in several preliminary trials (Rimm et al 1993; Stampfer et al 1993; Stephens et al 1996), but not in others (Mezzetti et al 2001; Pruthi et al 2001; Kromhout 2001; Kaul et al 2001). This is in contrast to other antioxidants such as beta carotene which has no effect on reducing oxidation of LDL or coronary events (Reaven et al 1993; Hennekens et al 1996; Omenn et al 1996).

### 1.3.2 Hypertension

Elevated blood pressure directly correlates with increased risk of CHD (Weijenberg et al 1996; Stokes et al 1989; Franklin 2001). Several clinical studies described a substantial decrease in CHD risk by successfully treating hypertension (Hansson et al 1998; Himmelmann et al 1998; Elliott et al 2001). Hypertension also has proinflammatory
properties by increasing the formation of hydrogen peroxide and free radicals such as superoxide anion and hydroxyl radicals in plasma (Griendling and Alexander 1997; Lacy et al 1998; Swei et al 1997). These substances in turn reduce the effect of vasodilation by nitric oxide on the endothelium (Vanhoutte and Boulanger 1995), increase leukocyte adhesion and increase peripheral resistance in the lumen of the artery (Swei et al 1997).

Angiotensin II, (a product of the renin-angiotensin cascade) is well recognized as a potent vasoconstrictor that causes atherosclerosis and hypertension (Dzau 1994; Walker et al 1979; Carluccio et al 2001). In addition to causing hypertension, angiotensin II can also contribute to atherogenesis by increasing smooth muscle contraction through increase in intracellular calcium, protein synthesis, smooth muscle cell hypertrophy and lipoxygenase activity, which promote inflammation through the oxidation of LDL (Campbell et al 1991; Gibbons et al 1992; Rossi et al 1995; Chobanian and Dzau 1996; Natarajan et al 1997). The expression of angiotensinogen, a precursor of angiotensin II is upregulated by IL-6 during the inflammatory process (Itoh et al 1989; Ohtani et al 1992; Takano et al 1993; Sherman and Brasier 2001). Angiotensin II acts through the angiotensin II type 1 receptor (AT1) involving the Janus kinase/signal transducers activator of transcription (JAK/STAT, also referred as Jak/STAT) pathway and intercellular calcium ions to upregulate IL-6 ((Funakoshi et al 1999; Han et al 1999), thus prolonging the inflammatory process.

Variations in genes which encode for proteins that are involved in the renin-angiotensin system have been shown to effect blood pressure levels and risk of hypertension (Allikmets et al 1999; Wang and Staessen 2000; Carluccio et al 2001). For example, allelic variations in the angiotensinogen and AT1 genes have been shown to be associated with early onset of hypertension (Schmidt et al 1995; Chiang et al 1997; Iso et al 2000; Takahashi et al 2000; Lajemi et al 2001). The insertion/deletion polymorphism in the angiotensin I converting enzyme (ACE) gene have also been shown to be independently associated with hypertension, particularly in Japanese men (Higaki et al 2000; Uemura et 2000) and risk the of MI (Cambien et al 1992; Tiret et al 1994; Bray et al 2001). Evidence to date suggests that elevated systolic and diastolic blood pressure causes greater
turbulence of blood flow through specific arterial sites, inducing injury to the endothelial lining of blood vessels (Gotlieb and Langille 1996). This injury induces platelet adhesion to the dysfunctional endothelium and stimulates proliferation and migration of smooth muscle cells to repair the damaged arterial wall (Ross 1993; 1999; Bombeli et al 1998). As a result, the rate of clearance of lipoproteins from the vessel wall may be reduced, therefore increasing the vessel wall susceptibility to plaque formation and escalating the risk of thrombosis (Mach et al 1997; Thompson et al 1995).

1.3.3 Infections

The infectious microorganisms, herpes virus and Chlamydia pneumonia have been implicated with atherosclerosis (Libby et al 1997; Jackson et al 1997). These organisms have been demonstrated at autopsy to be present in the atheromatous lesions in coronary arteries (Hendrix et al 1990; Jackson et al 1997) and titers of antibodies to these organisms are reported to be predictors of CHD risk in MI patients (Throm et al 1991; Melnick et al 1993; Gupta et al 1997). At present there is no direct evidence to suggest these organisms cause the lesion of atherosclerosis (Libby et al 1997; Hajjar et al 1986; Nicholson and Hajjar 1998). However, it may be possible that infection, combined with other factors, plays a pivotal role in the development of the atherosclerotic lesion (Libby et al 1997; Danesh et al 1997).

1.3.4 Smoking

Cigarette smoking is a major independent CHD risk factor and has synergistic effects when combined with other CHD risk factors (Gensini et al 1998; Seltzer 1989; Castelli 1990; Weintraub 1990). Smoking is strongly correlated with increased risk of acute coronary events in middle-aged smokers. These subjects have a 10 fold increased risk of sudden cardiac death and 3.6 fold greater risk of having an MI (Kannel et al 1984). CHD events rapidly decline on cessation of smoking (Gensini et al 1998; Weintraub 1990; Kuller et al 1991), with MI risk falling by ~50% within the first two years (Rosenberg et al 1985; Cook et al 1986).
Atherogenic effects from smoking may result from endothelial dysfunction. Endothelial damage to tissues in the lung from smoking can increase oxidative stress on plasma lipoproteins (Griendling and Alexander 1997; Lacy et al 1998; Jonasson et al 1986; van der Waal et al 1989). Smoking reduces levels of antioxidant vitamins and increases levels of oxidized LDL (Heitzer et al 1996a; 1996b; Harats et al 1989; 1990; Scheffler et al 1990; 1992; Morrow et al 1995), and is associated with lipid abnormalities, such as lower levels of HDL and ApoAI (Peacock et al 1997) and higher levels for LDL and triglycerides in smokers compared to nonsmokers (Craig et al 1989; Cuesta et al 1989). The incidence of arterial thrombosis is increased in smokers being associated with raised plasma fibrinogen, factor VII activity, prothrombin fragment 1+2 and impaired endogenous fibrinolysis (Rugman et al 1994; Miller et al 1998; Newby et al 1999; 2001) thereby favoring a procoagulant state. Finally, smoking also promotes atherogenesis via mediators of inflammation such as IL-6 and CRP. Elevated plasma levels of IL-6 and CRP are associated with risk of coronary events (Koenig et al 1999; Ridker et al 2000a; 2000b; Doggen et al 2000; Mendall et al 2000).

1.4 Inflammation and IL-6

Inflammation is a physiological response to tissue injury, trauma and infection and consists of a systemic reaction to combat further tissue damage, destroying infective organisms and activating repair processes. The early stage of inflammation, during which metabolic and catabolic changes occur in many organs, is known as the acute-phase response (APR). The APR is characterised by changes in the levels of serum acute-phase (AP) proteins, which are synthesised primarily in the liver. Serum concentrations of some AP proteins can increase as much as 1,000-fold several hours after the onset of the APR (Steel et al 1994). Activation of AP genes in hepatocytes is triggered by several inflammatory signals, including those of IL-6, IL-1, TNF-α and interferon gamma (IFN-γ) (Mackiewicz 1997; Poli and Ciliberto 1994). Of the numerous cytokines and growth factors that are involved in the upregulation of AP gene expression, IL-6 is considered to be the major mediator. This conclusion is supported by a correlation between increased
serum IL-6 and changes in AP gene expression during the inflammatory response, the large number of AP proteins synthesised in response to IL-6, and the observation that APR is impaired in mice lacking IL-6 (Poli and Ciliberto 1994).

Interleukin-6 (IL-6) is a multifunctional cytokine that participates in the regulation of acute-phase reactions, immune responses and haematopoiesis (Andus et al 1987; Gauldie et al 1987; Sehgal et al 1989). Abnormal expression of IL-6 may be involved in the pathogenesis of a variety of diseases including multiple myeloma, rheumatoid arthritis, post-menopausal osteoporosis, chronic autoimmune diseases, Castleman’s disease and acquired immunodeficiency syndrome (AIDS) (Akira et al 1993). The pleiotropic functions of IL-6 are summarised in table 1.1

Table 1.1 Pleiotropic Functions of IL-6 (adapted from Kishimoto et al 1995).

<table>
<thead>
<tr>
<th>Effect on B cells</th>
<th>Ig production</th>
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<tbody>
<tr>
<td></td>
<td>Proliferation of myeloma cells</td>
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<table>
<thead>
<tr>
<th>Effect on T cells</th>
<th>Proliferation of Epstein-Barr virus infected B cell</th>
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<tbody>
<tr>
<td></td>
<td>Differentiation of cytotoxic T lymphocytes</td>
</tr>
<tr>
<td></td>
<td>Induction of IL-2R expression and IL-2 production</td>
</tr>
<tr>
<td></td>
<td>Augmentation of NK activities</td>
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</tbody>
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<table>
<thead>
<tr>
<th>Effect on hematopoietic progenitor cells</th>
<th>Enhancement of multipotential hematopoietic colony formation</th>
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<table>
<thead>
<tr>
<th>Effect on macrophages</th>
<th>Growth inhibition of myeloid leukemic cell lines and induction of their macrophage differentiation</th>
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<table>
<thead>
<tr>
<th>Effect on hepatocytes</th>
<th>Acute-phase protein synthesis</th>
</tr>
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<table>
<thead>
<tr>
<th>Effect on bone metabolism</th>
<th>Stimulation of osteoclast formation</th>
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<tbody>
<tr>
<td></td>
<td>Induction of bone resorption</td>
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<table>
<thead>
<tr>
<th>Effect on blood vessels</th>
<th>Induction of platelet-derived growth factor (PDGF)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proliferation of vascular smooth muscle cells</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Effect on heart muscle cells</th>
<th>Negative Inotropic effect on heart</th>
</tr>
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<table>
<thead>
<tr>
<th>Effect on neuronal cells</th>
<th>Neural differentiation of PC12 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Support of survival of cholinergic neurons</td>
</tr>
<tr>
<td></td>
<td>Induction of adrenocorticotropic hormones synthesis</td>
</tr>
</tbody>
</table>

| Effect on placenta | Secretion of chorionic gonadotropin from trophoblasts |
Bowcock et al 1988; 1989, using restriction fragment length polymorphism (RFLP) have identified an AT rich polymorphic hypervariable region ~450bp from the polyadenylation site and an Alu repetitive DNA element in intron 4 of the human IL-6 gene. This hypervariable region is associated with bone mineral density in which elevated IL-6 levels correlate with greater bone mass loss (Murray et al 1997). Recently, Fishman et al 1998 reported several promoter polymorphisms in the IL-6 gene. This study reported a base G>C transition at position -174, where the C allele creates an NlaIII or Hsp92II restriction site. The frequency of the rare C allele in healthy Caucasians was 0.40 and was associated with lower IL-6 promoter activity in HeLa cells. The second reported polymorphism is the A<sub>n</sub>T<sub>n</sub> tract at position -395 to -375 with variable numbers of A and T runs. The A<sub>9</sub>T<sub>11</sub> is associated with -174C allele and the A<sub>9</sub>T<sub>11</sub>, A<sub>10</sub>T<sub>10</sub> and A<sub>10</sub>T<sub>11</sub> is associated with -174G allele.

The IL-6 -174G>C polymorphism was in almost complete linkage disequilibrium with the -597G>A polymorphism (delta=0.94) (Dr Faulds, personal communication and later reported by Terry et al 2000), where the -174G allele associated strongly with the -597G allele and the -597A allele with the -174C allele. Transient transfection of luciferase reporter constructs containing the -174G and -174C variants of the human IL-6 promoter (region -550bp to +61bp) in HeLa cells reveal modestly higher reporter expression with the -174G construct than the -174C construct under basal condition but significantly higher after stimulation with lipopolysaccharides (LPS) and IL-1 (see figure. 1.1) (Fishman et al 1998). This paper also reported that the IL-6 -174G>C promoter polymorphism was associated with IL-6 levels in healthy caucasians from a north London general practice (Goodinge study). Extrapolation of the data in the Goodinge study by Dr Faulds (personal communication) is shown in table 1.2.1. In this study, GG homozygous individuals had a significantly higher circulating IL-6 concentration compared to those homozygous for the C allele, an effect which was most strongly seen in smokers (p<0.01), but not in nonsmokers. Age, body mass index (BMI), blood pressure and lipid traits were not significantly different between the genotype groups. Individuals with the GG genotype have higher levels of plasma CHD risk factors for CRP and plasminogen activator inhibitor-1 (PAI-1) compared to those with the GC or CC genotypes, but this
was not statistically significant (CRP, p=0.161 and PAI-1, p=0.212 respectively). The data demonstrated here suggest that there is a genetically determined difference in the degree of IL-6 response to stressful stimuli between individuals and that the magnitude of this genotype effect is likely to be of biological significance risk of thrombosis and predisposition to CHD. This finding was however contradictory to a more recent report in patients with abdominal aortic aneurysms (AAA), where the -174C allele was associated with significantly higher IL-6 levels compared to those with either GC+CC alleles (see table 1.2.2) (Jones et al 2001) The IL-6 -174G>C genotype was not associated with levels for CRP and fibrinogen in these AAA patient. The frequency of the IL-6 -174C allele in the general population differs across ethnic groups, with the highest occurrences in Caucasians and lowest in Afro-Caribbean (Fishman et al 1998; Dr Faulds, personal communication) (see table 1.3).
Figure 1.1 Luciferase activities, measured as fold increase in light emission relative to the unstimulated -174G construct expressed in HeLa cells after the addition of LPS (10µg/ml) and IL-1 (10U/ml). Results are the mean of four transfections (+/- SD, where n=8), corrected for protein content and efficiency of transfection by β-galactosidase cotransfection (adapted from Fishman et al 1998).
Table 1.2.1 IL-6 -174G>C genotype and CHD risk factors levels in healthy Caucasians (Goodinge study) (Dr Faulds, personal communication).

<table>
<thead>
<tr>
<th>CHD risk traits</th>
<th>GG (n=35)</th>
<th>GC (n=45)</th>
<th>CC (n=22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 (pg/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-smokers</td>
<td>1.84</td>
<td>2.45</td>
<td>1.49</td>
</tr>
<tr>
<td>Smokers</td>
<td>5.54</td>
<td>3.06</td>
<td>1.62*</td>
</tr>
<tr>
<td>CRP (pg/ml)</td>
<td>1.20</td>
<td>1.09</td>
<td>0.80</td>
</tr>
<tr>
<td>PAI-1 (pg/ml)</td>
<td>106.3 (8.5)</td>
<td>95.1 (6.6)</td>
<td>82.9 (7.5)</td>
</tr>
</tbody>
</table>

*p<0.01

Table 1.2.2 IL-6 -174G>C genotype and CHD risk factors levels in patients with abdominal aortic aneurysms (AAA) (adapted from Jones et al 2001).

<table>
<thead>
<tr>
<th>CHD risk traits</th>
<th>GG (n=146)</th>
<th>GC (n=245)</th>
<th>CC (n=75)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 (pg/ml) median (IQR)</td>
<td>1.9 (0-106)</td>
<td>4.8 (0-126)</td>
<td>15.6 (0.5-215)*</td>
</tr>
<tr>
<td>InCRP (ng/ml)</td>
<td>4.1±0.9</td>
<td>4.2±0.9</td>
<td>4.1±1.0</td>
</tr>
<tr>
<td>Fibrinogen (g/l)</td>
<td>4.35±1.36</td>
<td>4.31±1.38</td>
<td>4.46±1.41</td>
</tr>
</tbody>
</table>

*p<0.047, IQR=interquartile range
Values for continuous variable are mean±SD, except for median IL-6, for which the Krustal-Wallis p value is given.

Table 1.3 Allelic frequencies of the IL-6 -174C in different ethnic groups (Dr Faulds, personal communication).

<table>
<thead>
<tr>
<th>Ethnicity</th>
<th>Frequency C allele</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caucasian</td>
<td>0.400</td>
<td>0.37-0.43</td>
</tr>
<tr>
<td>Gujarati Indian</td>
<td>0.15</td>
<td>0.09-0.18</td>
</tr>
<tr>
<td>Afro-Caribbean</td>
<td>0.05</td>
<td>0.02-0.07</td>
</tr>
<tr>
<td>Chinese</td>
<td>0.07</td>
<td>0.05-0.09</td>
</tr>
<tr>
<td>Primate (e.g. Gorilla and Chimpanzee)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
1.5 Structure and functions of the human IL-6 protein

1.5.1 Structure of IL-6

The human IL-6 (IL-6) is a glycoprotein molecule with a molecular mass of 21-28kDa, depending on the degree of glycosylation and cells specific expression (Tosato et al 1988; May et al 1988; May et al 1991; Santhanam et al 1989). The amino acid sequence deduced from a human cDNA clone consist of 212 amino acids residues (Zilberstein et al 1986; May et al 1986; Hirano et al 1986). Proteinase cleavage results in a mature protein of 184 amino acid residues. The 3-D X-ray crystallographic structure of the human IL-6 protein was elucidated by Somers et al 1997 and consists of four helices (A-D) plus a mini-helix (E) linked together by β-turns (figure 1.2).

![Ribbon representation of the IL-6 crystal structure](image)

Figure 1.2 Ribbon representation of the IL-6 crystal structure. The four main helices are labelled A, B, C and D. The extra helix in the final long loop labelled E. The missing part of the first cross-over connection is indicated by a dashed line (adapted from Somers et al 1997).

Arrangement of amino acid residues from the 3-D structure of the human IL-6 is summarised in table 1.4.
<table>
<thead>
<tr>
<th>Structure</th>
<th>Amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helix A</td>
<td>Ser21→Ala45</td>
</tr>
<tr>
<td>Type I β-turn</td>
<td>Ala68→Asp71</td>
</tr>
<tr>
<td>Disulphide bond</td>
<td>Cys73 and Cys83</td>
</tr>
<tr>
<td>Type II β-turn</td>
<td>Gln75→Phe78</td>
</tr>
<tr>
<td>Helix B</td>
<td>Gln80→Gln102</td>
</tr>
<tr>
<td>Helix C</td>
<td>Glu109→Lys129</td>
</tr>
<tr>
<td>Helix D</td>
<td>Gln156→Arg182</td>
</tr>
<tr>
<td>Helix E</td>
<td>Pro141→Gln152</td>
</tr>
</tbody>
</table>

Table 1.4 Location of amino acids residues in the IL-6 protein structure.

1.5.2 Molecular mechanism of IL-6 cytokine receptor signalling

IL-6, a major mediator of AP gene expression by the liver during inflammation, exerts its action via a unique cytokine receptor mechanism. This consists of two membrane proteins; an 80kDa glycoprotein ligand-binding chain called IL-6 receptor (IL-6R) and a 130kDa-glycoprotein signal transducer, gp130. Binding of IL-6 to IL-6R triggers the association of IL-6R and gp130, gp130 in turn transduces the signal (Taga et al 1989). However, soluble IL-6R (excluding transmembrane and cytoplasmic domains), can also associate with gp130 in the presence of IL-6 and transduce the signal through gp130 (Taga et al 1989).

To evaluate the therapeutic potential of IL-6 antagonists in the treatment of IL-6 related diseases, various independent investigators have constructed recombinant mutants of IL-6 in an attempt to elucidate its interactions with IL-6R and gp130. The results are summarised in table 1.5, and demonstrate that helices A and D on the IL-6 protein are essential for the activity and binding to IL-6R. This information was use in the constructions of anti-IL-6 monoclonal antibodies (anti-IL-6 mAbs) to treat patients with myeloma, Castleman disease and AIDS (Klein et al 1990; Daveau et al 1994; Emilie et al 1994), and has been shown to alleviate the symptoms of diseases. However, this effect is short term because these anti-IL-6 mAbs results in the increased production of IL-6 type cytokines by the immune system.

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Table 1.5 Analysis of IL-6 activity and binding properties by site-directed mutagenesis. +++a, active; ++a, reduced activity; +a, strongly reduced activity (<10%); -a, no activity; ++b, reduced binding to hIL-6R; +b, strongly reduced binding (<10%); -b, no binding. Δ, deletion (adapted from Grotzinger et al 1997)

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Location</th>
<th>Effect</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ 1-26</td>
<td>Helix A</td>
<td>+++a</td>
<td>Brakenhoff et al 1994</td>
</tr>
<tr>
<td>Δ 1-28</td>
<td>Helix A</td>
<td>++a</td>
<td>Brakenhoff et al 1994</td>
</tr>
<tr>
<td>Δ 1-30</td>
<td>Helix A</td>
<td>+a</td>
<td>Brakenhoff et al 1994</td>
</tr>
<tr>
<td>R30A</td>
<td>Helix A</td>
<td>-b</td>
<td>de Hon 1995</td>
</tr>
<tr>
<td>G77-E95 (human)→G77-H95 (murine)</td>
<td>Loop AB</td>
<td>-b</td>
<td>Ehlers et al 1994</td>
</tr>
<tr>
<td>F74E</td>
<td>Loop AB</td>
<td>-b</td>
<td>Kalai et al 1997</td>
</tr>
<tr>
<td>F78X, X=E, P</td>
<td>Loop AB</td>
<td>-b</td>
<td>Ehlers et al 1994; Kalai et al 1997</td>
</tr>
<tr>
<td>R168M</td>
<td>Helix D</td>
<td>-a</td>
<td>Fontaine et al 1994</td>
</tr>
<tr>
<td>L174V</td>
<td>Helix D</td>
<td>+b</td>
<td>Nishimura et al 1992</td>
</tr>
<tr>
<td>L174R</td>
<td>Helix D</td>
<td>-a</td>
<td>Fontaine et al 1994</td>
</tr>
<tr>
<td>S177R</td>
<td>Helix D</td>
<td>-b</td>
<td>Leebeek et al 1992a; 1992b</td>
</tr>
<tr>
<td>L178X, X=Q, R</td>
<td>Helix D</td>
<td>-b</td>
<td>Leebeek et al 1992a; 1992b</td>
</tr>
<tr>
<td>L178X, X=D, P</td>
<td>Helix D</td>
<td>-a</td>
<td>Fontaine et al 1993</td>
</tr>
<tr>
<td>R179K</td>
<td>Helix D</td>
<td>+a</td>
<td>Fontaine et al 1993</td>
</tr>
<tr>
<td>R179E</td>
<td>Helix D</td>
<td>-b</td>
<td>de Hon 1995</td>
</tr>
<tr>
<td>A180X, X=D, T, L, R</td>
<td>Helix D</td>
<td>+a, -b</td>
<td>Savino et al 1993; Leebeek et al 1992a</td>
</tr>
<tr>
<td>R182X, X=K, Q, A</td>
<td>Helix D</td>
<td>+a</td>
<td>Lutticken et al 1991</td>
</tr>
<tr>
<td>R182X, X=E, T, P</td>
<td>Helix D</td>
<td>+a</td>
<td>Lutticken et al 1991</td>
</tr>
<tr>
<td>H164-R182 (human)→Q164-R182 (murine)</td>
<td>Helix D</td>
<td>-b</td>
<td>Leebeek et al 1992a</td>
</tr>
<tr>
<td>H164-H182 (human)→Q164-R182 (rat)</td>
<td>Helix D</td>
<td>+b</td>
<td>Leebeek et al 1992a</td>
</tr>
<tr>
<td>Δ182-184</td>
<td>Helix D</td>
<td>-a</td>
<td>Kruttgen et al 1990</td>
</tr>
<tr>
<td>Δ183-184</td>
<td>Helix D</td>
<td>+a</td>
<td>Kruttgen et al 1990</td>
</tr>
</tbody>
</table>
Kalai et al 1997, using molecular modelling and site-directed mutagenesis reported the structure of the IL-6/IL-6R binding interface. This binding interface for IL-6 and IL-6R involves the association of hydrophobic and hydrophilic amino acids between the two proteins. The hydrophobic interaction occurs between Phe74 and Phe78 of IL-6 with Tyr188, Phe248 and Phe249 of IL-6R. This is then sealed by a hydrophilic core composing of amino acid residues Glu23, Asp26, Lys27, and Arg30 from helix A and Arg168, Arg179, and Arg182 from helix D of IL-6 and Arg250, Arg252, Glu254, Asp272, Glu296, Glu297, and Glu302 of IL-6R.

Molecular modelling-guided mutagenesis of the extracellular domain of gp130, revealed that amino acid residues located in the B'C' loop (Val252) and the F'G' loop (Gly306, Lys307) of domain 3 in the hinge region (Tyr218) connecting domains 2 and 3 were essential for complex formation with IL-6/IL-6R in COS-7/BaF3 cells (Horsten et al. 1997). This region has been shown to be part of the hydrophobic cluster formed by IL-6 and gp130 (Savino et al. 1994). To evaluate this, mutants Y218K, V252D, G306W and K307E of gp130, predicted from molecular modelling to destroy or caused sterical constraints of this hydrophobic cluster were tested. The result showed reduced complex formation capability compared to the wild-type (Wt) protein (i.e. Wt > Y218K ≈ K307E > G306W > V252D) (Horsten et al 1997).

The IL-6 receptor complex for IL-6 signalling proposed by Taga et al 1989 was originally thought to contain 1:1:2 stoichiometry of IL-6-IL-6R-gp130 with the initial binding of IL-6 to IL-6R being the crucial part of the signalling process (reviewed by Kishimoto 1995). In vitro studies utilising size exclusion chromatography and equilibrium centrifugation have shown that two molecules of IL-6 are needed to bind to two molecules of the soluble extracellular domain of IL-6 receptor (sIL-6R) to form a heterodimer (Ward et al 1994). In the presence of the soluble extracellular domain of gp130 (sgp130), a hexameric complex is formed and composed of IL-6, sIL-6R and sgp130 in a 2:2:2 stoichiometry (Ward et al 1994). Using evidence from structural, biochemical and mutagenic studies from various independent investigators (Fiorillo et al 1992; Fontaine et al 1993; Savino et al 1993; Savino et al 1994; Ehlers et al 1994; 1995; Hammacher et al 1994; de Hon et al...
1995; De Vos et al 1992; Somers et al 1994; Brakenhoff et al 1994; Yawata et al 1993; Ward et al 1994). Somers et al 1997 were able to propose a model for the hexameric complex of signal transduction based on the 3-D structure of IL-6 (figure 1.3). The first event in signal transduction is the binding of IL-6 to IL-6R (site 1) forming a heterodimer (Taga et al 1989). Then the IL-6-IL-6R heterodimeric complex binds to gp130 on the cell surface (site 2) to form a hetero-trimer. The final event involves the binding of two hetero-trimeric complexes mediated by interaction in sites 3 and 4 to form a hexameric signal transduction complex. This model is supported by previous evidence showing IL-6 mediated signal transduction occurs through clustering of two gp130 receptors by IL-6 (Murakami et al 1993; Wijdenes et al 1995).

Figure 1.3 Ribbon representation of the IL-6, IL-6R and gp130 hexamer signal model. The IL-6 crystal structure is shown in green, IL-6R in blue and gp130 in red. Site 1 is the site of IL-6-IL-6R interactions. Site 2 is the region where IL-6 interacts with gp130 in the trimer. Site 3 is the site of IL-6-gp130 interaction between trimers. Site 4 is the location of IL-6-IL-6 interaction between trimers (adapted from Somers et al 1997).
The IL-6-IL-6R-gp130 hexameric complexes which is believed to be involved in the activation of acute phase protein gene expression by the phosphorylation through signal transducer activator of transcription 3/acute phase response factor (STAT3/APRF) by the Janus kinase (Jak) pathway or via the serine/threonine phosphorylation of nuclear factor IL-6 (NF-IL6) (Nakajima et al 1993; Ihle et al 1995; Sasse et al 1997). Dimerization of STAT3/APRF and NF-IL6 by phosphorylation then leads to the induction of AP protein expression through the recognition of type 1 and type 2 IL-6 responsive elements (IL-6REs) (adapted from Akira and Kishimoto 1992) (figure 1.4).

Figure 1.4 Two pathways from gp130 to acute phase protein gene expression. Homodimerization of gp130 activates the STAT3/APRF pathway and Ras-MAPK cascade. An adapter molecule not yet identified is postulated to link the gp130 homodimer to Ras, leading to the critical threonine phosphorylation of NF-IL6 through MAPK activation. The STAT3 dimer and the NF-IL6 dimer bind to type 1 and type 2 IL-6REs, respectively, of the acute-phase protein gene (adapted from Kishimoto et al 1995).
A type 1 IL-6RE, characterised by the consensus sequence T(T/G)NNGNAA(T/G), is present in the promoter regions of AP genes (e.g. C-reactive protein). This element binds members of the CCAAT/Enhancer binding protein (C/EBP) family, of which NF-IL6 (also called C/EBPβ, AGP/EBP, LAP and IL-6DBP) and NF-IL6β (C/EBPβ) have been shown to be implicated in the regulation of AP proteins by IL-6 (Akira et al 1990; Poli et al 1990; Kinoshita et al 1992). Type 2 IL-6RE, consist of a hexanucleotide motif, CTGGGA, to which dimerization and activation of STAT3/APRF leads to the upregulation of the expression of AP proteins expression (e.g. fibrinogen) (Wegenka et al 1993). Table 1.6 shows examples of the acute phase genes regulated by type 1 and type 2 IL-6REs.

Table 1.6 IL-6 response elements identified in acute phase protein gene (adapted from Akira et al 1995).

<table>
<thead>
<tr>
<th>Type 1 Elements</th>
<th>Type 2 Elements</th>
</tr>
</thead>
<tbody>
<tr>
<td>rat AGP</td>
<td>TTGTGCAAG</td>
</tr>
<tr>
<td>TTGGCACAAT</td>
<td>human α1-antitrypsin</td>
</tr>
<tr>
<td>mouse</td>
<td>TTGGCACAAG</td>
</tr>
<tr>
<td>TTGGGAAAT</td>
<td>rat α-fibrinogen</td>
</tr>
<tr>
<td>rat angiotensinogen</td>
<td>TGGCACAAT</td>
</tr>
<tr>
<td>human CRP</td>
<td>TTGGATGTAAT</td>
</tr>
<tr>
<td>human C3</td>
<td>TGGACCAAG</td>
</tr>
<tr>
<td>human hemopexin</td>
<td>TGAATGTAAT</td>
</tr>
<tr>
<td>human haptoglobin</td>
<td>TGAAGCAAG</td>
</tr>
<tr>
<td>human fibrinogen</td>
<td>TTAATGCAAG</td>
</tr>
<tr>
<td>mouse SAA</td>
<td>TGGAGCAGAAT</td>
</tr>
<tr>
<td>Consensus</td>
<td>TTNNGNAAT</td>
</tr>
<tr>
<td>(G)</td>
<td>(G)</td>
</tr>
</tbody>
</table>

1.5.3 Negative regulation of the IL-6/IL-6R/gp130/Jak/STAT signalling pathway

The well-characterized negative regulation of IL-6 receptor signaling of AP (also known as APP) expression reported to date involves the Janus kinase/signal transducer activator of transcription (Jak/STAT or JAK/STAT) pathway. Inhibition of this pathway can occur by direct inhibition from protein inhibitor of activated STAT (PIAS) on STATs, inhibition of Jak/STAT signaling by suppressor of cytokine signaling (SOCS) or cytokine-inducible SH-2 containing protein (CIS) and endocytosis of the IL-6 receptor.
complex (see figure 1.5, for review, see Heinrich et al 1998; Hilton 1999; Krebs and Hilton 2001).

![Figure 1.5 Negative regulation of the IL-6-type cytokine signal transduction pathway (adapted from Heinrich et al 1998).](image)

1.5.3.1 Inhibition of STAT by PIAS

Recently, a protein inhibitor of activated STAT (PIAS) has been discovered in various human tissues (Chung et al 1997) which co-immunoprecipitates with tyrosine phosphorylation of STAT and blocks DNA binding of activated STAT as well as STAT mediated gene expression by IL-6 (Chung et al 1997). It is believed that there are specific PIAS for every STAT factor and the ratio of STAT and PIAS may determine the strength of the STAT signal. However, the regulation of PIAS is still not yet known.

1.5.3.2 Feedback inhibition of Jak/STAT pathway by SOCS proteins

The downregulation of Jak/STAT pathway by IL-6 induction has recently been reported to involved SOCS proteins such as SOCS 1-3 and CIS (Hilton 1999; Krebs and Hilton
Transcripts encoding SOCS 1-3 and CIS are often present in cells at low or undetectable levels, but are rapidly induced by a broad spectrum of cytokines, both *in vitro* and *in vivo*. For example, mRNA levels for SOCS and CIS are all induced in response to stimulation with interleukin-2 (IL-2), interleukin-3 (IL-3), interleukin-4 (IL-4), IL-6, interferon-γ (IFN-γ), erythropoietin, prolactin and growth hormone (Starr et al 1997; Naka et al 1997; Yoshimura et al 1995; Minamoto et al 1997; Cohney et al 1999; Pezet et al 1999; Davey et al 1999). There are several different molecular mechanisms by which SOCS family members can inhibit cytokine signaling (see figure 1.6 for an overview). SOCS 1 and SOCS 3 can directly interact with Jak and in doing so, inhibit their catalytic activity (Naka et al 1997; Endo et al 1997; Nicholson et al 1999; Yasukawa et al 1999), whereas CIS competes with STATs for the common Src-homology 2 (SH-2) or phosphotyrosine binding sites within the cytoplasmic domains of gp130 (Ram and Waxman 1999).

Figure 1.6 Molecular mechanism by which SOCS proteins negatively regulate cytokine signaling (adapted from Krebs and Hilton 2001).
1.5.3.3 Endocytosis of the IL-6 receptor complex

The rapid removal of inflammatory cytokines such as IL-6 from the circulation is essential for re-establishment of homeostasis (Castell et al 1988). The major organ in which the IL-6 receptor complex accumulates is the liver, where it is degraded (Castell et al 1990; Sonne et al 1990). After IL-6 binds to its receptor complex, it is efficiently internalized, thereby completely depleting the availability of the surface receptors within 30-60 minutes (Zohlnhofer et al 1992; Nesbitt and Fuller 1992) (figure 1.5). Thus, IL-6 downregulates its surface receptor and de novo protein synthesis is necessary to replenish IL-6 binding and signal transduction.

1.6 Expression of the human IL-6 gene

Studies using recombinant IL-6, anti-IL-6 antibodies and immunohistochemistry have demonstrated that IL-6 is expressed in a variety of cell types including monocytes and macrophages, T- and B-cells, fibroblasts, bone marrow stromal cells, endothelial cells, hepatocytes and smooth muscle cells (Kishimoto 1989). The human IL-6 gene is located on chromosome 7p21 (Sehgal et al. 1986; Ferguson-Smith et al. 1988; Bowcock et al. 1988), and consists of five exons and four introns (Yasukawa et al. 1987). The 5'-flanking region of the human IL-6 gene consist of two putative glucocorticoid response elements (GR, also known as GRE), an AT rich tract, a multiple response element (MRE), an NF-IL6, two AP1 consensus sites, an NFκB and two TATA sites (Tanabe et al. 1988) (figure 1.7).
1.6.1 Induction of IL-6 gene expression

IL-6 gene transcription is readily induced by bacterial or viral infections, serum, growth factors and inflammatory cytokines such as IL-1, tumor necrosis factor (TNF), platelet derived growth factor (PDGF), tumor growth factor-β (TGF-β) and interferons (Hirano et al 1986; Sehgal et al 1989; Sehgal et al 1990a, 1990b). Site directed mutagenesis has demonstrated that the region -225bp to +1 of the IL-6 promoter was sufficient for the induction of IL-6 expression (Ray et al. 1989; 1990; 1991). Figure 1.8 summarises the cis-acting elements and trans-acting factors that are essential for induction of IL-6 expression in response to inflammatory stimuli.
Ray et al (1989) demonstrated that a 23 bp IL-6 multi-response element (MRE) between -173 to -151 was responsible for the induction of IL-6 expression by IL-1, TNF, serum, protein kinase A and protein kinase C (phorbol ester). Using competitive gel retardation assay and methylation interference assay, Isshiki et al (1990) and Akira et al (1990) identified a DNA-protein complex at position -164 to -139, which they demonstrated to be a 14 bp palindromic sequence (ACATTGCACAATCT) termed, NF-IL6. Further analysis using chloramphenicol acetyl transferase (CAT) reporter construct demonstrated that the 14bp NF-IL6 binding sequence responded to IL-1 in glioblastoma cells SK-MG-4 (Isshiki et al 1990). Molecular cloning of NF-IL6 showed an intronless gene with a single open reading frame (Akira et al 1990). A search of the protein database showed that the C-terminal region is highly similar to a leucine zipper structure liver-specific nuclear factor, C/EBP (Landschulz et al 1988). Competitive analysis of NF-IL6 also showed that it binds to the same sequences as CCAAT/Enhancer binding protein (C/EBP), with the best fit consensus sequence T(T/G)NNGNAA(T/G) (Akira et al 1992).
Stimulation by LPS, IL-1, TNF and IL-6 was shown to increase NF-IL6 mRNA levels (Akira et al 1995). The NFkB site (-73 to -64) in the IL-6 promoter mediates IL-6 induction by IL-1, TNF and interferons (Shimizu et al 1990; Liberman and Baltimore 1990; Zhang et al 1990). Recently Kang et al (1996) using band shift and DNA footprinting assays showed Sp1 binds to the GC-rich region with the sequence CCACC in murine IL-6. The human IL-6 promoter contains two copies of this sequence. These data suggest that NFkB, NF-IL6 and Sp1 play an essential role in the induction of the IL-6 gene transcription by cytokines.

**1.6.2 Repression of IL-6 gene expression**

**1.6.2.1 Corticosteroids**

Glucocorticoids and 17-β Estradiol strongly inhibit IL-6 expression in many cell types (Tabibzadeh et al 1989; Pottratz et al 1994; Ray et al 1997; Sukovich et al 1998). Ray et al (1990) demonstrated that glucocorticoid receptor (GR)-mediated signal transduction was footprinted across the entire MRE region, the major TATA box and the major RNA transcription start site. Using the IL-6 promoter construct (region -225 to +1) linked to CAT reporter gene assay, gene transcription was repressed by dexamethasone in HeLa cells irrespective of the inducer used (Ray et al 1990). 17-β Estradiol interaction via the estrogens receptor also resulted in impaired IL-6 gene expression by blocking nuclear protein binding at the NFκB and NF-IL6 sites (Stein and Yang 1995; Galien and Garcia 1997).

**1.6.2.2 Regulation of IL-6 by hormones**

Elevation of plasma IL-6 levels during the process of inflammation and physiological stress can lead to the upregulation of plasma adrenocorticotropic hormone (ACTH) release from the anterior pituitary gland and subsequently results in elevated circulating cortisol levels (Jablons et al 1989; Fong et al 1989). Elevated levels of IL-6 and glucocorticoids (e.g. cortisol) during the acute phase response lead to induction of hepatocyte secretions of fibrinogen, complement factors, haptoglobin, hemopexin and C-
reactive protein (Sehgal et al 1989). Cortisol also helps prevent inflammation by stabilisation of lysosomal membranes, decreased migration of white blood cells into the inflamed area, phagocytosis of damaged cells, as well as suppression of the immune system and lowering the release of IL-1 from white blood cells (Guyton 1991). The downregulation of IL-6 by 17-β Estradiol in estrogen sensitive tissues such as stromal cells probably represents an additional negative feedback loop affecting circulating IL-6 in women (Tabibzadeh et al 1989).

1.6.2.2 IL-4 and IL-10

The proinflammatory cytokines, interleukin-4 (IL-4) and interleukin-10 (IL-10) produced by B cells, mast cells, monocytes, T cells and basophils (Rennick et al 1989; Paul 1991; Vellenga et al 1993) have been shown to inhibit the expression of IL-1β, IL-6, interleukin-8 (IL-8) and TNFα expression in monocytes (de-Waal-Malefyt et al 1991; Fiorentino et al 1991, Oswalds et al 1992; Wang et al 1994; Donnelly et al 1991; te Velde et al 1990; Standiford et al 1990). Both IL-4 and IL-10 inhibit LPS-induced IL-6 expression by inhibiting the IL-6 transcription rate. In the case of IL-4, this is accompanied by a reduction of AP-1 and NF-IL6 binding activity, whereas IL-10 only inhibited AP-1 binding activity (Dokter et al 1996). Therefore, IL-4 and IL-10 may play an important role in the inhibition of inflammation by monocytes.

1.6.2.3 Fibrates

Fibrates are lipid lowering drugs and ligands for peroxisome proliferator activator receptor α (PPARα) induction and have been implicated in the inhibition of IL-1 induced production of IL-6 via repression of NFκB signalling (Staels et al 1998). This has been demonstrated in studies of hyperlipidaemic patients treated with either fenofibrate or bezafibrate (Koenig et al 1994; Staels et al 1998), resulting in significantly lower levels of LDLc, but also lipoprotein (a) (Lp(a)), fibrinogen, CRP and IL-6. These data demonstrate a potential therapeutic value of fibrates in the activation of PPARα and the inhibition of AP protein during inflammation.
1.6.2.4 Statins

Inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, (or statins), are extensively used in medical practice as cholesterol-lowering drugs. Large clinical trials have demonstrated that statins greatly reduce cardiovascular related morbidity and mortality in patients with and without coronary disease (Shepherd et al 1995; Kobashigawa et al 1995; Herbert et al 1997; Pedersen 1999; Maron et al 2000; Vaughan et al 2000; Dotani et al 2000). Recent, in vitro findings indicate that statins, in addition to their cholesterol lowering effects, also have anti-inflammatory properties by lowering IL-1β and IL-6 mRNA expression and protein levels. They also induce the expression of PPARα from human umbilical vein endothelial cells (HUVEC) and hepatocytes (Vaughan et al 1996; Inoue et al 2000; Brull et al 2001b). This suggests a potential role for statins in both lipid lowering and anti-inflammatory treatment.

1.6.3 Animal model of IL-6 deficiency

To elucidate the unique function that IL-6 plays in vivo, Kopf et al 1994 constructed IL-6 deficient mice by homologous recombination. These IL-6 knockout mice developed normally, but failed to control infection by Vaccinia virus or Listeria monocytogenes efficiently. The inflammatory acute phase response following tissue damage or infection was severely compromised. These mice also demonstrate impaired liver regeneration characterised by liver necrosis and failure (Cressman et al 1996). This suggested that IL-6 is crucial in the activation of repair processes during tissue damage and the elimination of infective organisms during inflammation.

1.6.4 IL-6 concentrations in human

Circulating IL-6 can be found in the blood of normal individuals in the 1pg/ml range (D'Auria et al 1997; Yamamura et al 1998), with slight elevations during the menstrual cycle (Angstwurm et al 1997), modest elevations in certain cancers (melanoma)
(10pg/ml) (Mouawad et al 1996) and large elevations after surgery (30-430pg/ml) (Sakamoto et al 1994; Brull et al 2001a).

1.7 IL-6 and its implication in coronary heart disease (CHD)

There is strong evidence supporting the central role of IL-6 in the inflammatory response (Papanicolaou et al 1998; Woods et al 2000). Figure 1.9 demonstrate some of the pathways which links IL-6 in the inflammatory response that lead to the development of CHD. Several studies have found a direct linked between IL-6 levels and cardiovascular disease (Biasucci et al 1996; Harris et al 1999; Ridker et al 2000b) (figure 1.10). IL-6 has been implicated in the pathogenesis of CHD in patients with MI and unstable angina (Berk et al 1990; Miyao et al 1993; Liuzzo et al 1994; 1998). Elevated IL-6 levels appears to be predictive of future heart disease (Harris et al 1999), and are common in patients with unstable angina compared with those with angina, particularly in smokers (Bataille and Klein 1992; de Maat et al 1996; Biasucci et al 1996). IL-6 levels also showed strong correlation with levels for fibrinogen and CRP (de Maat et al 1996; Bataille and Klein 1992). Experimental studies indicate that vascular endothelial and smooth muscles cells from normal and aneurysms arteries produce IL-6 (Loppnow and Libby 1989a; 1989b; Szekanecz et al 1994), that IL-6 gene transcripts are expressed in human atherosclerotic lesions (Seino et al 1994; Rus et al 1996), and that IL-6 may have procoagulant effects (Mestries et al 1994; Van der Poll et al 1994; Stouthard et al 1996). Evidence from Ridker et al 2000b showed that median IL-6 concentrations were higher among men who subsequently had an MI compared to healthy men matched for age and smoking status. This study also demonstrates that the risk of future MI increased with increasing quartile of baseline IL-6 concentration such that men in the highest quartile had a relative risk 2.3 times higher than those in the lowest quartile with a 38% increased in risk for every quartile rise in IL-6 levels (table 1.7).
Figure 1.9 Central roles of IL-6 and its effect on the development of CHD (adapted from Wood et al 2000).

PSYCHOSOCIAL STRESS

MONOCYTES/MACROPHAGES
In inflammatory state
(smokers lung, atheroma plaque)

ADIPOSE TISSUE

INTERLEUKIN-6

LIVER
Acute phase response
↑Fibrinogen
↓HDL

PLATELETS
↑Aggregability

ENDOTHELium
↑Adhesion molecules

CHD
Figure 1.10 Median IL-6 levels in healthy men, MI survivors and unstable angina patients (adapted from Ridker et al 2000b; Biasucci et al 1996). IL-6 levels are from different studies and are only used here as illustration to show differences in MI and UA patients compared to normal healthy individuals.

Table 1.7 Adjusted relative risks of future myocardial infarction among apparently healthy men according to baseline level of IL-6 from the Physician Health Studies (Ridker et al 2000b).

<table>
<thead>
<tr>
<th>Quartile of IL-6 (range, pg/ml)</th>
<th>1 (&lt;1.04)</th>
<th>2 (1.04-1.46)</th>
<th>3 (1.47-2.28)</th>
<th>4 (&gt;2.28)</th>
<th>P for Trend</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total cohort</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RR</td>
<td>1.0</td>
<td>1.9</td>
<td>3.5</td>
<td>2.3</td>
<td>0.01</td>
</tr>
<tr>
<td>95% CI</td>
<td>-</td>
<td>0.9-3.9</td>
<td>1.8-6.9</td>
<td>1.1-4.6</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>-</td>
<td>0.08</td>
<td>0.001</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td><strong>Nonsmokers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RR</td>
<td>1.0</td>
<td>2.1</td>
<td>3.1</td>
<td>2.7</td>
<td>0.009</td>
</tr>
<tr>
<td>95% CI</td>
<td>-</td>
<td>1.0-4.5</td>
<td>1.6-6.7</td>
<td>1.1-5.7</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>-</td>
<td>0.06</td>
<td>0.002</td>
<td>0.02</td>
<td></td>
</tr>
</tbody>
</table>

RR indicates relative risk. P=p-values for RR compared to 1st quartile
Adjusted for total HDL cholesterol, BMI, diastolic blood pressure, diabetes, family history of premature coronary artery disease, alcohol use and exercise frequency
1.7.1 Impaired coagulation and fibrinolysis

Inflammation at the site of vessel wall injury, triggered by a combination of genetic and environmental risk factors can lead to low grade acute phase response and progressively shift the balance of haemostasis towards a prothrombotic state. This, coupled with plaque growth and fissuring, eventually triggers thrombosis and further amplifies the inflammatory response (Harrison et al 1997).

IL-6 has been well characterized as an inducer of acute phase proteins, synthesized from the liver, which are prothrombotic and promote increase blood viscosity (e.g. fibrinogen) (Lowe and Rumley 1999; Woodward et al 1999). Many studies have demonstrated a positive correlation between plasma fibrinogen levels and those of IL-6, thereby altering the haemostatic balance towards that of a prothrombotic state (Mendall et al 1997; Erren et al 1999; Koukkunen et al 2001). Elevated levels of fibrinogen have been shown to substantially influence the risk of venous and arterial thrombosis as well as cardiovascular disease risk in the general population (Kannel et al 1987; 1997; Koenig and Ernest 1993; Meade et al 1986; Thompson et al 1995; Koster et al 1994; Heinrich et al 1994; Folsom et al 1997; Woodward et al 1998; Eriksson et al 1999). Raised fibrinogen levels may also contribute towards chronic atherosclerotic plaque growth and risk of thrombosis by increasing plasma viscosity, red blood cell and platelet aggregation. Fibrinogen may be directly incorporated into the injured endothelium where it localises with LDL and promotes regional fibrin deposition (Ernst et al 1988a; 1988b Koenig and Ernest 1993; Smith and Thompson 1994; Woodward et al 1999). Coupled with increases in the levels for tissue factor (TF), plasminogen activator inhibitor 1 (PAI-1), thrombin generation, and antithrombin productions via activated protein C (APC), fibrin clot formation is favoured and the risk of thrombosis and MI increased (Neumann et al 1997; Samad et al 1994; Levi et al 1998; Kowal et al 1998; Hooper et al 1998).

1.7.2 CRP

The synthesis of CRP by the liver is largely regulated by IL-6 (Heinrich et al 1990). Several studies have shown that elevated plasma levels of CRP are associated with the
risk of ischaemic heart disease (IHD) and severity of atherosclerosis (de Maat et al. 1996; Berk et al. 1990; Woodhouse et al. 1994; Biasucci et al. 1996). Elevated CRP and IL-6 levels have been reported in patients with unstable angina (Biasucci et al. 1996) and predict risks of future cardiovascular disease in men and women (Ridker et al. 2000a; 2000b). It has also been shown that CRP levels are associated with CHD in apparently healthy subjects, both in a cross sectional study in general practice (Mendall et al. 1996), and longitudinally in the US Physicians Health Study (Ridker et al. 1997), the MONICA-Augsburg Cohort Study (Koenig et al. 1997) and the MRFIT Study (Kuller et al. 1996), where CRP levels predicted cardiovascular events or CHD mortality during a follow up of between 2 and 17 years. The precise role of CRP in cardiovascular disease is still debatable, but it is believed that CRP maybe more than just a marker of the acute phase response. Evidence suggests that CRP facilitates the uptake of lipids by macrophages accumulating in the atherosclerotic lesion (Hatanaka et al. 1995).

1.7.3 Connective tissue remodelling

Extracellular tissue remodelling is regulated by key matrix metalloproteinases (e.g. collagenases, elastases and stromelysin-1) and their inhibitors, ‘tissue inhibitor of metalloproteinases’ (e.g. TIMP-1) during tissue injury (Woessener 1991). The upregulation of TIMP-1 synthesis by IL-6 may shift the balance in favour of matrix proteins deposition leading to atherothrombosis and the progression of CHD (Kordula et al. 1992; Roeb et al. 1994; Ye et al. 1995; Humphries et al. 1998; de Maat et al. 1999). Evidence from immunohistochemistry showed IL-6 mRNA expression was localised to the human arterial atherosclerotic wall as cellular and extracellular deposits in the connective tissue matrix, with the fibrous plaque having significant higher level of IL-6 than the intima and media (Rus et al. 1996).

Data from studies of apolipoprotein E (ApoE) knockout mice model, which develop atherosclerotic plaques in the aorta also showed elevated levels of IL-6 mRNA predominate mainly in the plaque area compared to normal mice. The treatment of these
ApoE knockout mice with estrogens (e.g. 17-β-estradiol) demonstrate an anti-atherosclerotic effect where regression of plaque sizes was seen (Sukovich et al 1998).

1.7.4 Dyslipidaemia

Further evidence for the role of IL-6 in coronary heart disease came from studies showing secretion of IL-6 by subcutaneous adipose tissues (Mohamed-Ali et al 1997; Fried et al 1998) and IL-6 suppression of macrophage lipoprotein lipase secretion from the murine J774.2 cell line (Tenghu-Muhammad et al 1998). IL-6 has also been shown to reduce lipoprotein lipase (LPL) activity in adipose tissues, leading to reduced triglyceride uptake (Greenberg et al 1992). At present, little is known about the effects of IL-6 on adipose tissue. One possibility is that the downregulation of adipose tissue LPL results in increased hepatic triglyceride secretion (Nonogaki et al 1995) and may contribute to hypertriglyceridaemia. Evidence from Tanaka et al 1997 showed that C/EBPβ (formerly known as NF-IL6) is required for adipocyte differentiation in mice. C/EBPβ -/- mice did not accumulate lipid droplets. One can postulate that elevated levels of IL-6 facilitate the uptake of lipids by macrophages and IL-6 accumulation in the atherosclerotic lesions may be mediated through C/EBPβ, resulting in atherogenesis and greater CHD risk. The regulation and mechanisms underlying this relationship are poorly understood. It is however still not known if elevated IL-6 levels are a response to disease and/or promote disease progression.

1.8 Atherosclerosis-complex multifactorial disease

Cardiovascular disease such as atherosclerosis is a complex multifactorial disease (complex traits) because it does not follow simple Mendelian monogenic inheritance attributed to a single gene locus (Lander and Schork 1994). For example, the pathological processes that lead to progression of atherosclerosis involve multiple proteins which contribute unequally to the disease phenotype as well as modification from environmental factors. The identification of susceptibility loci that can contribute to atherosclerosis is of crucial importance in understanding the mechanism/etiology of the disease and
prevention (Humphries 1994). The current methods for genetic dissections of complex traits in humans are linkage-type and association studies (Lander and Schork 1994). Linkage studies test whether inheritance for a particular disease is correlated within families and have high power to identify ‘major gene effects’ and low power to detect genes with ‘modest effects’. However, association studies focus on population frequencies and are used in studies of unrelated subjects with high power to detect genes with ‘modest effect’. Thus for CHD risk and especially for CHD risk traits, a design not confounded by age, gender and particularly environmental factors is required. Association studies are therefore more appropriate than linkage studies for studying the effects of IL-6 polymorphisms on CHD risk and CHD risk traits because they have power to detect modest effects attributed by the IL-6 gene. The samples used in association studies should generally be as genetically homogeneous as possible (e.g. not to include subjects with different ethnic origins) and ideally should have both male and female individuals. A follow up prospective study with baseline sampling such as the Northwick Park Heart study II (NPHSII) would be ideal as it avoids the potential confounder of the presence of disease influencing levels of CHD risk traits. The main advantages of using the NPHSII study are that it consists of ~3000 healthy middle-age white caucasians men from the UK, followed for 6 years for CHD events. Lipids and coagulation risk traits implicated in CHD risks (e.g. cholesterol, triglyceride and fibrinogen) was made annually from these subjects to identify association between genetic and progression of disease. Therefore, this study would have power to detect modest effects on CHD risks attributed to polymorphisms in the IL-6 gene.

The HIFMECH study (Hypercoagulability and Impaired Fibrinolytic function: Genetics and environmental MECHanisms predisposing to myocardial infarction) was designed to look at CHD risks in the North and South of Europe. The advantage of this study is that it is a case-control of MI survivors and healthy subjects. Therefore, any association between CHD risk markers with candidate genes for CHD such as polymorphisms in the IL-6 gene can be identify in the different groups.
1.9 Aim of PhD

To investigate genetic variations in the human IL-6 gene and to examine the possibility that variations in IL-6 expression may predisposes to risk of coronary heart disease (CHD). To test this, I propose five specific aims:

**Aim 1:** To identify polymorphisms in the promoter region, 5'-UTR and the coding regions of the IL-6 gene using single stranded conformational polymorphism (SSCP) and DNA sequencing.

**Aim 2:** To develop methods for large scale genotyping of newly identified polymorphisms in population-based studies (i.e. NPHSII and HIFMECH).

**Aim 3:** To investigate the functional effects of IL-6 promoter polymorphisms using luciferase reporter gene assay in HepG2 and HuH7 cell lines. A time/dose response to dexamethasone and IL-1β plus dexamethasone stimulations will be carried out to look at gene expression of the different IL-6 promoter constructs.

**Aim 4:** To look for association between selected IL-6 genotypes and plasma concentrations of IL-6 and other inflammatory risk markers for CHD (e.g. fibrinogen and CRP) and possible interactions with environmental factors (e.g. smoking) in the NPHSII and HIFMECH studies.

**Aim 5:** To investigate possible mechanisms to explain the observations made with functional and association studies.
CHAPTER 2

IDENTIFICATION OF POLYMORPHISMS IN THE HUMAN IL-6 GENE BY SSCP AND DNA SEQUENCING
Chapter 2: Identification of polymorphisms in the human IL-6 gene by SSCP and DNA Sequencing

2.1 Introduction

Single stranded conformation polymorphism (SSCP) reported by Orita et al 1989 is widely used method for mutation detection because of its simplicity and versatility. In this method, the region of interest in the genome or cDNA is amplified by PCR, denatured to form single strands, and analyzed by non-denaturing polyacrylamide gel electrophoresis. Mutations are detected as differences of mobility of the DNA bands between control and test. Therefore strands which have either insertion/deletion, single or multiple base transitions will have unique secondary structures compared to the control and this can be seen as electrophoresis mobility shift patterns on non-denaturing polyacrylamide gel.

The enzymatic dideoxyribonucleotides (ddNTPs) chain terminator (Sanger et al 1977) methods using fluorescently ddNTPs (Perkin-Elmer, USA) can then use to identify nucleotide variations seen on SSCP.

The aim here was to identify polymorphisms in the coding regions and the promoter region of the human IL-6 gene by SSCP and DNA sequencing.

2.2 Materials

Taq DNA polymerase, 2'-deoxy-N-5'-nucleotide triphosphates (dATP, dCTP, dGTP and dTTP) and $\alpha^{32}$P-dCTP were obtained from Amersham Pharmacia Biotech (Amersham, UK), primers was from Gibco BRL (Life Technologies, UK). The ABI Prism dRhodamine Dye Terminator Cycle Sequencing Reaction kit and the Sequa gel XR sequencing gel was obtained from Applied Biosystems (Perkin-Elmer, Warrington, UK) and National Diagnostics (Hull, UK) respectively. All other chemicals and reagents were purchased from Merck Ltd (Dorset, UK). See appendix for additional information to the composition of solutions made or purchased.
2.3 Methods

2.3.1 DNA Extractions for Genetic analysis

DNA from whole blood for genetics analysis was extracted using the salting out method (Miller et al 1988) and a modification to the protocol from the Nucleon II kit (Scotlab, UK). All DNA samples were resuspended in TE buffer and stored at 4°C or -20°C (long term storage). This protocol is outlined as follows:

2.3.1.1 Cell lysis

5ml of frozen whole blood was thawed (at room temperature) and then transferred to a 30ml round bottom polypropylene tube (Sarstadt, UK). 20ml of cold (4°C) sucrose lysis buffer (10mmol/l Tris-HCl (pH 7.5), 5mmol/l MgCl₂, 1% (v/v) Triton X-100) was added to the centrifuge tube, inverted several times to mix and then left for 5 minutes on ice. Tube were centrifuged at 10,000rpm for 15 minutes at 4°C (Sorvall RC5C centrifuge using rotor SA-600) and the supernatant was then carefully discarded. The pellet was resuspended in 20ml of ice cold sucrose lysis buffer, spin down again at 10,000rpm for 15 minutes at 4°C and then supernatant removed. 2ml of nuclei lysis buffer (10mmol/l Tris-HCl (pH 8.2), 400mmol/l NaCl, 2mmol/l EDTA and 1% SDS) were added, briefly vortex and then allow to stand at room temperature for 10 minutes to lysed the nuclear membrane.

2.3.1.2 Deproteinisation

1ml of 5M Sodium perchlorate was added to the centrifuge tube mixed several times by inversion and then incubate at room temperature for 10 minutes. 2ml of cold (stored at -20°C) chloroform was added and then inverted several times to mix. The tube was then centrifuged at 3,000rpm for 3 minutes at room temperature and the upper aqueous layer transferred into a fresh 15ml polypropylene tube with a sterile Pasteur pipette.
2.3.1.3 DNA precipitation and storage

10ml of cold (stored at -20°C) 100% ethanol was added to the side while holding the tube at 45° angle and the tube inverted several times to precipitate the genomic DNA. The DNA precipitation occurs at the interface between the ethanol and the aqueous layers as a white woolly strands. The DNA was spooled with a sterile Pasteur pipette, washed in 70% ethanol, transferred into a sterile microtube containing 1ml TE buffer (10mmol/l Tris-HCl and 1mmol/l EDTA) and stored at 4°C or -20°C.

2.3.2 Polymerase Chain Reaction (PCR)

PCR amplifications was carried out using 50-100ng of DNA template, 10pmol each primer and 0.35 unit of Taq DNA polymerase in a final buffer of 1X Polmix: 50mM KCl, 10mM Tris-HCl (pH 8.3), 0.001% Gelatin, 0.2mM each 2'-deoxy-N-5'-nucleotide triphosphates (dNTPs) and 1.5mM MgCl2. The PCR reaction consist of initial denaturing temperature of 94°C for 4 minutes followed by 35 cycles of 95°C for 40 seconds, 55°C for 40 seconds and 72°C for 1½ minutes.

2.3.3 Single stranded conformational polymorphism (SSCP)

PCR for SSCP was carried out according to the method described by Orita et al (Orita et al 1989) with some modifications. 1/10th α32P-dCTP-labeled to cold dCTP were used to generate the radiolabelled α32P PCR product. 2µl of PCR product was added to 6µl of a 7:5 ratio of formamide dye mix (95% deionized formamide, 10mM EDTA, 0.02% (w/v) each of bromophenol blue and xylene cyanole and 0.1% (w/v) SDS). The PCR product was denatured at 95°C for 5 minutes and then placed on ice. 4µl of sample was loaded on a 7.5% nondenaturing polyacrylamide gel (49:1 acrylamide-bisacrylamide ratio) with 5% (v/v) glycerol, 1X TBE and 10mM EDTA (gel size 33 x 39cm) (Gudnason et al 1993). Electrophoresis was carried out at 300V in 1X TBE solution for 23hrs at 8°C. The SSCP gel was dried, exposed onto BioMax X-ray film (Eastman Kodiak, NY, USA) and then the results interpreted.
2.3.3.1 Screening for polymorphisms in the IL-6 gene coding regions

SSCP analysis described in section 2.3.3 was carried out on 94 randomly selected individuals from the third north Glasgow MONICA study (MONICA-3) using oligonucleotide primers in the introns to give coverage of the entire coding exons plus 15-20 bases of the intron-exon junctions from published human IL-6 DNA sequences (Yasukawa et al 1987) (figure 2.1 and table 1 in the appendix for PCR primers). The individuals selected for SSCP screening here are a mixtures of males and females that were classified as healthy subjects (at time of recruitment) and those with coronary heart disease.

![Figure 2.1 Schematic representations of the human Interleukin-6 (IL-6) gene coding regions. Arrows represent SSCP primers coverage of the all exons including the intron-exon boundaries of all the exons and the size of PCR products generated by each primer pairs.]

2.3.3.2 Screening for polymorphisms in the IL-6 promoter region

At the start of this project, no SSCP work had yet been carried out further upstream than the −597G>A polymorphism (Terry et al 2000 and Dr Gary Faulds, personal communication). Therefore, three sets of primers were designed for SSCP of the IL-6 promoter region from −1182 to −472 (Ray et al 1988) (figure 2.2 and table 2.2 in appendix for primer sequences) in same the 94 individuals selected randomly from the MONICA-3 study as described in 2.3.3.1.
Figure 2.2 Schematic representation of the IL-6 promoter region. The three known polymorphisms are shown (-597G>A, AnTn tract and -174G>C) and the three overlapping SSCP primers use for identifying SSCP variants.

2.3.4 Purification of PCR product for DNA sequencing

The PCR protocol mention in section 2.4 was used for generating DNA templates from samples with different SSCP variants prior to DNA sequencing. The QIAquick PCR Purification Kit protocol (Qiagen, UK) was then used to purify the double stranded PCR product from primers, nucleotide, polymerase and salts using a QIAquick spin column. The DNA was then eluted from the spin column with 30μl sterile water.

2.3.5 DNA Sequencing by Fluorescence Dye Terminator Technology

2.3.5.1 ABI Prism dRhodamine Dye Terminator Sequencing reaction

The dRhodamine dye terminator cycle sequencing chemistry kit (Perkin-Elmer, Warrington, UK) was used to perform all sequencing reactions, but with modifications. To a 0.2ml tube, 150ng PCR product, 4μl terminator ready reaction premix and 3.2pmol primer was added and the final volume was made up to 10μl with sterile dH2O. The mix was then overlaid with 20μl of mineral oil and the cycle sequencing carried out on the GeneAmp 9600 DNA thermal cycler (Perkin-Elmer, USA) with 25 cycles at 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes.
2.3.5.2 Purification of cycle sequencing products

The sequencing products were diluted with 10μl sterile dH₂O and then transferred to a fresh 0.5ml microcentrifuge tube containing 2μl 3M Sodium acetate (pH 4.6) and 50μl 95% ethanol. The contents were vortex briefly and then incubated at 4°C for 30 minutes and then centrifuge in a microcentrifuge at 12,000 rpm for 30 minutes at 20°C. The ethanol solution was then carefully removed with a Gilson micropipetter (Anachem, UK) and the pellet washed with 250μl 70% ethanol. Following 30 minutes spin (12,000 rpm) and aspiration of the 70% ethanol, the pellet was dried in a vacuum centrifuge for 10 minutes and stored at -20°C until gel loading.

2.3.5.3 Preparation of sequencing gel and loading of samples

The pellet was resuspended in 3μl of formamide loading buffer (25mM EDTA, pH 8.0 containing 50mg/ml Blue dextran in a ratio of 5:1 (v/v) deionized formamide to EDTA/Blue dextran), denatured at 95°C for 5 minutes and immediately placed on ice. 1.5μl of the denatured product was loaded onto a 36cm ABI denaturing gel (5% Sequal gel XR containing 1X TBE and 6M Urea). Electrophoresis was carried out for 7 hours on the ABI Prism 377 DNA Sequencer in accordance to the manufacturer instructions (Perkin-Elmer, Warrington, UK).

2.3.5.4 Sequencing analysis

All sequencing data collected was analyzed using the Sequencing Analysis 2.0 package (Perkin-Elmer, Warrington, UK).
2.4 Results

2.4.1 IL-6 coding exons

Screening of the human IL-6 coding exons for genetics variations using SSCP revealed a single SSCP variant spanning exon 1 region in 1 out of 94 individuals screened from the MONICA-3 study (figure 2.3(a), lane 2). DNA Sequencing of this variant in the forward and reverse directions with the primers: IL-6 Pro(7)F and IL-6(2)R (see table 2.1 in appendix for primer sequence) on several occasions was unsuccessful in identifying this variant and therefore are likely to be an SSCP artefact. Due to its very uncommon occurrence, it was not pursued further.

Three different SSCP variants were found in exon-5. An example of this is shown in figure 2.3(b) (see lanes 3, 6 and 10). In the 94 individuals screened, two samples had the SSCP pattern shown in lane 3, three individuals had the variant in lane 6 and four individuals had the lane 10 SSCP pattern. DNA sequencing of samples with SSCP variants in lanes 3 and 6 showed no sequence differences to those in lanes 1, 2 and 5 from the published sequence (Yasukawa et al 1987). These patterns were not seen in several repeated SSCP experiments and therefore were likely to be SSCP artifacts. However, the variants with the SSCP pattern shown in lane 10 have been successfully sequenced (figure 2.3(c)) using the forward primer (IL-6(9)F)) and then confirmed using the reversed primer (IL-6(10)R) (see table 1 in appendix for primer). At position 101 (figure 2.3(c)), a C>T base substitution resulted in a wobble position for codon F201 (TTT→TTC) in exon 5. Since, this did not alter the amino acid phenylalanine, it was unlikely to be of functional significance, and therefore was not studied further. No SSCP variants were found for exons 2, 3 and 4 (result not shown).
Figure 2.3(a) SSCP of IL-6 exon-1 gene. Mobility shift pattern for single stranded and double stranded DNA (ssDNA and dsDNA) are shown. Lane 2 contain an additional band.
Figure 2.3(b) SSCP of IL-6 exon-5 gene. Mobility shift pattern for single stranded and double stranded DNA (ssDNA and dsDNA) are shown.

Figure 2.3(c) DNA Sequencing of Exon-5 SSCP variant (lane 10). The base transition is shown in circle and represented by N.
2.4.2 IL-6 promoter region

Genetic screening for variants in the human IL-6 promoter regions using SSCP revealed no SSCP variation from -1182 to -910 and from -973 to -674 from the start site of transcription. Five different SSCP patterns were however seen from regions -766 to -472 (figure 2.4). All of these SSCP variants were very common, with the exception of those in lanes 9 and 22. DNA sequencing with primers IL-6(3)F and IL-6 Pro(6)R (see table 1 in appendix for primers) was only successful with the antisense strand and revealed a unique -572G>C polymorphism (figure 2.5(a) and (b)) and the common -597G>A polymorphism (figure 2.6(a) and (b)) (Dr Gary Faulds, personal communication and Terry et al 2000) plus the unreported -627C>A base transition variant (figure 2.7(a) and (b)). The -627C>A variant was found in one healthy women who was heterozygous for this genotype out of the 94 samples screen from the MONICA-3 study. This variant were extremely rare and uncommon, therefore it would be highly unlikely to have any effect on a common disease such as CHD.
Figure 2.4 SSCP of the human IL-6 promoter region –766 to –472. ssDNA and dsDNA variants are shown by the arrows. Lanes 14 to 16 are negative control and lanes 1, 2, 9, 12 and 22 are the 5 different SSCP variations.
Figure 2.5 DNA sequencing of IL-6 -572G>C promoter polymorphism. The sequences shown below are from the antisense strand with the -572 nucleotide position highlighted by a circle. (a) is the wild type G allele, denoted as C, and (b) is the heterozygous GC allele which is denoted as N.
Figure 2.6 DNA sequencing of the IL-6 -597G>A promoter polymorphism. The sequences shown below are from the antisense strand with the -597 nucleotide position highlighted by a circle. (a) is the wild type G allele, denoted as C, and (b) is the heterozygous GA allele which is denoted as N.
Figure 2.7 DNA sequencing of the IL-6 -627C>A promoter polymorphism. The sequences shown below are from the antisense strand with the -627 nucleotide position highlighted by a circle. (a) is the wild type C allele, denoted as G, and (b) is the heterozygous C/A allele which is denoted as N.
2.5 Discussion

2.5.1 SSCP sensitivity

The polymerase chain reaction (PCR) followed by single stranded conformational polymorphisms (SSCP) is widely use for the detection of mutation based on conformation differences of single stranded DNA (ssDNA) to migrates through polyacrylamide gel by electrophoresis. SSCP was chosen because it was considered the cheapest and most convenient method for large scale screening of samples in our laboratory for potential single nucleotide polymorphisms (SNPs) at the time of this project before the availability of high throughput capillaries DNA sequencing machines. The sensitivity of SSCP analysis to detect genetic variations (if any) in a single run is generally believed to be about 80% provided that the fragment are shorter than 300bp (Glavac and Dean 1993; Hayashi and Yandell 1993), and is also dependent on both the target sequences and experimental conditions (Liu and Sommer 1994; Humphries et al 1997; Jaeckel et al 1998; Nataraj et al 1999; Mitterski et al 2000). The disadvantages of SSCP is the sensitivity decreases with increasing fragment length (Sheffield et al 1993) and reproducibility can sometime be a problem (Hennessay et al 1998). A recent report has shown that the mobility shift of ssDNA was greatly improved by running the electrophoresis in low pH buffer such as Tris-MES-EDTA (TME) (Kukita et al 1997). However, in our hand, it was found that SSCP bands were clear and sharp when electrophoresis were carried out in TBE buffer, but was less well resolved in TME buffer (see figure 2.8).

SSCP cannot directly identify the exact position of change in the DNA sequence, therefore DNA sequencing is required in order to detect the different variations seen by SSCP.

Methods that can also be use for mutation detection includes denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), heteroduplex analysis and direct sequencing (Myers et al 1987; Cariello and Skopek 1993; Riesner et at
al 1992; Henco et al 1994; Gayther et al 1996; Prior et al 1994; Abernathy et al 1997; Sanger et al 1977). These methods were more expensive, inconvenient and were deemed not practical for high throughput screening of population based samples compared to SSCP.
Figure 2.8 SSCP of the human IL-6 promoter region -766 to -472 with (a) TBE buffer and (b) TME buffer. Samples in (a) are identical to those shown in (b). ssDNA and dsDNA variants are shown by the arrows.

(a) 1X TBE buffer

(b) 1X TME buffer
2.5.2 Genetic variations in IL-6

Genomic DNA from 94 individuals was chosen from the third north Glasgow MONICA (MONICA-3) study to screen for possible common variation in the human IL-6 promoter and coding regions by SSCP. These subjects include men and women aged between 24-74 years with and without the prevalence of CHD, and were randomly recruited from general GP practices in the north Glasgow region of the UK. The main reason for choosing 94 individuals instead of the normal 30-40 samples is because novel variants sometime are found more commonly in men than women or vice versa and may or may not be dependent on disease status. By picking these individuals, it gives greater chances of detecting common IL-6 variations. Using SSCP and DNA sequencing, two novel SNPs were detected, the base substitution located at position -627C>A, and the C>T in exon 5 which resulted in a wobble position for the amino acid phenylalanine plus the reported -597G>A and the -572G>C variants (Terry et al 2000; Humphries et al 2001). Due to the rare occurrence of both the -627C>A, and the exon-5 C>T variants, they were not pursued further. SSCP analysis of samples from ethnic groups such as those from Blacks and Chinese (not available at time of project) would also increase the detection of unique variation that may only be found in these groups. An example of this is the C>G substitution at nucleotide -634 in the IL-6 promoter (frequency of G allele=0.184), which is only found in the Japanese population (Ota et al 2001), but have not been reported in other ethnic groups.

Screening for genetic variations of the IL-6 promoter was not carried out beyond the DNA sequences published by Yasukawa et al 1987. This is because previous in vitro functional experiments have demonstrates the biological importance of this promoter were within 1.2kb upstream from the site of transcriptional start site, in particular, the -225bp region was sufficient for conferring responsiveness of the IL-6 promoter to cytokines such as IL-1, activator of protein kinase C and protein kinase A. (Ray et al 1990; 1994; Isshiki et al 1990). The IL-6 intronic regions were not screen for genetics variations in this study because none have been reported to date and also no evidence to suggest the introns have any biological significant in regulating IL-6 gene function. The IL-6 3'-untranslated region were also not screen because many groups have done so, and
only a polymorphic AT rich minisatellites repeat have been reported (Bowcock et al 1989) so far to date. The evidence to support the biological significant of this AT rich region is extremely poor. Only one report so far have found association between the AT rich minisatellites repeat with bone mineral density (Murray et al 1997). No other studies have reported this association (Huang et al 1999; Takacs et al 2000; Schmidt et al 2000; Pignatti et 2001.

2.5.3 Further aims

Interleukin-6 concentration has been implicated in the inflammatory process because elevated levels are found in patients with CHD and unstable angina, and also correlate with levels for C-reactive protein, fibrinogen and PAI-1 (Berk et al 1990, Liuzzo et al 1994, de Maat et al 1996, Biasucci et al 1996, Ridker et al 2000a; 2000b). Therefore, the mechanism of expression for IL-6 at the transcriptional level will be crucial in determining the final IL-6 protein concentration. Genetic variations such as single nucleotide polymorphisms (SNPs) identified here could be important in determining IL-6 expression at the transcriptional level. Therefore, the next stage would be to develop high throughput methods for screening these common IL-6 promoter polymorphisms (i.e. the -597C>A, -572G>C and the -174G>C) in population-based studies and to look for association with risk markers that predisposes to CHD.
CHAPTER 3

CLONING, SITE-DIRECTED MUTAGENESIS AND GENETIC REPORTER GENE ASSAY
3.1 Aims

In vitro functional studies were carried out in HepG2 cells to investigate and test the functional effects of the IL-6 -572G versus -572C and -597G/-174G versus -597A/-174C promoter variants on IL-6 gene expression levels (and hence predisposition to greater risk from CHD). The A<sub>n</sub>T<sub>n</sub> tract at positions -395 to -375 (A<sub>9</sub>T<sub>11</sub>) and the -627C>A (e.g. C variant) were kept constant for all variants so as to assessed only the function of these IL-6 variants. The aims therefore were to:

1. Create the different IL-6 promoter variants by site-directed mutagenesis, ligation and cloning (see table 3.1).
2. Establish conditions for optimal transient transfection of plasmid DNA into HepG2 cells.
3. Establish the optimum dose of IL-1β for experiment.
4. Examine if serum contains inhibitors.
5. Establish the optimum dose for repression by dexamethasone.

3.2 Materials

The pGEM-B672A plasmid (Yasukawa et al 1987) containing the cloned IL-6 promoter BamHI site at -1180 to the BamHI site in intron 3 (figure 3.1) was obtained from Dr Shizuno Akira via Professor Pat Woo. All restriction endonucleases were purchased from New England Biolabs UK Ltd (Hertfordshire, UK). The pBluescript SK(+-) phagemid, Pfu Turbo DNA polymerase, E.coli XL-1 blue subcloning or supercompetent cells and Quikchange 1-day site-directed mutagenesis protocol were obtained from Stratagene (La Jolla, USA). T4 DNA ligase and all reagents or solutions used in cell culture were purchased from Life Technologies Ltd (Paisley, UK). Dual
luciferase assay kit, pGL3 Basic vector, pSV-β-Galactosidase control vector plasmid and pRLTK vector was purchased from Promega (Southampton, UK). Interleukin-1β was obtained from R&D Systems (Oxon, UK). QIAquick gel extraction, QIAprep Spin Miniprep and Endotoxin free maxi plasmid prep kits were from QIAGEN Ltd (West Sussex, UK). All other reagents and solutions were either purchased from Merck Ltd (Dorset, UK) or from Sigma-Aldrich Ltd (Dorset, UK). See also appendix for compositions to reagents.

3.3 Methods

3.3.1 Cloning of the IL-6 promoter into pGL3 Basic gene expression vector

3.3.1.1 Restriction digestion and Gel extraction of IL-6 promoter

The full-length human IL-6 promoter sequences from -1180 to +13 with -627G/-597G/-572G/A9T11/-174G was obtained from the pGEM-B672A plasmid containing the IL-6 insert (figure 3.1). This was digested at 37°C for 3 hours with 5 units of BamHI restriction endonuclease in 1X BamHI buffer supplied and supplemented with 100μg/ml BSA. The digested plasmid was separated by electrophoresis (100volts for 1 hour) on a 1% low-melting agarose gel in 1X TAE buffer, excised from the gel under UV illumination, and then purified using the QIAquick gel extraction kit protocol. The final DNA was suspended in 30μl sterile water before further digestion with 5 units of Xhol restriction enzyme in accordance with manufacturer's instructions. A ~1.2kb IL-6 promoter DNA fragment was then excised following agarose gel electrophoresis and QIAquick gel extraction. The IL-6 fragments were eluted in 30μl sterile water for cloning into the firefly luciferase pGL3 Basic reporter gene vector.
Figure 3.1 The IL-6 insert (a) from -1180 to +2000 denoted as B672A cloned into (b) BamHI sites in the pGEM-3 vector (obtained from Dr Shizuno Akira from the published work by Yasukawa et al 1987).

(a) -1180 to +2000

BamHI

Exon 1

Exon 2

Exon 3

B672A

(b)
3.3.1.2 Ligation and transformation of IL-6 promoter into pGL3 Basic vector

The IL-6 promoter DNA fragment (-1180 to +13) obtained by BamHI and XhoI digestions of pGEM-B672A (figure 3.1(a)) lacks the restriction ends for direct ligation into the multiple cloning site (MCS) of the pGL3 basic vector. To overcome this, the IL-6 fragment was first cloned into the BamHI/XhoI site on the MCS pBluescript SK (+/-) phagemid to generate the noncomplementary BamHI/SacI restriction overhang ends for directional cloning into the MCS of pGL3 Basic vector (figures 3.2 and 3.3).

3.3.1.2.1 Ligation of IL-6 promoter into pBluescript SK (+/-) phagemid

The pBluescript SK phagemid vector was digested with BamHI and XhoI restriction endonucleases and then gel purified as described in section 3.3.1.1. For ligation, approximately 3:1 molar ratio of IL-6 promoter insert prepared in 3.3.1.1 to pBluescript SK vector DNA (i.e. 120ng IL-6 promoter insert DNA to 100ng pBluescript SK vector) in 1X ligase buffer was ligated with 8 units of T4 DNA ligase in a total volume of 20μl at 4°C overnight.

3.3.1.2.2 Transformation of pBluescript SK-hIL-6 promoter DNA

2μl ligation reaction (3.3.1.2.1) and 50μl Epicurian Coli XL-1 Blue subcloning-grade competent cells was mixed and incubated on ice for 20 minutes. The mixture was heat shocked at 42°C for 45 seconds and then incubated on ice for 2 minutes. 0.9ml SOC medium was added and then incubated at 37°C for 30 minutes with shaking at 225-250 rpm. 200μl transformation reaction was plated on LB plate containing 80μg/ml of fresh X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 20mM IPTG (isopropyl-1-thio-β-D-galacto-pyranoside). Several white colonies containing pBluescript SK phagemids with IL-6 promoter inserts was then selected for the mini plasmid preparation.
3.3.1.2.3 *p*Bluescript SK-IL-6 promoter DNA plasmid miniprep

5ml of LB medium containing 50μg/ml ampicillin was inoculated with a single white colony from section 3.3.1.2.2 and then incubated at 37°C with vigorous shaking (225 rpm) for 16 hours. The *p*Bluescript SK-IL-6 promoter phagemid was purified and resuspended in 30μl sterile water using the QIAprep Spin Miniprep Kit protocol. The insert was then verified by restriction mapping following BamHI and XhoI digestions.

### 3.3.1.2.4 IL-6 promoter in *p*GL3 Basic vector

To generate directional overhang ends for cloning the IL-6 promoter into *p*GL3 Basic vector multiple cloning sites, SacI and XhoI restriction digestion was performed on *p*GL3 basic vector and *p*Bluescript SK-IL-6 phagemid. Gel purification was performed as described in 3.3.1.1 to obtain the IL-6 insert and the linearised *p*GL3 Basic vector with their compatible ligation ends. The same ligation protocol (see section 3.3.1.2.1) was used to generate the *p*GL3 Basic IL-6 vector insert. Transformation was carried out as described in section 3.3.1.2.2 with selection by ampicillin resistance on LB plates. Several colonies were then picked for miniprep culture, followed by plasmid purification using the QIAprep Spin Miniprep kit. The final DNA plasmid was suspended in 30μl sterile water and 3μl was then use to generate a restriction map on a 1% agarose gel following SacI/XhoI digestions.
Figure 3.2 Subcloning of B672A (-1180 to +13) in pBluescript SK (+/-) phagemid vector. (a) IL-6 promoter DNA fragment (B672A) and (b) pBluescript SK were both cleaved by BamHI/XhoI, gel purified and then ligated together. Blue-white screening on LB plates with X-gal and IPTG used for selection of phagemid containing the IL-6 promoter insert.
Figure 3.3 Cloning of human IL-6 \(-627C/-597G/-572G/A\_Tn/-174G\) promoter construct into pGL3 Basic luciferase expression vector.
3.3.2 Site-directed Mutagenesis

The cloning of the human IL-6 promoter into pGL3 basic vector in section 3.3.1.2.4 produced the promoter variant -627C/-597G/-572G/A₉T₉/-174G (referred to in this thesis as either -572G or -597G/-174G). To investigate gene expression differences between the -572G versus -572C and -597G/-174G versus -597A/-174C variants (excluding the A₉T₉ tract), the variants: -627C/-597G/-572C/A₉T₉/-174G (referred to as -572C) and -627C/-597A/-572G/A₉T₉/-174C (referred to as -597A/-174C) were generated by site-directed mutagenesis (see table 3.1). All IL-6 promoter variants generated here drives the expression of firefly luciferase encoded by the pGL3 Basic expression vector.

3.3.2.1 Quikchange 1-day site-directed mutagenesis

Site directed mutagenesis to generate the IL-6 -572C variant was carried out using 125ng of each primer: IL-6 -572C Mut F and IL-6 -572C Mut R (see table 1 in appendix for primer sequences), 50ng of pGL3 Basic-IL-6 vector template, 1X reaction buffer, 0.25mM dNTP mix and 2.5units of Pfu turbo DNA polymerase in a 50µl reaction volume. The reaction was carried out using GeneAmp 9600 DNA thermal cycler (Perkin-Elmer, Warrington, UK) with an initial denaturation of 95°C for 30 seconds, followed by 12 cycles of 95°C for 30 seconds, 55°C for 1 minute and 68°C for 10 minutes in accordance to guidelines set out in the Quikchange site-directed mutagenesis protocol (see figure 3.4 for an overview of the method). Before the removal of nonmutated parental DNA template by digestion for 1 hour at 37°C with DpnI restriction enzyme, electrophoresis was carried out using 10µl of the amplified product on a 1% agarose gel to check for sufficient amplification product. Transformation of the DpnI treated pGL3 Basic-IL-6 samples was then carried out using XL-1 blue supercompetent cells as described in section 3.3.1.2.2 and selection was carried out on LB plates containing 50µg/ml ampicillin, followed by the mini culture and mini prep as previously described in section 3.3.1.2.3. The same procedure was used to generate the IL-6 -597A/-174G variant using the IL-6 -597G/-174G template and primers IL-6 -597A Mut F plus IL-6 -597A Mut R (see table 1 in appendix for sequences). This plasmid DNA template was
then used in site-directed mutagenesis to generate the IL-6 597A/-174C variant from primers, IL-6 -174C Mut F and IL-6 -174C Mut R (see table 1 in appendix for sequences). All IL-6 promoter constructs made for uses in *in vitro* functional studies are shown in table 3.1.

### 3.3.2.2 Sequencing IL-6 promoter constructs

All pGL3 Basic vectors with IL-6 inserts (i.e. IL-6 -572G referred to also as -597G/-174G, -572C and -597A/-174C) generated by cloning and site-directed mutagenesis from sections 3.3.1.2.4 and 3.3.2.1 were sequenced to check for integrity of the ligated insert and for potential errors using the protocol from chapter 2, section 2.3.5 and primers in table 2 of the appendix.

### 3.3.2.3 Purification of pGL3 Basic-IL-6 promoter constructs

#### 3.3.2.3.1 Endotoxin Free Maxi prep protocol

Plasmid DNA of pGL3 Basic IL-6 promoter constructs checked by DNA sequencing in section 3.3.2.2 was transformed into *E.coli* XL-1 blue subcloning competent cells and a mini culture preparation was made as described in sections 3.3.1.2.2 and 3.3.1.2.3. 200μl of the mini culture was added into a maxi culture containing 100ml of LB medium with 50μg/ml ampicillin (1/500th dilution). This was then grown for 16 hours at 37°C with vigorous shaking (225rpm). The bacterial cells were then harvested by centrifugation at 3,000rpm for 15 minutes at 4°C and the Endotoxin free maxi prep kit was used to obtain the purified plasmid DNA. This protocol involved resuspension of cells in P1 buffer, followed by alkaline lysis in buffer P2. After neutralization with buffer P3, the white precipitate containing proteins, genomic DNA, cell debris and SDS was separated from the lysate using the QIAfilter provided in the kit. The lysate was then incubated in buffer ER and applied to the QIAGEN tip 500 already primed by applying buffer QBT. After 2X 30ml wash with buffer QC, the plasmid DNA was eluted from the QIAGEN tip 500 with 15ml buffer QN. The eluted DNA was then obtained by precipitation with isopropanol.
(0.7 volumes) and centrifugation at 12,000rpm for 30 minutes at 4°C (Sorvall RC-5C centrifuge) and then washed with 5ml 70% endotoxin-free ethanol, centrifuged at 12,000rpm for 30 minutes. The pellet was air dried for 15 minutes and dissolved in 500µl endotoxin-free TE buffer.

### 3.3.2.3.2 Caesium chloride density gradient centrifugation

The plasmid DNA purified in section 3.3.2.3.1 was made up to 8ml with TE buffer and then 8g caesium chloride (CsCl₂) was added to each plasmid suspension (1g/ml gradient), followed by 50µl of ethidium bromide (10mg/ml). The solution was gently transferred to a Quickseal centrifuge tube (Beckman), using a syringe and wide needle (so as to prevent the plasmid DNA from shearing), the tubes precision balanced, sealed, and ultracentrifuged in a 70.1 VTi rotor (Beckman), at 60,000rpm for 20 hours at 20°C. The band of circularized plasmid was withdrawn from the tube using a 21 gauge needle (Terumo) and syringe transferred into a new Quickseal tube for repeat banding in 1mg/ml CsCl₂/TE mix, ultra-centrifuged as before. The double purified plasmid band was washed repeatedly with an equal volume of TE saturated butanol until the organic and aqueous phase became completely clear. An equal volume of water was then added, followed by 2 volumes of 4M ammonium acetate (pH 5.4), and ethanol to a volume of 70%. After incubation at 4°C for 10 min, the precipitated DNA was pelleted by centrifugation at 12,000rpm for 30 minutes at 4°C (Sorvall RC-5C centrifuge). The supernatant was discarded, the pellet washed twice with 70% ethanol, air dried and resuspended in 250µl endotoxin-free TE buffer. Plasmid concentration and purity were determined by UV spectrophotometry (Cecil), at 260nm (maximum absorption spectrum for DNA), and 280nm (max. absorption spectrum for protein). Only samples with a 260/280 OD ratio ≥ 1.8 were used for transfection. Plasmid identity was verified by PCR or restriction digestion or by both methods. The final purified plasmids were aliquoted and stored at –20°C.
Figure 3.4. Overview of the QuikChange 1-day site-directed mutagenesis method.

Step 1
Plasmid Preparation
Gene in plasmid with target site (●) for mutation

Step 2
Temperature Cycling
Denature the plasmid and anneal the oligonucleotide primers (●) containing the desired mutation (●)

Using the nonstrand-displacing action of PfuTurbo DNA polymerase, extend and incorporate the mutagenic primers resulting in nicked circular strands

Step 3
Digestion
Digest the methylated, nonmutated parental DNA template with Dpn I

Step 4
Transformation
Mutated plasmid (contains nicked circular strands)

Transform the circular, nicked dsDNA into XL1-Blue supercompetent cells

After transformation, the XL1-Blue supercompetent cells repair the nicks in the mutated plasmid

Legend
- Parental DNA plasmid
● Mutagenic primer
- Mutated DNA plasmid
Table 3.1 IL-6 promoter variants cloned into pGL3 Basic luciferase reporter gene expression vector.

<table>
<thead>
<tr>
<th>Name of IL-6 Clones</th>
<th>Construct length</th>
<th>-627 C&gt;A</th>
<th>-597 G&gt;A</th>
<th>-572 G&gt;C</th>
<th>-395 to -375 AnTn tract</th>
<th>-174 G&gt;C</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 -572G or -597G/-174G</td>
<td>-1180 to +13</td>
<td>C</td>
<td>G</td>
<td>G</td>
<td>A9T11</td>
<td>G</td>
</tr>
<tr>
<td>IL-6 -572C</td>
<td>-1180 to +13</td>
<td>C</td>
<td>G</td>
<td>C</td>
<td>A9T11</td>
<td>G</td>
</tr>
<tr>
<td>IL-6 -597A/-174C</td>
<td>-1180 to +13</td>
<td>C</td>
<td>A</td>
<td>G</td>
<td>A9T11</td>
<td>C</td>
</tr>
</tbody>
</table>

3.3.3 In vitro functional studies of IL-6 promoter in HepG2 cells

The three IL-6 promoter constructs (table 3.1) generated by cloning, subcloning and site-directed mutagenesis were used in an in vitro system to test for functionality. Expression of the firefly luciferase protein driven by the IL-6 promoter was assessed by transient transfection in HepG2 cells.

3.3.3.1 HepG2 Cell banks

The commercially obtained adherent human hepatocyte (HepG2) cell line (ECCAC, UK) was grown in Minimum Essential Medium (MEM) with Earle’s salts and L-glutamine, supplemented with 10% heat inactivated fetal bovine serum (FBS), 1% non-essential amino acids, 1% sodium pyruvate and 500 IU/ml Penicillin/Streptomycin at 37°C, 5% CO2 to generate cell stocks for subsequent transient transfection experiments. Trypsinization of cells in 1X Trypsin-EDTA (i.e. 1ml for 75cm² and 3ml for 150cm² TC flasks) was carried out following washing of cells in PBS solution. Serum growth medium was added to neutralize the trypsin-EDTA and following centrifugation (1,000rpm for 1 minute) the medium was discarded by aspiration. The cells were then resuspended in growth medium for replating or in cell freezing medium for cell storage. All cell stocks were initially stored in 1.5ml cryovial tubes with cell freezing medium containing serum-DMSO (Sigma, UK) in a polystyrene box with cotton wool overnight at -80°C, followed by rapid transfer into liquid nitrogen for long-term storage.
3.3.3.2 Cell counting

A hemocytometer containing two chambers (fig 3.5) was used for cell counting prior to plating. Cell concentration was determined by counting the number of cells within a defined area of the four major squares on the corners plus the central major square from both chambers to give 10 squares and then the average obtained. The number of cells was then multiplied by \(10^4\) to give number of cells/ml.

![Figure 3.5](image)

Figure 3.5 (a) One chamber of a hemocytometer slide under 10X objective and 10X ocular. The chamber is divided into 9 major squares. (b) Detailed view of one of the 9 major squares. Only cells that overlap the top and left borders of squares were counted to avoid overestimating the cell concentration. O: cells that should be counted and \(\varnothing\): cells that should be ignored.

3.3.3.3 Charcoal stripping of steroids from fetal bovine serum (FBS)

500mg activated charcoal and 50mg Dextran T70 was added to 500ml of heat inactivated fetal bovine serum. The mixture was then stirred for 30 minutes at room temperature, centrifuged at 1,500rpm for 15mins and the supernatant filter sterilized through a 0.22\(\mu\)m filter, under vacuum.

3.3.3.4 Transfection efficiency and X-gal staining of HepG2 cells

To determine optimal transient transfection protocol for HepG2 cells, 150\(\mu\)l of HepG2 growth medium (section 3.3.3.1) containing \(5 \times 10^5\) HepG2 cells were seeded into each well of a 96 well plate 30 hours prior to transfection. The cells (~50% density after 30
hours) were washed with 150μl PBS solution and then 40μl of serum free and antibiotic free growth medium added to each well, followed by 10μl of the transfection mix (pre-incubated for 15 minutes at room temperature) containing varying concentrations of pSV-β-Galactosidase control vector plasmid (0.05, 0.1, 0.15, 0.2 and 0.25μg/well) (figure 3.6(a)), 0.001μg of the pRL-TK Renilla expression vector (figure 3.6(b)) and 0.25μl LIPOFECTIN reagent in OptiMEM I (already incubated at room temperature for 45 minutes) in accordance with the manufacturer’s instructions (Life Technologies, Paisley, UK) with n=8 for 12 hours. Transfection medium was then replaced with HepG2 growth medium as described in section 3.3.3.1 for an additional 48 hours before brief washing in PBS solution. The cells were incubated with 50μl of PBS containing 0.5% glutaraldehyde for 15 minutes at room temperature, followed by brief washing with 3X 100μl PBS solution. 50μl of X-gal staining solution (in PBS: 5mM Potassium ferricyanide, 5mM Potassium ferrocyanide, 1mM MgCl₂ and 1mg/ml X-gal) was added to the cells in each well and incubated at 37°C overnight. Transfected cells that stained blue were viewed by light microscopy and the transfection efficiency was then estimated by the percentage of cells stained blue over 8 separate transfected wells at different DNA concentrations. The protocol with the highest transfection efficiency was then used in further subsequent transfections of the pGL3 Basic IL-6 promoter variants in HepG2 cells.
Figure 3.6 Circular map of (a) pSV-β-Galactosidase control vector and (b) pRL-TK Renilla vector. Both vectors contain the gene that encodes for ampicillin resistance (Amp'). The lacZ gene encodes β-galactosidase and is designed for monitoring transfection efficiencies of mammalian cells and the Rluc encodes for Renilla luciferase protein.
3.3.3.5 Transient transfection of IL-6 promoter constructs in HepG2 cells

The plating of HepG2 cells in 96 well plates and transient transfections were carried out as previously described in section 3.3.3.3, but only with 0.1µg per well of pGL3 Basic IL-6 promoter variants plus 0.001µg pRL-TK. After transfection, the cells were allowed to recover in growth medium containing 10% heat inactivated FBS or 10% charcoal stripped FBS for 24 hours, followed by the addition of stimulus. The cells were then lysed with 20µl 1X passive lysis buffer (PLB) (Promega, UK) for 20 minutes after washing with 2X 100µl phosphate buffer saline (PBS) solution. This procedure was used to test maximum promoter activities in response to different interleukin-1β (IL-1β) (0.1, 1, 10, 100 and 1000 WHO units/ml) and dexamethasone (5X10⁻⁷M, 5X10⁻⁶M and 5X10⁻⁵M) concentrations and also its activity over a 24 hour time course period. (0, 3, 6, 9, 12 and 24 hours)

3.3.3.6 Dual Luciferase Reporter Assay

The luciferase assay reagent II (LARIIL) and Stop & Glo reagents, freshly prepared in accordance with the manufacturer’s instructions (Promega Corp, UK), and the cell lysate were allowed to equilibrate to room temperature for 30 minutes prior to use. To assay for firefly luciferase, 50µl of LARIIL was added into each well of a 96 well plate containing 10µl cell lysate with a 2 seconds delay followed by 10 seconds measurement. This was then followed by 50µl addition of Stop & Glo with also the same delay and measurement times. The luminosity photon emission produced by the firefly luciferase and by the renilla luciferase was detected using a Tropix TR717 Microplate Luminometer plate reader (Perkin-Elmer). Values for the final firefly luciferase readings were calculated after adjusting for background luminescence (cells) and then normalization for transfection efficiency using the pRL-TK renilla luciferase reading. Mean firefly luciferase values were then calculated and any values that were more than two standard deviations from the mean were excluded.
3.4 Results

3.4.1 Transfection efficiency

The pSV-β-Galactosidase control vector DNA (concentrations ranging from 0.05 to 0.25μg/well), which encodes for β-galactosidase were transfected in HepG2 cells to optimize for transfection efficiency. Blue cells expressing β-galactosidase were visualized by microscopy following fixation with glutaraldehyde and incubation with the substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). As shown by the results in Table 3.2, 0.1μg/well pSV-β-Galactosidase control vector DNA (in 96-well plate) gave the highest transfection efficiency (23%) from the mean of 8 individually transfected wells. Higher concentrations of pSV-β-Galactosidase plasmid DNA did not significantly improved transfection efficiency in HepG2 cells. Therefore, this protocol was subsequently used in further transient transfection experiments.

Table 3.2 Transfection efficiency of pSV-β-Galactosidase control vector plasmid in HepG2 cells with LIPOFECTIN reagent. HepG2 cells were transfected in 96-well plate and efficiency of the transfection was derived from observation of blue stained cells following X-gal staining and microscopy.

<table>
<thead>
<tr>
<th>pSV-β-Galactosidase Conc. (μg/well)</th>
<th>Transfection efficiencies (%)</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>9 10 12 9 11 15 9 10</td>
<td>11</td>
</tr>
<tr>
<td>0.10</td>
<td>20 25 22 28 24 20 25 25</td>
<td>23</td>
</tr>
<tr>
<td>0.15</td>
<td>22 20 22 25 22 20 24 20</td>
<td>22</td>
</tr>
<tr>
<td>0.20</td>
<td>18 20 20 25 20 23 18 20</td>
<td>21</td>
</tr>
<tr>
<td>0.25</td>
<td>20 22 25 20 25 18 20 25</td>
<td>22</td>
</tr>
</tbody>
</table>

3.4.2 IL-1β dose response in HepG2 cells

To date, the maximal dose for IL-1β induction of IL-6 promoter variants in HepG2 cells are not known. Therefore to establish the IL-1β dose for maximum IL-6 induction, human recombinant IL-1β, ranging in concentration from 0.1 to 1000U/ml were added to the growth medium for 9 hours post transient transfection of the common wild-type IL-6 -
597G/-174G variant. A positive relative light unit gradient was seen for an IL-1β dose from 0U/ml (basal) to 1000U/ml, with the promoter activity peaking at 100U/ml (figure 3.7). Therefore this IL-1β concentration was selected for further transient transfection experiments.

Figure 3.7 IL-1β (also referred to as IL-1beta) dose response of pGL3 Basic IL-6 promoter variant -597G/-174G in HepG2. Mean values for relative light units are from 8 transfections following 9 hours IL-1beta stimulation calculated by subtraction of background luminescence and then normalized against Renilla luciferase reading.

3.4.3 Effects of Steroids on IL-6 expression in HepG2 cells

Steroids have been well documented to repress IL-6 gene expression (see section 1.6.2.1 and 1.6.2.2). Fetal bovine serum (FBS), an important constituent of HepG2 growth medium needed for cell growth and survival, contains various quantities of different steroids between different batches of FBS. This therefore introduces variability in the experiment that is difficult to control for and also could confound differences (if any) in IL-6 expression levels from one experiment to another by the IL-6 promoter variants under investigation. To eliminate this variability, activated charcoal and dextran T70 were used to remove steroids from FBS, and then the serum could be spiked with known amounts of steroids (e.g. dexamethasone, an analog of cortisol) when needed. The results
in figure 3.8(a) shows an example of the differences between the IL-6 -572G (also known as -597G/-174G) and -572C constructs in HepG2 cells supplemented with either normal FBS or charcoal stripped FBS. In growth media with either normal FBS or charcoal stripped FBS, whether in the presence or absence of IL-1β (IL-1beta) stimulus, the IL-6 -572C variant expression levels were higher than the IL-6 -572G variant. This reached significance in charcoal stripped FBS (p=0.007) and normal FBS plus IL-1β (p=0.022). The -572C variant gave 1.6 fold higher promoter expression levels than the -572G variant in media supplemented with charcoal stripped FBS (1.02 vs 0.63 relative light units). This difference was only 1.2 fold higher in normal FBS (0.68 vs 0.58 relative light units). The expression levels of the -572C variant were significantly higher in charcoal stripped FBS compared to normal FBS (1.02 vs 0.68 relative light units; p=0.001). No significant differences were observed for the -572C in normal and charcoal stripped FBS with IL-1β. The relative promoter activity differences for the -572G variant in normal FBS versus charcoal stripped FBS with or without IL-1β were not significant. Extrapolation of the results in figure 3.8(a) showing the fold difference of IL-1β stimulation (a potent inflammatory stimulus) versus basal (no IL-1β) for -572C and -572G variants in normal and charcoal stripped FBS media are presented in figure 3.8(b). In normal FBS, the -572C variant had ~59% greater fold difference than the -572G variant (3.5 fold vs 2.2 fold), but in charcoal stripped FBS, the -572G had ~10% greater fold difference than the -572C variant (2.9 fold vs 2.6 fold). Overall, the data presented here suggests that contaminants (e.g. steroids) within FBS are having a much greater repressor effect on the -572G than the -572C variant in the presence of IL-1β, but not for charcoal stripped FBS. To control for the presence of unknown steroid contaminants, all FBS used for in vitro work from now on was treated with activated charcoal and dextran T70 (charcoal stripped FBS).
Figure 3.8(a). Effects of steroids on promoter expression of IL-6 -572G and -572C variants in HepG2 cells containing 10% FBS and 10% Charcoal stripped FBS in the presence or absence of 100U/ml IL-1beta for 9 hours. Mean relative light units for -572G and -572C variants (n=8) was calculated by subtraction of background luminescence (cells) and then normalized against renilla luciferase reading. Fold difference was calculated by dividing relative light units of -572C from -572G. P-values are differences between -572C versus -572G for each conditions using t-test.

Figure 3.8(b). Fold difference of IL-6 -572G versus -572C promoter variants in HepG2 cells stimulated with 100U/ml IL-1beta from basal. Fold values were calculated by dividing the variant stimulated by IL-1beta from basal.
3.4.4 Dexamethasone dose response on IL-6 expression in HepG2 cells

Dexamethasone (DEX), a potent synthetic glucocorticoid analog of cortisol, has been reported to repress IL-6 gene transcription (see review in section 1.6.2.1), and was thus used in charcoal stripped FBS media as a steroid inhibitor for the repression of the wild-type IL-6 -572G/-174G variant in HepG2 cells. The maximum DEX dose for the repression of the wild-type IL-6 promoter variant on IL-6 gene expression in HepG2 cells was not known. However, the concentrations of DEX used on HepG2 cells reported in the literature range from 5X10^{-7}M to 5X10^{-5}M. Therefore three ethanol soluble DEX concentrations (i.e. 5X10^{-7}M, 5X10^{-6}M and 5X10^{-5}M) representing low, medium and high were selected. The results are shown in figure 3.9. There were no significant differences between promoter expression levels at basal with any of the DEX concentrations used. The lowest concentration of DEX (5X10^{-7}M) was chosen for further *in vitro* functional experiments.

Figure 3.9 Dexamethasone (DEX) dose response of the pGL3 Basic IL-6 -597G/-174G promoter variant in HepG2 cells. Cells were stimulated in DEX for 9 hours in 10% charcoal stripped FBS media. Mean relative light units value for each DEX concentration were calculated from 8 separate transfections.
3.4.5 IL-6 promoter expression of -572G and -572C variants in HepG2 cells

3.4.5.1 Effects of IL-6 -572G and -572C expression by stimuli

*In vitro* luciferase reporter gene assays were carried out in HepG2 cells to determine the functional effect of the -572G and -572C variants on IL-6 expression levels following repression with DEX and induction by IL-1β plus DEX. Transient transfection experiments demonstrated that the -572C variant gave higher promoter expression levels than -572G at basal, with DEX or IL-1β plus DEX (figure 3.10), reaching significance only in DEX (5X10^-7M) (p=0.031). The fold difference from basal for each variant showed that -572G expression was reduced by 21% in DEX compared to basal (0.79 vs 1; p=0.021), but the reduction was only 3% for the -572C (0.97 vs 1; p=0.502). However, the 22% fold higher expression of the -572C variant over the -572G in DEX stimulation (0.97 vs 0.79 fold) disappeared when IL-1β was also present with DEX in the growth media (1.95 vs 1.92 fold; p=0.155).

Figure 3.10 Dexamethasone (DEX) and IL-1β (IL-1beta) plus DEX stimulation of IL-6 -572G and -572C promoter variants in HepG2 cells for 8 hours. Relative light units are the mean (n=8) calculated by subtraction of background luminescence (cells) and normalized against renilla luciferase reading. Fold difference was calculated by dividing the relative light units of each variant from their basal reading.
Time course experiments (0, 3, 6, 9, 12 and 24 hours) of the -572G versus -572C in response to DEX or IL-1β plus DEX were carried out to determine whether there were differences in expression of the -572 variants over time following repression by DEX and induction with IL-1β plus DEX. The results in figure 3.11(a) demonstrate both variants had maximal IL-6 expression at 9 hours, with higher -572C expression (not significant) over the -572G construct at the 24 hours time period. However, in the presence of DEX and IL-1β plus DEX (figures 3.11(b) and 3.11(c)), the -572C variant was significantly higher than -572G at almost all time points (p<0.05), with the maximum expression occurring at 9 hours time point (for DEX: 1.7 (SEM±0.2) vs 1.0 (SEM±0.7); p=0.007 and for IL-1β plus DEX: 4.0 (SEM±0.3) vs 2.7 (SEM±0.2); p=0.006)). At this time point, IL-1β plus DEX stimulation gave a 3 fold induction in luciferase expression by the -572C construct from basal level compared to only 2 fold induction by the -572G (for -572C, 4.0 vs 1.3 and for -572G, 2.7 vs 1.3 relative light units respectively).

To account for the possibility that these results (figures 3.10 and 3.11) were due to chance or experimental errors such as the state of the HepG2 cells, purity of DNA plasmid preparation and transfection protocol handling, several time course experiments were performed using new aliquots of LIPOFECTIN reagent and freshly prepared DEX and IL-1β plus DEX. HepG2 cells were plated within one specified passage prior to all transfection experiments to minimize phenotypic changes to HepG2 cells. In addition, a second DNA preparation was made and used in transfection experiments in order to rule out artifact caused by impurities in the DNA. The results given in table 3.2.1 are the summary of several time course experiments at the 9 hour time point with two different plasmid preparations. Under basal condition, the -572C variant expression levels were higher than the -572G variant, with the exception of the second experiment using the second plasmid preparation. The same trend was also seen with DEX repression and IL-1β plus DEX induction, whereby the -572C gave higher promoter activities than the -572G construct. Therefore the higher promoter levels of the -572C compared to the -572G construct are consistent between DNA preparations and between experiments, though the absolute difference was not constant.
Figure 3.11 Time course of IL-6 -572G versus -572C promoter variants in HepG2 cells at (a) basal, (b) with DEX (5 X 10^-7M) or (c) IL-1β (IL-1beta)(100U/ml) plus DEX (5X 10^-7 M). Mean promoter activities (firefly luciferase reading) are from 8 transfections for each variant. P values were obtained using T-test.
Table 3.2.1 Mean (±SEM) promoter activities for the IL-6 -572G and -572C variants in HepG2 cells stimulated with DEX or IL-1β plus DEX for 9 hours.

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>DNA prep.</th>
<th>No. of Experiment</th>
<th>Relative light units -572G</th>
<th>Relative light units -572C</th>
<th>p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>1</td>
<td>1</td>
<td>1.11 ± 0.13</td>
<td>1.53 ± 0.14</td>
<td>0.016</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>1.16 ± 0.09</td>
<td>1.40 ± 0.08</td>
<td>0.030</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1</td>
<td>1.64 ± 0.86</td>
<td>2.81 ± 0.84</td>
<td>0.184</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2</td>
<td>1.83 ± 0.25</td>
<td>1.63 ± 0.11</td>
<td>0.244</td>
</tr>
<tr>
<td>Dexamethasone (DEX)</td>
<td>1</td>
<td>1</td>
<td>0.55 ± 0.04</td>
<td>0.61 ± 0.04</td>
<td>0.138</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1</td>
<td>3.15 ± 0.13</td>
<td>3.78 ± 0.35</td>
<td>0.183</td>
</tr>
<tr>
<td>IL-1β plus DEX</td>
<td>1</td>
<td>1</td>
<td>14.78 ± 0.94</td>
<td>19.60 ± 2.23</td>
<td>0.035</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>5.22 ± 0.44</td>
<td>7.27 ± 1.42</td>
<td>0.095</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1</td>
<td>2.53 ± 0.10</td>
<td>3.09 ± 0.22</td>
<td>0.023</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2</td>
<td>1.78 ± 0.15</td>
<td>2.11 ± 0.18</td>
<td>0.090</td>
</tr>
</tbody>
</table>
3.4.6 Expression of IL-6 -597G/-174G and -597A/-174C variants in HepG2 cells

3.4.6.1 Effects of -597G/-174G and -597A/-174C variants by stimuli

The naturally occurring IL-6 -597G/-174G and -597A/-174C promoter variants identified in Caucasian healthy men were used to test for functional differences in IL-6 expression levels in HepG2 cells. Results shown in figure 3.12 demonstrate the IL-6 -597A/-174C variant had significantly higher promoter activities than the -597G/-174G variant at basal (70%, p<0.005), with DEX (98%, p=0.006) and IL-1β plus DEX (126%, p=0.005). The fold difference (see figure 3.12) demonstrates a 21% reduction in expression level from the basal state for the -597G/-174G variant (0.79 vs 1 fold; p=0.032) compared to only 7% for the -597A/-174C (0.93 vs 1 fold; p=0.562) by DEX. There was an 18% higher expression for the -597A/-174C variant compared to the -597G/-174G (0.93 vs 0.79 fold; p=0.006), which then increased to 33% difference in the presence of IL-1β plus DEX (2.56 vs 1.92 fold; p=0.005).

Figure 3.12 Dexamethasone (DEX) and IL-1β (IL-1beta) plus DEX stimulation of the IL-6 -597G/-174G and -597A/-174C promoter variants in HepG2 cells for 8 hours. Relative light units are the mean from 8 transfections for each variant calculated by subtraction of background luminescence (cells) and normalized against renilla luciferase reading. Fold difference was then calculated by dividing the relative light units of each variant from their basal reading.
3.4.6.2 Time course response of the IL-6 -597G/-174G and -597A/-174C by stimuli

Using the same methods as described in section 3.3.3.5, a time course experiment (0, 3, 6, 9, 12 and 24 hours) was carried out on the IL-6 -597G/-174G and -597A/-174C variants to investigate possible differences in the response and expression levels post-stimulation with DEX or IL-1β plus DEX. The results from figures 3.13(a) to (c) demonstrate that the -597A/-174C variant had significantly higher expression level than the -597G/-174G variant at most time points (p=<0.05). The maximum induction for the -597A/-174C occurred at the 6 hours time point compared to 9 hours for -597G/-174G when cells were stimulated with IL-1β plus DEX (see figure 3.13(c)). At the 6 hours time point, the -597A/-174C variant expression level was significantly higher than the -597G/-174G variant at the basal state (38%, p=0.003), with DEX repression (139%, p=0.005) and with IL-1β plus DEX induction (384%, p=0.010). To account for the possibility of experimental errors, several time course experiments were carried out with these variants using a second plasmid preparation. These results (data not shown) demonstrated that both variants had a similar response to DEX or IL-1β plus DEX stimulation and the maximal expression occurring at the 9 hours time point. This suggests that the data from figure 3.13 may be due to experimental errors. The results in table 3.3 are a summary of the promoter activities of the -597G/-174G and -597A/-174C variants at the 9 hours time point from several time course experiments. Under all stimulus situations (basal, DEX and IL-1β plus DEX), the -597A/-174C variant gave higher promoter expression than the -597G/-174G variant. It should be noted that the absolute differences between the -597G/-174G versus -597A/-174C variants for the different experiments and DNA preparation were not significant.
Figure 3.13 Time course of transient transfections of the IL-6 -597G/-174G and -597A/-174C promoter variants in HepG2 cells at (a) basal, (b) with DEX (5 X 10^{-7}M) or (c) DEX (5X 10^{-7}M) and IL-1β (IL-1 beta)(100U/ml). Mean values for relative light units are from 8 transfection for each construct. P values were obtained using t-test.
Table 3.3 Mean (±SEM) promoter activities for the IL-6 -597G/-174G and -597A/-174C variants in HepG2 cells stimulated with DEX or IL-1β plus DEX for 9 hours. Relative light units values are from the means of 8 experiments.

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>DNA prep.</th>
<th>No. of experiment</th>
<th>Relative light units -597G/-174G</th>
<th>Relative light units -597A/-174C</th>
<th>p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Basal</strong></td>
<td>1</td>
<td>1</td>
<td>3.84 ± 0.49</td>
<td>4.89 ± 0.46</td>
<td>0.101</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1</td>
<td>1.11 ± 0.13</td>
<td>1.80 ± 0.12</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2</td>
<td>1.52 ± 0.18</td>
<td>1.83 ± 0.25</td>
<td>0.164</td>
</tr>
<tr>
<td><strong>Dexamethasone (DEX)</strong></td>
<td>1</td>
<td>1</td>
<td>0.55 ± 0.04</td>
<td>0.61 ± 0.04</td>
<td>0.138</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1</td>
<td>3.15 ± 0.13</td>
<td>3.78 ± 0.35</td>
<td>0.183</td>
</tr>
<tr>
<td><strong>IL-1β (IL-1beta) plus DEX</strong></td>
<td>1</td>
<td>1</td>
<td>1.78 ± 0.15</td>
<td>2.80 ± 0.51</td>
<td>0.039</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>5.22 ± 0.44</td>
<td>6.05 ± 0.48</td>
<td>0.114</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1</td>
<td>1.16 ± 0.08</td>
<td>1.33 ± 0.07</td>
<td>0.064</td>
</tr>
</tbody>
</table>
3.5 Discussion

3.5.1 Transfection efficiency of DNA plasmid in HepG2 cells

The liposome, LIPOFECTIN reagent (1:1 (w/w) liposome formulation of cationic lipid: N-[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA) and Dioleoyl phosphatidylethanolamine (DOPE)) has been widely used in the Cardiovascular Genetics laboratory and by others (Iwabuchi et al 1998; Bohm et al 1995; Lee et al 1996; Solway et al 1995; Guan et al 1995; Reddy et al 1995; Chen et al 1995) for the transient transfection of plasmid DNA into mammalian cells such as HepG2. At the start of these studies, there was no protocol available for the high throughput delivery of plasmid DNA into HepG2 cells for reporter gene studies using LIPOFECTIN reagent in a 96-well plate. Therefore in a 96 well plate, pSV-β-Galactosidase control vector DNA and LIPOFECTIN reagent were used to obtain optimum transfection efficiency, which could then be used for high throughput in vitro functional studies of the pGL3 Basic IL-6 promoter constructs (see table 3.1) in HepG2 cells. 0.1μg/well pSV-β-Galactosidase control vector cotransfected with 1/100th the concentration of pRL-TK vector gave the highest transfection efficiency (23%) after in situ staining with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). This was comparable to those reported in the literature (Iwabuchi et al 1998; Bohm et al 1995; Lee et al 1996; Solway et al 1995; Guan et al 1995; Reddy et al 1995; Chen et al 1995) for HepG2 cells using LIPOFECTIN reagent in which a transfection efficiency of 20-30% was recorded for 6, 12, 24 and 48 well transfection plates. Therefore this transfection protocol was acceptable for transfection of the IL-6 promoter constructs.

3.5.2 IL-1β dose response of IL-6 promoter activities in HepG2 cells

At the start of these studies, the concentration of IL-1β and IL-1α in vivo had not been reported. Therefore a dose stimulation of IL-1β (0.1-1000U/ml) (figure 3.7) was needed and was carried out using the wild-type IL-6 -597G/-174G variant to determine maximum expression of this variant in HepG2 cells. Recombinant human IL-1β was selected due to
its potency as a proinflammatory cytokine. It has been reported in the literature to be one of the major inducers of IL-6 gene transcription in many cell types (Hirano et al 1986). 100U/ml of IL-1β was shown to be sufficient to obtain maximum expression of this IL-6 promoter variant. It should be noted that the concentration of IL-1β used to mimic the inflammatory scenario for *in vitro* functional studies here may be artificial (100U/ml=500pg/ml). Since there is no *in vivo* data reporting plasma levels of IL-1β levels, the concentration of IL-1β used may not be physiologically a reflection of that seen *in vivo*.

### 3.5.3 Inhibition by steroids on IL-6 promoter expression in HepG2 cells

To establish whether steroid contamination in FBS may be affecting the expression levels of IL-6 -572G and -572C variants in HepG2 cells, growth medium containing either normal FBS or charcoal stripped FBS (no steroids) was used. Data from figures 3.8(a) and 3.8(b) strongly suggests that serum contains steroid contaminants, since the promoter activity of the -572C and -572G variants was significantly higher (p=0.007) in charcoal stripped FBS than in FBS. In addition, the presence of IL-1β in media with FBS significantly increased the fold difference between the -572C versus -572G compared to FBS with no IL-1β (1.9 fold vs 1.2 fold; p=0.005). This fold difference did not however alter in charcoal stripped FBS with or without IL-1β (1.6 fold vs 1.5 fold). This clearly demonstrates that FBS contain contaminants, such as steroids, whose concentration and composition may vary between different batches of FBS (Invitrogen, Paisley, UK; personal communication). This may therefore affect the expression of IL-6 promoter variants in a different manner and as a result would create potential artifacts in the level of promoter activity being recorded. To eliminate this experimental problem, charcoal stripped FBS and then the ability to spike the serum with a known concentration of a particular steroid such as dexamethasone was employed.
3.5.4 Dexamethasone dose response of IL-6 promoter expression in HepG2 cells

It has been well documented \textit{in vivo} that during times of stress or inflammation, IL-6 levels are increased, which in turn induce the release of corticotrophin-releasing factor (Navarra et al 1990; Lyson et al 1991). This in turn results in elevated systemic levels of corticosteroids (Kohase et al 1987; Feyen et al 1989; Nishida et al 1989), leading to inhibition of IL-6 production. Cortisol, a naturally occurring steroid \textit{in vivo} is known to be present in the circulation as an inhibitor of inflammation, being high during the early morning and low during the evening (Guyton 1991). To mimick an \textit{in vivo} state, dexamethasone (DEX) (an analog of cortisol) was used as a repressor of IL-6 expression. Three DEX concentrations (5X10^{-7}M, 5X10^{-6}M and 5X10^{-5}M) were use to test for the optimum dose for the repression of the wild-type IL-6 promoter variant in HepG2. All three concentrations of DEX tested showed slight repression (not significant) compared to basal (no DEX). A low dose of DEX (5X10^{-7}M) was chosen for subsequent \textit{in vitro} experiments because it marginally repressed the IL-6 -597G/-174G variant in HepG2 cells compared to basal (no DEX), and medium and high DEX concentrations (5X10^{-6}M and 5X10^{-5}M) did not significantly make any difference to the degree of repression seen. This may support earlier findings that IL-6 expression in hepatocytes is tightly regulated by glucocorticoids and estrogen (Ray et al 1989; 1990; 1997; Guyton 1991; Tabibzadeh et al. 1989; Jablons et al. 1989), which acts as a negative feedback loop to maintain homeostasis.

3.5.5 IL-6 promoter expression of -572G and -572C in response to stimuli

Several \textit{in vitro} studies looking at the relative promoter strength of the IL-6 -572G and -572C variants were carried out to test for promoter activity differences following DEX repression and IL-1\beta plus DEX induction. The significantly lower promoter activity of the -572G variant in DEX compared to that at basal (0.79 vs 1 fold; p=0.021) which was not seen for the -572C variant (figure 3.10), and the significantly higher promoter activity of the -572C variant compared to that of the -572G variant at the 24 hour time point with DEX in the presence or absence of IL-1\beta, suggests that DEX is having greater repression on the -572G than the -572C variant (figure 3.11). The mechanism for the -572C variant
having higher expression levels compared to the -572G in HepG2 cells could be explained by the fact that the G>C base transition at position -572 in the IL-6 promoter may reduce the affinity of the glucocorticoid receptor complex binding for the two putative GRE sites (-557 to -552 and -464 to -459). Hence, the -572C variants becomes less prone to repression by glucocorticoids. However, the results from these experiments must be interpreted with caution.

Experiments with a second plasmid preparation of the same IL-6 variants, using freshly prepared reagents and using 9 hours as the time point post-stimulation with DEX or IL-1β plus DEX, showed a smaller fold difference than for the first preparation, with levels for the C allele actually being lower than the G allele at basal conditions in one of the experiments. It should be noted however that the promoter activity of the C allele was higher than G in both experiments for IL-1β plus DEX, with the difference being statistically significant in one but not the other. The consistency of higher promoter activity of the -572C than -572G variant, with this trend being seen in four independent experiments, suggest this is likely to be a true observation. However, more repeats of this experiment would be required for a definitive interpretation.

3.5.6 IL-6 promoter expression of -597G/-174G and -597A/-174C by stimuli

The IL-6 promoter activity of IL-6 -597G/-174G versus -597A/-174C variants in response to stimuli such as DEX and IL-1β plus DEX was also investigated. The initial result (figure 3.12) showed the IL-6 -597G/-174G and -597A/-174C variants had a 21% and 7% reduction in expression levels in the presence of DEX from that at basal, with the -597A/-174C having an 18% higher expression than the -597G/-174G, increasing to 33% with IL-1β plus DEX suggesting the functional significance of this polymorphism. Evidence for this was supported by time course experiments showing the peak expression for the -597A/-174C variant induction by IL-1β plus DEX to occur at an earlier time point (6 hours) compared to that of the -597G/-174G variant (9 hours) (figure 3.13(c)). However, further subsequent time course experiments revealed that both variants had the same response to DEX and IL-1β plus DEX stimulation, with the maximum expression
occurring at the 9 hours time point for both. Although there was a degree of consistency between the two preparations of the plasmids, with the -597A/-174C variant showing higher activity than the -597G/-174G in all conditions used, only one of the differences was statistically significant at baseline and only one in IL-1β plus DEX. Although further repeat experiments may support the trend of the -597A/-174C having greater promoter activity, it cannot be concluded at this stage that this difference is biologically relevant or statistically robust. Since IL-6 expression levels in HepG2 cells are very low (Dr Mohamed-Ali, personal communication), variability arising between studies (inter experimental differences) would be sufficient to influence the result in favour of either positive or no significant difference between the -597A/-174C and -597G/-174G variants. To confirm the findings seen in HepG2 cells, another hepatocyte cell line (e.g. HuH7) which expresses higher IL-6 levels (Dr Mohamed-Ali, personal communication) would be useful. The experimental variability between experiments would be kept to a minimum by a higher promoter activity reading.
CHAPTER 4

IN VITRO FUNCTIONAL STUDIES OF IL-6 PROMOTER
CONSTRUCTS IN HUH7 CELLS
Chapter 4: *In vitro* functional studies of IL-6 promoter constructs in HuH7 cells

4.1 Aims

The aims for this chapter were to confirm findings seen in HepG2 cells (see chapter 3) by determining in another hepatocyte cell line (HuH7) the relative promoter strength of the IL-6 -572G, -572C, -597G/-174G (same as IL-6 -572G construct) and -597A/-174C promoter variants in response to dexamethasone repression and to IL-1β plus DEX induction over time. HuH7 cells were chosen here because it’s also a human cell line that closely resemble HepG2 cells, but was obtained from a much older caucasian female than that of HepG2.

4.2 Materials

All tissue culture media and transfection reagents were purchased from Invitrogen (Paisley, UK). HuH7 were from ECCAC and all other chemicals were from either Merck Ltd (Poole, UK) or Sigma (Poole, UK). The IL-6 plasmid constructs used for transient transfection of HuH7 were identical to those described in sections 3.3 and 3.4.

4.3 Methods

4.3.1 Transfection of IL-6 promoter in HuH7 cells

A cell bank of HuH7 cells was generated in growth medium identical to that of HepG2 cells as described in section 3.5.1. Cell counting, determination of transfection efficiency, transient transfection and dual luciferase assay was identical to that of HepG2 cells as described in sections 3.5.2 to 3.5.5. For HuH7, 1.25X 10⁴ cells were plated per well in 96-well plate prior to transfection. The pGL3 Basic luciferase plasmid vector containing the different IL-6 variants (prepared in section 3.3.2, see table 3.1) was used for transient transfection experiments, following optimization for transfection efficiency in HuH7 cell line. To allow direct comparison with HepG2 cells, 10% charcoal stripped FBS was used
in growth medium containing identical doses as described in sections 3.4.2 and 3.4.4 for dexamethasone (DEX) (5 \times 10^{-7} M) or IL-1\beta (100U/ml) plus DEX (5X \times 10^{-7} M).

4.4 Results

4.4.1 Transfection efficiency of HuH7 hepatoma cells

Transient transfections in HuH7 cells were carried out using the pSV-\beta-Galactosidase control vector DNA, pRL-TK vector and LIPOFECTIN reagent to optimize for transfection efficiency as described in sections 3.3.3.4 and 3.4.1. As shown from the results in table 4.1, 0.1\mu g/well of pSV-\beta-Galactosidase control vector DNA (in a 96-well plate) gave the optimum transfection efficiency (26\%) from the mean of 8 separate transfections experiments. Higher concentrations of plasmid DNA did not significantly alter or improve the transfection efficiency in HuH7 cells. Subsequently, 0.1\mu g pGL3 Basic IL-6 reporter DNA, 0.001\mu g of the pRL-TK vector and 0.25\mu g LIPOFECTIN reagent per well in a 96-well plate were used in further transient transfection experiments.

Table 4.1 Transfection efficiency of pSV-\beta-Galactosidase control vector DNA in HuH7 cells with LIPOFECTIN reagent.

<table>
<thead>
<tr>
<th>pSV-\beta-Galactosidase Conc. (\mu g/well)</th>
<th>Transfection efficiencies (%)</th>
<th>Mean (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>13, 18, 12, 10, 12, 15, 16, 14</td>
<td>13.8</td>
</tr>
<tr>
<td>0.10</td>
<td>24, 25, 25, 28, 25, 28, 27, 26</td>
<td>26.0</td>
</tr>
<tr>
<td>0.15</td>
<td>22, 25, 27, 25, 28, 25, 24, 28</td>
<td>25.5</td>
</tr>
<tr>
<td>0.20</td>
<td>22, 26, 22, 24, 25, 27, 22, 25</td>
<td>24.1</td>
</tr>
<tr>
<td>0.25</td>
<td>24, 22, 25, 25, 26, 23, 28, 24</td>
<td>24.6</td>
</tr>
</tbody>
</table>
4.4.2 IL-6 -572G and -572C expression in HuH7 cells

Time course experiments (0-24 hours) were carried out using HuH7 cells transiently transfected with the IL-6 -572G and -572C promoter constructs to determine promoter activities and response post-stimulation with DEX or IL-1β plus DEX. The results in figure 4.1 show that the IL-6 -572C construct gave significantly higher luciferase reporter gene expression levels compared to the -572G construct at most time points under basal conditions (no stimulus), in the presence of DEX repression, and with IL-1β plus DEX induction. The promoter activities of the -572G and -572C variants were repressed by dexamethasone and induced by IL-1β plus DEX compared to that at basal, and showed maximum levels for promoter expression in growth media containing DEX or IL-1β plus DEX after 12 hours. No significant (p>0.05) difference in luciferase fold induction was seen between the -572G or -572C constructs at the 12 hours optimal time point in the presence of IL-1β plus DEX stimulation compared to basal levels (-572G = 2 fold higher vs 1.9 fold higher for -572C).

To confirm that the results in figure 4.1 were not due to chance or experimental error, the same experiments were carried out using a second plasmid DNA preparation and the 12 hours as the time point of reference. The experiment were carried out in HuH7 cells plated within one specified passage prior to the transfection experiment to avoid possible phenotypic changes to the HuH7 cells from continuous cell division and passages. New aliquots of DEX and IL-1β plus DEX were used to stimulate the HuH7 cells and to reduce the possibility of reagent deterioration due to freeze-thawing. The results are given in figure 4.2. At basal, under DEX repression and IL-1β plus DEX induction, the IL-6 -572C construct gave higher promoter activity than the IL-6 -572G construct. The promoter activity for the IL-6 -572G construct was 10% lower under repression from DEX compared to basal activity (0.9 vs 1.0 fold). In the present of IL-1β plus DEX induction, the IL-6 -572G construct gave 2.2 fold compared to basal expression levels. For the IL-6 -572C construct, DEX had no effect on repression of promoter activity compared to basal (1.1 vs 1.0 fold). However, IL-1β plus DEX induction resulted in a 2.5 fold higher promoter activity than basal levels. Overall, these in vitro functional data
clearly show higher promoter activity of the -572C construct compared to the -572G construct. This was consistent between plasmid DNA preparations, although the absolute difference between the constructs is not constant.
Figure 4.1 Time course of IL-6 -572G and -572C promoter construct expression in HuH7 cells at (a) basal, (b) with DEX (5 X 10^-7M) or (c) IL-1β (100U/ml) plus DEX (5X 10^-7M). Mean promoter activities (firefly luciferase reading) were from 8 transfections for each IL-6 variant. P values were calculated using t-test.

(a) Basal

(b) DEX

(c) IL-1β plus DEX

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Figure 4.2 Promoter activities for pGL3 Basic IL-6 -572G and -572C promoter constructs (second DNA plasmid preparation) in HuH7 cells stimulated with DEX or IL-1β plus DEX for 12 hours. Mean promoter activities were from 8 transfections for each IL-6 variant. P values were calculated using t-test for significant difference between the -572G and -572C constructs.

![Graph showing promoter activities](image)

4.4.3 IL-6 -597G/-174G and -597A/-174C expression in HuH7 cells

The same time course experiments as described in section 4.4.2 were also carried out to investigate possible difference in expression levels for IL-6 -597G/-174G and -597A/-174C variants in response to stimulation from DEX or IL-1β plus DEX. Results in figure 4.3 demonstrate that the -597A/-174C construct gave significantly higher promoter activities compared to the -597G/-174G construct at almost all time points. HuH7 cells under DEX repression resulted in optimum expression levels at the 9 hours time point for the -597A/-174C construct and 12 hours for the -597G/-174G construct (see figure 4.3(b)). Under IL-1β plus DEX induction (figure 4.3(c)), the optimum expression for both constructs occurred at the 12 hours time point. Both constructs were induced upon the addition of IL-1β plus DEX (-597G/-174G = 2.1 fold and -597A/-174C = 1.2 fold higher compared to basal levels), but only the -597A/-174C construct was repressed by DEX in HuH7 cells.
Repeat of this experiment (3-24 hours time points only) using the same plasmid DNA preparation showed that overall, in the absence or presence of stimulus (DEX or IL-1β plus DEX), the IL-6 -597A/-174C construct still gave higher promoter expression levels compared to the -597G/-174G construct (see figure 4.4(a) to (c)).
Figure 4.3 Time course of IL-6 -597G/-174G and -597A/-174C promoter construct expression in HuH7 cells at (a) basal, (b) with DEX (5 X 10^(-7)M) or (c) IL-1β (100U/ml) plus DEX (5X 10^(-7)M). Mean promoter activities (firefly luciferase reading) were from 8 transfections for each variant. P values were calculated using t-test.
Figure 4.4 Time course of IL-6 -597G/-174G and -597A/-174C promoter construct expression in HuH7 cells at (a) basal, (b) with DEX (5 X 10^{-6}M) or (c) IL-1β (100U/ml) plus DEX (5X 10^{-6}M). Mean promoter activities (firefly luciferase reading) were from 8 transfections for each variants. P values were calculated using t-test.
4.5 Discussion

4.5.1 Transfection efficiency in HuH7 cells

The cationic liposome, LIPOFECTIN and the pSV-β-Galactosidase control vector previously described (see chapter 3, section 3.5.1) were used to optimize the transfection efficiency of plasmid DNA in HuH7 cells for the high throughput *in vitro* functional studies of IL-6 promoter variants in 96-well plates. 0.1μg/well pSV-β-Galactosidase control vector cotransfected with 1/100th the concentration of pRL-TK vector gave the highest transfection efficiency (26%) after *in situ* staining with X-gal. This was comparable to the results reported for HepG2 cells (23%) (Iwabuchi et al 1998; Bohm et al 1995; Lee et al 1996; Solway et al 1995; Guan et al 1995; Reddy et al 1995; Chen et al 1995) and was higher than the commercially available LIPOFECTAMINE reagent (10% efficiency) (Bichko et al 1994).

4.5.2 IL-6 -572G>C expression in response to stimulus

The relative promoter strengths of the IL-6 -572G and -572C constructs were estimated in response to DEX repression and IL-1β plus DEX induction. The higher promoter activity of the -572C construct compared to the -572G in response to DEX repression and IL-1β plus DEX induction confirm the findings in HepG2 cells (see chapter 3). This higher transcriptional activity of the -572C allele also consistent with that reported by Brull et al 2001, whereby subjects carrying this allele had significantly higher IL-6 levels compared to those with the -572GG genotype in patients undergoing coronary artery bypass grafting (CABG) as a model for acute inflammation. Interestingly, in healthy middle-aged men in the NPHSII study, the IL-6 -572G>C was not associated with higher CHD risk factors such as CRP and fibrinogen or CHD risk itself (see chapter 5). However, since IL-6 measurements were not available in the study, it is not possible to rule out effects of the genotype in healthy men, but the data imply that effects in a non-inflammatory situation may be small. Data from Professor Patricia Woo laboratory's using the same IL-6 -572G>C promoter constructs in HeLa cells however have demonstrated that the IL-6 -
572G variant resulted in a significantly greater IL-6 difference in relative promoter activity than the -572C variant (personal communication). This suggests that there is cell-specific expression of the IL-6 -572G>C promoter variants, being different in hepatocytes compared to epithelial cells.

The mechanism for the higher expression levels of the -572C variant compared to the -572G may be due to very weak repression from a negative feedback mechanism involving corticosteroids as described in section 3.5.5. One possible way to test the involvement of glucocorticoid with the -572 variant would be to knockout the two putative GRE sites at positions -557 to -552 and -464 to -459 (see figure 3.3, chapter 3) and test whether this directly affected IL-6 expression levels in response to repression from DEX and induction by IL-1β plus DEX over time (see chapter 7).

4.5.3 IL-6 -597G/-174G and -597A/-174C expression in response to stimulus

The relative promoter strength for the IL-6 -597G/-174G and -597A/-174C constructs was investigated in HuH7 in response to repression by DEX and induction by IL-1β plus DEX over time. Higher IL-6 promoter expression of the -597A/-174C construct compared to the -597G/-174G construct was found in two time course experiments using the same IL-6 plasmid preparation. Due to insufficient time available, it was not possible to repeat this experiment using a second IL-6 plasmid preparation of the same constructs in HuH7 cells in order to confirm the findings from the first plasmid preparation.

The higher promoter activity for the -597A/-174C construct compared to the -597G/-174G construct in HuH7 cells is consistent with observations in HepG2 cells (see chapter 3) and also in vivo data from the CABG study where the -597A/-174C variant was associated with higher IL-6 levels (Brull et al 2001). This finding has also been confirmed in other studies (Jones et al 2001; Jenny et al 2002). However experiments carried out in HeLa cells in Professor Patricia Woo laboratory’s using the same IL-6 constructs have demonstrated that the -597G/-174G variant resulted in higher promoter expression than the -597A/-174C variant in response to stimuli such as DEX, IL-1β and IL-1β plus DEX.
(personal communication). These data confirm observations reported from some *in vivo* studies where higher IL-6 levels was associated with the -597G/-174G variant (Fishman et al 1998; Fernandez-Real et al 2000; Rauramaa et al 2000).

The precise molecular mechanism to explain the functionality of the IL-6 -597/-174 variants is still not known. It appears that the expression of the IL-6 -597/-174 promoter variants are different in different cell type, since in HeLa cells, the -597G/-174G variant was associated with higher promoter activity whereas in hepatocytes (HepG2 and HuH7) the 597A/-174C variant resulted in higher promoter activity. To elucidate whether the functional affect of the -597G/-174G and -597A/-174C constructs was the result of the variant at positions -597 or -174 in hepatocytes, constructs are needed whereby the allelic association between the -597 and -174 position are eliminated (see chapter 7).
CHAPTER 5

GENOTYPING AND ASSOCIATION STUDIES
Chapter 5: Genotyping and Association studies

5.1 Introduction and Aim

Elevated IL-6 levels have been implicated in the inflammatory processes that lead to the pathogenesis of coronary heart disease (CHD), such as atherosclerosis (Sturk et al. 1992; de Maat et al. 1996; Biasucci et al. 1996; Ross et al. 1993, 1999; Berk et al. 1990, Liuzzo et al. 1994; Ridker et al. 2000b). Functional variations identified (see chapter 2) in the human IL-6 promoter may alter the regulation and expression levels of IL-6 in vivo, and therefore would be important in predisposing to CHD risk. The aim of this work was to develop a method for high throughput genetic screening of the human IL-6 -174G>C and the -572G>C promoter polymorphisms and to look for in vivo associations in a population-based study (e.g. Northwick Park Heart Study II) between these IL-6 polymorphisms with risk markers of inflammation that predispose to CHD risk.

5.2 Materials

The following materials were purchased: 30% acrylamide-bisacrylamide (19:1 ratio) solution (Severn Biotech, UK); Taq DNA polymerase and dNTPs (Amersham Pharmacia Biotech, UK) and restriction endonucleases (NlaIII and Mbol) (New England Biolab, and Helena Biosciences, UK respectively). All other chemicals and solutions were obtained from Merck Ltd (Dorset, UK). See appendix for compositions to all solutions made or purchased.

5.3 Methods

5.3.1 Genotyping assays for IL-6 promoter polymorphisms

5.3.1.1 Genotyping of the IL-6 -174G>C promoter polymorphism

IL-6 Pro 9A (5'-TGACTTCAGCTTTACTCTTG-3') and IL-6 Pro 10A (5'-CTGATTGAAACCTTATTAAG-3') PCR primers (Fishman et al. 1998) were designed
for the DNA amplification (190bp DNA fragment) and genotyping of the IL-6 -174G>C polymorphism. PCR reactions were carried out in 1X NH₄ Polmix PCR reaction buffer using 50-100ng genomic DNA, 10pmol of each IL-6 Pro 9A and 10A primers, 2mM MgCl₂ and 0.35 units of Taq DNA polymerase in a final volume of 20µl. The DNA amplification reaction was performed in a 96 well MJ Tetrad PTC-225 peltier DNA thermocycler (MJ Research, USA) with an initiation temperature of 95°C for 4 minutes followed by 35 cycles of 95°C for 40 seconds, 50°C for 40 seconds and 72°C for 1 minute. One unit of the restriction endonuclease, NlaIII was then added to the PCR product in a final digestion volume of 25µl and incubated overnight at 37°C in accordance with the manufacturer’s instructions (New England Biolab, UK). 7µl of the PCR-digest was mixed with 2.5µl formamide loading dye (98% formamide, 10mmol/l EDTA, 0.025% xylene cyanole FF, 0.025% bromophenol blue) and 7µl loaded directly onto an 8% nondenaturing pre-stained ethidium bromide (10µl of 10mg/ml EtBr per 100ml 1X TBE buffer for 15 minutes) MADGE gel. The gel were electrophoresed at 150V for 45 minutes in 1X TBE buffer and the DNA bands was then visualized under UV light.

5.3.1.2 Genotyping of the IL-6 -572G>C promoter polymorphism

For DNA amplifications of the IL-6 -572G>C polymorphism, the PCR primers: IL-6 Pro 5b F (5’-GGAGACGCCTTGAAGTAACTGC-3’) and IL-6 Pro 6 R (5’-GAGTTTCTCTGACTCCATCGCAG-3’) were used to generate a DNA fragment of 163bp. PCR for the IL-6 -572G>C genotypes was carried out in 1X Polmix PCR reaction buffer using 50-100ng genomic DNA, 1.5mM MgCl₂, 3pmol of each IL-6 Pro 5b F and 6R and 0.35 unit of Taq DNA polymerase. The PCR reaction was performed in an MJ Tetrad PTC-225 peltier thermocycler (MJ Research, USA) with an initial denaturation temperature of 94°C for 4 minutes followed by 35 cycles of 95°C for 40 seconds, 55°C for 40 seconds and 72°C for 1 minute. The IL-6 -572G>C DNA fragments were resolved by the MADGE high throughput method described in section 5.3.1.1 at 150V for 20 minutes following restriction endonuclease digestions with 2 units of MbiI in a final volume of 25µl in accordance to manufacturer instructions (Helena Bioscience, UK).
5.3.2 Population based study

To investigate whether the polymorphisms described predisposed to risk of CHD, the previously described methods (section 5.3.1) were used to obtain the genotyping data from the Northwick Park Heart Study II (NPHSII) study. Two independent observers read all genotypes and any discrepancies were resolved by repeating the genotyping experiment.

5.3.2.1 Subjects in NPHSII

The NPHSII study is a prospective study of 3053 healthy UK men aged 50 to 60 years from nine general medical practices (Miller et al 1996; Seed et al 2001). All individuals were free from a history of unstable angina, MI or evidence of a silent infarction, coronary surgery, aspirin or anticoagulant therapy, cerebrovascular disease, malignancy (except skin cancer other than melanoma), or any condition precluding informed consent. Each participant attending was non-fasting and instructed to refrain from smoking or vigorous exercise beforehand. Data for smoking habits (Medical Research Council Committee on Chronic Bronchitis and Questionnaire on Respiratory Symptoms 1995) and alcohol consumption were obtained by questionnaire. A standard 12-lead electrocardiogram (ECG) was recorded and coded according to the Minnesota criteria (Prineas et al 1982) at baseline and after 5 years. Height (m) was measured on a stadiometer and weight (kg) on a balance scale to calculate body mass index (BMI) (kg/m²), Blood pressure measurement was done twice using a sphygmomanometer after the subject had been seated for 5 minutes and the average taken. To date, data is available from survivors who were recalled on an annual basis for measurements of lipid concentrations yearly for 5 years. Coronary heart disease events taken as end points were fatal (sudden or not) and non-fatal MI based on WHO criteria (WHO 1976). The clinical information for each event was submitted to independent assessors who assigned the event to the appropriate category.
5.3.2.2 Biochemical measurements

Whole blood was taken from each subject and serum was then extracted by centrifugation at 3,500rpm for 15 minutes. The serum (top layer) was transferred into 2 ml Nunc screw cap vials and stored at -20°C pending assay. Serum cholesterol and triglyceride concentrations were determined by automated enzyme procedures with reagent from Sigma (Dorset, UK) and Wako Chemicals (Alpha laboratories, UK) respectively. Fibrinogen measurement was made using the Clauss method (Clauss 1957). In a subset of the NPHSII (North Mymms and Parkstone practices plus a randomly selected group from the entire cohort) CRP was measured using the CRP ELISA HS kit (Kordia, UK), with an inter assay CV of 9.7% and intra assay CV of 8.5%.

5.3.2.3 IL-6 genotyping in NPHSII

Genotyping data for the IL-6 -174G>C polymorphisms were obtained for 2720 samples, and for the IL-6 -572G>C from 2614 samples using the protocol described in sections 5.3.1.1 and 5.3.1.2.

5.4 Statistical methods

All genotyping data for the IL-6 -174G>C and -572G>C polymorphisms were entered onto Microsoft Excel 2000 spreadsheet (Microsoft Corp, Seattle, USA) and tested for deviation from Hardy-Weinberg equilibrium by using a χ² test. Statistical analysis was performed using the STATA (Intercooled Stata 6.0, STATA Corp) package. Concentrations of serum triglyceride, fibrinogen, IL-6, C-reactive protein, systolic and diastolic blood pressure and body mass index (BMI) were log transformed. Transformations were necessary to ensure that data required for statistical analysis was not skewed. Log transformed variables are presented as geometric means (antilog of the mean of log transformed values), standard deviation (SD) or standard error of the mean (SEM) given are approximate. The associations between genotype and plasma key traits were tested by ANCOVA using log transformed data where appropriate. The interactions of genotypes and smoking status with CHD risk were assessed by COX proportional
hazard model and results presented as hazard ratios with 95% confidence intervals (CI) using the GG genotype as the reference. Interaction terms were included to test for differences in the smoking effects by genotype and data for never and ex-smokers were combined for analysis. The Kaplan-Meier plot was used in the NPHSII study to estimate for survival probability over time in men with the different IL-6 -174G>C genotypes after adjusting for clinic, BMI, systolic blood pressure, triglyceride and fibrinogen levels at baseline. A p-value of <0.05 was taken as statistically significant. All statistical analysis described was carried out by Ms Emma Hawe, the statistician in the Cardiovascular Genetics group.

5.5 Results

5.5.1 IL-6 -174G>C and -572G>C genotypes

The MADGE high throughput method (Day et al 1995) described in section 5.3.1 was used for the genotyping of the IL-6 -174G>C and -572G>C promoter polymorphisms for subjects in the NPHSII study. An internal positive and negative control for both polymorphisms was used to eliminate the possibility of false results. For the -174G>C genotype, the rare -174C allele introduced (143/47bp), whereas the wild-type -174G allele destroys the restriction site for NlaIII (or Hsp92II). The DNA fragments of interests are the 190bp (G allele) and the 143bp (C allele) (figure 5.1), the 47bp fragment was too small to be detected on the MADGE gel system. In the case of the -572G>C genotype, the rare -572C allele destroyed the MboII recognition site (163bp), compared to the -572G allele which was cleaved to give 101/62bp fragments (figure 5.2).
Figure 5.1 IL-6 -174G>C promoter polymorphism genotype. Electrophoresis of samples plus the internal positive and negative controls were carried out at 150V for 45 minutes on the MADGE gel system. The -174C allele introduced an NlaIII restriction cleavage site (143/47bp) and the wild type G allele destroyed this site (190bp).

Figure 5.2 IL-6 -572G>C promoter polymorphism genotype. Samples plus internal positive and negative controls were electrophoresed at 150V for 20 minutes on the MADGE gel. The -572G allele introduced the naturally occurring MboI restriction cleavage site (101/62bp) and the -572C allele destroyed this site (163bp).
5.5.2 NPHSII studies

5.5.2.1 Risk characteristics of the sample

Genotyping for the IL-6 -174G>C and -572G>C promoter polymorphisms were carried out on all the NPHSII samples. The baseline characteristics for these healthy men with or without subsequent coronary heart disease event are shown in table 5.1. Compared with the event free (controls) group, those with coronary events had significantly higher mean BMI, systolic and diastolic blood pressure and higher concentration of plasma of fibrinogen, cholesterol and triglycerides. In those who had an event, 40.1% were smokers as compared with 27.5% of those who remained free of an event.

5.5.2.2 Genotype distribution in controls and cases

The genotype distributions and allele frequencies are shown in table 5.2. Genotype distributions in the non-event group were as expected for a sample in Hardy-Weinberg equilibrium for both polymorphisms. The frequency of the -174C allele was 0.43 (95% CI=0.42-0.44), and 0.05 (95% CI=0.04-0.06) for the -572C allele. For the -174G>C polymorphism, the genotype distribution in the event group was significantly different from Hardy-Weinberg proportions (p=0.01), and from the frequency distribution in the non-event group (p=0.05), although the frequency of the -174C allele (0.45, 95% CI=0.40-0.51) was not different from the non-event group. Compared to subjects with the -174GG genotype, the relative risk for coronary heart disease event for GC heterozygotes was 1.54 (95% CI=1.06-2.22), while for CC homozygotes this was 1.11 (95% CI=0.67-1.83). After adjusting for classical risk factors including systolic blood pressure, this remained substantially unaltered (1.55, 95% CI=1.06-2.25 and 1.07, 95% CI=0.65-1.77 respectively). The event rate of men by IL-6 -174G>C genotype is presented as a Kaplan-Meier survival probability plot (figure 5.3), demonstrating a reduced survival rate in men with the GC genotype as compared to the GG and CC groups. When subjects were divided into never and current+ex smokers combined, there was no significant evidence for genotype-smoking interactions with respect to risk (p=0.44). However, inspection of the risk estimate presented in figure 5.4, revealed higher risk in men with the GC
genotype was confined mostly to the smokers (compared to GG non-smokers, RR=2.66, 95% CI=1.64-4.32). As shown in table 5.2, the -572G>C polymorphism was not associated with a significant effect on coronary heart disease risk (compared to GG, hazard ratio for GC+CC=1.33, 95% CI=0.83-2.15).

Table 5.1 Mean (±SD) baseline characteristics by coronary heart disease event status in NPHSII.

<table>
<thead>
<tr>
<th>Risk Factors</th>
<th>Controls [n=2589]</th>
<th>Coronary heart disease Event [n=162]</th>
<th>p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>56.0 (3.4)</td>
<td>56.7 (3.6)</td>
<td>0.02</td>
</tr>
<tr>
<td>Body Mass Index (kg/m²)*</td>
<td>26.2 (3.4)</td>
<td>26.9 (3.4)</td>
<td>0.0074</td>
</tr>
<tr>
<td>Systolic Blood Pressure (mmHg)*</td>
<td>136.7 (18.6)</td>
<td>143.0 (20.3)</td>
<td>&lt;0.00005</td>
</tr>
<tr>
<td>Diastolic Blood Pressure (mmHg)</td>
<td>83.6 (11.3)</td>
<td>87.4 (11.3)</td>
<td>&lt;0.00005</td>
</tr>
<tr>
<td>Current smokers</td>
<td>27.5%</td>
<td>40.1%</td>
<td>0.001†</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>5.71 (1.0)</td>
<td>6.14 (1.07)</td>
<td>&lt;0.00005</td>
</tr>
<tr>
<td>Triglyceride (mmol/l)*</td>
<td>1.78 (0.94)</td>
<td>2.04 (1.07)</td>
<td>0.0005</td>
</tr>
<tr>
<td>Fibrinogen (g/l)*</td>
<td>2.70 (0.51)</td>
<td>2.85 (0.50)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

*For triglyceride, blood pressure measures, body mass index and fibrinogen means are geometric (approx SD)

Table 5.2 Carrier frequencies and means (±SD) of baseline characteristics by coronary heart disease event status in NPHSII

<table>
<thead>
<tr>
<th>Risk Factors</th>
<th>Controls [n=2589]</th>
<th>Coronary heart disease Event [n=162]</th>
<th>p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 -174G&gt;C genotype (GG/GC/CC)</td>
<td>827/1263/470</td>
<td>40/95/25</td>
<td>0.05†</td>
</tr>
<tr>
<td>IL-6 -174C freq. (95% CI)</td>
<td>0.43 (0.42-0.44)</td>
<td>0.45 (0.40-0.51)</td>
<td>0.32†</td>
</tr>
<tr>
<td>IL-6 -572G&gt;C genotype (GG/GC/CC)</td>
<td>2224/225/9</td>
<td>135/19/0</td>
<td>0.63†</td>
</tr>
<tr>
<td>IL-6 -572C freq. (95% CI)</td>
<td>0.05 (0.04-0.06)</td>
<td>0.06 (0.03-0.09)</td>
<td>0.63†</td>
</tr>
</tbody>
</table>

† p value from χ² test
Figure 5.3 Graph of the estimated survivor functions from the Cox’s proportional hazard model stratified by IL-6 -174G>C genotype. Adjustment has been made for clinic, BMI, systolic blood pressure, triglyceride and fibrinogen levels at baseline.

Figure 5.4 Hazard ratios (95% CI) associated with IL-6 genotypes in non-smokers and smokers after adjusting for age, clinic, BMI, systolic blood pressure, cholesterol, triglyceride and fibrinogen levels at baseline. Genotypes are shown with numbers of individuals in each group.
5.5.2.3 Genotypes and inflammatory risk markers

Analysis of baseline fibrinogen, systolic and diastolic blood pressure by IL-6 genotypes is shown in table 5.3. The IL-6 -174G>C promoter polymorphism was associated with a significant effect on baseline systolic (p=0.007) and diastolic (p=0.004) blood pressure, with the CC individuals having a mean systolic blood pressure approximately 2.5mmHg higher than the GG individuals. This polymorphism was not associated with a significant effect on fibrinogen or C-reactive protein levels. The IL-6 -572G>C polymorphism was not associated with a significant effect on blood pressure, fibrinogen or C-reactive protein levels.

To extend the analysis of the effect of IL-6 -174G>C polymorphism, data were analysed in current smokers compared to the group of ex+never smokers combined, and are presented in figure 5.5. It can be seen that the raising effect on blood pressure with the IL-6 -174C allele was of similar magnitude in both groups (genotype-smoking interaction, p=0.74), although the effect was only statistically significant in the group of nonsmokers (p=0.02). Fibrinogen levels were significantly higher in smokers compared to nonsmokers, but there was no significant evidence of an effect of the IL-6 -174G>C genotype on levels in either group. Since IL-6 is known to be secreted by adipose tissue (Mohamed-Ali et al 1997; Fried et al 1998), analysis was carried out to explore if the IL-6 -174C raising effect on systolic blood pressure was similar in lean and obese men. The relationship between IL-6 -174G>C genotype and systolic blood pressure with BMI tertiles is presented in figure 5.6. The raising effect of systolic blood pressure with IL-6 -174C allele was smaller in men in the lowest tertile of BMI (GC+CC 1.4 mmHg higher than GG, p=0.29) than in men in the middle or top tertile (2.9 mmHg, p=0.02 and 2.6 mmHg, p=0.05, respectively), but this interaction was not statistically significant (p>0.4). A similar effect was also seen for tertile BMI and diastolic blood pressure with -174G>C genotype, but again the interaction was not statistically significant (figure 5.7).
5.5.2.4 CRP, blood pressure and genotype

Plasma concentration of CRP was available from only 564 men and its correlations with coronary heart disease risk factors are shown in table 5.4. It can be seen that CRP levels showed a significant correlation with BMI, fibrinogen, triglyceride and systolic pressure. A weaker correlation with age and diastolic blood pressure, and no correlation with cholesterol were seen. Mean CRP levels were significantly higher (p<0.00005) in current smokers compared to never and ex-smokers combined (1.78 mg/l, 95% CI=1.50-2.11 vs 1.07 mg/l, 95% CI=0.97=1.19). In the group of men where CRP data were available, those with the IL-6 -174C allele had significantly higher systolic blood pressure (p=0.0017) (table 5.5). Although there was significant evidence of a relationship between CRP and systolic blood pressure (p=0.01), after adjusting for BMI, age, smoking and clinical practices, this effect was no longer statistically significant (p=0.17). However, after adjusting for CRP, there remained significant association between systolic blood pressure and genotype (p=0.04).
Table 5.3 Mean (±SEM) for fibrinogen, systolic and diastolic blood pressure and C-reactive protein in NPHS II men.

<table>
<thead>
<tr>
<th>Traits</th>
<th>-174 G&gt;C</th>
<th></th>
<th></th>
<th></th>
<th>-572 G&gt;C</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen (g/l)</td>
<td>2.71 (2.67-2.74) [863]</td>
<td>2.71 (2.69-2.74) [1349]</td>
<td>2.70 (2.66-2.75) [493]</td>
<td>0.92</td>
<td>2.70 (2.68-2.73) [2347]</td>
<td>2.74 (2.67-2.81) [244]</td>
<td>2.61 (2.17-3.14) [9]</td>
<td>0.56</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>135.5 (134.3-136.7) [865]</td>
<td>137.9 (136.9-138.9) [1358]</td>
<td>138.0 (136.3-139.8) [495]</td>
<td>0.007</td>
<td>137.0 (136.3-147.3) [2357]</td>
<td>136.8 (134.6-139.0) [244]</td>
<td>136.7 (118.2-158.0) [9]</td>
<td>0.97</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>82.8 (82.1-83.6) [865]</td>
<td>84.2 (83.6-84.8) [1358]</td>
<td>84.7 (83.6-85.7) [495]</td>
<td>0.004</td>
<td>83.8 (83.3-84.2) [2357]</td>
<td>83.5 (82.0-84.9) [244]</td>
<td>82.6 (74.7-91.3) [9]</td>
<td>0.87</td>
</tr>
<tr>
<td>C-reactive protein</td>
<td>1.14 (0.96-1.36) [170]</td>
<td>1.26 (1.10-1.44) [244]</td>
<td>1.43 (1.12-1.83) [80]</td>
<td>0.31*</td>
<td>1.24 (1.12-1.38) [436]</td>
<td>1.29 (0.87-1.91) [38]</td>
<td>2.13 (0.24-1.91) [3]</td>
<td>0.69</td>
</tr>
</tbody>
</table>

*p values from ANCOVA after adjustment for age, smoking habit and BMI. * P value for linear trend =0.13
Figure 5.5 Relationship between -174G>C genotype and (a) systolic blood pressure, (b) diastolic blood pressure and (c) fibrinogen levels at baseline in smokers and nonsmokers (never+ex). P values represent differences between the genotypes.
Figure 5.6 Relationship between systolic blood pressure and -174G>C genotype men in different tertiles of BMI. For statistical analysis data from GC and CC groups were combined.

![Systolic Blood Pressure Distribution](image)

Figure 5.7 Relationship between diastolic blood pressure and IL-6 -174G>C genotype men in different tertile of BMI.

![Diastolic Blood Pressure Distribution](image)
Table 5.4 Correlation between CRP (C-reactive protein) and coronary heart disease risk factors in 564 NPHSII men.

<table>
<thead>
<tr>
<th>CRP*</th>
<th>Age</th>
<th>BMI*</th>
<th>Fibrinogen*</th>
<th>Systolic BP*</th>
<th>Diastolic BP*</th>
<th>Triglyceride*</th>
<th>Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.08</td>
<td>0.22</td>
<td>0.44</td>
<td>0.10</td>
<td>0.07</td>
<td>0.16</td>
<td>0.04</td>
</tr>
<tr>
<td>p-values</td>
<td>0.06</td>
<td>&lt;0.00005</td>
<td>&lt;0.00005</td>
<td>0.01</td>
<td>0.09</td>
<td>0.0001</td>
<td>0.40</td>
</tr>
</tbody>
</table>

*Correlations relate to log transformed data.

Table 5.5 Mean (±SEM) CRP and systolic blood pressure in men with different IL-6 -174G>C genotype.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>CRP (mg/l)* [n]</th>
<th>Systolic BP (mmHg)* [n]</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>1.14 (0.96-1.36) [170]</td>
<td>135.5 (134.3-136.7) [865]</td>
</tr>
<tr>
<td>GC+CC</td>
<td>1.30 (1.16-1.46) [324]</td>
<td>137.9 (137.0-138.8) [1853]</td>
</tr>
</tbody>
</table>

p-values | 0.21 | 0.0017 |

Means are geometric (SEM are approximate).

*Analysis on log transformed data.

CRP data were available from only 494 men.
5.6 Discussion

5.6.1 Single nucleotide polymorphism (SNP) genotyping

The major strategy for high throughput genotyping SNPs such as the IL-6 -174G>C and -572G>C in our laboratory is based on RFLP (Botstein et al 1980). Firstly, PCR amplification of the target sequences containing the IL-6 SNPs are carried out and this is followed by restriction endonuclease digestion (e.g. NlaIII or Mbol), and resolution of DNA fragments on the MADGE gel system (Day et al 1995). This technique had been demonstrated to be a rapid and robust method for genotype the IL-6 polymorphisms described. The advantages of the MADGE gel are that DNA fragments from 96 samples can be loaded rapidly using a multichannel pipette. This takes less than 5 minutes and reduces the possibility of mistakes made in the loading process. Electrophoresis run time is relatively short (20-40 minutes) due to small fragment size, and gels can be stacked on top of one another (5 gels) in a conventional horizontal gel tank after briefly running the sample into the polyacrylamide gel. The main disadvantage of the MADGE gel system is the time consuming processes in gel preparations and staining prior to a high throughput electrophoresis runs (i.e. >10 gels). To overcome this, MADGE gels can be made, stained with EtBr in 1X TBE and stored in the dark at room temperature 1-2 weeks in advance prior to electrophoresis of samples. The accuracy of the PCR-RFLP-MADGE genotyping method for screening IL-6 polymorphisms in a population study such as the NPHSU is estimated to be >99% provided that positive and negative controls are present in the same 96 well plate as samples being genotype from DNA amplification, restriction digestion and electrophoresis of DNA fragment on MADGE gel.

Other strategies for genotyping SNPs include heteroduplex analysis (Gayther et al 1996; Prior et al 1994; Abernathy et al 1997), denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) (Myers et al 1987; Cariello and Skopek 1993; Riesner et al 1992; Henco et al 1994). However, with the recent explosion of information on SNPs (The International SNPs Map Working Group 2001), there is a growing awareness for a more robust, automatable and scaleable SNPs genotyping techniques. These newly devised strategies for SNPs scoring are derived from
techniques either involving post-PCR enzymatic steps or allele-specific oligonucleotide hybridization (ASOH) (Stoneking et al 1991). Examples of this include high-density chip arrays for ASOH (Yershov et al 1996), the 5’ nuclease (TaqMan) assay (Livak et al 1995), the template directed dye terminator incorporation (TDI) assay (Chen et al 1997, 1999a, 1999b), the molecular beacon ASOH assay (Tyagi and Kramer 1996), single base extension (Syvanen et al 1990) and the real time pyrophosphate sequencing (Ahmadian et al 2000).

PCR based RFLP technique is extensively used in genetic epidemiology and is considered to be a very reliable method. In fact, it is the workhorse of all laboratories involved in large-scale molecular genetics epidemiological research into genetics variation, such as bi-allelic polymorphisms within gene, which is believed to underpin genetic susceptibilities to many common diseases.

There are no generally accepted procedures for quality control of the genotyping procedure, except for the individual practices put in place in each laboratory. In the cardiovascular genetics (CVG) department, these include double checking data entry, and repeat of PCR on the whole plate rather than individual samples in case of dropouts. In fact, several times that the PCR/RFLP protocols were repeated for any plate where dropout were observed for all of the IL-6 polymorphisms genotyped, the genotypes assigned never changed, thereby validating the accuracy of the genotyping procedure. The highly standardised procedures used in Professor Steve Humphries laboratory ensure that errors from incorrect interpretation of RFLP results and data handling for the genotyping of IL-6 -174G>C and -572G>C polymorphism in large scale population studies such as the NPHSII are minimised, if not completely eliminated. Further reduction in mis-genotyping was achieved by the use of two independent RFLP assays for the same polymorphism, followed by comparison of the results and further testing of any discrepancies.
5.6.2 IL-6 genotype and risk traits

5.6.2.1 NPHSII study

The NPHSII study was used to investigate the hypothesis that variations in the promoter of the IL-6 gene (i.e. -174G>C and -572G>C) may influence plasma IL-6 or tissue concentration, thus affecting plasma levels of CHD risks factors and CHD risk itself. The advantage of the NPHSII study is that it is a prospective study of GP-based UK Caucasian healthy middle-aged men. This study was designed to look at late onset of CHD with measurements of coagulation factors (Miller et al 1996; 1997) and 5 years of follow up where lipid measurements were also made. However, the limitation of this study is the limited number of events to date (162) and thus low power. CHD events in this study are defined as fatal and non fatal MI, CABG, and ‘silent ischaemia’ detected by ECG changes from baseline to the 5 year follow up visit. It is therefore possible that different processes underlie these types of events.

5.6.2.2 IL-6 -572G>C genotype

The IL-6 -572G>C promoter polymorphism described in chapter 3 and by Terry et al 2000 was investigated in the NPHSII study for association with plasma levels of CHD risk factors and CHD itself. No significant association between the IL-572G>C genotype with any CHD risk traits. Since the frequency for the rare -572C allele is low; this sample only had power to detect a 1.9 fold greater risk in carriers. The results from this study imply that this polymorphism has negligible or no effect on CHD risk traits in healthy middle-aged men. However, in vitro data in HeLa cells from Terry et al 2000 where the -572C allele was demonstrated to have no major impact on reporter expression levels compared to the -572G allele either untreated and treated with IL-1. Although there were no significant effect of the -572G>C polymorphism with any of the risk traits in this study, Brull et al 2001 recently demonstrated, using the coronary artery bypass graft surgery (CABG) study as a model for acute inflammation that, individuals that are carriers of the -572C allele had significantly higher plasma IL-6 levels 6 hours post-CABG compared to those homozygous for the GG allele. This suggests that the effect of
the -572G>C genotype on CHD risk traits may only be seen in an acute inflammatory scenario. These men in the NPHSII study were healthy when measurements of CHD risk traits were made, and they were made months or even years before CHD events had occurred. Therefore one can speculate that any CHD risk traits differences between -572GG subjects compared to carriers of the -572C allele are subtle, and may only be detected under acute inflammation. It is possible to speculate that the mechanism for higher expression levels of the -572C allele compared to the -572G allele may be due to an alteration in the binding affinity of glucocorticoids and estrogen receptors for the GRE sites at -557 to -552 and -464 to -459 in response to stimuli. If the GRE sites are important in the expression of IL-6, then the effect of the -572G>C genotype may differ in women compared to men because of hormonal differences or in response to infections. This needs to be investigated in a large prospective population-based study of healthy women.

5.6.2.3 IL-6 -174G>C genotype

The IL-6 -174G>C promoter polymorphism reported by Fishman et al 1998 to be associated with IL-6 expression levels in HeLa cells under acute stimulations with LPS or IL-1 was investigated in healthy middle-aged men from the NPHSII study to examine the relationship between this genotype and plasma levels of CHD risk factors and CHD risk. The major novel finding in the NPHSII study was the association of blood pressure with IL-6 -174G>C genotype. A 2.4-2.5mmHg higher mean systolic blood pressure was seen in healthy men homozygous for the -174C allele compared to subjects' homozygotes for the -174G allele. This effect, although modest, appeared to be robust in the smokers and nonsmokers, in the middle and top BMI tertile and was seen over the 5 years of follow up. Elevated systolic blood pressure has been shown to be a major risk factor for CHD in the NPHSII study, where a 1mmHg systolic blood pressure was estimated to result in a 2% increase in the risk for MI (Seed et al 2001). The size of the risk estimate was similar to other studies (Kannel 2000; Vaccarino et al 2001; Fernandex-Real et al 2001; Chae et al 2001) and suggests that healthy men in the NPHSII study with the CC allele would
have approximately 5% greater risk of having an MI than those homozygous for the G allele.

The second finding of this study was that smokers that are carriers for the -174C allele had a relative risk for CHD of approximately 2.7 fold greater than nonsmokers with the GG genotype. This risk effect was essentially unaltered after adjusting for classical risk factors such as age, BMI, systolic blood pressure, cholesterol, triglyceride and fibrinogen levels at baseline. This effect on risk however must be interpreted with caution. The number of events in this study is relatively small (n=162) and the confidence interval on the risk estimate is large. Power calculations indicates that this study only has the power to detect a relative risk of 1.6 in carriers of the -174C allele and 2.2 in smokers who represent 28% of the sample size. The relative risk in individuals homozygous for the -174C allele was smaller than in those with the GC alleles although the confidence intervals overlap, thus the pattern of risk is unlikely to be acting through co-dominant model predicted for IL-6 genotype on plasma IL-6 levels. A higher risk of CHD mortality for carriers of the -174C allele was also observed in the Small Aneurysm Trial of abdominal aortic aneurysms subjects (Jones et al 2001), the CABG study (Brull et al 2001), Etude Cas-Temoin de l'Infarctus du Myocarde (ECTIM) (Georges et al 2001) and the MONICA-Augsburg Cohort study (personal communication, data not published). Table 5.6 demonstrates CHD risks by -174 GC+CC vs GG genotype in population studies to date. It can be seen that the -174C allele was associated with CHD risk compared to -174GG homozygotes allele in all studies. Since the confidence interval (CI) for GC+CC for all studies overlap, there is no significant heterogeneity in CHD risk between the NPHSII and the other studies. Systolic blood pressure were higher in individuals with the -174GC+CC genotypes compared to those with GG genotype over the 5-years follow-up, reaching significant in years 1, 2, 4 and 5 (see figure 5.8).
Table 5.6 CHD risks of IL-6 -174 GC+CC vs GG genotypes in population studies.

<table>
<thead>
<tr>
<th>Studies</th>
<th>CHD Risk (-174 GC+CC vs GG)</th>
<th>p-values</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPHSII</td>
<td>RR 1.54 (95% CI=1.00-2.23)</td>
<td>0.05</td>
<td>Humphries et al 2001</td>
</tr>
<tr>
<td>AAA</td>
<td>OR 1.93 (95% CI=0.91-4.09)</td>
<td>0.09</td>
<td>Jones et al 2001</td>
</tr>
<tr>
<td>ECTIM</td>
<td>OR 1.33 (95% CI=1.04-1.69)</td>
<td>0.03</td>
<td>Georges et al 2001</td>
</tr>
<tr>
<td>CHS</td>
<td>OR 1.54 (95% CI=1.08-2.19)</td>
<td>0.02</td>
<td>Jenny et al 2001</td>
</tr>
</tbody>
</table>

Northwick Park Heart Study II (NPHSII)
Abdominal aortic aneurysms (AAA)
Etude Cas-Témoin de l'Infarctus du Myocarde (ECTIM)
Cardiovascular Health Study (CHS)

Figure 5.8 Systolic blood pressures in healthy men at baseline and 5 years follow up

5.6.3 *IL-6 -174G>C and in vitro reporter gene expression*

The data presented here in the NPHSII study appear to be contradictory to the earlier finding in 102 healthy men and women from the UK, where GG homozygotes had approximately 2 fold higher circulating IL-6 concentration compared to homozygotes for
the -174C allele (Fishman et al 1998). The same study also demonstrated using a luciferase reporter gene assay in HeLa cells, that the IL-6 -174G construct had significantly higher expression compared to the -174C construct when cells were stimulated with IL-1 or LPS. This finding has been confirmed in recent work from Professor Woo laboratory’s (personal communication, data unpublished) using the same constructs and cell line as reported by Fishman et al 1998 and with constructs of a larger human IL-6 promoter fragment (-1180bp to +13bp) (Yasukawa et al 1987). However, data in HeLa’s and ECV304 cells support the -174C allele as having higher promoter expression than the -174G allele after these cells were stimulated with IL-1 or IL-1 plus dexamethasone (Terry et al 2000). Transient transfection experiments carried out in our laboratory (data unpublished) using HepG2 and HuH7 cells (see chapters 3 and 4) and the full length constructs taking into account the allelic association at position -597 also demonstrate the -174C allele as having higher promoter expression than the -174G allele after stimulation with IL-1β plus dexamethasone. Overall, evidence from in vivo and in vitro experiments support the -174C allele as having higher IL-6 expression levels than the -174G allele. Although one cannot rule out the possibility that the promoter strength of the -174G and -174C constructs might be cell-specific due to the regulation complexity of IL-6 by many factors that are known to switch on and off transcription such as IL-1, LPS, glucocorticoids and estrogen (17-β Estradiol) (Shalaby et al 1989; Nordan and Potter 1986; Fried et al 1998; Pottratz et al 1995; Ray et al 1997). IL-6 itself can also upregulate the expression of gene such as suppressor of cytokine signaling (SOCS) (Hilton 1999; Krebs and Hilton 2000; Starr et al 1997; Nicholson et al 1999) which then act by negative feedback inhibition on its own signal transduction cascade via the JAK-STAT pathway (Heinrich et al 1998). It is worth noting that the IL-6 promoter constructs used in reporter gene assay in Professor Humphries laboratory’s (and others) would lack other important regulatory sequence that exist further upstream from the start site of transcription which maybe in allelic association with the -174 variants. Thus the data from functional in vitro reporter gene assays for the -174G and -174C variants are only an approximation to that expected in vivo. It is possible that the -174C allele having higher promoter expression maybe due to the longer lasting effect from an acute inflammatory response by the stimulation of IL-1 compared to the -174G allele.
5.6.4 IL-6 -174G>C mechanism of effects on CRP, fibrinogen and blood pressure

5.6.4.1 CRP and fibrinogen

In the NPHSII study, CRP levels were only available on a sub-sample out of a total of ~3000 healthy men. Although there was a significant positive correlation between CRP levels and systolic blood pressure, no significant association between CRP levels with either of the two IL-6 promoter polymorphisms was observed. This implies that the effect of genotype on blood pressure is unlikely to be acting through systemic inflammation which would be seen as elevated CRP levels, but rather the production and effect of IL-6 is through local cells that are present in the vessel wall which determining blood flow. The trend for association between the two IL-6 polymorphisms with CRP levels (not significant), but not with fibrinogen levels suggest that CRP gene expression may be more sensitive to changes in IL-6 levels than fibrinogen gene expression. This may be explained by the fact that the promoter for the CRP gene contains both type-1 and type-2 IL-6 responsive elements through which IL-6 can exert its signal transduction in the induction of acute phase gene expression (Akira et al 1995) while the fibrinogen promoter contains type-2 elements (Akira et al 1995). It is also possible that men in this study could be taking aspirin which would have a direct affect as an anti-inflammatory and anti-platelets aggregation drug on lowering fibrinogen level and inflammation as a whole (Pickart and Thaler 1990) regardless of IL-6 levels or genotype. Data from the Cardiovascular Health Study (Jenny et al 2002, in press) showed the subjects that are carriers of the IL-6 -174C allele was associated with higher values for CRP (11% higher, p=0.02) and fibrinogen (3% higher, p=0.02). Other studies, however, have not found associations between IL-6 -174G>C genotype with plasma concentrations for fibrinogen or CRP (Burzotta et al 2001; Margaglione et al 2001; Jones et al 2001).

5.6.4.2 Blood pressure

There are numerous possible mechanisms by which IL-6 could affect blood pressure (see figure 5.9). Several cells are known to secrete IL-6 including monocytes and macrophages, T- and B-cells, fibroblasts, bone marrow stromal cells, endothelial cells,
hepatocytes, smooth muscle cells and adipose tissues (Kishimoto 1989; Rus et al. 1996; Keller et al. 1996; Mohamed-Ali et al. 1997; Fried et al. 1998). Of these, subcutaneous adipose tissue appeared to be a major secretor of circulating IL-6 in healthy men and women, accounting for up to ~30% of the net plasma IL-6 concentration which increases with adiposity (Mohamed-Ali et al. 1997). BMI was also shown to correlate positively with IL-6 concentration and blood pressure (Mohamed-Ali et al. 1997; Iwai et al. 1995). This support our finding that the raising effect associated with the IL-6 -174C allele on systolic blood pressure was found only in men in the middle and top tertile for BMI but not in the lower tertile of BMI. Therefore increasing amount of adipose tissue (high BMI) will lead to high plasma circulating IL-6 levels and thus prolonging the inflammatory processes through a genetic-specific manner.

A second mechanism would involve the renin-angiotensin cascade via the effect on expression of angiotensinogen. Angiotensinogen is an acute phase protein and its plasma concentration increases upon stimulation by IL-6 (Itoh et al. 1989; Ohtani et al. 1992; Takano et al. 1993; Sherman and Brasier 2001), leading to higher concentrations of angiotensin II which is a potent vasoconstrictor of arterial walls. In addition to causing hypertension, it can contribute to atherogenesis by stimulating smooth muscle contraction and growth, and the formation of free radicals that promote the oxidation and deposition of low density lipoprotein (LDL) (Chobanian and Dzau 1996; Griendling and Alexander 1997; Lacy et al. 1998; Swei et al. 1997) in the area of inflammation. Angiotensin II can also stimulate further IL-6 production from vascular smooth muscle cell through pleiotropic activation of nuclear factor-kappa B transcription factor (Funakoshi et al. 1999; Han et al. 1999) thereby prolonging the inflammatory processes. Levels of angiotensinogen have been positively correlated with blood pressure in subjects with a family history of hypertension (Kataoka et al. 1996; Schorr et al. 1998; Taittonen et al. 1999), and polymorphisms in the angiotensinogen gene have been reported to affect blood pressure in some, but not all studies (Gharavi et al. 1997; Hegele et al. 1994, 1997, 1998; Hingorani et al. 1996; Ishigani et al. 1999; Winklemann et al. 1999; Bigda et al. 1997; Niu et al. 1998; Niu et al. 1999). Unfortunately, in the NPHSII study, levels of
angiotensinogen and its products, angiotensin I and angiotensin II were not available to test the effect on blood pressure with IL-6 genotypes.

A third possible mechanism would be through connective tissue remodelling of the vessels wall by IL-6. IL-6 has been reported to be involve in the increase of vessel wall collagen synthesis (Choi et al 1994; Greenwel 1995; Duncan and Berman 1991) and the reduce degradation of collagen by matrix metalloproteinase (MMPs) via tissue inhibitor of metalloproteinase (TIMPs) (Sato et al 1990; Silacci et al 1998; Roeb et al 1995). It is therefore possible that over time, the remodelling of vessel wall as a results of high levels of IL-6 (either systemic or locally at the site of atherosclerosis) would effect vascular compliance and therefore blood pressure.

Given that the IL-6 -174C allele appears to be associated with higher plasma IL-6 levels (Jones et al 2001; Brull et al 2001; Jenny et al 2002), and of CRP (this study; Jenny et al 2002). A fourth possible mechanism involving IL-6 would be through the induction of acute phase proteins synthesis such as fibrinogen which promote increase blood viscosity (Lowe and Rumley 1999; Woodward et al 1999). Elevated plasma fibrinogen levels have been shown to promote increases in plasma viscosity and red blood cell aggregation, hence whole blood viscosity and as a result higher blood pressure (Woodward et al 1999; Ernst et al 1988a; 1988b). However, this mechanism is unlikely because there was no effect of the two IL-6 polymorphisms with fibrinogen levels in the NPHSII study.

5.6.5 IL-6 -174G>C mechanism of effect on CHD risk

It is very likely that the pathogenic processes that lead to CHD such as atherosclerosis have already been well established in these middle aged men from the NPHSII study. However, factors that determine rapid plaque progression and plaque rupture are of critical importance in determining the timing of CHD events. The inflammatory process resulting in elevated IL-6 concentrations in vivo at the site of injury on the vessel wall could promote plaque growth and rupture by increasing expression of several key genes that are important in tissue remodeling. Immunohistochemical staining of the human
arterial atherosclerotic wall has found high levels of IL-6 mRNA expression in areas around the fibrous plaque compared to the intima or media (Rus et al. 1996). Studies in ApoE knockout out mice, which develop atherosclerotic plaque in the aorta, also show that elevated IL-6 mRNA expression was found predominantly in the plaque area (Sukovich et al. 1998). In addition, the upregulation of inhibitors of matrix metalloproteinases such as TIMP-1 by IL-6 (Roeb et al. 1994) could shift the balance of matrix tissue remodeling in favour of matrix deposition, thereby favoring plaque formation and progression of atherosclerosis leading to plaque rupture.

Cigarette smoking is known to cause endothelial dysfunction by favoring the formation of free radicals and modified LDL particles, and also the recruitment of monocytes-derived macrophages (Griendling and Alexander 1997; Lacy et al. 1998; Jonasson et al. 1986; van der Waal et al. 1989). Smoking has been shown to be associated with increased plasma levels of inflammatory markers such as IL-6, CRP and fibrinogen (Ridker et al. 2000a; 2000b; Woodward et al. 1991; 1999), therefore inducing the endothelium to have procoagulant instead of anticoagulant properties, and to form vasoactive molecules, cytokines and growth factors (Hirano et al. 1986; Sehgal et al. 1989; 1990a; 1990b) which then further aggravate injury to the vascular wall. One can speculate that the association between IL-6 -174C allele with increased CHD risk compared to those with the -174G allele may be from a greater response over time to the inflammatory effect of smoking. Overall, this data support the role of inflammation in the development of CHD.

Although there was an affect of the -174G>C genotype on blood pressure and CHD risk in these healthy middle aged men, we need to confirm this observation in another large prospective study of healthy men or in a case-control study. It is important to note that observations made here only refer to male subjects, and since IL-6 levels are lower in women as a results of the downregulation by estrogen (17-β-estradiol) (Pottratz et al. 1994), there may be additional negative feedback loop affecting circulating IL-6 in women. At present, all the evidence seem to indicate the link between IL-6 levels with blood pressure (i.e. systolic and diastolic) plus pulse and mean arterial pressure only in healthy men but not in women (Femandex-Real et al. 2001; Chae et al. 2001).
Unfortunately, due to the design nature and how the samples from the NPHSII study were gathered, it was impossible to obtain accurate and reliable plasma IL-6 concentrations in these healthy men to study possible effect with IL-6 genotype and blood pressure. Therefore, further *in vivo* and *in vitro* studies are required to examine the molecular mechanisms of these effects.
Figure 5.9 Purposed mechanisms for the involvement of IL-6 on blood pressure.

- Dietary lipids
  - Adipose tissue
  - IL-6

- Infections
  - Monocytes, macrophages, T- and B-cells
  - CRP
  - Liver
    - Angiotensinogen
    - Renin
    - Angiotensin I
    - ACE
    - Angiotensin II

- Heart
  - Smooth muscle cells contraction
  - Blood pressure

- Kidney
  - Salt retention

- Lumen of Artery
  - Collagen production and artery wall stiffness
  - Vascular smooth muscle cells growth
  - Vasoconstriction
  - Blood pressure
CHAPTER 6

IL-6 POLYMORPHISMS AND CORONARY HEART DISEASE (CHD) RISK IN THE HIFMECH STUDY
Chapter 6: IL-6 polymorphisms and Coronary heart disease (CHD) risk in the HIFMECH study

6.1 Introduction and aims

Several studies have directly examined the role of IL-6 in the pathogenesis of CHD (Berk et al 1990; Miyao et al 1993; Liuzzo et al 1994; 1998). Elevated IL-6 levels appear to be predictive for future CHD events (Harris et al 1999), and are common in patients with myocardial infarction (MI) and unstable angina (Ridker et al 2000b; Biasucci et al 1996). IL-6 is an inducer of acute phase protein synthesis from the liver and its concentrations are also positively correlated with that for inflammatory risk markers such as fibrinogen and C-reactive protein (CRP) which have been shown to be associated with risk of CHD (de Maat et al 1996; Bataille and Klein 1992; Ridker et al 2000a). Follow-up data from the Physicians’ Healthy Study of healthy men demonstrate that the risk of future MI increased with increasing quartiles of baseline IL-6 concentration, such that men in the highest quartile at entry had a relative risk 2.3 times higher than those in the lowest quartile (Ridker et al 2000b). The same study also showed that for each quartile increase in IL-6 levels, there was a 38% increase in CHD risk. Genetic variations in the human IL-6 promoter reported by Fishman et al 1998 and that found by SSCP screening as described in chapter 2 of this thesis would therefore be ideal in explaining the relationship between genotypes with levels of IL-6 and other inflammatory risk markers known to be associated with risk for CHD.

The aim for this chapter is to investigate the possible association if any, between the IL-6 -174G>C and -572G>C genotypes with plasma concentrations for the inflammatory risk markers of IL-6, fibrinogen and CRP, and with CHD risk itself, in a study of MI survivors, matched for age with healthy subjects, from four European centres (the HIFMECH study). The finding in the NPHSII study (see chapter 5) of an association between IL-6 -174G>C genotype and blood pressure will also be investigated.
6.2 Materials and Methods

6.2.1 IL-6 genotyping

The methods used for genotyping the IL-6 -174G>C and -572G>C promoter polymorphisms is described in chapter 5, section 3.2.

6.3.2 Subjects: HIFMECH study

The HIFMECH study (Hypercoagulability and Impaired Fibrinolytic function: Genetics and environmental MECHANisms predisposing to myocardial infarction) was designed to examine genetic and environmental mechanisms contributing to the differences in cardiovascular risk between Northern and Southern Europe. The study comprised of MI survivors and healthy controls caucasian men under 60 years of age from two high risk regions (Stockholm, Sweden and London, UK) and two from low risk regions (Marseille, France and San G Rotondo, Italy). MI survivors were recruited between 3-6 months after an event and matched for age with population-based healthy subjects in each region. The participants were asked to fill out a questionnaire listing past medical history, current medication, smoking status and alcohol consumption. Patients and controls subjects were examined in parallel in the morning after an overnight fast. A clinical examination was carried out where weight, height, and systolic and diastolic blood pressure were taken as described in chapter 5, section 5.3.2.1. Venous blood was drawn from subjects into either Na₂EDTA or trisodium citrate vacutainer tubes for respective measurements of lipids, procoagulant and fibrinolytic factors, inflammatory risk traits and DNA extraction. These samples were stored at -20°C and shipped in dry ice to the European collaborators involved in the HIFMECH study for various biochemical measurements.

6.3.3 Biochemical measurements

Plasma concentrations of lipid traits, coagulation and fibrinolytic factors in the HIFMECH study was determined by enzyme-linked immunosorbent assays (Hamsten et al 2002). IL-6 concentrations were measured using the human IL-6 Quantikine HS
ELISA kit (R&D System, Abingdon, UK). Inter- and intra-assay coefficients of variation were 5% and 3%, respectively. CRP and fibrinogen measurements were made using ELISA methods as described by Hamsten et al 2002.

6.3.4 Genotyping the IL-6 -174G>C and -572G>C

Genomic DNA from the whole blood of subjects (collected in Na₂EDTA tubes) was extracted for genetic analysis as described in section 2.3.1. The IL-6 -174G>C and -572G>C genotyping data were obtained from all subjects recruited into the HIFMECH study using PCR, restriction enzyme digestion and the MADGE gel system as described in chapter 5, sections 5.3.1.1 and 5.3.1.2.

6.3.5 Statistical analysis

All statistical analysis for the HIFMECH study was carried out by Ms Emma Hawe, the statistician in the Cardiovascular Genetics group, Dept Medicine, UCL using the STATA (Intercooled Stata 6.0) statistical package. Due to small sample size in some centres, and to avoid potential problems with multiple comparisons, the centres were combined into Northern Europe (Stockholm and London) and Southern Europe (Marseille and San G Rotondo) for analysis. For a number of variables (blood pressure, weight, BMI and plasma triglyceride), square root or log transformations were applied because distributions of variables were skewed. Means listed for these variables are the square of the mean of square root measurements or the exponential of the mean of log transformed values. Standard deviations (SD) for all transformed values are therefore approximate. Differences of variables by centres were examined either by analysis of variance (ANOVA) or analysis of covariance (ANCOVA). Differences in the frequency distribution of categorical variables by North-South status were analyzed by chi-squared test ($\chi^2$). Case-control differences were examined by conditional logistic regression analysis, hence considering the matching of controls to cases. Coefficients presented for continuous variables are given for a one SD change. Smoking status in the HIFMECH study was classified into smokers (ex + current) and nonsmokers, coded with dummy
variable of 1 and 0, respectively for statistical analysis. Current and Ex smokers were grouped together for statistical analysis because it was found that large numbers of the recruits had lied on their questionnaire form about their smoking status.

6.4 Results

6.4.1 Personal and lipid risk characteristics in HIFMECH

The North-South case-control study of MI survivors and healthy matched control men from four European centres (Stockholm, London, Marseille and San G Rotondo) (the HIFMECH study) described in section 6.3.1 was used to investigate the association between IL-6 genotypes with plasma concentrations of inflammatory risk markers and CHD risk. The baseline characteristics of these subjects are shown in table 6.1. In the controls (healthy men) and cases (MI survivors), subjects from North European centres (Stockholm and London) were approximately 2 years older than those from the South (Marseille and San G Rotondo). BMI was significantly higher in healthy control men in the South of Europe compared to the North (2.3%; p=0.02). There was a highly significant case-control difference for BMI in the North (27.2kg/m² vs 25.8kg/m², p<0.00005), but this was not seen in the South. No significant difference in smoking status was seen between the North and South for healthy control men. However, a significantly higher percentage of current+ex smokers was seen in the cases compared to the control groups in both the North and South of Europe (76.8% vs 62.1%; p=0.0001 and 86.3% vs 62.4%; p<0.00005 respectively). Systolic blood pressure was significantly higher in the North as compared to the South for both controls (3%) and cases (2.5%), but there were no North or South case-control differences. Diastolic blood pressure was significantly higher in controls compared to cases in both North (2.2%) and South (3.4%) regions, but no North-South differences was seen for either the healthy matched controls subjects or MI survivors. Cholesterol concentrations were significantly higher in the North compared to the South in both controls (5.9%) and cases (9.3%), with case-control significant differences only found in the South (5.18 vs 5.39 mmol/l; p=0.02), but not in the North. Plasma triglyceride concentrations were also significantly higher in the North.
compared to the South in both controls (9.4%) and cases (11.2%). The North and South case-control differences for plasma triglyceride levels were both highly significant (p<0.00005), such that MI survivors had higher triglyceride levels than the healthy matched controls men (31% vs 29% respectively).

6.4.2 IL-6, fibrinogen and CRP in cases and control by North-South and by centres

Plasma concentrations for IL-6, fibrinogen and CRP in controls and cases in the North and South of Europe are shown in figure 6.1(a) to (c). MI survivors also had significantly higher plasma concentrations for C-reactive protein (85% vs 52%), fibrinogen (10% vs 8%) and IL-6 (37% vs 85%) than in the controls in the North and South of European. Stratification of the results from figure 6.1 was then carried out to determine if there were consistent cases-control differences in each of the four centres. As shown in figure 6.2, plasma IL-6 concentration was significantly higher in MI survivors than in the healthy control men from each of the four centres. Fibrinogen and CRP plasma levels were also higher in MI survivors than the healthy matched control subjects in all four centres, but the differences were only significant in Stockholm, London and San G Rotondo. As expected, there was a significant positive correlation of IL-6 levels with that for fibrinogen (r=0.30; p<0.00005) and C-reactive protein (r=0.36; p<0.00005) (see figures 6.2.1 and 6.2.2).
Table 6.1 Baseline characteristics of healthy men (controls) and MI survivors (cases) (Mean (±SD) Number) in HIFMECH study.

<table>
<thead>
<tr>
<th>Risk factors</th>
<th>Controls North (N)</th>
<th>Controls South (S)</th>
<th>Controls Difference</th>
<th>Cases North (N)</th>
<th>Cases South (S)</th>
<th>Cases Difference</th>
<th>North Case-Control Difference</th>
<th>South Case-Control Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>52.7 (5.0)</td>
<td>50.5 (5.6)</td>
<td>&lt;0.00005</td>
<td>53.1 (5.1)</td>
<td>51.0 (5.6)</td>
<td>&lt;0.00005</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (Kg/m^2)</td>
<td>25.8 (3.1)</td>
<td>26.4 (3.2)</td>
<td>0.02</td>
<td>27.2 (3.4)</td>
<td>26.9 (3.3)</td>
<td>0.39</td>
<td>&lt;0.0005</td>
<td>0.13</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current+Ex</td>
<td>157 (62.1%)</td>
<td>201 (62.4%)</td>
<td>0.93</td>
<td>179 (76.8%)</td>
<td>259 (86.3%)</td>
<td>0.004</td>
<td>0.0001</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Never</td>
<td>96 (37.9%)</td>
<td>121 (37.6%)</td>
<td></td>
<td>54 (23.2%)</td>
<td>41 (13.7%)</td>
<td>0.04</td>
<td>0.34</td>
<td>0.99</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>130.2 (15.8)</td>
<td>126.2 (13.1)</td>
<td>0.009</td>
<td>129.5 (17.7)</td>
<td>126.4 (16.2)</td>
<td>0.04</td>
<td>0.34</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>84.0 (8.6)</td>
<td>84.2 (8.6)</td>
<td>0.77</td>
<td>82.2 (9.8)</td>
<td>81.4 (10.7)</td>
<td>0.40</td>
<td>0.03</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>5.71 (0.99)</td>
<td>5.39 (0.94)</td>
<td>0.0001</td>
<td>5.66 (1.22)</td>
<td>5.18 (1.11)</td>
<td>&lt;0.00005</td>
<td>0.31</td>
<td>0.02</td>
</tr>
<tr>
<td>Triglyceride (mmol/l)</td>
<td>1.52 (0.60)</td>
<td>1.39 (1.61)</td>
<td>0.01</td>
<td>1.99 (0.83)</td>
<td>1.79 (0.72)</td>
<td>0.003</td>
<td>&lt;0.00005</td>
<td>&lt;0.00005</td>
</tr>
<tr>
<td>C-reactive protein (mg/l)</td>
<td>1.30 (1.43)</td>
<td>1.55 (1.36)</td>
<td>0.05</td>
<td>2.41 (2.88)</td>
<td>2.35 (2.24)</td>
<td>0.81</td>
<td>&lt;0.00005</td>
<td>&lt;0.00005</td>
</tr>
<tr>
<td>Fibrinogen (g/l)</td>
<td>3.42 (0.71)</td>
<td>3.39 (0.68)</td>
<td>0.55</td>
<td>3.77 (0.84)</td>
<td>3.66 (0.98)</td>
<td>0.16</td>
<td>&lt;0.00005</td>
<td>&lt;0.00005</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>1.29 (0.74)</td>
<td>1.20 (0.81)</td>
<td>0.23</td>
<td>1.77 (1.21)</td>
<td>2.22 (1.47)</td>
<td>0.01</td>
<td>&lt;0.00005</td>
<td>&lt;0.00005</td>
</tr>
</tbody>
</table>

1Means given are the antilog of the mean of log transformed values and standard deviations are approximate.
2P-values for categorical variables are from chi-squared tests.
3P-values for continuous variables are from analysis of variance.
4P-values are from conditional logistic regression.
6.4.3 IL-6 genotype distribution and risk in HIFMECH

6.4.3.1 IL-6 -174G>C and -572G>C allele distribution

Genotyping of the IL-6 -174G>C and -572G>C promoter polymorphisms was carried out on the HIFMECH samples. The genotype distribution and allele frequencies are shown in table 6.2. For both polymorphisms, the genotypes distribution in the controls and cases were all in Hardy-Weinberg equilibrium. The carrier frequency for the -174C allele in both healthy control men and MI survivors were significantly higher in the North than in the South (controls: 0.46, 95% CI=0.42-0.51 vs 0.28, 95% CI=0.24-0.31; p<0.00005 and cases: 0.39, 95% CI=0.35-0.44 vs 0.29, 95% CI=0.25-0.33; p=0.002 respectively). When stratifying the -174C allele distribution into the different centres, a frequency gradient was observed. Stockholm had the highest frequency for the C allele, followed by London and then Marseille, with San G Rotondo having the lowest frequency (figure 6.3) in the healthy control men and MI survivors. This -174C allele frequency distribution gradient in healthy control men and MI survivors by the different centres was highly significant (controls: p<0.0005 and cases: p<0.032).

For the -572G>C polymorphism, the frequency of the rare C allele was higher (not significant) in the South of Europe compared to the North in both healthy control men and MI survivors (controls: 0.08, 95% CI=0.06-0.10 vs 0.05, 95% CI=0.03-0.07 and cases: 0.09, 95% CI=0.06-0.11 vs 0.06, 95% CI=0.03-0.08) (table 6.2). Stratification of the -572C allele distribution by centres also demonstrated a case-control gradient as was seen for the rare -174C allele (figure 6.4). However, the gradient for the distribution of the rare -572C allele was not significant between centres.

6.4.3.2 IL-6 genotypes and CHD risk

The association of IL-6 genotypes with CHD risk was investigated in the HIFMECH study. Overall, there was no North-South case-control difference for either polymorphism. However, as seen in figure 6.5, individuals from the North region of Europe who had the IL-6 -174GC+CC genotypes were significantly protected against
CHD risk compared to those with the GG genotype (odds ratio (OR)=0.64; 95% CI=0.43-0.96; p=0.03). This protective effect in the North was similar in Stockholm and London (heterogeneity of effect, p=0.82). Individuals from the South of Europe with the IL-6 -174GC+CC genotypes had slightly higher CHD risk compared to those with the -174GG genotype (OR=1.12; 95% CI=0.81-1.54), but the difference was not significant. The interaction between North-South status and IL-6 -174 genotype on CHD risk was significant (p=0.03). The -572G>C genotype was not associated with CHD risk in the North or the South (p=0.95) or in any of the different centres.

6.4.3.3 IL-6 genotypes, smoking status and risk

CHD risk in smokers and nonsmokers was investigated in subjects with different IL-6 genotypes from the North and South of Europe using -174GG nonsmokers as the reference group. The result is shown in figure 6.6. Smoking history (i.e. current+ex) was associated with higher CHD risk in both the North (OR=2.30, 95% CI=1.48-3.55) and in the South of Europe (OR=3.60, 95% CI=2.34-5.52). Although the effect of smoking on risk was greatest in the South compared to the North, there was no significant evidence for North-South interaction (p=0.15). In the North, smoking increased risk by 2.42 fold in those with the IL-6 -174GG genotype and 1.39 fold in the -174GC+CC genotype group compared to GG nonsmokers. The effect, however, was only significant in GG smokers (p=0.012). In the South of Europe, smokers in the GG group had a 4.36 fold higher risk and the GC+CC group a 4.30 fold higher risk compared to GG nonsmokers. The difference was significant in both group (p<0.0005 and p<0.0005 respectively). Overall, CHD risk by IL-6 -174G>C genotype was not significantly different in the North or the South. After adjusting for classical risk factors (e.g. alcohol consumptions, exercise and BMI), the CHD risk estimates remained essentially unchanged (table 6.2a). It was not possible to examine the effect of smoking and CHD risk by North-South status for the IL-6 -572G>C genotype due to the low numbers of subjects with the -572C allele.
Figure 6.1 Unadjusted mean (±SEM) plasma concentrations for (a) IL-6, (b) fibrinogen and (c) CRP in controls and cases North and South of Europe.
Figure 6.2 Mean (±SEM) plasma concentrations for (a) IL-6, (b) fibrinogen and (c) CRP in controls and cases from Stockholm, London, Marseille and San G Rotondo.

(a) IL-6 concentrations

(b) Fibrinogen concentrations

(c) CRP concentrations

Means are geometric (antilog of log transformed values) ±SEM
Figure 6.2.1 Correlation of IL-6 levels with fibrinogen levels in the HIFMECH study.

Figure 6.2.2 Correlation of IL-6 levels with CRP levels in the HIFMECH study.

*Log transformations are used for both variables.
Table 6.2 IL-6 -174G>C and -572G>C genotype distribution in the HIFMECH study.

<table>
<thead>
<tr>
<th>Risk factors</th>
<th>Controls</th>
<th>Cases</th>
<th>North (N)</th>
<th>South (S)</th>
<th>Difference between N &amp; S</th>
<th>North (N)</th>
<th>South (S)</th>
<th>Difference between N &amp; S</th>
<th>North case-control Difference</th>
<th>South case-control Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 -174G&gt;C (GG/GC/CC)</td>
<td></td>
<td></td>
<td>71/120/53</td>
<td>169/120/28</td>
<td>&lt;0.00005*</td>
<td>89/100/40</td>
<td>138/119/21</td>
<td>0.002*</td>
<td>0.09*</td>
<td>0.48*</td>
</tr>
<tr>
<td>IL-6 -174C Freq. (95% CI)</td>
<td>0.46</td>
<td>0.28</td>
<td>(0.42-0.51)</td>
<td>(0.24-0.31)</td>
<td></td>
<td>0.39</td>
<td>0.29</td>
<td>(0.35-0.44)</td>
<td>(0.25-0.33)</td>
<td></td>
</tr>
<tr>
<td>IL-6 -572G&gt;C (GG/GC/CC)</td>
<td>215/26/0</td>
<td>260/43/3</td>
<td>199/23/1</td>
<td>234/48/0</td>
<td>0.24*</td>
<td>0.06</td>
<td>0.09</td>
<td>0.21*</td>
<td>0.87†</td>
<td>0.75†</td>
</tr>
<tr>
<td>IL-6 -572C Freq. (95% CI)</td>
<td>0.05</td>
<td>0.08</td>
<td>(0.03-0.07)</td>
<td>(0.06-0.10)</td>
<td></td>
<td>0.06</td>
<td>0.09</td>
<td>(0.03-0.08)</td>
<td>(0.06-0.11)</td>
<td></td>
</tr>
</tbody>
</table>

* P value from chi-square test
† P value obtained from GG vs GC+CC genotypes
Figure 6.3 Rare allele frequencies (95% CI) by centres for IL-6 -174C in HIFMECH study.

IL-6 -174G>C (control)
- Stockholm: P<0.0005
- London: 44
- Marseille: 31

IL-6 -174G>C (cases)
- Stockholm: 40
- London: 33
- Marseille: 32
- San G Ron: 25

Relative rare allele frequency (95% CI)
Figure 6.4 Rare allele frequencies (95%) by centres for IL-6 -572C in HIFMECH study

IL-6 -572G>C (control)
- Stockholm
- London
- Marseille
- San G Ron

IL-6 -572G>C (cases)
- Stockholm
- London
- Marseille
- San G Ron

p=0.17
p=0.053

Relative rare allele frequency (95% CI)
Figure 6.5 Odds ratio (95% CI) associated with IL-6 genotypes in HIFMECH study by North or South of Europe.

Figure 6.6 Odds ratio (95% CI) associated with IL-6 -174G>C genotype in nonsmokers and smokers (current+ex) by North or South regions in the HIFMECH study.
Table 6.2a Unadjusted and adjusted odds ratio (95% CI) associated with IL-6 -174G>C genotype in smokers and nonsmokers (current+ex) by North and South regions in the HIFMECH study in healthy subjects (controls) and MI survivor (cases).

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Cases</th>
<th>OR (95% CI) unadjusted</th>
<th>OR (95% CI) adjusted*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>North</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GC+CC smokers</td>
<td>109</td>
<td>109 (50.0%)</td>
<td>1.39 (0.75, 2.26)</td>
<td>1.68 (0.86, 3.28)</td>
</tr>
<tr>
<td>GC+CC non smokers</td>
<td>64</td>
<td>31 (32.6%)</td>
<td>0.61 (0.31, 1.19)</td>
<td>0.72 (0.35, 1.48)</td>
</tr>
<tr>
<td>GG smokers</td>
<td>41</td>
<td>66 (61.7%)</td>
<td>2.42 (1.21, 4.83)</td>
<td>3.04 (1.42, 6.50)</td>
</tr>
<tr>
<td>GG non smokers</td>
<td>30</td>
<td>23 (43.4%)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>South</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GC+CC smokers</td>
<td>98</td>
<td>121 (55.3%)</td>
<td>4.30 (2.36, 7.82)</td>
<td>4.28 (2.30, 7.94)</td>
</tr>
<tr>
<td>GC+CC non smokers</td>
<td>50</td>
<td>19 (27.5%)</td>
<td>1.19 (0.54, 2.63)</td>
<td>1.28 (0.56, 2.92)</td>
</tr>
<tr>
<td>GG smokers</td>
<td>99</td>
<td>120 (54.8%)</td>
<td>4.36 (2.36, 8.07)</td>
<td>4.21 (2.23, 7.95)</td>
</tr>
<tr>
<td>GG non smokers</td>
<td>70</td>
<td>18 (20.5%)</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

*adjustment is made for alcohol (ex+current vs never), exercise (sedentary+moderate) vs (active+fit)
6.4.4 Genotypes and inflammatory risk markers

6.4.4.1 IL-6 -174G>C

The association between levels of IL-6, fibrinogen, CRP, diastolic and systolic blood pressure for the IL-6 -174G>C polymorphisms in the HIFMECH study is shown in table 6.3. IL-6 levels were not significantly associated with the -174 genotype, but a weak trend was seen in MI survivors from the Southern region of Europe. Stratification of this data into the different centres (Marseille and San G Rotondo) demonstrated that higher IL-6 levels were seen in subjects with the CC genotype compared to the GG genotype in both centres (figure 6.7). This difference was almost statistically significant in Marseille (p=0.06), but not in San G Rotondo. Fibrinogen concentration was significantly higher in healthy control men with the CC genotype compared to those with the GG or GC genotypes from the South of Europe (p=0.02 for difference by genotype) (table 6.3). After adjusting for centres and age, this effect was no longer significant (p=0.12). No associations between IL-6 -174G>C genotype with plasma concentration for fibrinogen was seen in cases. When stratified into the different centres (Marseille and San G Rotondo), healthy control men homozygous for the C allele had higher fibrinogen levels than those homozygous for the -174G allele, but the difference was not significant (figure 6.8). There was also no significant difference between IL-6 -174G>C genotype and levels of CRP, diastolic and systolic blood pressure in post MI infarct survivors or healthy control men by the North or South (table 6.3) or by centres (not shown).

6.4.4.2 IL-6 -572G>C

Association between plasma concentrations of inflammatory risk markers and blood pressure measurements with IL-6 -572G>C genotype was also investigated in the HIFMECH study, and the result is shown in table 6.4. Subjects with the -572GC+CC genotypes had higher systolic blood pressure in healthy control men from both the North and South of Europe compared to those homozygous for the -572G allele. This difference was significant in the South (130.6mmHg vs 125.7mmHg, p=0.03), and was of similar magnitude and almost significant in the North (136.0mmHg vs 129.4mmHg, p=0.06).
Stratification of this data into the different centres by IL-6 -572G>C genotype is shown in figure 6.9. It can be seen that the higher systolic blood pressure of the -572GC+CC genotype compared to the -572GG men was seen in Stockholm, Marseille, San G Rotondo, but not in London which was the smallest group. The effect was significant in Stockholm (140.5mmHg vs 130.2mmHg, p=0.02), and was almost significant in San G Rotondo (133.1mmHg vs 128.6mmHg, p=0.06). After adjusting for the raising effect of smoking on systolic blood pressure, this difference between the -572GC+CC genotypes compared to -572GG genotype was essentially the same (Stockholm, p=0.02; London, p=0.64; Marseille, p=0.27; San G Rotondo, p=0.06; and all combined, p=0.04).

There was a trend towards higher diastolic blood pressure in the healthy controls, with the -572GC+CC genotype compared to individuals homozygous for the GG genotype in the North and South, reaching statistical significance in the South (p=0.05). No genotype effect on systolic blood pressure was seen in MI survivors in either the North or the South. Levels of IL-6, fibrinogen and CRP were not associated with the IL-6 -572G>C genotype.

6.4.5 IL-6 genotypes and smoking, blood pressure and IL-6 levels

The effect of smoking on systolic and diastolic blood pressure and on levels of IL-6 with the IL-6 -174G>C and -572G>C genotypes was investigated in the healthy control men. The results in figure 6.10 showed that healthy men with the -174GC+CC genotype had slightly higher plasma IL-6 levels than those with the -174GG genotype in smokers (current+ex) and nonsmokers (never), but the difference was not significant. Diastolic and systolic blood pressure levels were also not significantly different in smokers or nonsmokers in healthy men by IL-6 -174G>C genotype (data not shown). For the IL-6 -572G>C genotype in healthy men, there was no significant difference in IL-6 levels between this genotype in smokers and nonsmokers (see figure 6.11). However, smokers with the -572GC+CC genotypes had significantly higher diastolic and systolic blood pressure compared to those homozygous for the -572G genotype (diastolic BP: 3.4% higher, p=0.03 and systolic BP: 5% higher, p=0.004) (see figure 6.12). However, no
significant genotype effect was seen in nonsmoker for diastolic or systolic blood pressure. Levels of inflammatory risk markers was not associated with the IL-6 -572G>C genotype in smokers or nonsmokers in the healthy men (data not shown).

The association of -174G>C and -572G>C genotypes with blood pressure and CHD risk markers was not examined in MI survivors due to the potentially confounding effects of lipid lowering drugs (fibrates and statins), blood pressure lowering drugs (ACE inhibitors, calcium antagonists and anti-diuretic), as well as anti-inflammatory drugs (statins) that these post MI infarct patients had been prescribed (see table 6.5).

6.4.6 IL-6 genotype, IL-6 levels and BMI tertile

Since adipose tissue is known to be a major source of plasma IL-6 (Mohamed-Ali et al 1997), analysis was carried out to explore whether there was any association between the IL-6 genotype and plasma IL-6 levels by BMI tertile in healthy control men. After adjusting for the effect of smoking and differences between centres, healthy men with the -174GC+CC genotypes had higher plasma IL-6 levels than those with the GG genotype across all tertiles of BMI (13%, 3% and 5% respectively), but the difference was not significant (data not shown). There was also no significant different between IL-6 -174 genotype with systolic and diastolic blood pressure across all tertiles for BMI (data not shown). For the IL-6 -572 genotype, after adjusting for the effect of smoking and difference between centres, healthy men with the GG allele had higher plasma IL-6 levels than GC+CC individuals only in the middle (7%) and top (18%) tertile for BMI. Overall, no significant difference was seen between the -572 genotype and IL-6 levels for all tertiles of BMI (data not shown). Individuals with the -572GC+CC genotypes had higher systolic blood pressure compared to those homozygous for the G allele only in the middle (7%) and top (2.3%) tertile of BMI, reaching statistical significance only in the middle tertile of BMI (125.8 vs 117.5mmHg, p=0.002) (figure 6.13). The same observation was also seen for diastolic blood pressure, which was almost statistically significant in the middle tertile of BMI (-572GC+CC=3.4% higher than -572GG, p=0.07) (figure 6.14).
Table 6.3 Unadjusted mean (±SD) concentrations for plasma IL-6, fibrinogen, CRP, and diastolic and systolic blood pressure with IL-6 -174G>C genotype in healthy controls and MI survivor (cases) by North-South in the HIFMECH study.

<table>
<thead>
<tr>
<th>Traits</th>
<th>North (Stockholm/London)</th>
<th>South (Marseille/San G Rotondo)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
<td>Cases</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG [n]</td>
<td>1.21</td>
<td>(0.67)</td>
</tr>
<tr>
<td>GC [n]</td>
<td>1.31</td>
<td>(0.76)</td>
</tr>
<tr>
<td>CC [n]</td>
<td>1.24</td>
<td>(0.67)</td>
</tr>
<tr>
<td>P</td>
<td>0.61</td>
<td></td>
</tr>
<tr>
<td>Fibrinogen (g/l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG [n]</td>
<td>3.45</td>
<td>(0.74)</td>
</tr>
<tr>
<td>GC [n]</td>
<td>3.36</td>
<td>(0.65)</td>
</tr>
<tr>
<td>CC [n]</td>
<td>3.50</td>
<td>(0.76)</td>
</tr>
<tr>
<td>P</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>C-reactive protein (mg/l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG [n]</td>
<td>1.30</td>
<td>(1.47)</td>
</tr>
<tr>
<td>GC [n]</td>
<td>1.18</td>
<td>(1.36)</td>
</tr>
<tr>
<td>CC [n]</td>
<td>1.51</td>
<td>(1.47)</td>
</tr>
<tr>
<td>P</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG [n]</td>
<td>83.70</td>
<td>(8.57)</td>
</tr>
<tr>
<td>GC [n]</td>
<td>83.81</td>
<td>(8.57)</td>
</tr>
<tr>
<td>CC [n]</td>
<td>83.83</td>
<td>(8.02)</td>
</tr>
<tr>
<td>P</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG [n]</td>
<td>130.09</td>
<td>(15.45)</td>
</tr>
<tr>
<td>GC [n]</td>
<td>129.87</td>
<td>(16.52)</td>
</tr>
<tr>
<td>CC [n]</td>
<td>130.39</td>
<td>(14.97)</td>
</tr>
<tr>
<td>P</td>
<td>0.98</td>
<td></td>
</tr>
</tbody>
</table>

Means given are geometric from log transformed values and SD is approximate.

*p values for fibrinogen, IL-6, CRP, diastolic and systolic blood pressure after log transformation.*
Figure 6.7 Mean (±SEM) concentrations of plasma IL-6 with IL-6 -174G>C genotype in MI survivors (cases) from Marseille, San G Rotondo and both centres combined (ALL) in the HIFMECH study.

![Figure 6.7](image)

Figure 6.8 Mean (±SEM) concentrations of serum fibrinogen with IL-6 -174G>C genotype in MI survivors (cases) from Marseille, San G Rotondo and both centres combined (ALL) in the HIFMECH study.

![Figure 6.8](image)
Table 6.4 Unadjusted mean (±SD) concentrations for plasma IL-6, fibrinogen, CRP, diastolic and systolic blood pressure for IL-6 -572G>C genotype in healthy controls and MI survivors (cases) by North-South in the HIFMECH study.

<table>
<thead>
<tr>
<th>Traits</th>
<th>North (Stockholm/London)</th>
<th>South (Marseille/San G Rotondo)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
<td>Cases</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.29 (0.75) [194]</td>
<td>1.32 (0.67) [22]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.89</td>
<td></td>
</tr>
<tr>
<td>Fibrinogen (g/l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.42 (0.70) [196]</td>
<td>3.38 (0.68) [24]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.83</td>
<td></td>
</tr>
<tr>
<td>C-reactive protein (mg/l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.29 (1.45) [171]</td>
<td>1.20 (1.31) [21]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.77</td>
<td></td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>83.68 (8.29) [215]</td>
<td>85.08 (8.81) [24]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>129.38 (14.89) [215]</td>
<td>136.01 (22.38) [24]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.06</td>
<td></td>
</tr>
</tbody>
</table>

Means given are geometric from log transformed values and SD is approximate.
P values for fibrinogen, IL-6, CRP, and diastolic and systolic blood pressure after log transformation.
Figure 6.9 Mean (±SEM) systolic blood pressure in healthy control men with IL-6 -572G>C genotype by centres and all centre combined (ALL) in the HIFMECH study.

Figure 6.10 Plasma IL-6 concentrations in nonsmokers and smokers (current+ex) by IL-6 -174G>C genotype adjusted by centres.
Figure 6.11 Plasma IL-6 concentrations in nonsmokers and smokers (current+ex) by IL-6 -572G>C genotype adjusted by centres.

Means are geometric (antilog of log transformed values) ±SEM.
Figure 6.12 Systolic and diastolic blood pressure levels in nonsmokers and smokers (current+ex) by IL-6 -572G>C genotype adjusted by centres.

(a) Systolic blood pressure

Means are geometric (antilog of log transformed values)SEM

(b) Diastolic blood pressure

Means are geometric (antilog of log transformed values)SEM
Table 6.5 Proportion of MI survivors in the HIFMECH study on lipid lowering, blood pressure lowering or anti-inflammatory drugs in the North and South of Europe.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Proportion of MI survivors on drugs</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>North</td>
<td>South</td>
</tr>
<tr>
<td>Aspirin</td>
<td>91.4% (n=213)</td>
<td>83.7% (n=239)</td>
</tr>
<tr>
<td>Lipid lowering</td>
<td>26.7% (n=62)</td>
<td>27.2% (n=77)</td>
</tr>
<tr>
<td>B-Blockers</td>
<td>84.1% (n=195)</td>
<td>40.6% (n=115)</td>
</tr>
<tr>
<td>Calcium antagonists</td>
<td>9.1% (n=21)</td>
<td>22.7% (n=64)</td>
</tr>
<tr>
<td>Nitrates</td>
<td>33.6% (n=78)</td>
<td>45.1% (n=127)</td>
</tr>
<tr>
<td>ACE inhibitors</td>
<td>30.6% (n=71)</td>
<td>31.2% (n=88)</td>
</tr>
<tr>
<td>Fluros</td>
<td>16.4% (n=38)</td>
<td>3.9% (n=11)</td>
</tr>
<tr>
<td>Diuretics</td>
<td>3.9% (n=9)</td>
<td>4.6% (n=13)</td>
</tr>
<tr>
<td>Other anti-hypertensive</td>
<td>3.0% (n=7)</td>
<td>1.8% (n=5)</td>
</tr>
</tbody>
</table>
Figure 6.13 Relationship between systolic blood pressure and IL-6 -572G>C genotype in healthy control men by tertile of BMI (adjusted for smoking and centres).

![Graph showing the relationship between systolic blood pressure and IL-6 -572G>C genotype by tertile of BMI.](image)

Mean are geometric (antilog of log transformed values) and standard errors are approximate.

Figure 6.14 Relationship between diastolic blood pressure and IL-6 -572G>C genotype in healthy control men by tertile for BMI (adjusted of smoking and centres).

![Graph showing the relationship between diastolic blood pressure and IL-6 -572G>C genotype by tertile of BMI.](image)

Mean are geometric (antilog of log transformed values) and standard errors are approximate.
6.5 Discussion

6.5.1 HIFMECH study and CHD risk

The HIFMECH study was designed to examine genetic and environmental mechanisms contributing to the differences in mortality rate from CHD risk between Northern and Southern Europe (Hamsten et al 2002). Measurements of lipid, coagulation and fibrinolytic factors, as well as inflammatory risk markers were available. The advantages of using the HIFMECH study is that it is a case-control comparisons of MI survivors and healthy aged-matched caucasian control men from two high risk centres (Stockholm and London) and two low risk centres (Marseille and San G Rotondo). This study was ideal in trying to confirm findings in the NPHSII study of the association between IL-6 -174G>C genotype and blood pressure and also to test the hypothesis that IL-6 genotypes (e.g. -174G>C and -572G>C) may influence plasma IL-6 levels, thus affecting plasma concentrations of CHD risk factors (e.g. fibrinogen and CRP) and CHD risk itself. The limitation of this study is that it lacks the power to detect CHD risk associated with the -572 genotype due to the low frequency of the -572C allele (5-9%). All advantages and disadvantages concerning the use of the MADGE technique for genotyping have already been described in chapter 5, section 5.6.1.

The HIFMECH study showed that the risk of CHD associated with higher IL-6 levels was seen more strongly in men in the South compared to the North region of Europe (South cases 85.0% higher than controls; North 37.2% higher). The mechanism to explain the greater CHD risk of IL-6 in the South compared to the North is unlikely to be through smoking because the majority of these individuals had stopped smoking by the time the sample were taken. The lower risk in the North compared to the South is also unlikely to be explained by the uses of lipid-lowering drugs such as statins or fibrates due to similar proportion of MI survivors take these drugs (North=26.7% vs South=27.2%; p=0.902). However, a possible explanation is that smoking, which causes chronic inflammation can leads to greater susceptibility to endothelial dysfunction (Griendling and Alexander 1997; Lacy et al 1998), may be more of an influence in individuals from the South of Europe compared to those in the North. This may be because of differences in the types of
cigarette used (tar content, filters etc), but no data were available in the HIFMECH study to examine this possibility. It could also be explained by the fact that a significantly greater proportion of MI survivors in the North compared to the South are taking aspirin (91.4% vs 83.7%; p=0.01), and beta blockers (84.1% vs 40.6%; p=<0.00005) (see table 6.5). The HIFMECH study also found elevated levels of the well-known CHD risk factors such as fibrinogen and CRP in MI survivors compared to healthy control men, and supports the role for fibrinogen and CRP as independent risk factors for CHD (Lowe 1995; Berk et al 1990; Harris et al 1999; Ridker et al 2000a). The raising effect of higher IL-6 levels on plasma fibrinogen and CRP concentration is likely to be through gene activation involving IL-6 type-1 or type-2 elements (Akira et al 1995). This finding is supported in the HIFMECH study where IL-6 levels showed a positive correlation with both plasma fibrinogen and CRP levels (r=0.30, p=<0.00005 (n=316) and r=0.36, p=<0.00005 (n=383) respectively) in healthy control men (figures 6.2.1 and 6.2.2) and also supports observations reported by other studies (de Maat et 1996; Berk et al 1990; Woodhouse et al 1994; Biasucci et al 1996).

6.5.2 IL-6 genotype and CHD risk

The association of the IL-6 -572G>C and -174G>C polymorphisms with CHD risk was examined in the HIFMECH study. No significant effect of -572G>C polymorphism with CHD risk was detected in either the North or South of Europe. This is due in part to the lack of power in the HIFMECH study as a result of the low frequency of the -572C allele in this study. The low frequency (5% in controls and 9% in cases) suggests that this genotype is unlikely to be common enough to be genetically significant for CHD risk in the general population. Calculation shows that this study of 532 cases and 574 controls has the power (α=0.05, β=0.8) to detect an effect on risk of 1.44 fold for the -174G>C, and 1.88 fold for the -572G>C.

The “protective” effect from risk of having an MI (36% lower) in carriers with the -174C allele (North of Europe) is contradictory to that found in the NPHSII study and others (Humphries et al 2001, as described in chapter 5; Brull et al 2001; Georges et al 2001;
Jones et al 2001; Jenny et al 2002). One possible explanation for the observed protective effect in the group that are carriers of the -174C allele in the North could be that a higher proportion of these individuals have died of an MI and therefore would not be available for recruiting as "MI survivors". To explore the likelihood of this scenario, modeling has been carried out using the data from figure 6.5. Assuming that on average, one third of MI events are fatal (Volmink et al 1998), and given the genotype distribution seen in the controls in the North (GG=29%, GC=49% and CC=22%), the genotype distribution seen in the MI survivor would be obtained if the proportion of fatal events in GG subjects was 0.29, in GC subjects was 0.39 and CC subjects was 0.53. The data from the AAA study (Jones et al 2001) and the small amount of data from the 6 months mortality in the CABG study (Brull et al 2001) support such an effect (mortality in the Brull sample; GG=0/47, GC=2/67 and CC=3/13).

Smoking is universally recognized as a major independent risk factor for CHD risk (Gensini et al 1998; Seltzer 1989; Castelli 1990; Weintraub 1990) and was also seen in HIFMECH (2.9 fold higher in current+ex smokers compared to nonsmokers). Although the NPHSn study reported that the association of the -174G>C genotype with CHD risk was higher in smokers (Humphries et al 2001), other studies have not found this interaction (George et al 2001). In the HIFMECH study, the impact of IL-6 -572G>C and -174G>C genotype on CHD risk was investigated in smokers versus nonsmokers. Any possible difference in the effect of the -572G>C genotype on CHD risk in smokers and nonsmokers could not be determined in the HIFMECH study because the low frequency of individuals that are carriers for the -572C allele in the smoking group made statistical analysis invalid. For the IL-6 -174 genotype, higher CHD risk was associated with the GG and GC+CC smokers in the North and South of Europe, but the -174GC+CC nonsmokers were protected from CHD risk compared to GG nonsmokers (although the difference not significant). This finding in the HIMECH study is unexpected. One possible explanation could be that men in the GC+CC group have a lower survival rate after an MI, with the effect seen most clearly in the North of Europe where the C allele frequency is high.
It is still unclear as to the functionality \textit{in vivo} of the IL-6 -174G\textgreater{}C promoter polymorphism with CHD risk due to inconsistent published data. Some studies have reported that carriers of the -174C was attributed to risk from CHD (Jones et al 2001; Brull et al 2001; George et al 2001; Jenny et al 2002) (see table 5.6 in chapter 5), while others have not found risk association with the -174G\textgreater{}C polymorphism (Burzotta et al 2001; Margaglione et al 2001; Elghannam et al 2001). The mechanism for the IL-6 -174GG and GC+CC genotypes with CHD risk in smokers compared to GG nonsmokers is probably through the effect of smoking that lead to endothelial damage by promoting inflammatory mediators such as IL-1 via NF\kappa{}B (Shimizu et al 1990; Liberman and Baltimore 1990; Zhang et al 1990), which is the main inducer of IL-6 gene transcription and hence higher IL-6 levels. IL-6 then induces the expression of proinflammatory genes such as fibrinogen and CRP which promote by thrombosis (Woodward et al 1991; 1999) in the face of plaque instability.

\textit{6.5.3 IL-6 genotype and plasma IL-6, fibrinogen and CRP}

The association of IL-6-572G\textgreater{}C and -174G\textgreater{}C genotypes with plasma levels of IL-6, fibrinogen and CRP was examined in the HIFMECH study. Although a trend for higher IL-6 levels was seen in the -174CC men compared to GG subjects in the South, IL-6 genotypes did not significantly affect levels of IL-6 in MI survivors or in the healthy control men in the North or South of Europe or in any of the four centres. The same observations were also found for the -572G\textgreater{}C genotype. The most like explanation is that the greatest difference expected between the -174G\textgreater{}C and -572G\textgreater{}C genotypes with levels for IL-6 would be found in MI survivors who had inflammation. However, since these post-infarct men are taking anti-inflammatory medications that are known to reduce plasma IL-6 concentration (e.g. aspirin and statins), this would reduce the power of this study to detect large differences associated with the IL-6 genotypes. The findings in the HIFMECH study are however in agreement with finding by Brull et al 2001 in post-CABG subjects where the C allele was associated with higher IL-6 levels and also in the WOSCOPS study (Basso et al 2002) which showed higher IL-6 levels (not significant) in CC subjects who subsequently had a cardiovascular event.
The significant effect of the -174 genotype in the determination of fibrinogen levels in the South is likely to be through effects on plasma IL-6 levels could be by chance. This is because no significant association were found between genotype in each centre (Marseille & San G Rotondo) with fibrinogen levels and also the -174 genotype and IL-6 levels. Also the HIFMECH study does not take into consideration the dinurnal variations of IL-6 levels which have been shown in patients with rheumatoid arthritis to peak in the morning, while being low in the afternoon and evening (Arvidson et al 1994). Therefore a single measurement would be a poor estimate of IL-6 levels, and any small difference in IL-6 levels caused by IL-6 genotypes would not be detected easily.

No effect of IL-6 genotypes was also seen for CRP levels. This observations seen in the HIFMECH study support findings reported by Margaglione et al 2001 involving 598 healthy subjects aged 22-66 years (250 men and 348 women) where the IL-6 -174G>C polymorphism was not associated with levels of IL-6, fibrinogen, CRP, total cholesterol and triglyceride.

6.5.4 IL-6 -174G>C and -572G>C, plasma IL-6, blood pressure and BMI

In the HIFMECH study, a positive correlation was found between BMI and plasma IL-6 concentration (r=0.17, p=0.0003). This confirms the report by Mohamed-Ali et al 1997 in which increasing obesity resulted in higher levels of IL-6, and demonstrates the significant role which adipose tissue plays in the determination of plasma IL-6 concentration in humans. The greatest effect on IL-6 levels by the -174GC+CC genotype compared to the GG group (differences not significant) which were seen most strongly in the lowest tertile for BMI, but less so in the middle or top tertile, suggests that when adipose levels are low, the genotype association maybe observed more easily due to IL-6 production from non-adipose tissues being genotype dependent (Kishimoto 1989). Since there was less difference in plasma IL-6 concentrations between the IL-6 -174G>C genotypes in the top by tertiles of BMI, it suggest that the -174 genotype is not functionally active in adipose tissue, with the (modest) genotype effect being swamped by IL-6 secretion from adipose tissue. The same positive correlation between BMI with
systolic (r=0.30, p=<0.00005) and diastolic blood pressure (r=0.30, p=<0.00005) in the HIFMECH study (data not shown) was also seen and has been reported elsewhere (Hekman et al 1990; Masana et al 1996; Hirose et al 2000) but was not the direct result of the -174 genotype.

The IL-6 -572G>C genotype in the HIFMECH study was also investigated for association between plasma IL-6 levels or blood pressure (systolic and diastolic) by tertile for BMI. The higher systolic blood pressure in the middle and top (significant only in the middle tertile for BMI), but not the lower tertile in healthy control men for the -572GC+CC compared to -572GG genotypes suggests that the -572 effect on systolic blood pressure is greatest with increasing adiposity. This trend was also seen for diastolic blood pressure. The data from the HIFMECH study fit well with the concept already described in the NPHSII study in that increasing adiposity leads to higher plasma IL-6 levels in carriers of the -572C allele and suggest possible -572 functionality in adipose tissue.

6.5.5 IL-6 genotype and blood pressure

The effect of smoking and IL-6 -174G>C genotype on systolic blood pressure reported in the NPHSII study (see chapter 5 and reported by Humphries et al 2001) is not confirmed in the HIFMECH study or by others (Margaglione et al 2001; Elghannam et al 2001). The most likely explanation for not seeing any difference in healthy control men from the HIFMECH study is that this study has low power to detect modest effects compared to the NPHSII study (~550 vs >2000 healthy men). Analysis of blood pressure in MI survivors in the HIFMECH study was not feasible because the post infarct men were on blood pressure lowering drugs (e.g. beta blocks, ACE inhibitors, calcium antagonists and anti-diuretic) (see table 6.5).

The effect of IL-6 -572G>C genotype on blood pressure levels (systolic and diastolic) in smokers and nonsmokers was also investigated. The significant association of higher systolic and diastolic blood pressure only in smokers (current+ex) for the -572GC+CC genotype compared to smokers with the -572GG genotype suggests three possible
mechanisms by which this may occur (already described in greater detail in chapter 5). The first mechanism could involve increased blood viscosity through higher fibrinogen concentration from the inflammatory effect of smoking (Griendling and Alexander 1997; Lacy et al 1998), the second mechanism could be through the renin-angiotensin system involving the potent vasoconstrictor, angiotensin II (Chobanian and Dzau 1996) and the final mechanism could involve vessel wall stiffness as a result of increased collagen synthesis (Choi et al 1994; Greenwel 1995; Duncan and Berman 1991). Since there was no significant association between the IL-6 -572G>C genotype with fibrinogen levels in smokers and nonsmokers, it seems unlikely that the -572G>C genotype could effect blood pressure through this pathway. This study was not designed to test for other possible mechanisms in detail.

The rapid isolation of serum from whole blood after sample collection has been demonstrated to be critical in the accurate determination of IL-6 levels in healthy individuals (Flower et al 2000). In this study, it was shown that extraction of serum from whole blood immediately after collection gave approximately 14% higher IL-6 concentration compared to samples in which serum were obtained many hours after blood collection when using the HS IL-6 ELISA kit from R&D System (as was used for HIFMECH). At the time when the HIFMECH study was set-up and blood sample collections taken (1996-1997), this was not known. This minor difference in the protocol used to collect and store samples may have increased the imprecision of the IL-6 assay and reduced the power of the study to detect modest genotype effects. Also, as shown by Brull et al 2001 on the CABG study as a model for acute inflammation, IL-6 levels rise and then fall quickly after bypass surgery, leading towards baseline levels 5 days after the acute inflammatory response. Since MI survivors in the HIFMECH study were recruited between 3-6 months after an infarct had taken place, the IL-6 levels would have fallen near to baseline levels when these subjects were recruited. Any small difference in IL-6 levels by IL-6 genotypes would therefore be difficult to detect.
CHAPTER 7

MOLECULAR MECHANISMS OF THE IL-6 572G>C PROMOTER POLYMORPHISM IN HEPATOCYTE
Chapter 7: Molecular mechanism of the IL-6 -572G>C promoter polymorphism in hepatocyte

7.1 Introduction and Aims

In the cytoplasm, glucocorticoid receptors (GRs) are sequestered as inactive complex by two heat shock protein (HSP-90), and other cytosolic proteins (Oakley et al 1999). Upon binding of glucocorticoids (GCs) to their respective GRs, a conformational change occurs that allow it to dissociate from HSP-90. The GC-GR complex then translocates to the nucleus where it transiently associates with HSP-56 and later binds as a dimer to conserved palindromic DNA sites called GREs (GGTACAnnnTGTTCT). Depending on the gene, the GC-GR may stimulate (transactivation), or alternatively inhibit (transrepression) gene transcription. IL-6 contains two putative GRE hexamer DNA binding half sites for glucocorticoid repression (AGAACA) located at -557 to -552 (GRE 1) and -464 to -459 (GRE 2) (see figure 7.1).

Figure 7.1 Schematic representation of the human IL-6 promoter

In vitro functional data in HepG2 and HuH7 cells using the dual luciferase reporter gene assay system have shown that the IL-6 -572C variant resulted in higher relative promoter activity compared to the -572G variant in the presence of DEX and IL-1β plus DEX (see chapters 3 and 4). One possible mechanism to explain this finding would be through direct glucocorticoid repression of IL-6 gene transcription containing the -572G variant and not for the -572C variant (see figure 7.2).
Figure 7.2 Purposed mechanisms for repression of IL-6 gene transcription of (a) -572G and (b) -572C variants by glucocorticoids (GCs)-glucocorticoid receptor complex in hepatocytes.

(a) IL-6 -572G

(b) IL-6 -572C
The aim of this chapter is to elucidate the function (if any) of the two putative GRE sites on the IL-6 -572G and -572C variant to DEX repression. To study this, knockouts of the two GRE sites for the -572G and -572C constructs were generated by site-directed mutagenesis and the relative promoter strength determined in HuH7 cells in response to the DEX repression and IL-1β plus DEX induction at a 12 hours time point.

7.2 Materials

Hepatocyte cell line (HuH7) was obtained from ECACC (UK), Pfu Turbo DNA polymerase and supercompetent cells were from Stratagene (UK). All reagents and media used for the cell culturing and transfections were purchased from Invitrogen (Paisley, UK). Dexamethasone (DEX) was from Sigma (Dorset, UK) and IL-1β from R&D Systems (Oxon, UK).

7.3 Methods

7.3.1 GRE knockouts for the IL-6 -572G and -572C constructs.

Mutagenic primer pairs: IL-6 GRE1 F plus IL-6 GRE1 R and IL-6 GRE2 F plus IL-6 GRE2 R (see table 3 in appendix) were created using MathInspector V2.2, TRANSFAC 4.0 software (http://transfac.gbf.de/). These primers were then used to knockout the putative GRE1 and GRE2 for the IL-6 -572G and -572C variants using the IL-6 -572G and -572C constructs from chapter 3, table 3.1. The protocols for this were identical to those described in sections 3.3.2.1 to 3.3.2.3. Table 7.1 below shows the normal and GRE knockouts for the -572 constructs that were successfully made.
Table 7.1 IL-6 -572G>C promoter variants and glucocorticoid responsive element (GRE) knockout for -572G>C variants.

<table>
<thead>
<tr>
<th>Construct length</th>
<th>-597 G/A</th>
<th>-572 G/C</th>
<th>-395 to -375 AₙTₙ tract</th>
<th>-174 G/C</th>
<th>GRE knockout</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1180 to +13</td>
<td>G</td>
<td>G</td>
<td>A₉T₁₁</td>
<td>G</td>
<td>-</td>
</tr>
<tr>
<td>-1180 to +13</td>
<td>G</td>
<td>G</td>
<td>A₉T₁₁</td>
<td>G</td>
<td>GRE1</td>
</tr>
<tr>
<td>-1180 to +13</td>
<td>G</td>
<td>C</td>
<td>A₉T₁₁</td>
<td>G</td>
<td>-</td>
</tr>
<tr>
<td>-1180 to +13</td>
<td>G</td>
<td>C</td>
<td>A₉T₁₁</td>
<td>G</td>
<td>GRE1</td>
</tr>
<tr>
<td>-1180 to +13</td>
<td>G</td>
<td>C</td>
<td>A₉T₁₁</td>
<td>G</td>
<td>GRE2</td>
</tr>
</tbody>
</table>

7.3.3 Functional studies

7.3.3.1 GRE knockout of -572 variant

HuH7 cells were plated for transfection in 96-well TC plates as described in section 4.3.1. Transient transfection of the pGL3 Basic IL-6 -572G, -572C and the GRE knockout constructs from table 7.1 was carried out in HuH7 cells using LIPOFECTIN reagent and then stimulated by the additions of DEX (5X 10⁻⁷M) or IL-1β (100U/ml) plus DEX (5X 10⁻⁷M) for 12 hours in accordance with the protocol for HepG2 cells as described in section 3.3.3.5. The relative promoter activities of samples containing the various IL-6 constructs were then determined using the protocol described in section 3.3.3.6.
7.4 Results

7.4.2 Functional effects of the GRE knockouts for the IL-6 -572G and -572C variants

7.4.2.1 Effects of the IL-6 -572G versus -572C

Firstly the higher relative promoter strength for the -572C variants compared to the -572G variant seen in chapter 4 were reconfirmed in HuH7 cells. The results presented in figure 7.3 shows that the IL-6 -572C construct has higher relative promoter strength than the -572G construct under DEX inhibition (54%) and IL-1β plus DEX induction (46%), although in both experiments, the differences between the constructs was not statistically significant (p=0.079 and p=0.149 respectively). Both constructs were induced by IL-1β plus DEX from basal levels (-572G=1.6 fold and -572C=2.4 fold), repression by DEX was only seen for the -572G variant (0.6 fold), but not for the -572C variant (1.1 fold).

Figure 7.3 Promoter activities of IL-6 -572G vs -572G constructs in HuH7 cells. Cells were stimulated with DEX (5X10-7M) and IL-1β (100U/ml) plus DEX (5X10-7M) for 12 hours. Mean relative light units are from 8 separate transfection for each construct, fold value are from basal and p-value using t-test.
7.4.2.2 IL-6 -572G vs GRE knockout of -572G

*In vitro* functional studies of the wild-type IL-6 -572G variant with the intact putative GRE site versus GRE knockout for the -572G was carried out in HuH7 cells to investigate the function of the GRE1 or GRE2 sites (-557 to -552 and -464 to -459) upon DEX repression. The results in figure 7.4 shows the comparison between the common allele -572G variant containing the intact putative GRE1 site and the GRE1 knockout for -572G. It can be seen that -572G GRE1 knockout has higher promoter activity than the wild type -572G at basal, in the presence of DEX repression and IL-1β plus DEX induction, with this difference reaching significance in the present of DEX repression (p=0.049). The addition of DEX to the HuH7 cells for 12 hours resulted in lower (36%) promoter activity for -572G wild-type compared to that at basal (p=0.001). No repression by DEX was seen for the -572G GRE1 knockout variant. This indicates that impairment of the GRE1 site eliminated repression by DEX. The promoter activities of both constructs was induced by the addition of IL-1β plus DEX, with the greatest fold difference from basal level seen for the -572G GRE1 knockout compared to the wild-type -572G construct (2.2 fold vs 1.6 fold respectively). Comparison with the -572G GRE2 knockout or the GRE double knockout for the -572G variants could not be made because the generation of these variants was unsuccessful.
Figure 7.4 Promoter activities of -572G vs -572G Gre1 knockout in HuH7. Cells were stimulated with DEX (5x10^{-7}M) and IL-1β (100U/ml) plus DEX (5x10^{-7}M) for 12 hours. Mean relative light units are from 8 separate transfection for each construct, fold value are from basal and p-value using t-test.

7.4.2.3 **IL-6 -572C vs GRE knockouts for -572C**

*In vitro* experiments were carried out as described in section 7.4.3 using the -572C, -572C Gre1 and -572C Gre2 variants to determine the functionality of the two GRE sites in response to DEX repression and IL-1β plus DEX induction. The results are presented in table 7.2, and are expressed as percentage difference from the wild-type -572G variant at basal. It can be seen that the promoter activity of the -572C, -572C Gre1 and -572C Gre2 variants was not repressed by DEX and the presence of DEX appeared to result in the *induction* of the IL-6 promoter expression activity for the -572C Gre1 and -572C Gre2 variants by 113% and 118% respectively. IL-1β plus DEX induction resulted in increased promoter activities of all the variants tested (-572G Gre1=175%, -572C, -572C Gre1 and -572C Gre2=135%, 63% and 46% respectively). There was no major difference in promoter activity between the two -572C constructs containing the knockout for Gre1 or Gre2. The -572C construct showed greater induction than either the -572C Gre knockout variants. However when this lower basal activity is taken into account, the activity is similar i.e. 163% to 183% and 146% to 247% compared to 235%.
Table 7.2 Relative promoter strength of GRE knockout for IL-6 -572G and -572C promoter variants (n=8) in HuH7 cells. Values are expressed as percentages and comparison made relative to the -572G variant at basal (set at 100%).

<table>
<thead>
<tr>
<th>Allele</th>
<th>Glucocorticoid responsive element (GRE) knockout</th>
<th><strong>Stimuli</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GRE1</td>
<td>GRE2</td>
<td>Basal</td>
</tr>
<tr>
<td>-572G</td>
<td>-</td>
<td>-</td>
<td>100%</td>
</tr>
<tr>
<td>-572G</td>
<td>X</td>
<td>-</td>
<td>123%</td>
</tr>
<tr>
<td>-572C</td>
<td>-</td>
<td>-</td>
<td>98%</td>
</tr>
<tr>
<td>-572C</td>
<td>X</td>
<td>-</td>
<td>89%</td>
</tr>
<tr>
<td>-572C</td>
<td>-</td>
<td>X</td>
<td>56%</td>
</tr>
</tbody>
</table>

X=GRE knockout

*P-values were calculated using t-test from -572G (Basal)
7.5 Discussion

7.5.1 IL-6 -572G wild-type versus GRE knockout in HuH7 cells

GRE1 knockout (-557 to -552) for the IL-6 -572G constructs were used to explore the hypothesis that the lower promoter activity of the -572G construct compared to that of the -572C constructs in HuH7 cells (see chapters 3 and 4) by IL-1β plus DEX was due to greater repression through the GRE site. The significantly higher relative promoter strength of the -572G construct containing the GRE1 knockout over that of the wild-type -572G in the presence of IL-1β plus DEX from that at basal (174% vs 61%) and the inability to be repressed by DEX (+33% vs -36%) indicate that this GRE1 site may be important in determining IL-6 gene expression. Since the GRE2 and the double GRE knockout of the -572G construct were not successful, the result from this one pilot study must be interpreted with caution. Also because of time limitation, the experiment was done only once using one plasmid DNA preparation. Further repeat experiments are required, to include double GRE knockout and the GRE2 knockout for the -572G variant. Work should also be repeated in another liver cell type such as HepG2 with different plasmid preparations in order to confirm this finding.

7.5.2 IL-6 -572C versus GRE knockout of -572C in HuH7 cells

GRE knockouts were made to test the function of the two GRE sites relative to the -572C constructs from DEX repression or IL-1β plus DEX induction in HuH7 cells. The mechanism to explain the higher promoter activity of the -572C GRE1 (113%) and GRE2 (118%) knockout compared to that of the wild-type -572C construct in the presence of DEX repression is not certain. However, it is known that glucocorticoids can activate as well as inhibit the transcription of some genes (see review by Almawi et al 2002).

The modestly lower promoter activity of the -572C GRE1 knockout (11%) and greater effect for the GRE2 knockout (44%) in the presence of IL-1β plus DEX induction compared to DEX was unexpected. One would hypotheses that knocking out the two putative GRE binding sites would enhance the induction or make no difference to IL-6
gene transcription. Since this experiment is only an initial pilot experiment, it is possible that this is a chance finding. However, due to insufficient time, further experiments could not be done to check for this.

Overall, the data from this chapter, if repeatable, suggests that the GRE1 and GRE2 sites in the human IL-6 gene is functional to repression by glucocorticoids and is critical in the determination of IL-6 expression and CHD risk. The substitution from G>C at position -572 therefore could impair the binding of the GC-GR complex to either the GRE1 or GRE2 site resulting in lower repression of IL-6 expression.
CHAPTER 8

CONCLUSIONS
Chapter 8: Conclusions

The work presented in this thesis is an investigation of the effects of IL-6 promoter polymorphisms on gene transcription rate, and on plasma levels of IL-6 and related traits for CHD risk. IL-6 is known to exert an overall pro-atherogenic effect through its upregulation of numerous genes that encode for acute phase proteins involved in the pathogenesis of atherosclerosis. The aim of this study was to examine genetic variations in the human IL-6 gene in the determination of IL-6 gene expression and its association with CHD risk in the general population of healthy middle aged men and also in a case-control study of MI survivors.

Previous work jointly carried out in ours (Dr Faulds) and in Professor Woo’s laboratories (Drs Fishman & Jeffrey) had resulted in the identification of numerous IL-6 promoter variants (-174G>C, -597G>A and the AnTn tract). In chapter 2, three novel variants were identified in addition to those already found namely, a G>C at position -572, C>A at -627 and a wobble position in codon F201 (TTT>TTC) (frequency of rare allele=0.05, 0.01 and 0.04 respectively) by single stranded conformation polymorphism (SSCP) and DNA sequencing. High-throughput genotyping methods using RFLP and the MADGE gel system for the more common variants -174G>C (NlaIII), -597G>A (FokI) and -572G>C (Mbol) was developed for population screening for these polymorphisms. The -174G>C and -597G>A variants were in strong allelic association with each other (delta=0.94).

Functional studies of the IL-6 -174G>C and -572G>C variants in HepG2 and HuH7 cells using the dual luciferase reporter assay system showed that the -174C variant was associated with higher relative promoter strength than the -174G variant over a 24 hour time course period after dexamethasone (DEX) repression or IL-1β plus DEX induction in both HepG2 and HuH7 cells. The -572C variant also gave higher promoter activity than the -572G variant in response to the same stimulus and time periods in these cells. When the glucocorticoid response elements (GRE) at position -557 to -552 were destroyed by mutation, the -572G promoter construct in HuH7 cells no longer showed repression by DEX and had higher induction from IL-1β plus DEX compared to the wild-type -572G variant. This suggests that the GRE is functional when in combination with
the -597G/-572G and -174G variant. Knockout of GRE1 and to a greater extent of GRE2 reduced basal expression of the -597G/-572C/-174G construct, but not repression or induction. Possible mechanism could be that the C variant at position -572 interferes with the induction/repression by GR-mediated nuclear binding factors or it creates a binding site for unknown nuclear transcription factors which in turn blocks GC-GR complex-mediated repression of IL-6 gene transcription. No promoter activity difference was found for the -572C GRE knockout compared to the -572C wild type variant. This suggests that the impact of the -572G>C variant is not acting via GRE-related mechanisms.

The IL-6 -174G>C and -572G>C promoter polymorphisms were examined in the second Northwick Park Heart Study (NPSHII) of ~3000 prospective healthy middle-aged men from the UK (frequency of rare allele 0.43 95% CI=0.42-0.44 and 0.05 95% CI=0.04-0.06 respectively). The -174C allele was not associated with fibrinogen levels, but was significantly associated with higher systolic blood pressure in smokers and non-smokers, an effect which was greater in men in the top two tertiles for body mass index (>24.68kg/m²). Compared to those with the GG genotype, men carrying the -174C allele had a relative risk of CHD of 1.54 (95% CI=1.0-2.23, p=0.048) and this effect was greatest in smokers (RR=2.66, 95% CI=1.64-4.32). These effects remained statistically significant even after adjusting for classical risk factors including systolic blood pressure (p=0.04). The -572C allele was not associated with a significant effect on blood pressure, fibrinogen or relative risk for CHD. To confirm the finding of the NPHSII study, the same two polymorphisms were examined in MI survivors and healthy aged-matched control from four European centres (HIFMECH study). A significant gradient of declining allele frequency was seen in the controls (p=<0.005) and the cases (p=0.032) for the -174C allele, but not for the -572C allele. There were no significant differences in genotype distribution or allele frequency between cases and controls in the North or the South. Significantly higher plasma concentrations for IL-6, fibrinogen and CRP were seen in MI survivors compared to the healthy controls in the North and South of Europe (p=<0.00005). Smoking was associated with greater risk in the South of Europe (OR=3.60, 95% CI=2.34-5.52) compared to the North (OR=2.30, 95% CI=1.48-3.55), but
the North-South interaction was not significant. The -174G>C genotype was not significantly associated with levels of IL-6, fibrinogen, CRP or blood pressure in MI survivors or healthy control men from the North or South of Europe. Carriers for the -572C allele who were smokers had significantly higher systolic (5% higher, p=0.004) and diastolic blood pressure (3.4% higher, p=0.03) compared to -572GG genotype, an effect not seen in the nonsmokers. The lack of confirmation of the CHD risk associated with the functional -174C allele in the HIFMECH study and the novel associations seen with the -572C allele may be a result of the case-control design.

Overall, the findings in this thesis support recent in vivo primary prevention trial data from the WOSCOPS study looking at the effectiveness of pravastatin in reducing morbidity and mortality from CHD (Basso et al 2002). In this study, hypercholesterolemic men homozygous for the -174C allele had higher CHD risk compared to those with the GG+GC genotype (placebo), and were better responder to pravastatin treatment in reducing the risk from CHD.
CHAPTER 9

FUTURE WORKS
Chapter 9: Future works

9.1 Functional studies

The inherent problem associated with transient transfection studies is the variability due to the state and nature of these cells pre and post transient transfection procedures that results in an inconsistency in relative promoter strength for the -174G>C and the -572G>C variants between experiments. The construction of stable cell lines expressing the different IL-6 variants investigated here would allow for selection of cells that contain the IL-6 construct and therefore reduce to a large extent the experimental variations that are seen. The use of high IL-6 expression cell lines such as monocytes, macrophages (THP-1 and RAW264) and adipocytes cell lines (3T3-L1 and F442A adipocytes) compared to relatively low IL-6 expressers such as hepatocytes (HepG2 and HuH7) (Dr Mohamed-Ali, personal communication) would be better in reducing relative promoter strength variability between IL-6 constructs and experiments. An alternative approach to investigate the functionality would be direct RNA quantitation using RT-PCR from monocytes of subjects selected specifically for the IL-6 -174G>C and -572G>C genotypes. Treatments of the cell ex vivo with dexamethasone or IL-1β plus dexamethasone would also allow relative promoter strength differences between the genotype to be examine and would therefore give an indication of IL-6 protein expression.

9.2 Association studies

The NPHSII and the HIFMECH studies were not designed for looking specifically at associations between IL-6 genotype with IL-6 levels and CHD risk. The inappropriate natures of sample collections make accurate measurement for IL-6 concentration impossible. Therefore the association of the -174C allele with CHD risk only in the NPHSII study and the -572C only in the HIFMECH study needs to be confirmed in several other larger longitudinal and case-control studies.
CHD risk attributed to IL-6 -174G>C and -572G>C genotypes have only been studied in caucasians, mostly in men and not in other ethnic groups (NPHSII and HIFMECH studies). It is known that the frequency of the rare -174C allele is different in the different ethnic groups (Fishman et al 1998, see table 1.3). Therefore CHD risk assessments are needed in these groups to find out whether CHD risk association are the same as that seen in caucasians.

9.3 IL-6 A₈Tₙ tract

In vitro data reported by Terry et al 2000 in HeLa and ECV304 cells have found evidence for the functionality of the A₈Tₙ tract (-395 to -375). However, no in vivo data have yet been published concerning the A₈Tₙ tract. It is hypothesed that the composition of As and Ts that make up this A₈Tₙ tract could determine the nature of its unfolding, and this in turn will effect the binding of nuclear transcription factors that promote or inhibit IL-6 gene transcription, and hence affect IL-6 levels and thus CHD risk susceptibility. Possible methods for population screening of the A₈Tₙ tract include PCR amplification with Pfu Turbo DNA polymerase (do not add As at the end of PCR product), followed by restriction digestion with Tsp509 I (recognition sequence ‘AATT’) and then GeneScan analysis (ABI biosystem) of the cut and uncut samples. The second method and most accurate would be using DNA sequencing.
References


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Medical Research Council Committee on Chronic Bronchitis and Questionnaire Respiratory Symptoms. 1995


Van der Wal, A.C., Becker, A.E., van der Loos, C.M., and Das, P.K. (1994) Site of intimal rupture or erosion of thrombosed coronary atherosclerotic plaques is characterized by an inflammatory process irrespective of the dominant plaque morphology. Circulation 89: 36-44.


Appendix

Table 1 PCR primers for amplification of exons 1-5 in the human IL-6 gene

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primer</th>
<th>Sequence</th>
<th>PCR size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IL-6(1)F</td>
<td>5'-CCCATGAGTCTCAATATTAGAGTC-3</td>
<td>284</td>
</tr>
<tr>
<td></td>
<td>IL-6(2)R</td>
<td>5'-CGAGCGCAGGGTGACTGAC-3'</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>IL-6(3)F</td>
<td>5'-CCCCAGCTGCTGCTGTCGTAC-3'</td>
<td>296</td>
</tr>
<tr>
<td></td>
<td>IL-6(4)R</td>
<td>5'-GGAGGGAGACGCATCGC-3'</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>IL-6(5)F</td>
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<td>IL-6(6)R</td>
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<td>IL-6(10)R</td>
<td>5'-GGACAGCTTCTGACCAGAAGG-3'</td>
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</table>

Table 2 SSCP primers for amplification of the human IL-6 5'-untranslated region

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<td></td>
<td>IL-6 Pro(2)R</td>
<td>5'-CAGGCGAGAAAGGGGAGATTAC-3'</td>
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<tr>
<td>-973 to -674</td>
<td>IL-6 Pro(3)F</td>
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<tr>
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<td>294</td>
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<td>IL-6 Pro(6)R</td>
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Table 3  Site-directed mutagenesis oligonucleotide primers of human IL-6 promoter region

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 -572C Mut F</td>
<td>5' - GTTCTACAACAGGCCCTCACAGGGAGGCC-3'</td>
</tr>
<tr>
<td>IL-6 -572C Mut R</td>
<td>5' - GCTTCCTCCTGTGAGGCTGTTGTAAC-3'</td>
</tr>
<tr>
<td>IL-6 -174C Mut F</td>
<td>5' - CTTGTTGCTTTGAGATCGTACACCTG-3'</td>
</tr>
<tr>
<td>IL-6 -174C Mut R</td>
<td>5' - CAAATGCGAGCTCTTTGACATGGCCAAGACAATAGCAG-3'</td>
</tr>
<tr>
<td>IL-6 -597A Mut F</td>
<td>5' - CTGCACGAAAATGGTAGGAGGCCAGGACTTCTAC-3'</td>
</tr>
<tr>
<td>IL-6 -597A Mut R</td>
<td>5' - GATAGAATGGCCAGAGATTCGCTCCTTACATGAGCAG-3'</td>
</tr>
<tr>
<td>IL-6 GRE1 F</td>
<td>5' - CAGAGAGGAGGAGACAAATTTCAGAAGACTCAGATGACTGG-3'</td>
</tr>
<tr>
<td>IL-6 GRE1 R</td>
<td>5' - CAGTCATCTAGTTTCTGTGAATTTGTACCCTCTTCGG-3'</td>
</tr>
<tr>
<td>IL-6 GRE2 F</td>
<td>5' - CAGAGGAAACTCGAGCTGATCCTTTGTTTACAAATAC-3'</td>
</tr>
<tr>
<td>IL-6 GRE2 R</td>
<td>5' - GTATTGTGAAAAACCAAAGATCAGCTGAACCTGATTCTCTG-3'</td>
</tr>
</tbody>
</table>

Notes:
IL-6 -572G (Wt) AGCCGCTCA; IL-6 -572C (Mut) AGGCCTCA
IL-6 GRE1 (Wt) CCAGAAACAGAAG; IL-6 GRE1 (Mut) CCAGAATTCAGAAG
IL-6 GRE2 (Wt) AGTTCAGACATCTTTGG; IL-6 GRE2 (Mut) AGTTCAGACTGCCTTTGG

Table 4  IL-6 inserts sequencing oligonucleotide primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rvprimer3</td>
<td>5'-CTAGCAAAATAGGCTGCC-3'</td>
</tr>
<tr>
<td>Glprimer</td>
<td>5'-CTTTATGTTTGGGCCTCC-3'</td>
</tr>
<tr>
<td>IL-6 Pro(2)R</td>
<td>5'-CAGGGCAGAAAGGGGAGATTAC-3'</td>
</tr>
<tr>
<td>IL-6 Pro(3)F</td>
<td>5'-CAAGGTCCCTCCCTTGACATCCCC-3'</td>
</tr>
<tr>
<td>IL-6 Pro(4)R</td>
<td>5'-GTCTGACATTTTCTCCTACCTGCTCC-3'</td>
</tr>
<tr>
<td>IL-6 Pro(6)R</td>
<td>5'-GATTTTCTCTGACCTCCATCGAC-3'</td>
</tr>
<tr>
<td>IL-6 Pro(5b)F</td>
<td>5'-GGAGACGGCAGCTGAAGTAACTGC-3'</td>
</tr>
<tr>
<td>IL-6 Pro(8)R</td>
<td>5'-CAGCAGTTTTGGGATGTTCTTGAC-3'</td>
</tr>
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<td>IL-6 Pro 9A</td>
<td>5'-TGACTTCAGCTTTACTCTTGT-3'</td>
</tr>
<tr>
<td>IL-6 Pro 10A</td>
<td>5'-CTGATTTGGAAACCTTATTAAG-3'</td>
</tr>
</tbody>
</table>
Electrophoresis buffer solutions

**10X TBE (Tris-borate EDTA) buffer (1L)**
107.8g Tris
55g Boric acid
7.44g EDTA (disodium ethylenediaminetetraacetate, dihydrate)

**10X TAE (Tris-acetate EDTA) buffer**
0.4M Tris-acetate
10mM EDTA

Polymerase chain reaction (PCR) buffers

**10X Polmix**
500mM KCl
100mM Tris (pH 8.3)
0.01% Gelatin
2mM each dNTPs

**10X NH₄ Polmix**
160mM (NH₄)₂SO₄
670mM Tris (pH 8.4)
0.001 v/v Tween 20
2mM each dNTPs

**10X Pfu DNA polymerase reaction buffer**
100mM KCl
100mM (NH₄)₂SO₄
200mM Tris-HCl (pH 8.8)
20mM MgSO₄
1% Triton X-100
1mg/ml Nuclease-free bovine serum albumin (BSA)
Cloning buffers

**10X Ligase buffer**
500mM Tris-HCl (pH 7.5)  
70mM MgCl₂  
10mM Dithiothreitol  
10mM rATP

Transfection reagents, buffers and staining solutions

**LIPOFECTIN reagent**
1:1 (w/w) liposome formulation of cationic lipid:
- N-[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA)
- Dioleoyl phosphotidylethanolamine (DOPE)

**PBS (Phosphate Buffer Saline)**
137mM NaCl  
2.7mM KCl  
4.3mM Na₂HPO₄  
1.47mM KH₂PO₄

**Solutions for X-gal staining**
5mM Potassium ferricyanide (K₃Fe(CN)₆)  
5mM Potassium ferrocyanide (K₄Fe(CN)₆.3H₂O)  
1mg/ml X-gal in dimethylformamide  
1mM MgCl₂  
Dissolve in PBS

**1X Trypsin-EDTA solution**
0.05% (w/v) Trypsin  
0.53mM EDTA
Bacterial growth media

**LB Medium (1L)**

- 10g Bacto-tryptone
- 5g Bacto-yeast extract
- 10g NaCl

Add deionized water to approximately 1L. Adjust to pH 7.5 with 10N NaOH and autoclave.

**LB Plates (1L)**

- 10g Bacto-tryptone
- 5g Bacto-yeast extract
- 10g NaCl
- 20g Bacto-agar

Add water to a final volume of 1L. Adjust to pH 7.0 with 5N NaOH and autoclave.

**SOB Medium (1L)**

- 20g Bacto-tryptone
- 5g Bacto-yeast extract
- 0.5g NaCl

Autoclave, add 10ml of 1M MgCl₂ and 10ml of 1M MgSO₄/litre of SOB medium prior to use. Filters sterilize.

**SOC Medium (per 100ml)**

SOC Medium

Add 1ml of a 2M filter-sterilized glucose solution or 2ml of 20% (w/v) glucose prior to use & then filter sterilize.

**NZY⁺ Broth (1L)**

- 10g NZ amine (casein hydrolyase)
- 5g Yeast extract
- 5g NaCl

Adjust to pH 7.5 using NaOH. Autoclave and then add the following supplement prior to use: 12.5ml of 1M MgCl₂, 12.5ml 1M MgSO₄ and 10ml of 2M filter-sterilized glucose solution or 20ml of 20% (w/v) glucose. Filter sterilize
Solutions for DNA extraction

(1) **Reagent A (1L)**
- 109.5g Sucrose
- 5ml 1M MgCl₂
- 10ml 1M Tris (pH 7.5)
- 10ml Triton X 100
Make up to 1L with deionized water

(2) **Reagent B (100ml)**
- 1ml 1M Tris-HCl (pH 8.2)
- 2.34g NaCl
- 0.4ml 0.5M EDTA (pH 8.0)
Make up to 90ml with deionized water, autoclave & add 10ml 10% SDS

(3) **5M Sodium perchlorate (100ml)**
- 70.24g Sodium perchlorate
Make up to 100ml with deionized water

(4) **TE buffer (pH 7.6)**
- 1.21g Tris
- 0.37g EDTA
Make up to 1L with deionized water, pH with Conc HCl & autoclave

(5) **Other solutions**
- (a) Chloroform stored at -20°C
- (b) 100% ethanol stored at 4°C or -20°C
- (c) 70% ethanol stored at room temperature
**Restriction endonucleases buffers and BSA**

### 10X NEBuffer BamHI
- 1.5M NaCl
- 100mM Tris-HCl
- 100mM MgCl₂
- 10mM Dithiothreitol (pH 7.9)

### 10X NEBuffer 4
- 500mM Potassium acetate
- 200mM Tris-acetate
- 100mM Magnesium acetate
- 10mM Dithiothreitol (pH 7.9)

### 100X BSA
- 1mg/ml Bovine serum albumin (BSA)

### 10X NEBuffer 1
- 100mM Tris-Propane-HCl
- 100mM MgCl₂
- 10mM Dithiothreitol (pH 7.9)

### 10X Buffer Y⁺/Tango
- 33mM Tris-acetate (pH 7.9 at 37°C)
- 10mM Magnesium acetate
- 66mM Potassium acetate
- 0.1mg/ml BSA
Microtitre array diagonal gel electrophoresis (MADGE) gel mix and loading dye

The MADGE gel consists of an open faced horizontal system (100 x 150mm, 2mm deep with 8 x 12 wells). The wells are at an angle of 71.6° to the row of axis of the array, but perpendicular to the edge of the Perspex formers (Day and Humphries et al 1994) with a maximum track length of 26.5mm.

To prepare a MADGE gel, a glass plate (100 x 150 x 2mm) was cleaned with detergent followed by ddH₂O rinsing and then dried. One side of the glass plate was coated with 1ml of 'sticky silane' (0.5% (v/v) glacial acetic acid, 0.5% (v/v) γ-methacryloxypropyltrimethoxysilane) and allowed to air dry for 5 minutes (this enables the polyacrylamide gel to adhere to the glass plate). In the meantime, an 8% nondenaturing MADGE gel (30ml) solution was prepared from 30% stock acrylamide-bisacrylamide solution (19:1 ratio of acrylamide-bisacrylamide) containing 1X TBE buffer, 150μl N’N’N’N'-Tetramethylethlenediamine (TEMED), 150μl of 25% ammonium persulphate (APS) and ddH₂O. The gel mix was then thoroughly mixed and then poured all over the MADGE former. A sticky silane side of the glass plate was then gently placed over the MADGE former-gel mix and then a 250g weight placed on top. The gel was allowed to polymerize at room temperature for 20 minutes. After polymerization, the glass plate was removed from the MADGE former and any excess polyacrylamide gel was scraped off the edge of the glass plate. The gel can either be used immediately or stored in 1X TBE buffer for several weeks.

**Formamide loading dye**

<table>
<thead>
<tr>
<th>%</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>98%</td>
<td>formamide</td>
</tr>
<tr>
<td>10mmol/l</td>
<td>EDTA</td>
</tr>
<tr>
<td>0.025%</td>
<td>Xylene cyanol FF</td>
</tr>
<tr>
<td>0.025%</td>
<td>Bromophenol blue</td>
</tr>
</tbody>
</table>

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Single stranded conformational polymorphism (SSCP) gel

Gel preparation

Two plates were used to pour a gel, one larger plate (33 x 42cm) and one smaller plate (33 x 39cm). The larger plate was labeled ‘out’ on one side and the smaller plate was labeled ‘in’. Before use, plates were washed thoroughly in detergent, rinsed in ethanol (100%) and distilled water and wiped dry. The unmarked surfaces of the two-labelled plate were coated with Repelcote (2ml). The plates were arranged such that the larger ‘out’ plate rested on a leveled platform with the side labeled ‘out’ closest to the platform. The ‘in’ plate was then placed on top with the side marked ‘in’ facing out. This plate was aligned perfectly with the ‘out’ plate forming a straight labeled edge at the bottom. A pair of 0.4mm x 10mm x 400mm spacers supported both sides of the glass plates. The plates were clamped at the side with bulldog clips. The gel was poured by injection between the glass plates using a 50ml syringe using the gel mixtures shown below. The plates were tapped firmly to aid the flow of the gel mix under capillary action to prevent the formation of bubbles. Gels were left for two hours to allow polymerization to complete before use.

Gel mixtures

To prepare a 7.5% non-denaturing SSCP polyacrylamide gel (75ml), the following reagents was used:

- 11.25ml 50% acrylamide-bisacrylamide solution (49:1 acrylamide-bisacrylamide)
- 7.5ml 10X TBE buffer
- 3.75ml Glycerol
- 1.5ml 0.5M EDTA
- 115μl 25% Ammonium persulphate
- 115μl TEMED
Minimun Essential Medium (MEM) (Cat no. 31095-029)
With Earle’s Salts and L-glutamine

- 264mg/l CaCl₂.2H₂O
- 400mg/l KCl
- 200mg/l MgSO₄.7H₂O
- 6.8g/l NaCl
- 2.2g/l NaHCO₃
- 158mg/l NaH₂PO₄.2H₂O
- 1g/l D-Glucose
- 10mg/l Phenol Red
- 126mg/l L-Arginine.HCl
- 24mg/l L-Cystine
- 292mg/l L-Glutamine
- 42mg/l L-Hisidine HCl.H₂O
- 52mg/l L-Isoleucine
- 52mg/l L-Leucine
- 73mg/l L-Lysine.HCl
- 15mg/l L-Methionine
- 32mg/l L-Phenylalanine
- 48mg/l L-Threonine
- 10mg/l L-Tryptophan
- 36mg/l L-Tyrosine
- 46mg/l L-Valine
- 1mg/l D-Ca Pantothenate
- 1mg/l Choline Chloride
- 1mg/l Folic Acid
- 2mg/l i-Inositol
- 1mg/l Nicotinamide
- 1mg/l Pyridoxal HCl
- 0.10mg/l Riboflavin
- 1mg/l Thiamine HCl
3T3-L1 cell growth media

*Dulbecco’s Modified Eagle Medium (D-MEM) (Cat No. 22320-022)*
With L-glutamine, 1000mg/ml D-Glucose, Sodium Pyruvate & 25mM HEPES

264mg/l CaCl$_2$.2H$_2$O  
0.10mg/l Fe(NO$_3$)$_3$.9H$_2$O  
400mg/l KCl  
200mg/l MgSO$_4$.7H$_2$O  
3.5g/l NaCl  
3.7g/l NaHCO$_3$  
141mg/l NaH$_2$PO$_4$.2H$_2$O  
1g/l D-Glucose  
15mg/l Phenol Red  
5.96g/l HEPES  
110mg/l Sodium Pyruvate  
84mg/l L-Arginine.HCl  
48mg/l L-Cystine  
580mg/l L-Glutamine  
30mg/l Glycine  
42mg/l L-Histidine HCl.H$_2$O  
105mg/l L-Isoleucine  
105mg/l L-Leucine  
146mg/l L-Lysine HCl  
30mg/l L-Methionine  
66mg/l L-Phenylalanine  
42mg/l L-Serine  
95mg/l L-Threonine  
16mg/l L-Tryptophan  
72mg/l L-Tyrosine  
94mg/l L-Valine  
4mg/l D-Ca pantothenate  
4mg/l Choline Chloride  
4mg/l Folic Acid  
7.2mg/l L-Inositol  
4mg/l Nicotinamide  
4mg/l Pyridoxine HCl  
0.4mg/l Riboflavin  
4mg/l Thiamine HCl
Publications


