UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

School of Medicine

Tissue Specific Genetic Regulation of Interleukin 6

by

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ABSTRACT

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES
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TISSUE SPECIFIC GENETIC REGULATION OF IL6

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Interleukin 6 (IL6) is associated with arterial disease development, progression and surgical outcome. Raised levels of IL6 may play a causal role in disease development or may be the effect of pathology. An *IL6* single nucleotide polymorphism (SNP) G-174C has been identified and reported to associate with IL6 expression. However, conflicting results have emerged and both the relationship between IL6 and vascular disease and the precise effect of SNP G-174C in vivo in humans remains obscure.

The aim of this study was to establish the effect of SNP G-174C in humans, in vivo in different tissues. Varicose vein surgery patients donated adipose tissue, skeletal muscle, vein and blood samples. Patients were genotyped for SNP G-174C. A new pre-mRNA assay was developed, using gel electrophoresis, restriction digest and fluorescence quantification, to measure the ratio of heterozygous allelic pre-mRNA transcription. IL6 mRNA expression in different tissues was also measured using relative real time PCR (RT-PCR) to assess effect of tissue type on expression profiles. mRNA expression within tissues was compared between G-174C genotypes, to further quantifying the association of SNP G-174C with transcript levels.

The pre-mRNA assay showed higher expression for the C-allele, though not statistically significant. The pre-mRNA assay needed to detect low levels of intron retaining allelic pre-mRNA isoforms. Replicates and controls for residual genomic DNA were used to monitor assay precision. Adipose tissue gave the greatest precision in the pre-mRNA assay. In the RT PCR assay adipose tissue expressed significantly more IL6 mRNA than all other tissues examined. In vein and leukocytes subjects with the CC genotype expressed significantly higher levels of IL6 mRNA than subjects with GC or GG genotypes. There was a trend towards higher expression for the CC genotype in all tissue types. A significant though weak correlation between IL6 mRNA expression and age was demonstrated for vein and leukocytes.

Adipose tissue may be an important source of IL6 compared to other tissues. This may be relevant for obesity associated diseases. Subjects with G-174C genotype CC showed a trend towards higher IL6 RNA expression. Further studies are necessary to clarify the effect of genotype on IL6 expression.

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I, Sabine Sonnenberg declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

TISSUE SPECIFIC GENETIC REGULATION OF IL6

I confirm that:

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   Sonnenberg S, Shearman CP, Baxter S, Morris GE, Cumming DV, Montgomery H, Rose-Zerilli MJ, Day IN; Level of ex vivo interleukin 6 expression in human peripheral fat compared with other tissues. Eur J Vasc Endovasc Surg. 2008 Mar; 35(3):314-9.

   S Sonnenberg, et al., The Regulation of Interleukin 6 in Surgical Tissues, Society of Academic and Research Surgery, SARS Yearbook P.93, 2007 (Abstract)

Signed: ……

Date: 22.07.2009…….

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I dedicate this thesis to

Nick, Emma, Albert, Hannah and George

Abbreviations

AAA	abdominal aortic aneurysm
AGTR-1	angiotensin II receptor type 1
ARDS	adult respiratory distress syndrome
ASA	American society of Anaesthesiologists
Bp	base pairs
Dest 1	de-stained once
Dest 2	de-stained twice
CAD	coronary arterial disease
cDNA	complimentary DNA, synthesised from RNA
cDNA-Ex4	cDNA synthesised using specific primers annealing in exon 4
cDNA-In4	cDNA synthesised using specific primers annealing in intron 4
cDNA-In3	cDNA synthesised using specific primers annealing in intron 3
cDNA-In2	cDNA synthesised using specific primers annealing in intron 2
CVD	cardiovascular disease
DNA	desoxyribonucleic Acid
DSU	day surgery unit
EB	ethidium bromide
ELB	erythrocyte lysis buffer
ERK	extracellular signalling regulated kinase
EVAR	endovascular aneurysm repair
hnRNA	heteronuclear RNA
ICAM-1	intercellular adhesion molecule 1
IL6	interleukin 6 (protein or RNA)
IL6	interleukin 6 gene (italics are used by convention)
IL6R	interleukin 6 receptor
IMT	intima-media thickness
IRS	insulin receptor substrate
JAK	janus kinase
LD	linkage disequilibrium
LDL-R	low density lipoprotein receptor
LOX	lecithin like oxLDL receptor
LPS	lipopolysaccharide (endotoxin)
MADGE	microplate array diagonal gel electrophoresis

MAPK	mitogen activated protein kinase
MCP1	monocyte chemotactic protein
MI	myocardial infarction
M-MLV	Maloney Murine Leukaemia Virus
MMP	matrix metalloproteinase
mRNA	messenger RNA
NADH	nicotinamide adenine diphosphate
NADPH	nicotinamide adenine dinucleotide phosphate
NF	necrosis factor
NO	nitric oxide
NOS	nitric oxide synthase
OD	ratio of absorbance at 260 nm to absorbance at 280 nm in spectrophotometry; used to assess purity of nucleic acid quantified
oxLDL	oxidised low density lipoprotein
PAOD	peripheral arterial occlusive disease
RNA	ribonucleic Acid
rRNA	ribosomal RNA
ROS	reactive oxygen species
rpm	revolutions per minute
RT	reverse transcription
RT-PCR	real time PCR
SMC	smooth muscle cells
SOCS	suppressor of cytokine signalling
STAT	signal transducer and activator of transcription
Taq	thermus aquaticus (thermophilic bacterium)
TIG	label given to this study to allow identification of study samples in the laboratory (samples labelled as TIG X; X=Patient number)
TNFα	tumour necrosis factor alpha
ul	microliter
ug	microgram
UTR	untranslated region
VEGF	vascular endothelial growth factor
VCAM-1	vascular cell adhesion molecule 1

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S Sonnenberg, et al., The Regulation of Interleukin 6 in Surgical Tissues, Society of Academic and Research Surgery, SARS Yearbook P.93, 2007 (Abstract)

Presentations

Interleukin 6 expression in adipose tissue and effect of single nucleotide polymorphism G-174C, Norman Tanner/ MIA Price Meeting December 2007 RSM, London

Level of ex vivo Interleukin 6 expression in human peripheral fat compared to other tissues, South West Vascular Meeting, March 2007, Swansea

The regulation of Interleukin 6 in surgical tissues (Poster), SARS Conference, January 2007, Cambridge

Genetic regulation of Interleukin 6 and it's relevance in cardiovascular disease, Medical, Health & Life Sciences Conference, June 2006, Southampton

1 Introduction

1.1 Interleukin 6 (IL6)

Interleukin 6 (IL6) is regarded by many as one of the most important cytokine in the acute phase response (1-3). Besides its physiological role in immune defence, it has been associated with a wide range of diseases such as atherosclerosis (4), cancer, diabetes, asthma, rheumatic diseases, inflammatory bowel disease and a host of other pathological processes (5-9).

Cytokines such as IL6 are proteins that act as intercellular mediators. They are produced by a variety of tissues and cells and generally act locally in a paracrine or autocrine manner. IL6 is a predominantly pro-inflammatory cytokine. It is expressed by macrophages/monocytes, T-lymphocytes, adipocytes, endothelial cell, vascular smooth muscle cells, fibroblasts, osteoblasts, chondroblasts and myocytes (10-12). IL6 plays a central role in host defence through the induction of acute phase response proteins, maturation of B-cells into antibody producing cells and stimulation of proliferation and differentiation of cytotoxic T-cells (13). In addition to this role in immune response, IL6 has a positive effect on haematopoiesis and plays a role in regulating cell numbers and neuronal function in the central nervous system (14;15).

This study focused on IL6 because of its potential role in vascular disease development as well as outcome from vascular surgery. Numerous association studies have linked raised levels of interleukin 6 to the development and progression of atherosclerosis, however the causal nature of this association is still in doubt (see section 1.5). Genetic variation may have an effect on circulating levels of IL6; therefore genetic studies could potentially be used to investigate causality (see section 1.7). The experimental design of this study aimed to clarify the effect of a genetic variant on *IL6* gene expression.
The following introduction will summarise the background information important in understanding the potential regulatory mechanisms of this protein. It will summarise existing evidence of association between IL6 and aspects of vascular disease development as well as outcome from vascular surgery.

1.2 The IL6 Gene

The gene for IL6 was first mapped to chromosome 7 in 1986 (16). Subsequent studies further localised the gene to the short arm of the chromosome (position 7p21-p15) (17;18). The gene contains five exons. Exons are regions of a gene that are expressed i.e. are translated into protein (19) (see Figure 1-1).

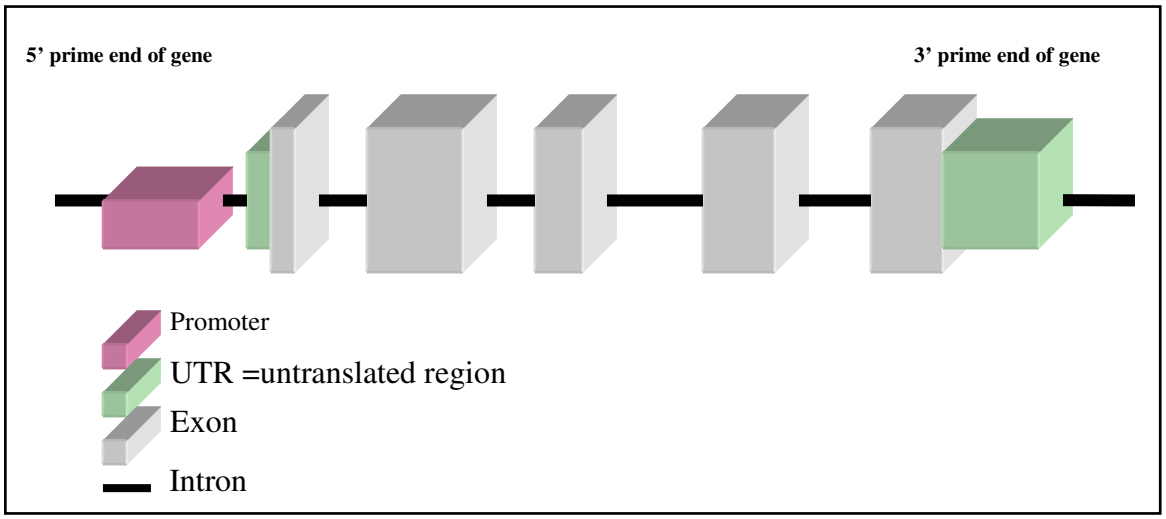


Figure 1-1: Schematic representation of IL6 gene (not to scale) showing promoter region (control point for regulated gene transcription), untranslated regions (region of gene transcribed into RNA but not translated into protein), introns (region of the gene transcribed into RNA but spliced out before translation into protein occurs) and exons (protein coding regions).

1.3 IL6 Expression

The plasma level of IL6 is determined by the rate of IL6 gene expression and subsequent release into the circulation as well as the plasma clearance rate (20;21). The activity and half-life of circulating IL6 is affected by the presence of interleukin 6 receptor (IL6R) which in turn is determined by factors such as exercise (22;23).
Thus the levels of biologically active circulating IL6 are regulated at various steps of which gene expression is the first. Gene expression is the process by which the DNA sequence of a gene is converted into a functional protein starting with transcription during which the coding DNA strand sequence is copied into ribonucleic acid (RNA) and ending with post translational changes of the protein substrate.
Expression of IL6 is induced by a variety of inflammatory stimuli such as interleukin 1β (IL1β), tumour necrosis factor α (TNFα), platelet derived growth factor,

transforming growth factor β, bacterial products (e.g. lipopolysaccharides) and viral infection (12;24). IL6 expression by monocytes is inhibited by IL4 and IL13 (25).

Gene expression is regulated at various levels. The first and most important is the primary control of transcription. Subsequent levels of regulation are alternative splicing, RNA editing, regulation of translation and post translational changes of the protein substrate (25;26).

Transcription is tightly regulated by transcription factors. Transcription factors form part of the transcription initiation complex which binds to the promoter region of the gene. They can act as activators, such as nuclear factor IL6 (NF-IL6) and nuclear factor κB (NF-κB) or as inhibitors, e.g. glucocorticoid receptor (24). Experiments have shown the region -180 to -123 in the promoter region of the IL6 gene to be important for transcription induction by IL1 (24;27;28). Genetic variation within this region may therefore affect gene expression.
Primary transcription of the gene results in a precursor RNA copy of the DNA sequence called heterogeneous nuclear RNA (hnRNA). hnRNA contains untranslated regions (UTRs) as well as exons and introns. Before RNA is translated into protein it undergoes splicing. Splicing removes introns from the RNA sequence and binds consecutive exons together. This process occurs in the nucleus. Splicing is catalysed by the spliceosome, a large RNA/protein complex composed of small nuclear ribonuclear proteins (snRNP) [snRNP= small nuclear ribonucleic acids (snRNA) and ribonuclear proteins (RNP)]. Catalysis results in a series of biochemical reactions between the RNA nucleotides leading to the removal of introns from the RNA message and ultimately to the formation of messenger RNA (mRNA) (25;26) (see Figure 1-2).

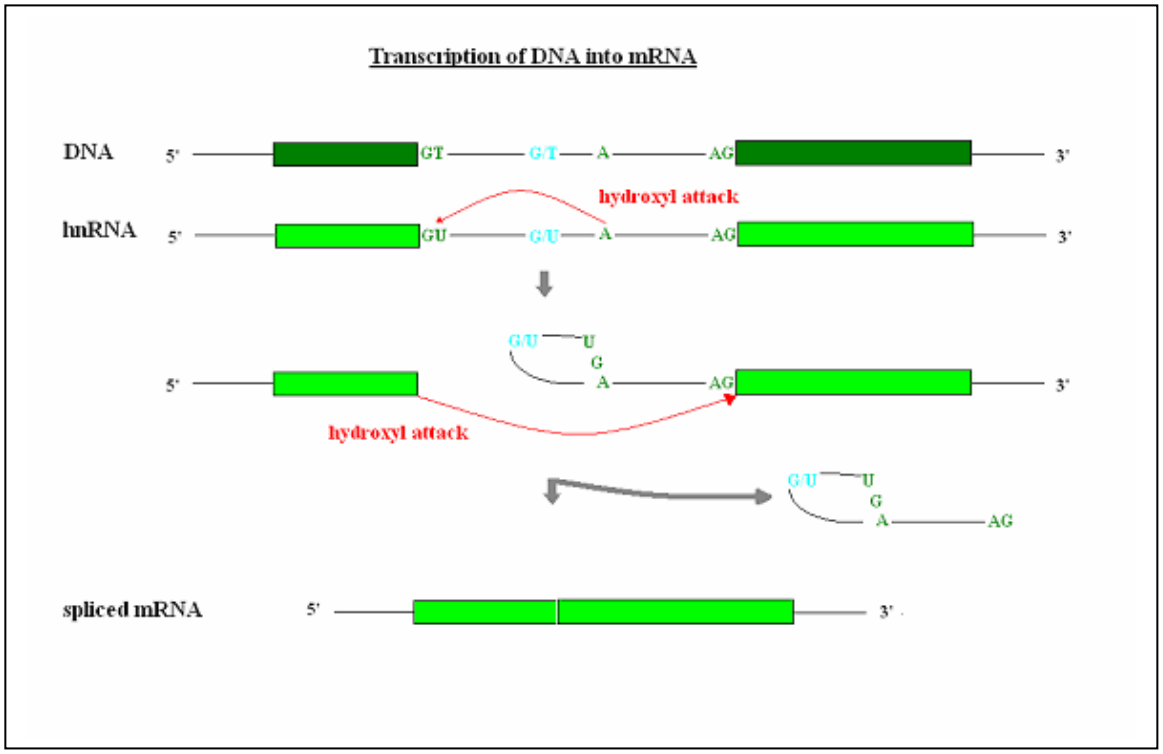


Figure 1-2: Primary transcription of DNA into hnRNA followed by cleavage of 5' splice site with lariat formation and cleavage of 3' splice site. End product is mRNA which does not contain the intron region. The spliced intron is debranched and degraded.

In the human transcriptome alternative splicing is a frequent phenomenon. There are four major types of alternative splicing: exon skipping, alternative 3' splice site, alternative 5' splice site and intron retention (29;30). Exon skipping seems to be the most frequent type of alternative splicing (31). During exon skipping exons are removed together with the intronic regions. This leads to non-consecutive exons being joined together. Alternative splicing allows multiple mRNA and in turn protein isoforms to be generated from one gene. This process is important as the alternative products may or may not maintain their biological activity. Whether or not they do, will depend on the function of the domain that was lost/maintained during alternative splicing and on how the tertiary structure of the protein is altered.
To date several alternative splice products for IL6 have been described, though the relative abundance of the different isoforms has not been clearly established.
Alternative splicing in IL6 predominantly leads to the loss of exon two. Two distinct isoforms seem to exist, both missing exon two (IL6Δ2). One of these exerts IL6 inhibitory properties on proliferation and differentiation (32), while the other does not appear to serve as a significant inhibitor to IL6 (33). IL6Δ2 encodes a predominantly intracellular protein which could provide an explanation for the cell

surface receptor-independent IL6 signalling seen in a variety of cell systems (21;34). There is no evidence to date that intron retention occurs in IL6 RNA, however other isoforms, missing part of exon 2, arising from alternative promoters and truncated versions have been described (35).

RNA editing and translation occurs in eukaryotes. RNA editing involves chemical modification of mRNA (2). Following any post-transcriptional changes the mature mRNA is translated into protein. Translation of mRNA may be affected globally by phosphorylation of eIF-2a, a translation factor or by transcript specific regulatory mechanism (2), however these regulatory mechanisms have not been specifically investigated in IL6.

The IL6 protein consists of 184 amino-acids and a 28 amino-acid-hydrophobic signal sequence (36). The protein undergoes post translational changes involving glycosylation and phosphorylation which affect the tertiary structure and the biological activity of IL6. These post translational changes appear to be tissue specific (37;38). As a result of the post transcriptional as well as the posttranslational changes, IL6 is secreted as a set of proteins that are heterogeneous, with varying molecular mass (39).

1.4 Signaling Pathways for IL6

There appears to exist one intracellular and one extracellular IL6 inducible signalling pathway. The details of the intracellular pathway are yet to be defined, but alternative splice products (see section 1.3), have been postulated to play a crucial role (21;34).

The extracellular pathway is known to act via the IL6 receptor (IL6R) complex containing two distinct transmembrane molecules: the ligand binding subunit IL6 receptor and the signal transducing protein gp130 (40-42). The IL6 receptor complex is under the control of several regulatory mechanisms. The IL6R subunit is up regulated by dexamethasone and down regulated by IL6 and granulocyte macrophage colony-stimulating factor (43). Tumour necrosis factor, IL1 and dexamethasone up regulate gp130 (43).

The IL6R exists in membrane bound and soluble form (sIL6R). sIL6R is generated either by proteolytic cleavage (shedding) of the membrane bound IL6R or by alternative splicing of the IL6R mRNA (40;44;45). Many cells, e.g. endothelial cells, are unresponsive to IL6 as they miss the membrane bound IL6 receptor(45). However the presence of the soluble IL6 receptor (sIL6R) enables these cells to

mount an inflammatory response to IL6 via the interaction of the soluble IL6 receptor/ IL6 complex (sIL6R/IL6) with membrane bound gp130 (45). This process is called trans-signalling (46). Gp130 trans-signalling is not exclusive to IL6. Other members of the IL6 family also trans-signal through gp130, a phenomenon termed receptor redundancy (47;48). It is important to take receptor redundancy into account, when considering IL6 blockade for therapeutic purposes. Gp130 may be activated by other members of the IL6 family, thus rendering isolated IL6 blockade ineffective.

Recent evidence has been found for a soluble gp130 receptor which may exhibit antagonistic properties. However neither the in vivo control nor the functional significance of soluble receptors is clearly understood (49).

Signal transduction via the gp130 receptor results in activation of the janus kinase (JAK)/signal transducers and activator of transcription (STAT) pathway (12) (see Figure 1-3). Gp130 receptors associate with members of the janus kinase family. Binding of IL6/IL6R to the gp130 receptor induces dimerisation of the liganded receptor, which induces tyrosine phosphorylisation of the associated JAKs. The JAKs in turn phosphorylate tyrosine residues in the receptor endodomain. These phosphorylated tyrosines are recognized by the SH2 domains of STAT1, STAT3 and SPH2 tyrosine phosphatase which are recruited to the activated receptor complex. Once a STAT molecule is bound to the receptor, JAK catalyses its tyrosine phosphorylation, which leads to hetero or homo dimerisation. The dimerised STAT is translocated to the nucleus. Here it binds to a specific DNA response element in the promoter region of the target gene, activating gene expression (50). Seven types of STATs have been identified. Of these, STAT3 appears to be vital for the signalling of IL6 (51).

On the other hand SPH2 has a negative regulatory effect on STAT activation. In addition it acts as an adaptor protein that links IL6 to the Ras/ERK pathway. ERK has a positive effect on STAT nuclear translocation and activates transcription factors such as NF-IL6 and ELK1.

Schematic representation of the IL6 signalling pathway

(from http://www.biocarta.com/pathfiles/h_il6Pathway.asp):

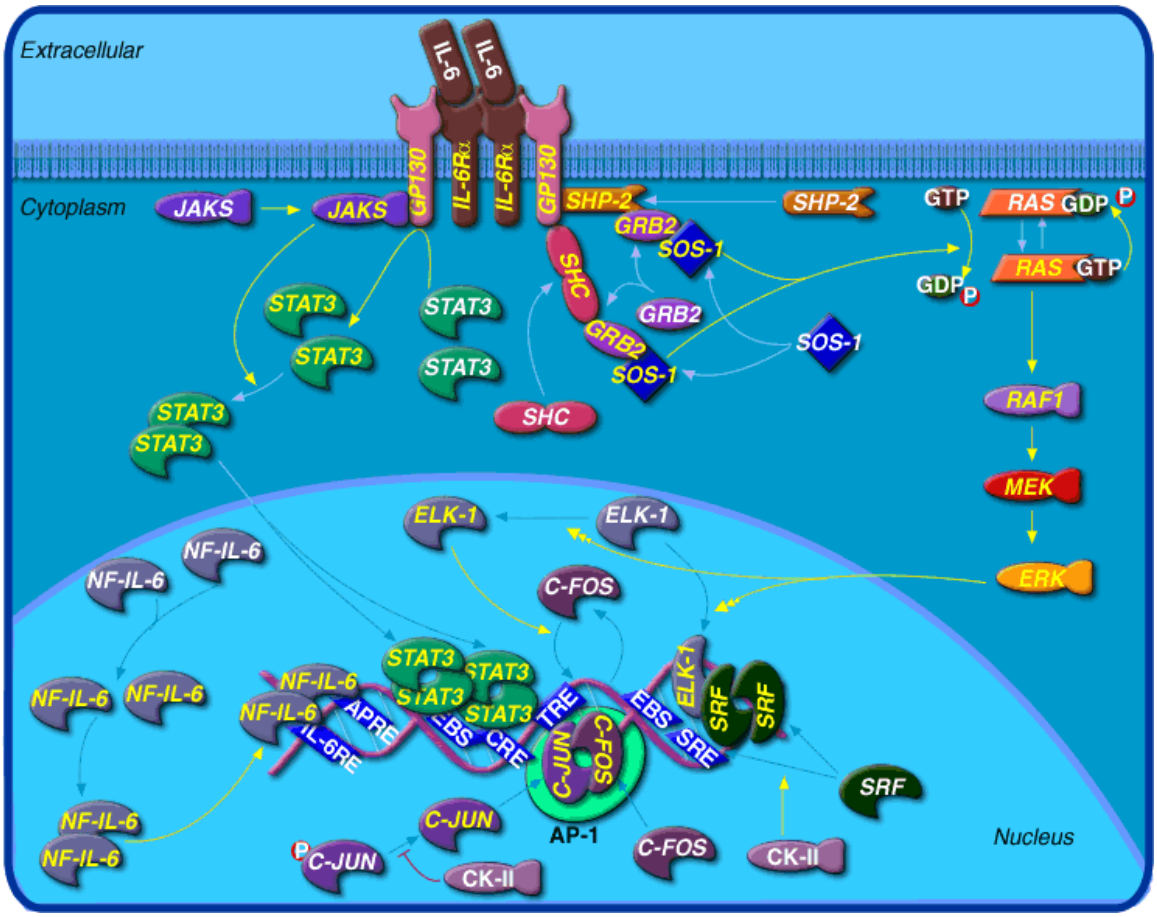


Figure 1-3: The IL6R/IL6 complex is shown binding to the gp130 receptor subunit. This leads to activation of JAK, which in turn leads to activation of STAT3, which dimerises and translocates to the nucleus were it binds to the response unit of target genes. The activated JAK also induces a SHP2/GAB mediated ERK/MAPK pathway leading to activation of ELK1 and inhibition of the JAK/STAT pathway. (Abbreviations: JAK=Janus Kinase; STAT= signal transducers and activators of transcription; SPH2=a Src homology 2-containing tyrosine phosphatase; GAB= Grb2 associated binder; ERK= extracellular-signal-regulated kinase; MAPK= mitogen-activated protein kinase APRE=acute phase response element; EBS=EST binding site; CRE= cAMP regulatory element)

The JAK/STAT signalling cascade has been studied extensively (52). If adequate control of this IL6 signalling pathway is to be maintained a negative regulatory mechanism for the cytokine response must exist. Functional studies have provided evidence that suppressors of cytokine signalling (SOCSs) may play an important role (53).

Having summarised the regulatory mechanisms of transcription and IL6 signalling pathways, the following chapters will discuss the relevance of IL6 to arterial disease.

1.5 IL6 and Aspects of Arterial Disease Development

1.5.1 IL6 and Atherosclerosis

IL6 has been implicated in a variety of diseases, one of which is atherosclerosis (54). Atherosclerotic disease is the most common cause of death in the western world (4). It represents a chronic inflammatory process which has been shown to be associated with increased levels of circulation pro-inflammatory cytokines such as IL6, IL1β and TNFα (12). IL6 has been named as the most consistent biomarker of peripheral arterial disease to date (55). Many of the effects of IL6 relating to the vascular system are known and it is possible to delineate pathways through which IL6 may causally affect the development of atheroma.

IL6 pathways in atherosclerosis

The earliest manifestation of the atherosclerotic lesion is the 'fatty streak' (56). Fatty streaks are characterized by the presence of macrophages and T-cells in the intima. Cell adhesion molecules are implicated in leukocyte recruitment into the fatty streak and have been shown to be up regulated in lesion prone sites in the arterial tree (57). These include vascular cell adhesion molecule 1 (VCAM1), intercellular adhesion molecule 1 (ICAM1) and e-selectin (58;59). Both IL6 and TNFα can induce expression of these adhesion molecules in endothelial cells (12;60;61). VCAM1, ICAM1 and e-selectin interact with specific ligands on circulating leukocytes to facilitate their adhesion to the vessel wall (12). After binding to the endothelium, leukocytes migrate into the sub-endothelial space. This migration is driven by chemoattractants such as oxidised low density lipoproteins (oxLDL), lipoprotein(a) (Lp(α)),monocyte chemotactic protein 1 (MCP1), IL1 and tumour necrosis factor alpha (TNFα) (62). IL6 is able to induce MCP1 and influence levels of TNFα and may therefore exert some control over this migration process. (see Figure 1-4)

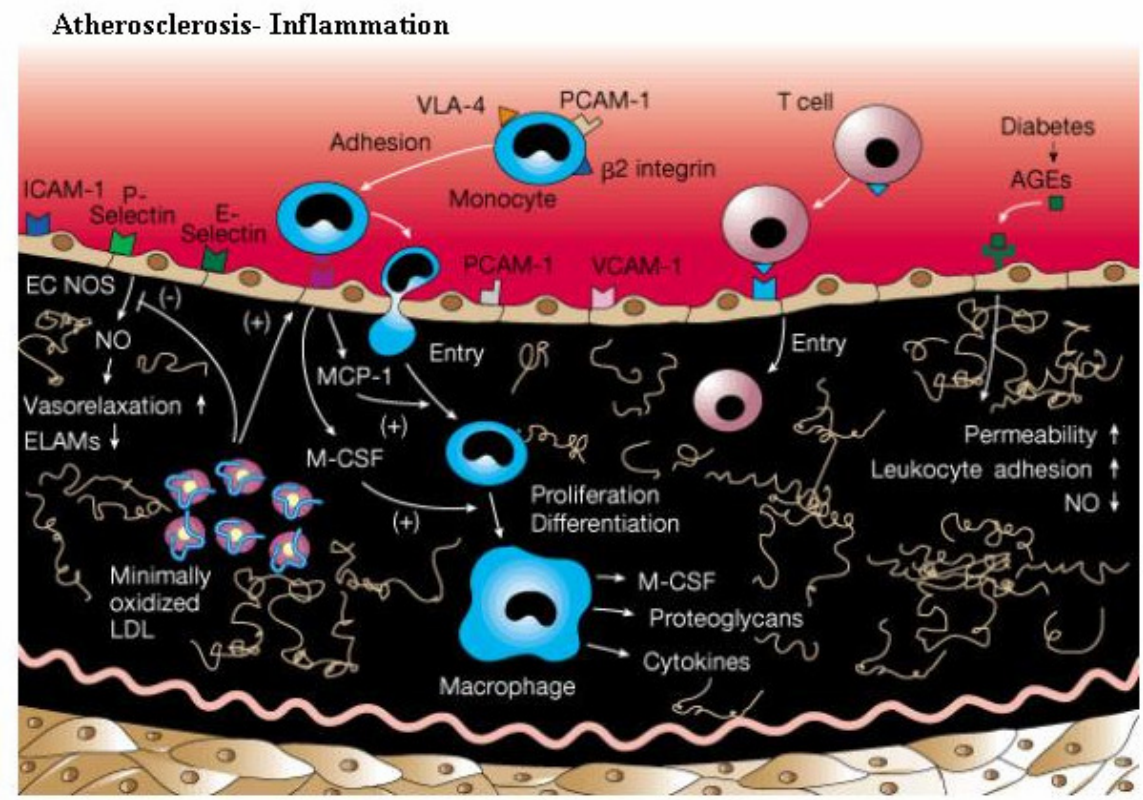


Figure 1-4: From AJ Lusis, Nature 2000. Inflammatory pathways in atherosclerosis.

Once inside the intima, monocytes differentiate into macrophages. This process is partially controlled by IL6 and ultimately leads to the formation of lipid laden cells called foam cells. Foam cells are one of the characteristic components of the atherosclerotic plaque. The transition of macrophages into foam cells through lipid uptake is mediated by TNFα, IL1β and IFNγ which activate monocyte-macrophages to increase their mobility, phagocytosis and enzyme levels (12).

The more advanced atherosclerotic lesion contains macrophages, T-cells, smooth muscle cells (SMC), extracellular lipid and matrix deposit (63). The cellular components of the advanced plaque contribute to the ongoing inflammatory process that drives plaque progression by expressing a variety of mediators involved in intercellular signalling. These mediators are involved in the proliferation of smooth muscle cells, a characteristic histological feature of a mature atherosclerotic lesion. IL6 and its soluble receptor have been shown to be released from human macrophages. The ensuing IL6/sIL6 receptor complex stimulates up regulation of gp130 in cultured human SMC and ultimately drives these cells into a proliferative state (64). As both macrophages and smooth muscle cells are a characteristic

histological feature of a mature atherosclerotic lesion, it is possible that IL6 plays an important role in determining the cellular structure of the mature plaque.

IL6 down regulates TNFα and IL1 expression, whereas transcriptional activity of IL6 is increased in response to TNFα (12;24). IL6 and TNFα both have an effect on the expression of other mediators of atherosclerosis. IL6 and TNFα induce, among others, MCP1, a powerful chemo attractant involved in leukocyte adhesion and migration. Thus IL6 may have a direct effect on plaque development and an indirect effect through modulation of the expression of other mediators.
In addition to the autocrine and paracrine effects of IL6 within the arterial lesion itself, IL6 is able to induce hepatic acute phase response proteins such as C-reactive protein (CRP) and fibrinogen. Increased levels of fibrinogen lead to a state which favours atherothrombosis (65).

Besides this evidence for the involvement of IL6 in molecular processes leading to atherosclerosis, clinical studies also suggest a role for IL6 in the atherosclerotic disease process.

IL6 and Atherosclerosis: Clinical Evidence

Interleukin gene transcripts have been found to be expressed in human atherosclerotic lesions with atherosclerotic arteries showing a 10-40-fold higher level of IL6 mRNA than non-atheromatous vessels (54), suggesting a fundamental involvement of IL6 in plaque development (46). Elevated levels of circulating IL6 are found in patients with abdominal aortic aneurysms and chronic limb ischemia (66-71). Furthermore raised levels of IL6 are associated with coronary, peripheral and carotid atherosclerosis (72) and risk of death in patients with cardiovascular disease (9;73;74).
Low echogenicity of carotid plaques is a known risk factor for plaque rupture and ischaemic stroke. IL6 levels have been shown to be inversely related to plaque echogenicity (75). This association could relate to IL6 induced monocyte recruitment with subsequent expression of matrix metalloproteinases leading to plaque rupture (76;77). Thus IL6 seems to be associated with both chronic vascular disease and acute vascular events.

Raised IL6 levels also appear to have some predictive value for vascular disease. Circulating levels of IL6 have been shown to be associated with evidence for sub-

clinical atherosclerosis, independent of traditional risk factors (78). In the Edinburgh Artery Study serum IL6 at baseline was found to be the most reliable predictor of arterial disease progression as assessed by ankle brachial pressure index (ABPI) of all the inflammatory markers examined (79). ABPI in turn predicts future cardiovascular risk as has been shown in several large scale trials (80;81). Elevated levels of circulating IL6 are also associated with an increased risk for future myocardial infarction in healthy individuals (82). A prospective study of healthy men compared 202 participants who developed myocardial infarction within the follow up period of 6 years with 202 asymptomatic matched controls. A correlation between raised IL6 levels at baseline and increased cardiovascular risk over a prolonged period of time was found (82) (see Table 1.9-1 & Figure 1-5).

Table 1.9-1: Table from PM Ridker (Circulation 2000) showing crude and adjusted relative risk (RR) of future myocardial infarction among apparently healthy men according to baseline level of IL6. (Fully adjusted models additionally controlled for total and HDL cholesterol, body mass index, diastolic blood pressure, diabetes, family history of premature coronary artery disease, alcohol use, and exercise frequency.)

	Quartile of IL-6 (range, pg/mL)				
	1 (<1.04)	2 (1.04–1.46)	3 (1.47–2.28)	4 (>2.28)	*P* for Trend
Crude analysis					
Total cohort					
RR	1.0	1.4	2.8	2.3	<0.001
95% CI	...	0.7–2.6	1.6–5.1	1.3–4.3	
P	...	0.3	0.001	0.005	
Nonsmokers					
RR	1.0	1.4	2.5	2.6	0.001
95% CI	...	0.7–2.7	1.4–4.7	1.3–4.8	
P	...	0.3	0.003	0.01	
Total and HDL cholesterol adjusted					
Total cohort					
RR	1.0	1.5	2.9	2.3	0.003
95% CI	...	0.7–2.9	1.6–5.4	1.2–4.3	
P	...	0.3	0.001	0.01	
Nonsmokers					
RR	1.0	1.6	2.6	2.5	0.003
95% CI	...	0.8–3.1	1.4–5.1	1.3–5.1	
P	...	0.2	0.004	0.01	
Fully adjusted					
Total cohort					
RR	1.0	1.9	3.5	2.3	0.01
95% CI	...	0.9–3.9	1.8–6.9	1.1–4.6	
P	...	0.08	0.001	0.03	
Nonsmokers					
RR	1.0	2.1	3.1	2.7	0.009
95% CI	...	1.0–4.5	1.6–6.7	1.1–5.7	
P	...	0.06	0.002	0.02	

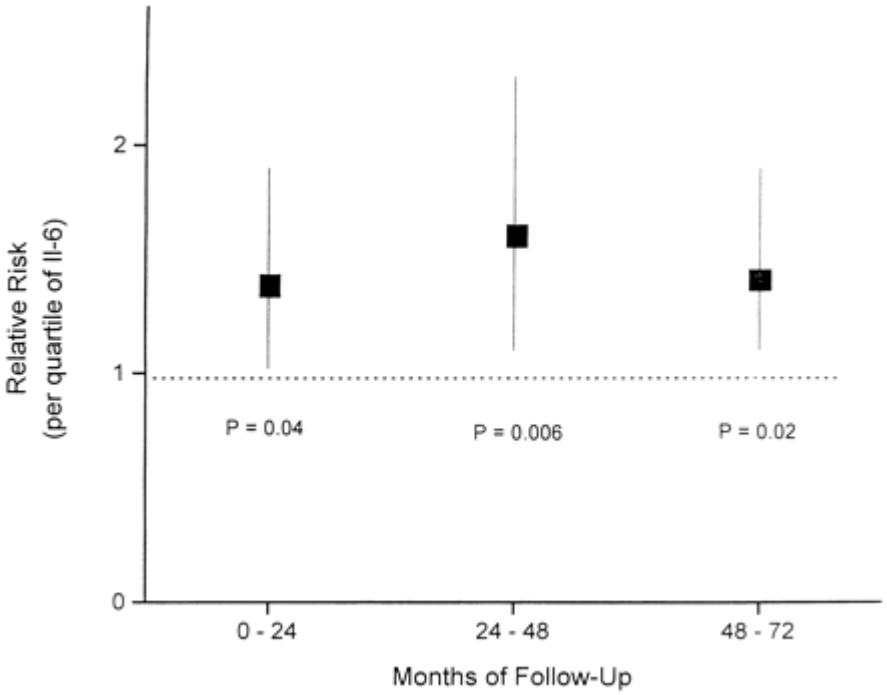


Figure 1-5: Graph from PM Ridker (Circulation 2000) showing relative risk of future myocardial infarction associated with each increasing quartile of baseline IL-6 concentration according to months of follow-up. The figure demonstrates an increased risk associated with increased IL6 levels, which is stable over a prolonged time period.

The fact that raised levels of IL6 can precede clinical disease development or progression could suggest that IL6 is causally involved in atherosclerosis and thus could be clinically relevant. However it could equally be argued that an increase in circulating IL6 may merely be the result of an inflammatory process in the arterial wall which has not yet become clinically detectable. Thus the question of causality remains unanswered.

One facet of the potentially causal relationship between IL6 and atherosclerosis is the suggestion that IL6 may form part of a link between risk factors and atherogenesis.

1.5.2 IL6 and Risk Factors for Atherosclerosis

It has been hypothesised that IL6 is not only associated with arterial disease itself but may in fact be part of the pathway that links risk factors with arterial disease development.

Hypertension

Clinical trials have shown a significant association between hypertension and atherosclerosis however the exact underlying mechanism for the association remains uncertain (83-86). With increased understanding of the cellular and molecular processes involved, it has become apparent that certain mechanisms are common to both hypertension and atherosclerosis (83). It has been suggested that the bridge connecting these two conditions is inflammation (84). In this respect Angiotensin II, part of the Renin Angiotensin System (RAS) important in blood pressure (BP) control, appears to play a major role (87-94). Angiotensin II stimulates nicotinamide adenine dinucleotide phosphate (NADPH)/nicotinamide adenine dinucleotide (NADH) oxidase in the components of the vessel wall, leading to the generation of reactive oxygen species. This in turn, leads to up regulation of NF-κB which stimulates IL6 expression and thus contributes to a pro-inflammatory state (83;84;89;92;95-98). Therefore factors involved in initiating hypertension such as Angiotensin II may via pro-inflammatory factors such as IL6 also influence the development of atherosclerosis. A recent paper has defined a positive feed back loop, that demonstrates how vascular inflammation can become self sustaining (87). The local production of Angiotensin II in the vessel wall leads to the induction of IL6 expression which in turn leads to up regulation of angiotensinogen production by the liver via the Janus Kinase (JAK)/signal transducer and activator of transcription (STAT) pathway. This supplies more substrate to the activated vascular renin angiotensin system leading to further production of Angiotensin II - perpetuating vascular inflammation (87).

The importance of the IL6 mediated effects of the RAS on the atherosclerotic process is demonstrated by clinical studies showing that a variety of antihypertensive agents can exert an anti-atheromatous effect independent of blood pressure reduction (87;89;99;100). This effect has been put down to inhibition of the expression of inflammatory mediators such as IL6. Other clinical studies have shown that high IL6 levels correlate with increased blood pressure in men (82;101). Recent studies in IL6 knock out mice have demonstrated hypertension caused by angiotensin II and high salt diet as well as the acute hypertensive response to stress significantly depends on the presence of IL6 (102;103). This suggests that IL6 may mediate both the hypertensive effect as well as the atherosclerotic effect of Angiotensin II.

Type II Diabetes

Atherosclerotic disease occurs at a significantly higher rate in individuals suffering from diabetes than in the general population (104-107). Atherosclerotic disease has been regarded as the sequel of longstanding diabetes. However, recent evidence shows that markers of sub clinical vascular disease can predict future risk of Type II diabetes and that cardiovascular disease (CVD) can precede the onset of diabetes by up to 15 years (108;109). This has lead to the theory that both Type II diabetes and CVD share a common pathophysiology, possibly reduction in nitric oxide (NO) (110) (111). Reduction in NO appears to occur in hyperglycaemia and insulin resistance as well as arterial plaque development (111-114). It occurs via down regulation of endothelial nitric oxide synthase and chemical reaction of NO with reactive oxygen species (115). The local production and release of nitric oxide is crucial to normal endothelial function, as NO is a powerful vasodilator and inhibitor of platelet aggregation, leukocyte adhesion and vascular smooth muscle cell proliferation (116;117). Decreased production of NO has been shown to be associated with the increased activation of transcription factor NF-κB (118-122). NF-κB in turn increases expression of pro inflammatory cytokines such as IL6, IL1 and TNF thereby creating a pro-atherogenic environment (116).

A rise in IL6 can also promote insulin resistance. In human hepatocytes insulin receptor signal transduction is impaired by IL6 and in fat cells IL6 may promote insulin resistance via induction of suppressor of cytokine signalling (SOCS)-3 (123;124). Therefore reduction in NO may via increase in IL6 create both an athero- and a diabetogenic environment. However, the effect of IL6 on glucose metabolism may be tissue specific. Inhibition of insulin receptor substrate 1 (IRS-1) and induction of SOCS-3 thought to induce insulin resistance could only be demonstrated in the liver but not the muscle of IL6 treated mice (125). In fact IL6 may improve glucose uptake in muscle cells (125). Thus the overall effect of IL6 on glucose metabolism remains controversial, as does the theory of a joint developmental pathway for diabetes and vascular disease via IL6.

Hypercholesterolemia

The association between raised cholesterol levels and vascular disease is well described. Oxidised low density lipoprotein (oxLDL) can decrease endothelial nitric oxide synthase (eNOS) expression and thus nitric oxide bioavailability which leads to endothelial dysfunction (126;127). One pathway through which oxLDL may mediate reduction in eNOS expression is through binding to the lecithin-like oxLDL receptor 1

(LOX1) present on human endothelial cells. The binding of oxLDL to LOX1 on endothelial cells leads to activation of intracellular signalling pathways such as mitogen-activated protein kinase (MAPK) (128) which may induce inhibition of eNOS expression (129). It has also been shown in bovine aortic endothelial cells that activation of the LOX1 receptor by oxLDL leads to increase in the production of reactive oxygen species (ROS) such as superoxide anions (130). These can react with and deactivate nitric oxide also leading to a reduction in its bioavailability. Another effect of binding of ox-LDL to LOX-1 is an increase in NF-κB expression. This may also be mediated via the MAPK cascade or possibly an increase in ROS (128;131). NF-κB in turn leads to up regulation of IL6 and other inflammatory mediators, as well as endothelial vasoconstrictors and growth factors producing a pro-atherogenic environment in the vascular wall (117). Hypercholesterolemia may therefore induce atherosclerosis in part via a pathway involving IL6.
Clinical evidence supports the view that inflammation and its mediators are involved in the association between hypercholesterolemia and arterial disease (132). Statin drugs, which are known to reduce the morbidity and mortality from cardiovascular events, appear to have anti-inflammatory activity (133;134) (135;136). Carotid plaques from patients receiving statin therapy have been shown to contain less IL6 than plaques from untreated patients (132). Simvastatin and atorvastatin have been shown to reduce IL6 levels in the first 7 days following the start of treatment (137;138) and the beneficial effect of statins on cardio-vascular disease are thought to be in part mediated by this reduction (139).

Smoking

Though the underlying mechanism is not fully understood, smoking is associated with accelerated atherosclerosis and cardiovascular disease (140;141). One possible mechanism is via increased generation of reactive oxygen species (ROS), which has been shown to occur in smokers (141;142). This encourages oxidation of low density lipoproteins which have been shown to have a pro-atherogenic effect (see above) in part through reducing nitric oxide (NO) bioavailability resulting in endothelial dysfunction as well as induction of IL6 expression (141;143-145). Indeed serum levels of IL6 in current smokers and former smokers have been found to be significantly higher than in non-smokers (140;146-148). Other factors that may potentially mediate the atherogenic effect of smoking are increased platelet turnover and activation and increase in circulating fibrinogen (149;150). It has been shown that smoking increases fibrinogen levels in healthy volunteers and in patients with

coronary artery disease (CAD) (151-154). IL6 is important in regulating fibrinogen levels, leading to up regulation of fibrinogen synthesis in the liver (152). An IL6 response element exists in the promoter region of the β-fibrinogen gene and, as production of β-fibrinogen is thought to be the rate limiting step in fibrinogen synthesis, IL6 may directly influence the rate of fibrinogen production (155) (156;157). It is therefore possible that the increase in fibrinogen in smokers is mediated through IL6. One study investigated fibrinogen levels in healthy controls and patients with coronary artery disease (CAD) (152). Both groups were further subdivided into smoker and non smokers. This study demonstrated a significant difference in IL6 levels between smoking and non smoking CAD patients. No difference was seen in healthy volunteers (152). However, coronary artery disease had a greater effect on fibrinogen levels that smoking (152). Thus smoking may increase IL6 levels in patients who already suffer from arterial disease, but the increase in fibrinogen in these patients does not appear to be mediated entirely through IL6 (152).

Smoking may increase the development of vascular disease and its complications through a variety of pathways, however the relative importance of the individual effects is not clear (141).

Obesity

Obesity, particularly visceral obesity is associated with an increased risk for cardiovascular disease (158). Adipose tissue has traditionally been regarded as a passive energy store, however more recently it has become apparent that fat is an important endocrine organ (159). It secretes a variety of bioactive peptides involved in glucose and fat metabolism. These include leptin, adiponectin, resistin as well as interleukin 6 (160-162). Furthermore IL6 levels are increased in obese subjects and IL6 levels increase with increasing adiposity (163;164). It has therefore been suggested that obesity represents a low grade inflammatory state and that the associated increased levels of IL6 are the link between obesity and atherosclerosis (49;160;165;166).

Studies looking at site of production of circulating IL6 suggest a greater contribution from visceral fat than from peripheral adipose tissue (167;168). Assuming IL6 represents a link between atherosclerosis and obesity, this would correlate with the observation that visceral obesity constitutes a greater risk factor for atherosclerosis than peripheral adiposity (167;168).

It appears that IL6 is closely associated with arterial disease development. There is evidence for a potential role for IL6 in the cellular processes of plaque formation, as well as a link between risk factors and vascular disease. In addition IL6 may affect surgical treatment outcome and relevant evidence is discussed in the following chapters.

1.6 IL6 and Vascular Surgery

1.6.1 IL6 Response to Surgery in the early Post-operative Period

1.6.1.1 Local IL6 Response

IL6 may be involved in wound healing through stemming of infection and removing cellular debris through leukocyte infiltration (169). It may further be involved in angiogenesis through induction of vascular endothelial growth factor (VEGF) and may modulate matrix metalloproteinase (MMP) expression (170-172). It is unclear whether IL6 overall plays a positive or negative role in wound healing as its effects are complex. MMPs are enzymes capable of degrading extracellular matrix deposits and thus play a major role in tissue remodelling and can potentially delay wound healing. IL6 may stimulate expression of matrix metalloproteinase 9 (MMP9), which when present in increased amount is associated with poor wound healing and chronic ulcers (173-176). However, delayed wound healing in glucocorticoid treated mice can be partially reversed by administration of recombinant (rm)IL6 (173). It is thought that rm-IL6 acts through suppressing MMP10 production, which is increased by glucocorticoids (177).

Wound healing in IL6 knock out mice is significantly delayed when compared with wild type mice (170) and restored by administration of recombinant mouse IL6, while IL6 antibodies and (rm)IL6 delayed wound healing in wild type mice (178-180). It is therefore possible that physiological levels of IL6 are necessary for normal wound healing, but raised IL6 levels have a negative effect on re-establishing tissue integrity following injury.

IL6 may further influence wound healing by suppressing albumin production in hepatocytes (181). Low albumin levels in turn are thought to be associated with poor wound healing.

1.6.1.2 Systemic IL6 Response

Systemic Inflammatory Response Syndrome and Multi Organ Failure

Major surgery, such as aortic surgery, is accompanied by a systemic inflammatory response associated with an increase in circulating cytokines (182). The most consistently identified rise in cytokines is that of circulating IL6 (177;183). Studies into the IL6 response following aortic surgery, found that IL6 levels were significantly associated with major postoperative complications (184). A study looking at levels of TNFα, IL1β and IL6 following abdominal aortic aneurysm (AAA) repair, found that those with adult respiratory distress syndrome (ARDS)/Multi-organ failure had significantly higher levels of IL6 then those without (177;183). An exaggerated post-operative IL6 response can precede the onset of clinical complications by 12 to 48 hours (71;169;185-187). Whilst consistent with a role for IL6 in causing post-operative complications, it remains possible that this association derives from a confounding effect, or that post-operative complications drive IL6 synthesis before they become clinically manifest.

Systemic inflammatory Response in Endovascular Aortic Repair

It would appear that the characteristics of the surgical procedure influence the extent of the post-operative IL6 response. Both the extent of tissue trauma and the duration of the procedure have been cited as the determinant of the postoperative IL6 levels with evidence that the main determinant is extent of tissue injury (188). This is of interest when considering the impact of emerging endovascular techniques in which the reduction of tissue trauma could convey a major advantage. It has been shown that post-operative IL6 levels are reduced after endovascular aneurysm repair (EVAR) when compared to open AAA repair (189-193). However IL6 is not only release from injured tissues; the aortic mural thrombus itself has been shown to contain high amounts of IL6 which could be released during manipulation inside the thrombus during endovascular repair (194). Thus increases in IL6 may be part of the post-operative inflammatory response in both open and endovascular surgery, though one may induce a smaller response than the other.

Ischaemia Reperfusion Injury

Ischaemia reperfusion injury initiates an inflammatory response which is reflected in an increase in circulating cytokines, including IL6 (195). Hypoxia has been

demonstrated to induce expression of IL6 in endothelial cells and a study looking at lower limb bypass surgery showed that circulating levels of IL6 were markedly raised following reperfusion compared to levels measured prior to clamping (71;192;196) . Studies on ischaemia reperfusion injury have also demonstrated high levels of cytokines in both the legs (IL6,IL8,IL10) and the gastrointestinal tract (IL6) compared with systemic levels following aortic clamping (197).
Tissue ischaemia then results in accumulation of high levels of inflammatory mediators such as IL6, which on reperfusion are released into the systemic circulation (198-201). The activation of inflammatory pathways can result in injury to distant organs such as the lung and kidney through the vascular sequestration of activated neutrophils and damage by activated resident neutrophils (202).
There is evidence from mouse models that IL6 directly contributes to the organ injury caused by ischaemia reperfusion I/R induced systemic inflammatory response. A study looking at renal ischaemia in IL6 knockout mice compared to wild type mice found evidence that IL6 enhances the degree of renal injury, dysfunction and inflammation following reperfusion (203). It was also shown that inhibition of NF-κB can prevent lung injury induced by intestinal I/R in mice (71). As NF-κB induces expression of IL6 and TNFα, it is possible that these play a causal role in the development of distal organ injury leading to multiple organ failure following I/R.

Immunosuppression

It is known that major surgery can lead to an immune suppressed state (185;204-206). Major surgery is associated with an early hyper-inflammatory response characterised by the release of pro-inflammatory cytokines such as TNFα, IL1 and IL6 (43;205-207). It is thought that the rise in IL6 may paradoxically play a role in the post-operative immune depressed state (185). Although IL6 is generally regarded as a pro-inflammatory cytokine it also displays anti-inflammatory properties which are thought to be important in its function as an immune regulatory cytokine (43). The release of IL6 leads to up-regulation of major anti-inflammatory mediators e.g. by stimulation of macrophage expression of interleukin-1-receptor antagonist and a soluble TNF receptor (208). In addition IL6 is also able to directly inhibit expression of TNFα and IL1 (209-211). Another pathway through which IL6 can contribute to the post-operative immune suppression is by stimulating the expression and release of strong anti-inflammatory mediators such as transforming growth factor β (211), prostaglandin E2 and glucocorticoids (210-212).

TNFα, IL1 and IL6 are all raised in the immediate postoperative period (183;204;213). However whereas the rise in TNFα and IL1β is relatively short lived (183;207;213), IL6 levels remain elevated for up to 7 days following surgery (183;214). This prolonged release of IL6 may lead to an imbalance between pro- and anti-inflammatory cytokines and ultimately contributes to the immune suppressed state seen after major surgery. This theory is supported by a study showing that high levels of IL6 in the early postoperative phase after oesophagectomy are associated with a late phase suppression of cellular immunity (204). Increased IL6 levels on day one following major tumour resection also correlate with Interleukin 1 receptor antagonist (IL1ra) levels (183;215;216).

Besides its role in the early postoperative period IL6 also appears to influence long term outcome from vascular surgery.

1.6.2 IL6 and Long Term Outcome after Vascular Surgery

1.6.2.1 Graft Patency/Intimal Hyperplasia

Intimal hyperplasia complicates all forms of arterial reconstruction (217;218). It is the principal cause of failure in vein and synthetic grafts and in atherosclerotic arteries that have been stented. Intimal hyperplasia involves a process in which smooth muscle cells migrate into the intima were they proliferate, accompanied by the secretion of matrix proteins. This leads to an increase in arterial wall thickness and narrowing of the lumen. It has been suggested that cytokines such as IL6 and TNFα might contribute to the mesenchymal cell recruitment, proliferation and intimal hyperplasia seen in vascular grafts (219). IL6 and its soluble receptor have been shown to lead to cellular proliferation in cultured human smooth muscle cells. (220). IL6 has also been shown to induce VEGF (vascular endothelial growth factor) (220-222). Though the role of VEGF in neointimal hyperplasia is still controversial, it may have a role to play, as neovascularisation is part of the intimal hyperplastic process in models of angioplasty, arterial stenting and vein bypass grafting (223-225). Also, administration of VEGF has been shown to increase intimal hyperplasia in an animal model of arterial injury (226).

Studies in rats also suggest a role for IL6 in intimal hyperplasia. It was shown that in vivo induction of toll like receptor 2 (Tlr2) leads to increased intimal hyperplasia (227). Toll like receptors use a downstream NF-κB signalling pathway inducing the production of pro-inflammatory cytokines and chemokines (228-230). This is

demonstrated in a study showing that vascular cells stimulated with a synthetic Tlr2 ligand express significantly more IL6, IL8 and MCP1 than control (231). Another study investigating the NF-κB signalling pathway showed that inhibition of NF-κB in vivo reduces the development of intimal hyperplasia in rats (232). It is therefore likely that the intimal hyperplasia induction by Tlr2 is in part mediated via IL6. Further evidence for this comes from pharmacological studies. In a rat model the combined treatment with pravastatin and clopidogrel significantly decreased intimal hyperplasia following carotid endarterectomy. Pravastatin has been shown to reduce the expression of IL6 by smooth muscle cells (233). In addition endothelial cells pre-treated with pravastatin show reduced IL6 synthesis (218). Clopidegril in turn could, through its antiplatelet effect, decrease cytokine release from activated platelets (218). It has therefore been proposed that the reduction in intimal hyperplasia seen in rats treated with pravastatin and clopidegril may in part be mediated by a reduced expression of cytokines such as IL6 (234).

1.7 Proof of Causality and Mendelian Randomisation

It has been demonstrated that IL6 levels are raised in patients suffering from atherosclerosis and that raised levels are associated with increase risk of developing cardiovascular disease as well as with poor outcome in the atherosclerotic patient. Pathways exist through which IL6 can modulate plaque development and become the link between risk factors for atherosclerosis and arterial disease itself. IL6 plays a major role in the inflammatory response to surgery and thus short term patient outcome, as well as long term outcome in terms of vascular graft patency. Thus IL6 appears intricately involved in all aspects of atherosclerosis and vascular surgery, however final proof of causality has not been established.

The association between increased in IL6 levels with aspects of the vascular disease process does not prove causality, as raised levels could equally be the result rather than the cause of pathology. Furthermore, much of the knowledge about IL6 has been gained from in vitro experiments. Although these provide good models, the in vitro effects cannot be assumed to be of relevance in vivo. The extent of redundancy in the cytokine system and the complexity of biological pathways involved in vivo, make it difficult to single out one mediator as the fulcrum in the disease process. Equally convincing arguments could be made for other mediators such as TNFα, IL1,

NF-κB etc. being the likely essential link in the chain of events. Therefore before considering using immune modulation of IL6 to improve outcome for the vascular patient, IL6 has to be shown to have a causal effect that is clinically relevant. Studies aimed at showing causality using mouse models have given some insight; though interpreting the results is complex. A study on atherosclerosis prone mice *(ApoE-/-)* demonstrated that exogenous administration of recombinant mouse interleukin-6 significantly enhanced fatty lesion development (235;236). This suggests that IL6 could indeed play a causal role in the development of vascular disease. However other studies, again using *ApoE* knock out mice but with an additional knock out of *IL6* showed that life long deficiency of IL6 in *ApoE* knock out mice enhances atherosclerotic lesion development and increases cholesterol levels (237). This suggests that a certain level of IL6 is required to maintain a healthy vasculature but exogenous or excessive administration might be harmful. It is not clear whether exogenously administered IL6 will have the same effect as an intrinsically raised IL6 level and it is the intrinsic levels which are relevant in clinical terms. These considerations make interpretation of mouse models difficult.

The ideal study to investigate the contribution of IL6 to arterial disease or outcome from arterial surgery would be to control IL6 levels in individuals over a prolonged period of time and assess the association of prolonged increased levels with increased plaque formation or increased complications form vascular surgery. Clearly, such a study could not be performed. However, in order to mimic the above conditions, scientists have recently employed Mendelian randomisation. Mendelian randomisation is the term applied to the random assortment of alleles at the time of gamete formation (238). This random assortment can lead to different individuals being genetically programmed to produce more or less of certain mediators at baseline or after stimulation. This variation in gene expression can be caused by polymorphisms within the gene. Polymorphisms are variations within a gene that occur in more than 1% of the population (239). Polymorphisms can affect the ultimate level of gene product in a variety of ways, e.g. promoter polymorphisms may affect the binding of the transcription initiation complex and thus the rate of primary transcription. Therefore if a genotype could be defined that would cause an individual to produce more IL6 than a person of a different genotype, this could be used to study the effects of IL6 in vivo.

Using the example of IL6, Mendelian randomisation studies are based on the following principle: The level of IL6 has been found to correlate with arterial disease and disease/treatment outcome. However it is not possible to establish whether

raised IL6 levels are cause or effect (reverse causation). If it can be shown that an individual's genotype may influence levels of IL6 expression and proof is also found that this genotype is associated with disease development and outcome, than a strong case is made for a causal role of IL6 in this disease. The proof of causality relies on the fact that the association of genotype and disease is unidirectional i.e. must be based on the genotype influencing likelihood of disease outcome and progression. In other words – Genotype can influence disease, but disease cannot influence genotype.

IL6 contains several common genetic variants but interest has focused on a single nucleotide polymorphisms (SNP) in the promoter region of the gene, which was defined in 1998 (238) (see Figure 1-6). This variant is a single nucleotide polymorphism (SNP) 174 base pairs upstream from the start of the untranslated region (UTR) were a guanine base is substituted for a cytosine base (SNP G-174C) (rs1800795). Although many studies have investigated SNP G-174C, its precise effect on *IL6* gene expression remains unclear (see section 1.7.1). The reported allele frequencies vary widely in different populations (for the G allele 0.48 in the Finish population to 0.74 in an Italian population) (240). According to data from the HapMap project, genotype frequency in the Utah population of northern and western European ancestry is 0.53 for the G allele and 0.47 for the C allele. http://www.hapmap.org/cgi-perl/snp_details_B35?name=rs1800795&db=hm20 . However data from SeattleSNPs http://pga.gs.washington.edu/finished_genes.html gives the frequency as 0.26 for the C allele and 0.74 for the G allele for a European/Afro American panel.

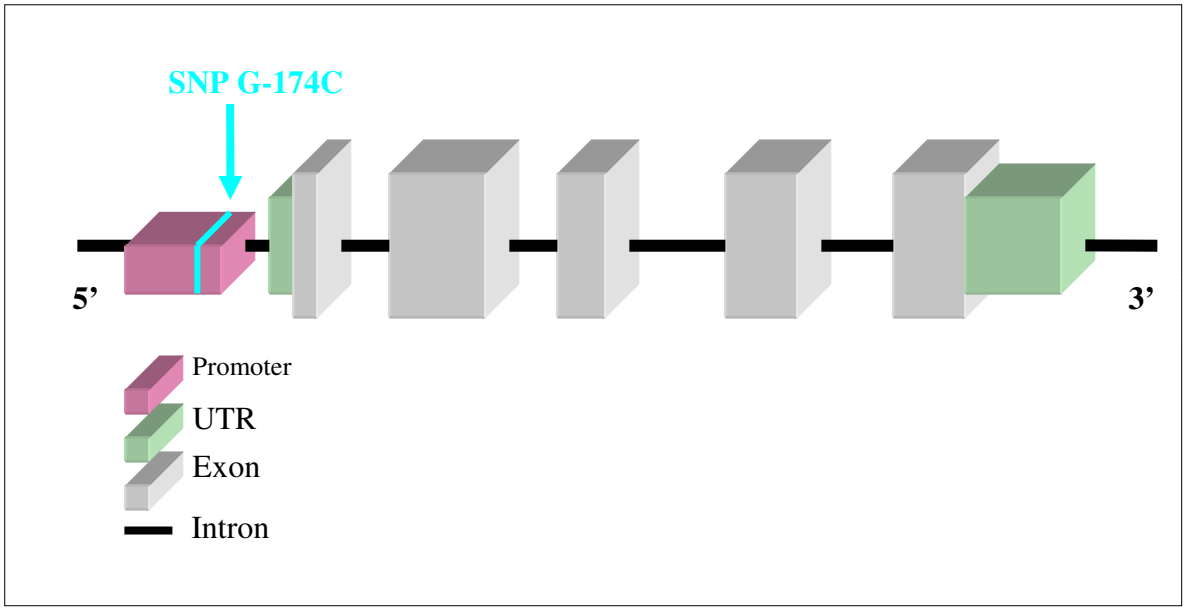


Figure 1-6: Schematic representation of the IL6 gene. The position of the promoter polymorphism is marked in light blue.

The SNP G-174C promoter variant was shown to affect gene transcription in HeLa cells transfected with a construct of the 5' region of the *IL6* gene and a luciferase reporter gene. The -174C construct showing a 0.624 fold lower expression than the -174G construct both with and without stimulation (238). The same study showed that circulating levels of IL6 were about twice as high in healthy subjects with the GG genotype then the CC genotype. A host of clinical studies followed the discovery of SNP G-174C, examining the association of the SNP and IL6 levels in human subjects as well as association of SNP G-174C genotype with disease development and progression. These have shown conflicting results.

1.7.1 Mendelian Randomisation Studies: IL6 Promoter SNP G-174C

A number of studies have tried to find evidence of an association between SNP G-174C, varying *IL6* expression and cardiovascular disease. Conflicting evidence has emerged from these studies.

A number of studies confirmed the in vitro finding (238), that the GG genotype was associated with increases in IL6 expression. Circulating levels of IL6 were found to be higher in healthy subjects carrying the G-allele (238). The GG genotype was also found to be associated with higher levels of IL6 in patients who had undergone coronary artery bypass grafting (CABG) (241) and in Type II diabetic patients (242).

If IL6 plays a causal role in arterial disease development and the G-allele is associated with increased IL6 expression, then one would expect the GG genotype to be found more commonly amongst patients with vascular disease. Indeed it was shown that the combined genotype of SNP G-174C GG and another polymorphism in the promoter region of *MMP3* (6A6A) was strongly associated with increased carotid intima media thickness, a marker for atherosclerosis, as assessed by ultrasound (243;244). A study which assessed endothelial function by means of reactive dilation of the brachial artery in a random number of individuals aged 20-28 found a trend towards poorer endothelial function in individuals carrying the G-allele (245). A study which assessed endothelial function by means of reactive dilation of the brachial artery in healthy young men found that smoking had a more detrimental effect on endothelial function in the GG genotype group than in the CC genotype (245). In a study comparing patients with peripheral arterial disease (PAD) with healthy controls found that the GG genotype as well as the G allele were significantly more common in patients with PAD (242;246). The GG genotype was more common in Type II diabetics with PAD then in those without (242). From this evidence it would seem that IL6 may indeed be causally involved in arterial disease development and that it is the SNP G-174C GG genotype that conveys a greater risk of atherosclerosis.

The evidence in the literature however is conflicting. Other studies have shown that the C-allele is associated with higher plasma IL6 levels (247-249). In patients who underwent CABG surgery and in hypercholesterolaemic patients, those with the C-allele were shown to have higher plasma levels of IL6 than those with the GG-genotype (250;251). It has been suggested that the C-allele confers a risk of myocardial infarction in the elderly (252). There are a variety of studies which found the C-allele to be associated with an increased risk of cardiovascular disease, among them a study on 1109 random Australian subjects which showed a significant association between the C-allele and increased carotid intima-media thickness (IMT) (248;249;253-255). It was also found that in healthy men the C-allele was associated with 2.4-2.5% increase in blood pressure, equating to a 5% increase in risk for MI in genotype CC, when compared to subject carrying only the G-allele (249).

Equally some studies have found no correlation between IL6 G-174C genotype and IL6 levels or disease. A study examining the interaction between the IL6 G-174C promoter polymorphism and risk of MI in male smokers, found that there was no association after adjustment for multiple comparisons (256). A study investigating if

IL6 G-174C genotype affects IL6 production in vivo following elective aortic aneurysm repair, found no significant difference between genotypes (182). In a large population based study no difference in genotype distribution between a group of MI patients and a group of controls could be demonstrated (138). Moreover there was no association between cardiovascular risk factors and IL6 genotype (138). A large study on 6434 participants showed no influence of SNP G-174C on plasma levels of IL6 (257). The same study found no association between *IL6 G-174C* genotype and risk of CVD or MI (257). Similarly other studies failed to show a significant influence of IL6 G-174C genotype on risk of vascular disease (138;243;247;258;259).

A clinically relevant causal role of IL6 in arterial disease processes cannot be assumed in the face of this evidence. However, neither can such a role be excluded, as there are a variety of reasons why these discrepancies may have occurred.

Possible Reasons for conflicting Results from Genotype Studies

When the IL6 G-174C genotype is used in association studies to prove causality, an assumption is made that the effect of this polymorphism in vivo is fully understood. There is no study to date however, that has satisfactorily investigated the in vivo effect of SNP G-174C on gene expression. The effect of the polymorphism has mainly been demonstrated in culture using constructs of the promoter region transfected into cell lines i.e. in an in vitro model (24;238).

When using an in vitro model, potential regulatory sites may not form part of the constructs i.e. the model accounts for only part of the gene as adjacent areas, not transfected into the model, might influence gene expression in vivo e.g. by providing binding sites for inhibitory factors. This is demonstrated by two studies which investigated gene expression in HeLa cells transfected with different length constructs of the promoter region of *IL6* (24;238) which showed discrepant results, possibly due to exclusion of a regulatory region from the construct by one or other study (182).

Another limitation of in vitro studies is the use of known inducers of IL6 expression such as LPS. It is possible that the artificial induction of cytokines in vitro exerts a different effect on the cell as the induction of IL6 in vivo (24).

The effect of SNP G-174C on gene expression in vivo has been investigated by measuring plasma levels of IL6 in subjects of different genotypes. Though these

studies provide valuable in vivo data, one has to take into account certain factors which may influence the results.

The plasma level of IL6 is the result of IL6 expression in a variety of tissues such as endothelium, leucocytes, adipose tissue and muscle. If the promoter SNP G-174C is functional, then the circulating levels of IL6 would be influenced by the sum of the effect of SNP G-174C in contributing tissue types. The result can only be interpreted accurately with regards to SNP G-174C effect, if the effect is the same in all tissue types. However it has not been established whether SNP G-174C influences transcription in vivo in humans equally in different tissues/cell types. It is possible that the effect of SNP G-174C on gene transcription is modulated by the tissue type in which the gene is expressed; thus circulating IL6 levels may give misleading results. It is likely that transcription is regulated differently in different tissue types through e.g. varying expression of transcription factors in different tissues (24). It is therefore difficult to draw conclusions from the overall plasma level to the exact effect of SNP G-174C.

Another drawback of using plasma levels and genotype is that gene expression is regulated at several levels. The first and most important regulatory level is the primary control of transcription. The promoter variant SNP G-174C is likely to affect expression only at this level. However, subsequent levels of regulation with the capacity to influence final protein levels exist. Therefore a study investigating circulating protein levels takes into account all these levels of regulation as well as the effect of SNP G-174C on the primary transcription. Studying primary RNA expression patterns rather than measuring final protein product with regards to genotype would allow more precise insight into the effects of the promoter polymorphisms.

Another reason for the discrepant observations may be that SNP G-174C was studied in isolation. The effect one polymorphism exercises on gene expression may be influenced by base pair changes at other polymorphic sites i.e. by haplotype. It is therefore possible that haplotype analysis rather than genotype analysis would give more consistent results. Other polymorphisms in the IL6 gene that may be investigated as part of a haplotype analysis, are SNP G-597A, G-572C (252;260-263). These are also thought to affect gene transcription. However G-597A is in complete linkage disequilibrium (see final paragraph section 1.7.1) with G-174C (24). The G-572C polymorphism is relatively rare; making it therefore of use only in studies with large sample numbers (24).

Further possible reasons for the discrepant observations in association studies is the fact that the association of the promoter polymorphism appears to be age dependent, therefore studies looking at population with a different age distribution may show conflicting results (252).
Apart from these practical experimental considerations the inherent limitations of Mendelian randomisation also have to be taken into account when interpreting genetic association studies. The Mendelian randomisation approach may give erroneous results if the marker under examination is in linkage disequilibrium with another variant influencing disease risk (24;264). Linkage disequilibrium (LD) describes a scenario were two genetic variants are inherited together. If the marker SNP G-174C was in LD with another marker affecting a gene other than IL6, also able to influence disease risk, then demonstrating an association between SNP G-174C and disease may not reflect a causal role of IL6 in the disease process; rather it would relate to the effect of the marker in LD with SNP G-174C. It has not been shown that linkage disequilibrium exists for SNP G-174C with a marker in another gene. However such a marker may be discovered in the future. As IL6 is located within a relatively gene poor part of the genome, and linkage disequilibrium is less common with increasing physical distance, this scenario appears unlikely.

1.8 Summary

IL6 is a cytokine that is associated with atherosclerotic risk factors, vascular disease development and progression and outcome from surgery and thus appears to play an important role in all aspects of vascular disease. Modulation of this protein could therefore offer a promising therapeutic strategy. Before such a therapeutic option can be entertained, proof of causality needs to be established. Epidemiological studies employing the principle of Mendelian randomisation promise to be a useful tool to answer the question of causality, however they require the definition of a genotype that affects gene expression in vivo. SNP G-174C is thought to affect expression, but epidemiological studies have given conflicting results. This may be partially due to the methodology employed in investigating the effects of the IL6 promoter SNP. Cell culture experiments may not provide adequate models for in vivo SNP effects on IL6 expression and protein levels are influenced by various levels of regulation, whereas promoter SNP G-174C is only likely to affect primary transcription. RNA expression studies on human tissues may provide a more

accurate answer to the effect of promoter SNP G-174C on gene expression. In addition, plasma levels do not take into account possible variation of the promoter SNP effect in different tissues. Plasma levels of IL6 are the result of IL6 secretion in a variety of tissues and cells. It is therefore important to understand the regulation of gene expression in the individual tissue types and also the relative contribution the individual tissues make to the circulating levels of IL6. Lastly, inter-individual comparisons are subject to confounding variables which may mask the effect of promoter SNP G-174C and may explain some of the discrepant observations in the literature.
It is necessary to delineate the in vivo effect of this promoter polymorphism further, employing *in/ex vivo* investigations in human tissues.

1.9 Hypothesis, Study Aims and Objectives

Hypothesis:
1. The magnitude of *IL6* expression is determined by IL6 G-174C genotype.
2. The magnitude of *IL6* expression is determined by the tissue type in which the gene is expressed.

Aims: The aim of this study was to investigate the effect of SNP G-174C genotype on IL6 gene expression in vivo in humans in different tissues. Delineating this effect could be useful in future studies investigating the nature of the IL6/arterial disease association. The second study aim was to assess levels of relative IL6 RNA expression in humans in different tissues.

Objectives: Blood, vein, adipose and skeletal muscle tissue was to be collected from individuals free of diseases known to affect IL6 expression. The collected samples were to be investigated for levels of IL6 mRNA expression in the different tissues. The effect of SNP G-174C genotype on expression in different tissues was to be investigated. A new assay was to be designed which would allow measurement of the relative IL6 hnRNA expression from the two alleles in heterozygous individuals.

2 Patients, Materials and Methods

Please see Appendix A: for a list of materials used in this study.

2.1 Study Outline

The following sections will outline the study (see Figure 2-1) and the reasons for conducting the study in the manner in which it was performed.

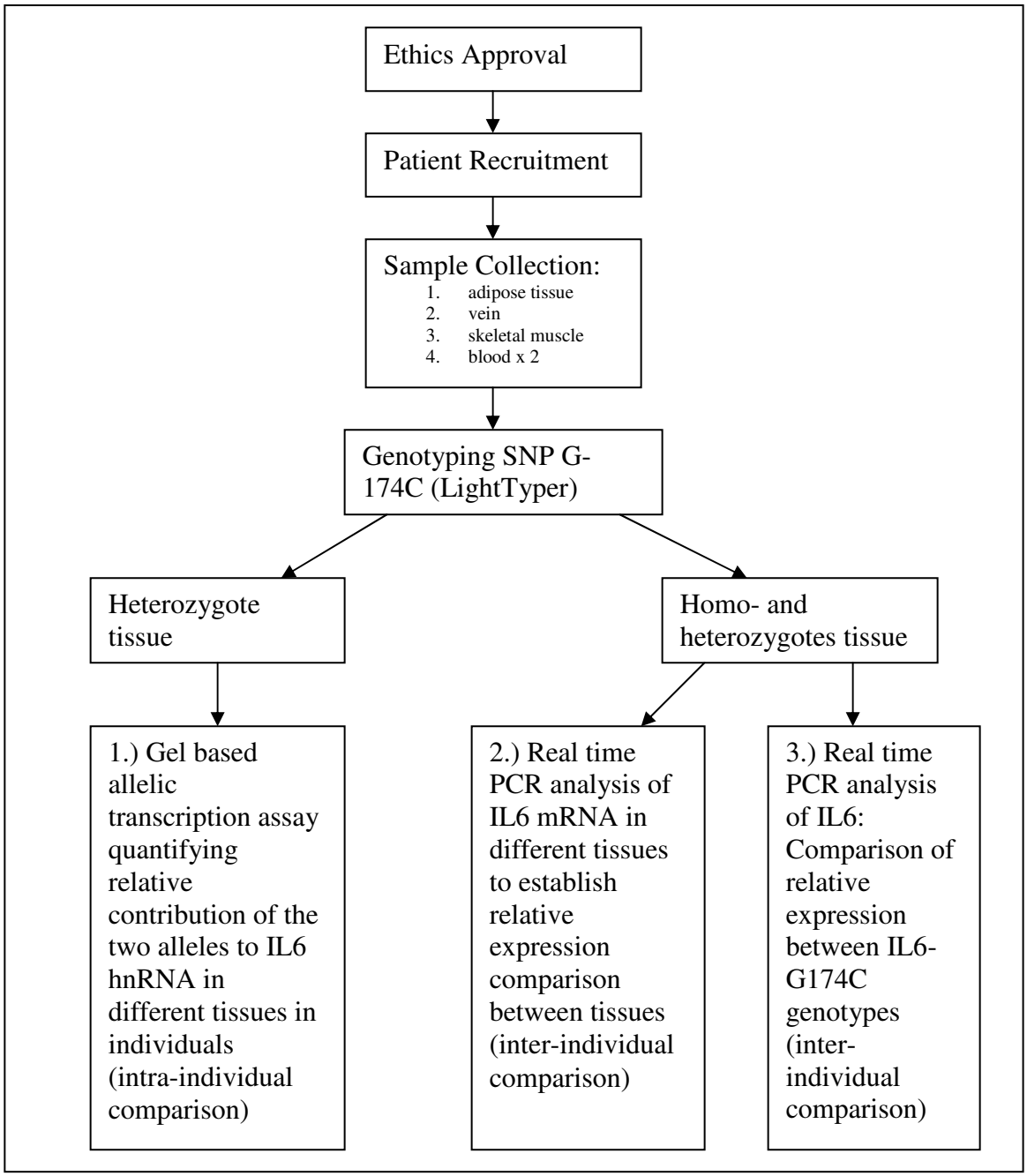


Figure 2-1: Flow chart of study.

2.1.1 Patient Recruitment and Tissue Selection

Cell culture is only an approximate model of in vivo conditions (see section 1.7.1). In order to achieve an accurate measure of in vivo RNA expression *ex vivo* tissue samples were used in this study. Cardiovascular disease and a variety of other pathologies, such as acute inflammatory or malignant diseases, may affect IL6 tissue expression. The study therefore aimed to investigate individuals free of these systemic diseases. To study the effect of tissue type interaction with SNP G-174C genotype on IL6 expression, a variety of tissues had to be investigated. Tissues both known to express IL6 and accessible in patients treated in the vascular unit (vein, subcutaneous adipose tissue, muscle and leukocytes) were chosen for the investigation. Other tissues known to express IL6 i.e. neuronal tissue, cartilage and bone where not included in this study as access to these samples was not available in the study patients. Leukocytes rather than mononuclear cells were used, as the processes involved in isolating individual cell types from leukocytes may have caused alteration in the RNA profile of the cells. Most isolation methods involve prolonged centrifugation in a gradient solution. The RNA profile of cells once harvested may change over time. This study aimed to assess the in vivo RNA profile, thus avoiding time delay was important for accurate results. Patients undergoing varicose vein surgery in the day surgery unit were chosen for recruitment to the study for the following reasons:

1. Patients with significant systemic disease are precluded from day surgery. Therefore these patients are free from significant cardiac disease.
2. The groin dissection performed during the tying of the sapheno-femoral junction for varicose vein surgery allows access to vein, adipose tissue and skeletal muscle without extension of the skin incision.

Therefore varicose vein patients attending the Day Surgery Unit were ideally suited to donate samples for this study.

Muscle and adipose tissue biopsies had not been previously performed routinely in varicose vein patients in this unit and concern was raised that these may lead to an increase in post-operative pain and bleeding complications. A follow up of the initial patients was therefore performed.

2.1.2 Effect of Promoter SNP G-174C on IL6 RNA Expression

The promoter SNP G-174C is likely to affect transcription of DNA into RNA rather than translation of RNA into protein, therefore this study examined RNA expression levels rather than protein expression levels. Real time PCR analysis is a well described method for the analysis of RNA expression levels and this technique has previously been used to compare RNA expression between individuals of different genotypes. This inter-individual comparison however, is susceptible to confounding variables, such as age, smoking habits and medication. Therefore the initial stage of this study aimed to design an assay that would minimise the effect of such confounding variables. This assay was based on preferential allelic transcription.

2.1.2.1 Preferential Allelic Transcription

Diploid organisms have two copies of a gene, one maternal and one paternal copy. These two DNA coding sequences are referred to as alleles. Most genes are expressed from both alleles, except for genes subject to X-chromosome silencing or genomic imprinting (265). However, expression does not always occur in equal amounts from both alleles (266;267). A study looking at 602 genes showed that 326 of these had preferential expression from one allele (265). Assuming SNP G-174C affects gene transcription one would expect an imbalance of expression from the two alleles in heterozygote individuals. The advantage of quantifying allelic transcription in heterozygous tissues lies in the fact that inter-individual confounding variables affect both alleles and thus are unlikely to influence the transcription ratio between the two alleles. Therefore measuring allelic transcription may provide an insight into the effect of promoter SNP G-174C on transcription while eliminating the influence of confounding variables.

2.1.3 Ex Vivo Expression of IL6 RNA in different Tissues

IL6 RNA expression may not only be influenced by genetic polymorphisms but also by the tissue type in which the gene is expressed. Raised *circulating* levels of IL6 have been linked to aspects of vascular disease (see 1.5). IL6 is expressed in a variety of cells including monocytes/macrophages, adipocytes, endothelial cells, fibroblasts and myocytes. The *circulating* levels of IL6 are therefore the result of its secretion from various tissues. The relative contribution individual tissues make to the plasma IL6 level however is not known. Definition of the relative IL6 expression

of individual tissues would allow research to focus on tissues expressing the most IL6 and thus are more likely to influence circulating levels.
Levels of expression of IL6 have previously been investigated in tissue culture with and without stimulation. Levels of expression in individuals suffering from a variety of diseases have also been studied (24;268-271). The results obtained in these studies cannot be compared to assess the relative contribution individual tissues make, as experimental conditions varied, as did the samples used (i.e. cell culture, ex vivo tissue samples, plasma etc.). To date there is no study allowing a direct comparison of baseline IL6 expression in different tissues in vivo in the same individual.
This study therefore aimed to gain further insight into the relative RNA expression of IL6 in different tissues by comparing relative *IL6* transcription in a variety of tissue types in a single individual.

2.1.4 Effect of SNP G-174C Genotype on IL6 RNA Expression in different Tissues

In addition to the design of a preferential allelic transcription assay, the effect of SNP G-174C on IL6 mRNA expression in different tissues was also measured using real time PCR analysis. It was hoped that this would provide clues as to the interaction of genotype with tissue type. Expression of IL6 mRNA in adipose tissue, vein, muscle and leukocytes was measured and analysed according to G-174C genotype of the patient. This analysis consisted of an inter-individual comparison and was thus subject to potential confounding variables.
Plasma levels of IL6 have been shown to vary with age. Smoking has also been shown to influence IL6 expression (140;272;273). The association of IL6 expression with age and smoking was therefore investigated in vein, adipose tissue, skeletal muscle and leukocytes.

The following sections will provide a more detailed account of the methods used in this study.

2.2 Methods in Detail

2.2.1 Ethics Consent

Ethics approval was obtained from the Southampton & South West Hampshire Research Ethics Committees on the 01.03.2004 REC Reference: 040/04/w to collect samples of vein, skeletal muscle, subcutaneous adipose tissue as well as blood from anaesthetised patients undergoing varicose vein surgery at the Day Surgery Unit Southampton General Hospital/Royal South Hants Hospital (at this time the majority of varicose vein treatments were conventional surgery). Permission for genotyping and subsequent usage of samples in genetic studies was obtained.

2.2.2 Patient Recruitment

Patient recruitment was performed by the researcher. 108 patients were approached about the study and 95 agreed to take part. Of these 95 patients, 67 ultimately donated tissue samples which were used in the study (see Figure 2-2). Patients for recruitment were identified by review of the hospital waiting lists and theatre lists. Patients with ASA Grade 1 or 2 were included in the study (ASA=American Society of Anaesthesiologists: ASA I=no disease; ASA 2=mild systemic disease but no effect on daily activity; ASA 3= Symptomatic illness but minimal restriction on life; ASA 4= symptomatic illness causing severe restriction on life; ASA 5=moribund). Patients with ASA grade III or above or significant cardiovascular disease, acute inflammation/infection or untreated carcinoma were excluded from the study, as were patients currently taking steroids. Patient notes were reviewed and relevant data were collected on all patients who were recruited to the study. Ethnic origin, age, gender, type of operation, CEAP classification (Clinical, etiological, anatomical and pathological: 0=no visible venous disease, 1=telangiectasia or reticular veins, 2=varicose veins, 3=oedema, 4=skin changes, 5=healed ulcer, 6= active ulcer), past medical history, current medication, smoking status and family history was recorded. Patients were recruited in the pre-assessment clinic after routine pre-operative assessment. An information leaflet was given to the patient to read. The study was then explained to the patient in a private interview room. Possible side effects and complications were explained, including the potential risk of bleeding, bruising and pain. The patient was given the opportunity to ask questions before being given a copy of the consent form. In all cases it was made clear that signing the consent form would not preclude the patient from leaving the study at a later stage. A further copy of the consent form and the patient information sheet was

given to the patient to take home. After one month it became clear that many patients currently on the waiting list for surgery had already been seen in the pre-assessment clinic and that recruitment in the pre-assessment clinic was ineffective. Therefore ethics approval for a further method of recruitment was sought (Southampton & South West Hampshire Research Ethics Committee, Amendment Number 2 Ref. No: 040/04/w, approval granted 09th February 2005). Patients recruited by this second method had already been allocated an operation date. They were sent a patient information sheet and a copy of the consent form in the post with an explanatory letter attached. This was followed up with a telephone call during which the study was explained and the patient was given the opportunity to ask questions. In these cases written consent was taken on the day of operation.
Due to changes in the provision of services in the Southampton Region, the Day Surgery Unit at the Royal South Hants Hospital was due to be closed and very few varicose vein operations were carried out at this unit in the later part of patient recruitment. It was therefore decided to attempt sample collection on the Isle of Wight where the through-put of varicose vein patients had remained unaffected. Ethics consent was obtained to include the Day Surgery Unit at St. Mary's Hospital, Newport, Isle of Wight in this study. The Isle of Wight Healthcare NHS Trust Research and Development Committee granted approval on the 28th July 2005.

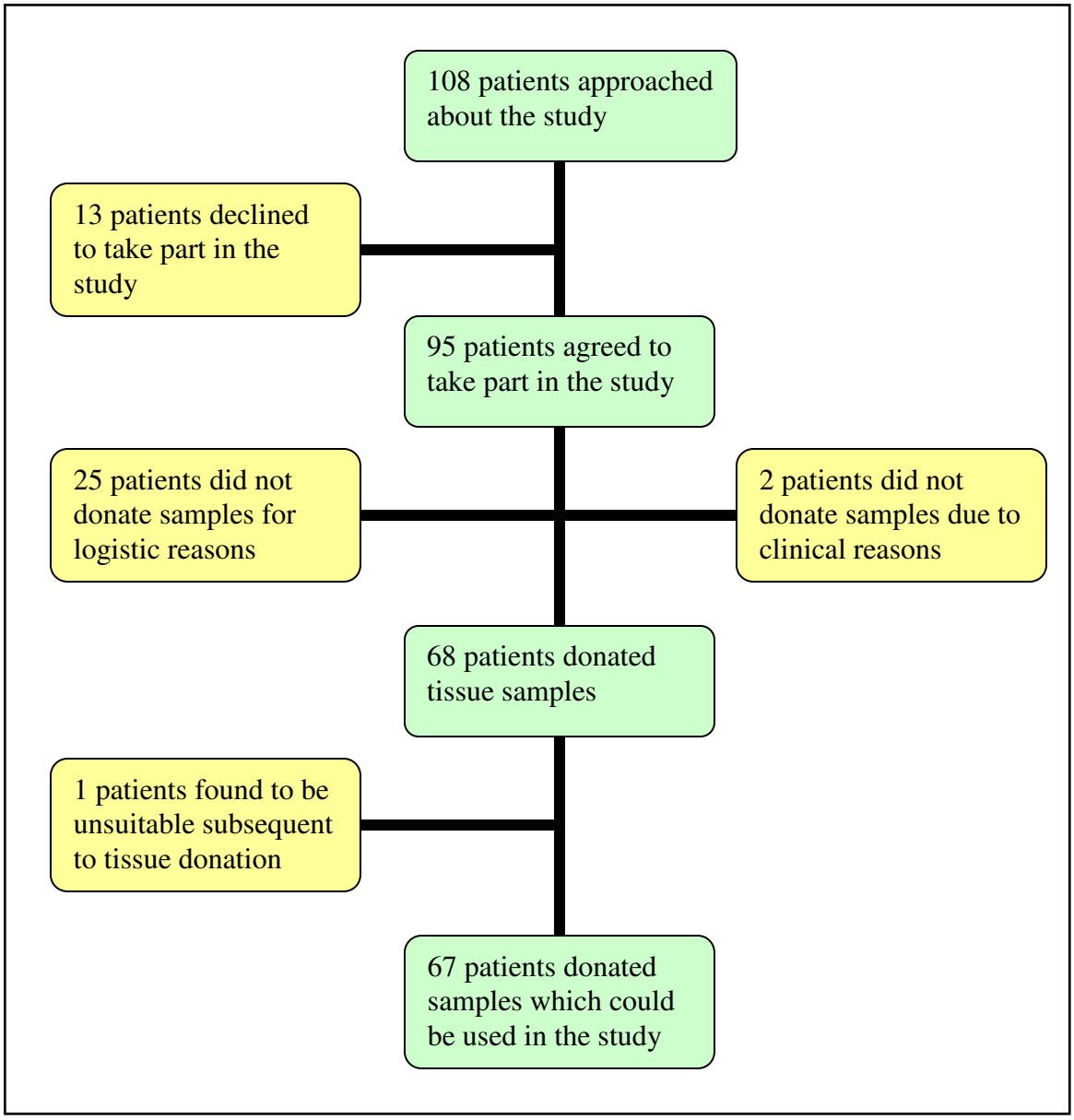


Figure 2-2: Total number of patients asked to take part in the study (108): Patients who agreed to take part (95); patients who declined to take part (13). Of the 95 patients who agreed to take part in the study 68 donated tissue samples to the study. 25 did not donate samples due to logistic reasons and two did not donate samples for clinical reasons. One patient was found to have taken steroids prior to his varicose vein surgery and was therefore excluded from the study.

Patients were not required to attend any additional appointments for the study. The patient's GP was informed of their patient's participation in this study by letter which also contained a copy of the patient information sheet.

2.2.3 Sample Collection

Samples were collected by the researcher from anaesthetised patients. All samples were handled using universal precautions of infection control, including the wearing of gloves and avoiding contamination of any surfaces. A peripheral blood sample was collected by the researcher. Blood was collected with a Safety-Lok™ blood collection system and a standard BD Vacutainer™ needle holder. The first sample for leukocyte DNA analysis for genotyping was collected into a nine millilitre EDTA plastic vacutainer tube. The second sample, for extraction of Leukocyte RNA, was collected using the PAXgene™ Kit (PreAnalytiX). Tissue samples were collected during the operation (see Figure 2-3).

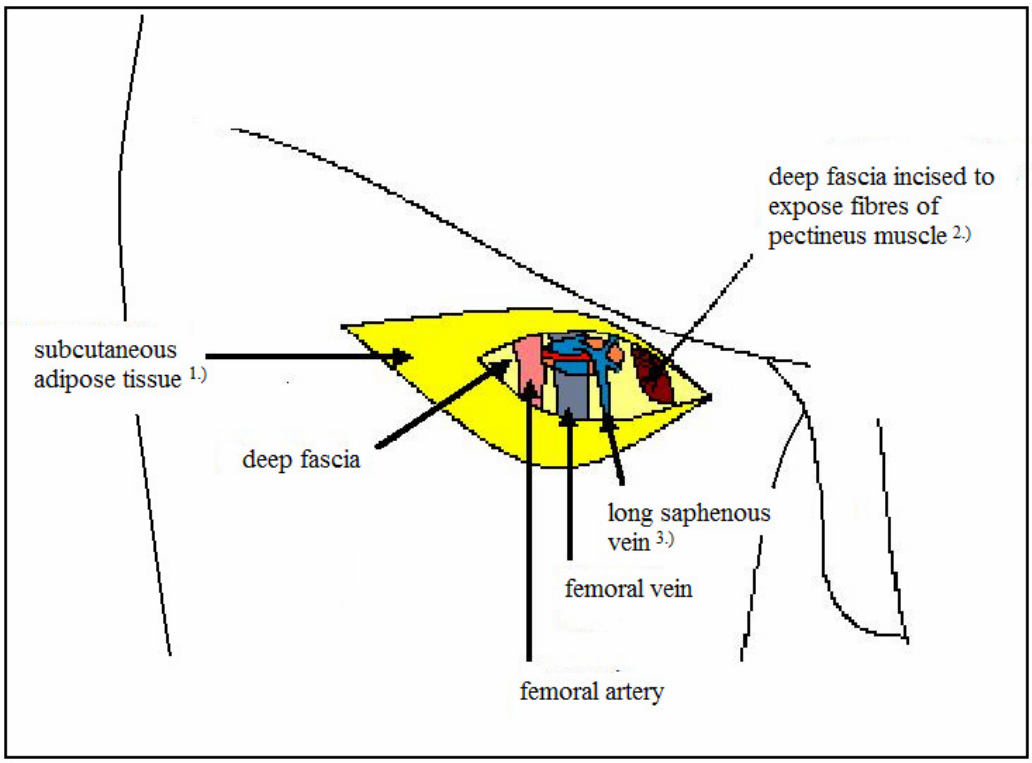


Figure 2-3: Schematic representation of the sapheno-femoral junction during groin dissection for high long saphenous vein tie. A subcutaneous adipose tissue sample[1.] was taken from the edge of the wound, as convenient. Skeletal muscle[2.] was taken as a biopsy from pectineus muscle. The long saphenous vein[3.] was stripped as part of the varicose vein operation and a segment used for the study. (The diagram exaggerates the scale of the incision and in no patient was the incision enlarged to obtain the muscle specimen.)

All tissue samples were harvested by the operating surgeon and handed to the researcher for further processing. Vein was harvested from the long saphenous vein which had been removed using a plastic stripper, except in two cases - in which

short saphenous vein and 5 cases - in which vein material from avulsions was collected. Short saphenous vein was harvested by formal surgical dissection and excision of the proximal portion, as was standard clinical practice. Vein material from avulsion was obtained using a vein hook and mosquito forceps. Adipose tissue (1cm cube) was surgically excised from the subcutaneous tissue of the wound edges. Access to the pectineus muscle was gained through dissection of the medial part of the incision. The subcutaneous fat was dissected off the deep fascia overlying pectineus muscle. The pectineal fascia was opened and a 0.5cm length of superficial muscle fibres excised. Any resulting bleeding was treated with diathermy. Fat and muscle samples were collected in succession, either immediately before or after removal of the vein. Blood samples were collected during the same time period as the tissue samples.

Tissue samples were placed on a single use sterile plastic container and immediately submersed in RNA*later*® (Sigma-Aldrich) to maintain the in vivo RNA profile (274). Sterile metal forceps and scalpel were used for processing. The section of vein was agitated in the RNAlater solution to remove adherent blood cells. It was then freed of adherent connective and adipose tissue with the help of a scalpel. The vein was dissected into sections of approximately 0.1 cm to allow easy penetration of the RNA stabilising solution into the tissue. The fat biopsy was dissected and inspected to ensure no lymphoid tissue was included before dissection into 0.3cm cubes. The muscle biopsy was equally dissected into 0.3cm cubes and any adipose tissue was removed. All tissue samples were transferred into 1.5ml microtubes containing 1.3ml of RNAlater™.

EDTA blood collection tubes were stored at -80°C for future analysis. PAXgene™ tubes were incubated overnight at room temperature before being placed at -80°C for storage. All tissue samples were stored in the fridge at -4°C overnight before being transferred to -80°C until further analysis could take place.

2.2.4 Patient Follow-Up

The reason for follow up was initial concern about potential complications from added surgical procedures. The collection of vein tissue from patients did not represent an additional procedure for patients undergoing varicose vein surgery. Adipose tissue is often adherent to the stripped vein and thus the excision of fat from the area surrounding the sapheno-femoral junction was considered unlikely to

put the patient at risk of additional complications. However it was unclear whether muscle biopsy could lead to additional complications, namely bleeding and prolonged recovery form operation due to pain. Patient follow up was therefore performed in the initial stages of the study. Further reason for follow up was to establish whether the change from personal recruitment to recruitment by post was acceptable to the patients. Patients were followed up with a telephone call and asked to answer a number of questions (for questionnaire see Appendix D:). They were asked about their satisfaction with the study recruitment process and whether they would consider taking part in clinical trials in the future. Any post-operative complications were noted, as was the time scale of return to normal daily activity following the surgery. Patients were asked to grade their pain in the first two postoperative days on a scale of one to ten, and finally whether they felt that they had suffered any ill effects from taking part in the study.

This concluded the clinical part of the study. The following sections will describe the laboratory work carried out.

2.2.5 DNA Extraction

DNA was extracted from 9ml whole blood according to a method by Miller et al (275). Blood samples had all been stored at -80°C. Samples were defrosted on a roller mixer for at least 1 hour at 4°C. The blood was then transferred into 50ml conical tubes and 30ml of erythrocyte lysis buffer (ELB) was added. After repeated inversion the tubes were placed on ice and mixed gently on a rotating mixer for 15 minutes. The tubes were centrifuged at 1500rpm for 10 minutes at 4°C and the supernatant poured off leaving a white cell pellet at the bottom of the tube. This ELB wash was repeated two more times. The white cell pellet was then washed into a 15ml conical tube using 3ml nucleic lysis buffer (NLB). The white cell pellet was re-suspended and 250ul 10% sodium dodecyl sulphate (SDS) and 150ul protease (40mg/ml) was added. The samples were placed in a shaker incubator overnight at 37°C. Samples were allowed to cool to room temperature before adding 1ml saturated NaCl to each tube, which was shaken vigorously for 15 seconds. The samples were centrifuged at 4000rpm for 20 minutes at room temperature. The supernatant was transferred to a fresh tube and exactly twice the volume of absolute cold alcohol was added. The tube was inverted until the DNA formed a pellet. The pellet was removed and washed in 1ml of 70% alcohol in a 1.5ml micro

tube. The samples were pulse centrifuged and the ethanol poured off. The DNA was allowed to dry at room temperature before adding 500ul of TE buffer. Samples were left overnight to allow the DNA to dissolve prior to storage. Samples were transferred to screw top tubes and stored at -20°C.

2.2.5.1 Quantification of Genomic DNA

Quantification of DNA was kindly performed by Nikki Graham (Laboratory Technician). The method employed, used PicoGreen®, an ultra-sensitive fluorescent nucleic acid stain which allows quantification of double-stranded DNA (dsDNA). Fluorescence of samples was measured against a standard curve created from standard DNA of known concentration.

2.2.6 Genotyping for G-174C

Genotyping was performed using white cell DNA from EDTA blood samples and an Odyssey LightTyper assay.

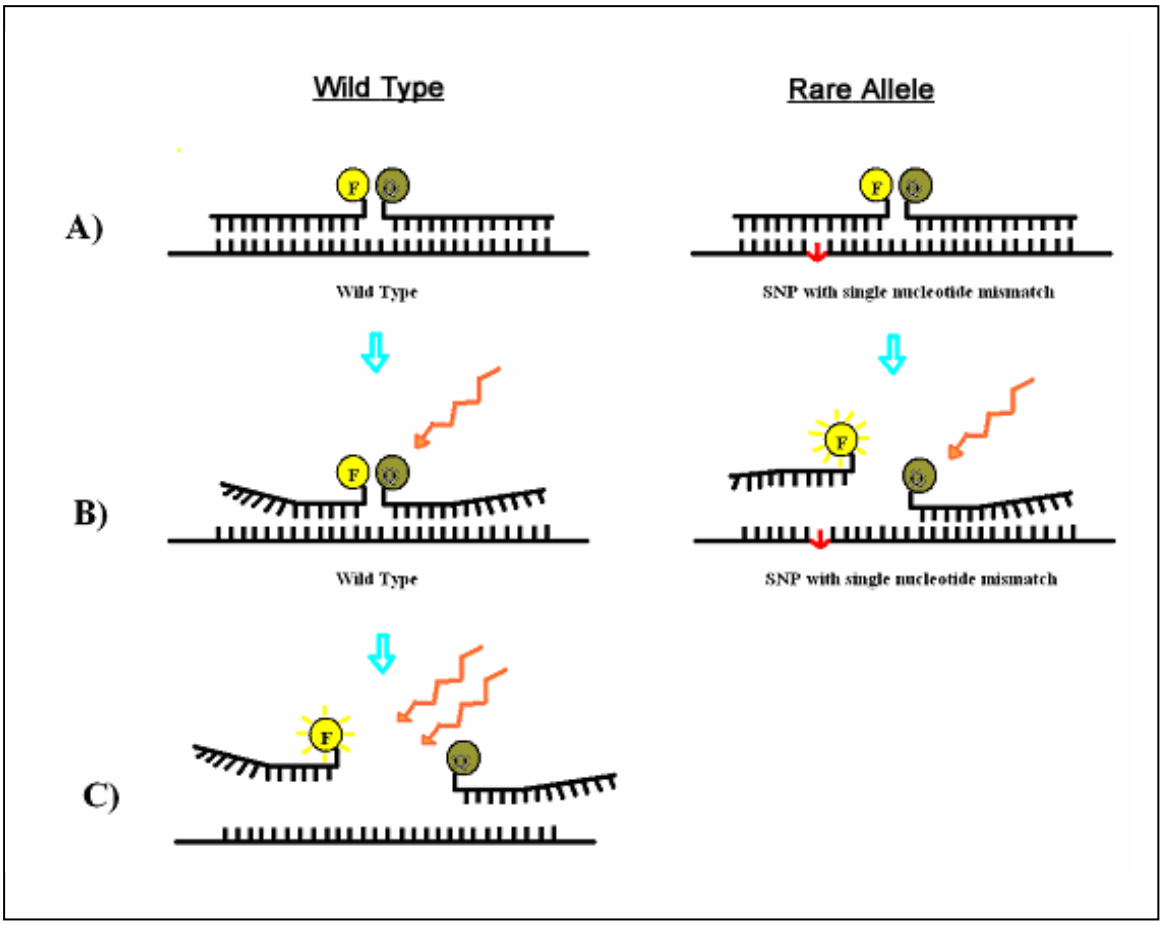


Figure 2-4: Principle of melt curves analysis: (A) A fluorescent labels probe[F] and a quencher probe[Q] bind to a nucleotide sequence, one wild type and one mutant containing the SNP. (B) As the temperature is raised, the complimentary strands are denatured, the fluorescent probe and the quencher probe are separated and fluorescence can be detected. This occurs at lower temperatures for the imprecisely bound probe. (C) Higher temperatures are required to separate the probe bound to the wild type. This leads to two separate peaks on the melt curve.

This assay uses the principle of 'melt curves' (see

Figure 2-4). A fluorescent labelled probe is designed to anneal to the precise sequence of one of the possible SNP alleles. A second quencher probe is designed to anneal to the adjacent sequence. Both probes are included in the PCR master mix as is a primer pair that will amplify the region surrounding the SNP. PCR amplification is then performed in the usual manner. Following PCR the samples are heated up to a

set temperature. During the heating process the imprecisely bound probe (i.e. the probe bound to the alternative allele) melts off the DNA at a lower temperature than the precisely bound probe. As the fluorescent probe is separated from the quencher probe the detectable fluorescence (F) changes. The change in fluorescence [F(n+1)-F(n)] is measured and plotted against temperature to produce a melt curve. This reveals individual peaks corresponding to each of the two alleles, allowing genotyping of template DNA (Figure 2-5).

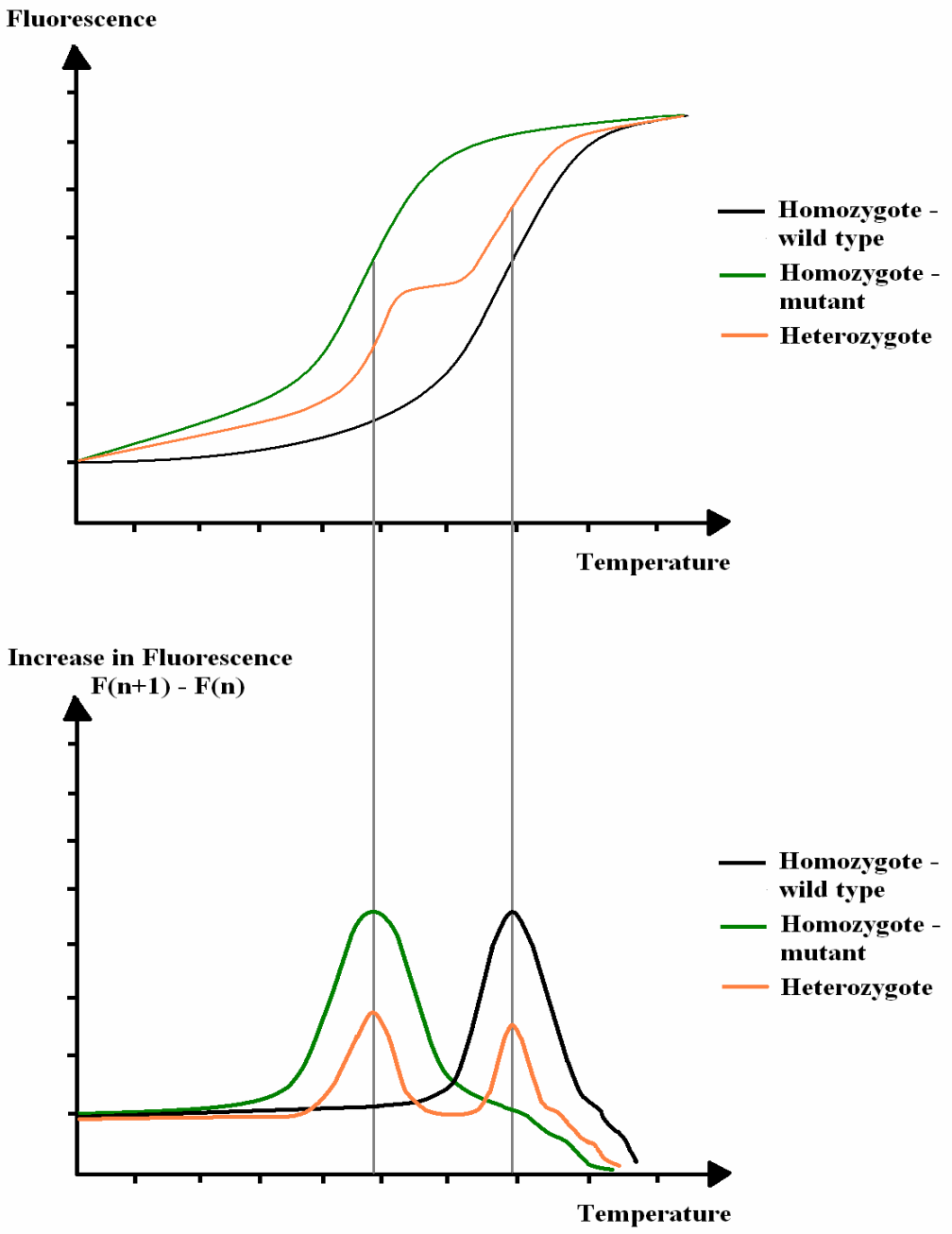


Figure 2-5 : Schematic representation of a LightTyper melt curves for the three different genotypes. The top graph shows detectable fluorescence according to ramp temperature. The bottom graph is a derivative of the top graph depicting increase in fluorescence at a given temperature. The prominenet peaks of the bottom graph allow allocation of genotype. Double peaks correspond to heterozygote template. Single peaks correspond to homozygote templates, both wild type and mutant, differentiated by temperature.

An assay for genotyping of G-174C had previously been designed in the laboratory (Ryan Waters). The sequences for the forward and reverse primer, as well as the fluorescent and quencher probe for the LightTyper assay are shown below (see Sequences 1).

Genotyping assay primer sequences:

G-174C	Forward	5'- cgctagcctcaatgacga - 3'
	Reverse	5'– tctttgttggagggtgagg - 3'

Genotyping assay probe sequence:

FITC Probe (fluorescent probe):	5'-ctttagcatCgcaagacac- 3'
DABCYL Probe (quencher):	5'-aaccttattaagattgtgcaatgtgacg- 3'

Sequences 1: Sequence of forward and reverse primers annealing in areas of the gene adjacent to SNP G-174C . PCR using these primers will lead to amplification of the sequence surrounding the SNP G-174C. The sequence of the FITC probe and the quencher probe is also given. The FITC probe dimerises with the SNP itself (annealing base shown in red) and its neighbouring bases. The quencher probe anneals in close proximity to the FITC probe. Separation of the fluorescent probe from the quencher probe during the melt cycle occurs at different temperatures for the precisely bound and the imprecisely bound probe, allows detection of fluorescence peaks at different temperatures for different alleles (see text for a more detailed description of this assay).

2ul of genomic DNA extracted from leukocytes was placed onto a 384 well plate and 10ul of PCR master mix was added before placing the plate onto the thermal cycler. Below is a list of reagents for the PCR reaction for SNP G-174C genotyping using the Odyssey LightTyper:

PCR Reagents	**10ul Final Concentration**
10x PCR Buffer	1ul (1xPCR Buffer)
10mM dNTPs	0.2l (200uM)
25mM Mg^{2+}	0.6ul (1.5mM)
Primers(10pmol/ul) Forward	0.5ul (500nM)
Reverse	0.1ul (100nM)
FITC probe (10pmol/ul)	0.2ul (200nM)
DABCYL probe (10pmol/ul)	0.2ul (200nM)
Promega Taq (5U/ul)	0.04ul
H_2O	7.16ul

Below are the timings for the PCR program for the SNP G-174C genotyping assay:

PCR cycling program:	
Initial denaturation step	94ºC for 2 minutes
Denaturation step	94ºC for 20 seconds
Primer annealing step	59ºC for 30 seconds
Extension step	72ºC for 30 seconds
	go to step 2 and repeat cycles 49 times
Final extension step	72ºC for 2 minutes

Following PCR the samples were covered with 5ul of Chill-Out™ wax and melted from 35°C to 70°in the 384 well Odyssey LightTyper. LightTyper software was used to analyse the change in fluorescence during melting and to assign genotypes to individual melt curves. Melt curves were visually inspected to ensure accurate assignment of genotype by the software.

The Genotype assay was performed in triplicate on three separate PCR plates. All samples had at least 2 valid genotyping results.

2.2.7 Methods for RNA Extraction

2.2.7.1 RNA extraction using TRI Reagent®

Sample Preparation

RNA extraction was carried out using TRI Reagent® according to the manufacturer's instruction with some modifications to increased RNA yield. If possible RNase free disposable plastic ware was used. Glassware was washed with ethanol and diethylpyrocarbonate (DEPC)-treated water and baked overnight.

Tissue samples were defrosted and weighed. Between 60-100mg of vein and muscle tissue and 150-200mg of fat tissue was taken and homogenised separately by repeated cutting with a disposable scalpel blade on a baked glass slide. The sample was then transferred to a 1.5ml micro tube. A 1.5ml micro tube holds a maximum of 100mg of tissue; therefore fat samples were processed in two tubes.

Homogenisation was continued using a disposable homogenisation pestle inserted into the micro tube while mixing the sample on a vortex. TRI Reagent® was gradually added during the homogenisation process. A total of 1.5ml of TRI Reagent® was added. Vein, muscle and fat tissue samples were then repeatedly aspirated with a needle and syringe. Samples were left to stand at room temperature for 5 minutes before centrifuging the homogenate at 13,000rpm in a refrigerated centrifuge for 10 minutes at 4°C. The clear supernatant was transferred to a fresh tube. The adipose samples developed a fatty layer on top of the supernatant at this stage. This layer was carefully removed by pipette aspiration before the supernatant was transferred to a fresh tube. Samples were allowed to stand for 5 minutes at room temperature before adding 0.3ml of chloroform. Samples were then covered and shaken vigorously for 15 seconds and were allow to stand for 5 minutes at room temperature, before centrifuging at 13,000rpm for 15 minutes at 4°C. The colourless upper aqueous phase containing RNA was transferred to a fresh tube.

RNA Isolation

A total of 0.75ml of isopropanol was added to the aqueous phase and samples mixed thoroughly by repeated inversion. Samples were left to stand for at least 30 minutes at -20ºC before centrifuging at 13,000 x g for 18 minutes at 4°C.

The supernatant was removed and the RNA pellet was washed by adding 1.5ml of 75% ethanol and vortexing the sample. The RNA pellet was reformed by centrifugation at 13,000 x g for 18 minutes at 4°C. All ethanol was removed from

the samples by decanting and pipette aspiration and the RNA pellet was then air dried for 10 minutes.
Vein and muscle RNA was dissolved in 20-30µl of RNA storage solution. Fat RNA was dissolved in 10ul of RNA storage solution. To facilitate dissolution, samples were mixed by repeated pipetting at 55ºC for 10-15 minutes. The two fat RNA samples were merged.

2.2.7.2 RNA Isolation using PAXgeneTM Kit

All buffers, spin columns and proteinase K used in RNA isolation from blood were purchased as part of a kit from PreAnalytiX (Qiagen). Bloods were thawed in an open rack. The PAXgene™ blood RNA tubes were centrifuged for 10 minutes at 3000-5000 x g using a swing out rotor. The supernatant was removed by decanting. 5ml RNase free water was added to the pellet, a lid was applied and the sample was vortexed before centrifuging for 10 minutes at 3000-5000 x g. Again the supernatant was removed. 360ul of Buffer BR1 was added and the sample was vortexed until the pellet was visibly dissolved. Samples were pipetted into a microcentrifuge tube and 300ul Buffer BR2 was added before adding 40ul Proteinase K. Samples were vortexed then incubated for 10 minutes at 55ºC using a shaker incubator. Samples were then centrifuged at a minimum of 10,000 x g for 3 minutes and the supernatant transfered to a fresh 1.5ml microcentrifuge tube. 350ul 99% ethanol was added and samples mixed by vortexing before centrifuging for 1-2 seconds at ≤1000 x g. 700ul of the sample was added to the PAXgene™ spin column place in a 2ml processing tube and centrifuge for 1 minute at ≥8000 x g. The PAXgene™ spin column was placed in a new 2ml processing tube. The remaining sample was added to the PAXgene™ spin column and the spin column was centrifuged for 1 minute at ≥8000 x g. The spin column was placed in a new 2ml processing tube. Then 700ul of Buffer BR3 was added to the PAXgene™ spin column and centrifuged for 3 minutes at ≥ 8000 x g. The PAXgene™ spin column was placed into a new processing tube before adding 500ul Buffer BR4 and centrifuging for 1 minute at ≥8000 x g. The Spin column was placed in a new 2ml processing tube and 500ul Buffer BR4 was added before centrifuging for 3 minutes at maximum speed to dry the spin column membrane. The PAXgene™ spin column was placed in a new 2ml processing tube and again centrifuged for 1 minute at full speed. The spin column was placed in a 1.5ml elution tube and 40ul Buffer BR5 was added before centrifuging for 1 minute at ≥8000 x g to elute the RNA. A further 40ul of Buffer BR5 was added and the spin column again centrifuged for 1 minutes at ≥8000 x g. The eluate containing RNA

was incubated for 5 minutes at 65ºC in a heating block then chilled on ice immediately.

2.2.7.3 RNA Quantification

RNA was quantitated using spectrophotometry. A spectrophotometer measures light intensity as a function of wave length. In order to quantitate RNA concentration the absorbance of light with a wave length of 260nm is measured as it passes through the solution containing RNA.

$$A=log_{10}(I_0/I_1)$$

A=Absorbance; I_0=Intensity of incident light; I_1=Intensity of light after passing through material

The concentration is then calculated according to the Beer-Lambert Law:

$A=\alpha lc$ or $c=A/\alpha l$ where: $\alpha=4\pi k/\lambda$

α= absorption coefficient specific to absorbing material; l=path length; c=concentration of absorbing species in material; k=extinction coefficient; λ=wavelength

For RNA the concentration is = Absorption at 260nm times 40ng/ul (assuming a path length of 1cm.)

This way of measuring does not distinguish between DNA and RNA.
To check the RNA concentration of the study samples using spectrophotometer, 4ul of RNA solution was added to 156ul of tris HCL buffer pH=8.0.

All RNA was diluted to 50ng/ul for cDNA synthesis in all experiments.

2.2.7.4 RNA Purity

In order to check for RNA purity the relative absorption at 260nm and 280nm wave length was measured for all samples using spectrophotometry. Maximum absorption for RNA/DNA is at 260nm wave length. Protein is usually measured at 280nm. A

ratio greater than 1.8 indicates little protein contamination. The ideal A260/A280 ratio for RNA samples is regarded as 1.9-2.1.

2.2.7.5 RNA Quality

In order to ensure that the laboratory techniques and storage condition employed did not lead to degradation of RNA a few samples of control RNA (0.75ug) extracted from blood were checked for quality. A number of RNA samples which were known to be of good quality were analysed at the same time (kindly supplied by Jana Kralovicova).

Ribosomal RNA (rRNA) makes up almost 80% of total RNA. The integrity of the major mammalian rRNA species (28S and 18S) can therefore be used to give an indication of RNA quality. The 28S and 18S bands should be clearly visible on an ethidium bromide gel for the RNA to be minimally degraded (276).

Samples were mixed with gel loading buffer (50:50) and denatured by heating to 65°C for 3-5 minutes. The samples were loaded onto the denaturing urea acrylamide gel run at 400V, 40mA, 9W for 1 hour. The gel was then stained in ethidium bromide for 10 minutes before imaging on Typhoon fluorimager. Clear 28S and 18S bands indicate minimal degradation of RNA.

2.2.8 Preferential Allelic Transcription

The study plan was to design an assay to investigate the effect of promoter SNP G-174C on primary transcription minimising inter-individual confounding variables by examining the allelic transcription ratio in heterozygous individuals.

2.2.8.1 Allelic Transcription Assay - Theory

Assuming SNP G-174C affects gene transcription, one would expect one of the alleles to be preferentially expressed in heterozygote individuals (preferential allelic transcription). Using RNA extracted from heterozygous tissue to make cDNA, yields cDNA with equal allelic ratio to the RNA template (Figure 2-6).

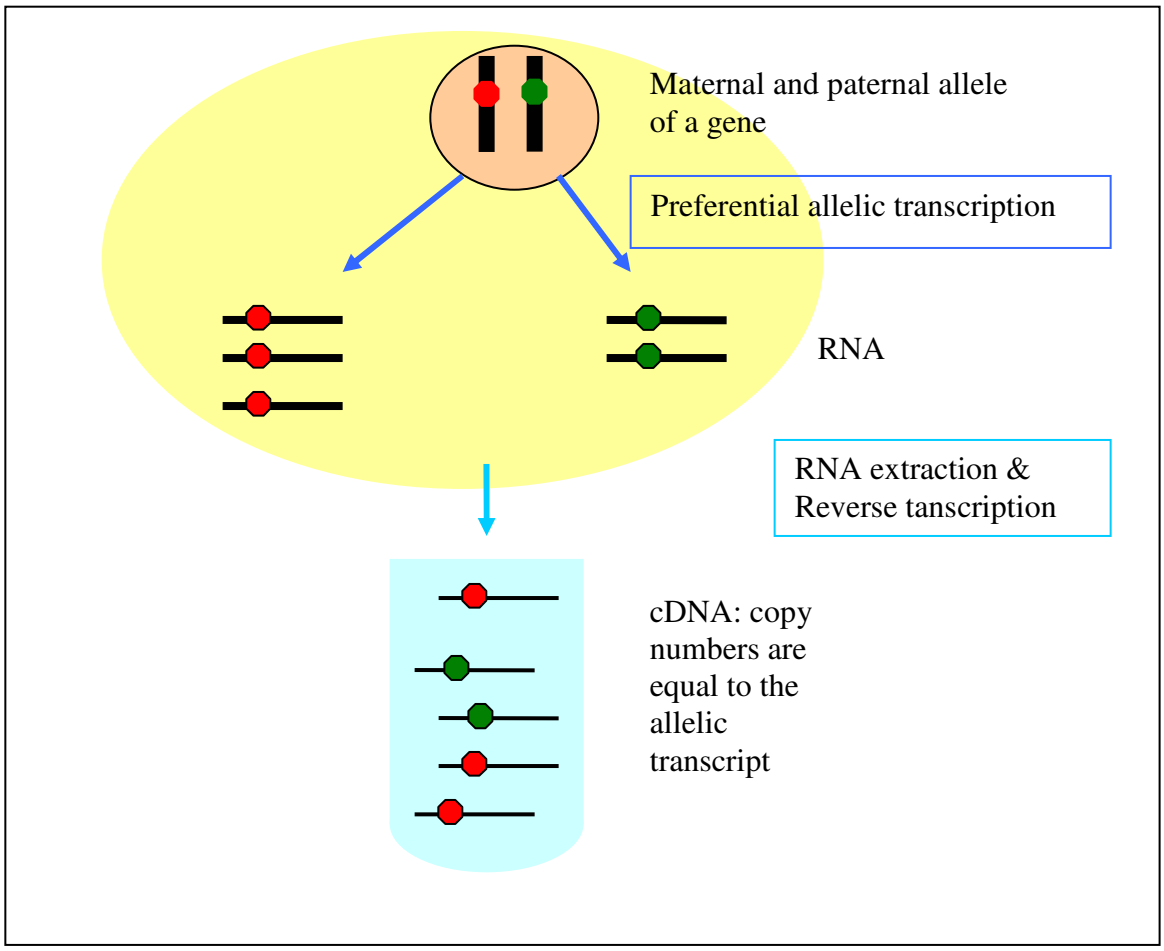


Figure 2-6 : Preferential expression of one allele in a heterozygote individual. RNA extraction from heterozygous tissues and reverse transcription yields cDNA with equivalent allelic ratio to the extracted RNA .

When this cDNA is used as template in PCR amplification, copy number should increase while maintaining the original allelic ratio. Subsequent restriction digest of the PCR product with the appropriate enzyme results in the enzymatic digestion of only one of the two allelic amplicons. Subjecting the digest product to gel electrophoresis allows separation of undigested and digested PCR product (see Figure 2-7). Intensity of fluorescence of individual bands is proportionate to the amount of digest product present. The ratio between cut and uncut bands therefore represents the original allelic RNA ratio.

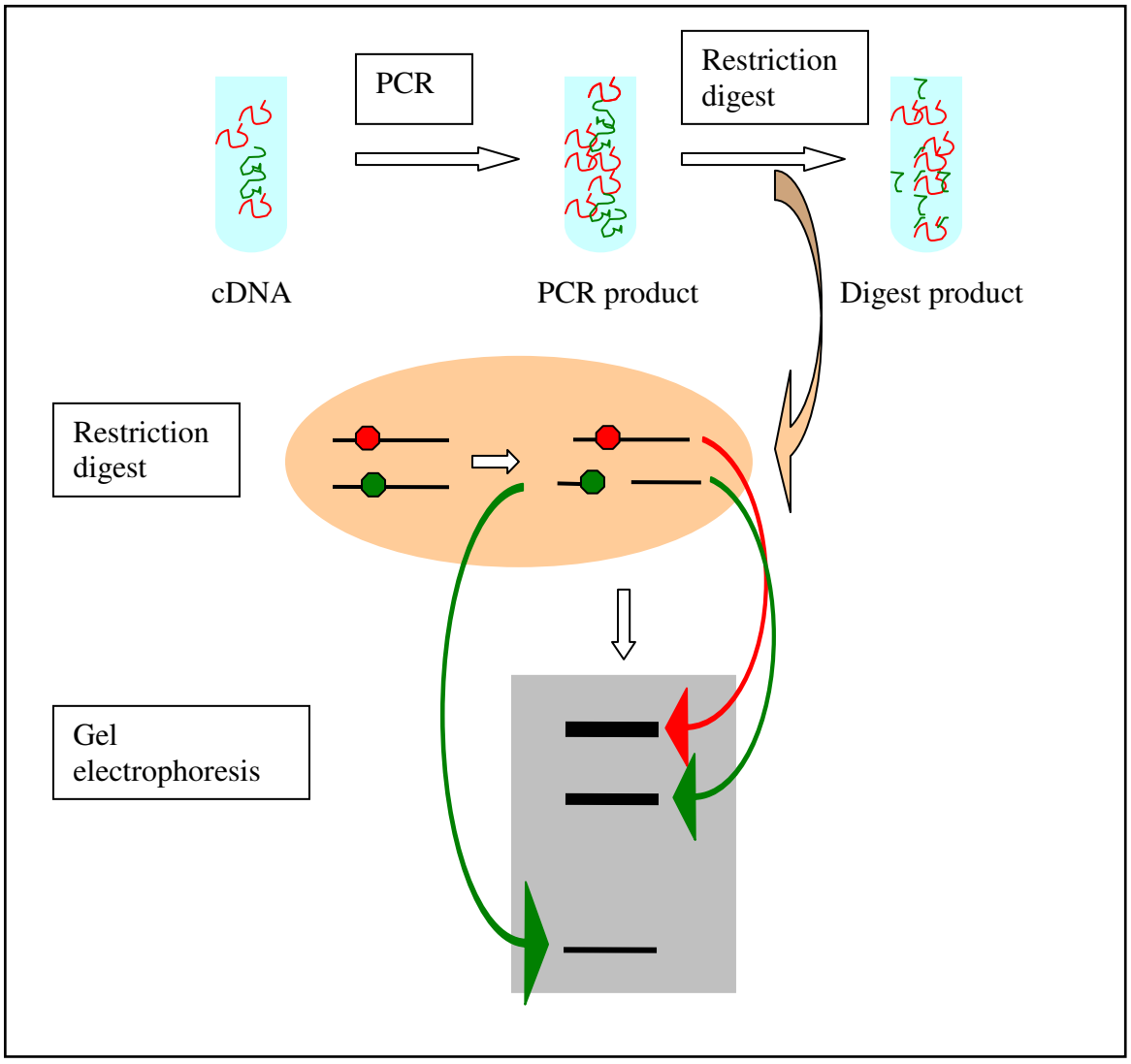


Figure 2-7: Schematic representation of the allelic transcription assay: Restriction digest of PCR amplicons and subsequent separation of digest products on a gel using electrophoresis. The intensity of fluorescence is proportional to the quantity of digest product present. This allows measurement of allelic transcription ratio through measurement of fluorescence intensity.

2.2.8.2 Identifying a Marker for SNP G-174C

To assess allelic expression differences in heterozygotes with reference to a polymorphism, it is necessary to identify from which allele the RNA molecules were originally transcribed. This poses a problem in the case of promoter polymorphisms as the promoter sequence is not transcribed into RNA. To allow allocation of RNA molecules to the relevant allele, a marker has to be identified within the transcribed region of the gene that is co-inherited with the promoter polymorphism.
SeattleSNPs Haplotype Data Phase Output Version 2.0
http://pga.gs.washington.edu/data/il6/ilkn6.phase.out was used to identify possible markers. There were no markers within exon regions but three intron SNPs were identified, which were in linkage disequilibrium with SNP G-174C, one each in intron 1, 2 and 3.
Intron regions are not present in mRNA (Figure 2-8). It was therefore necessary to use primary transcript (hnRNA) as a template in this allelic expression experiment. To assess which of the intron SNPs was the best marker, linkage disequilibrium had to be assessed between individual SNPs by looking at haplotypes present in the population. This data was gained from SeattleSNPs Haplotype Data Phase Output Version 2.0 http://pga.gs.washington.edu/data/il6/ilkn6.phase.out.

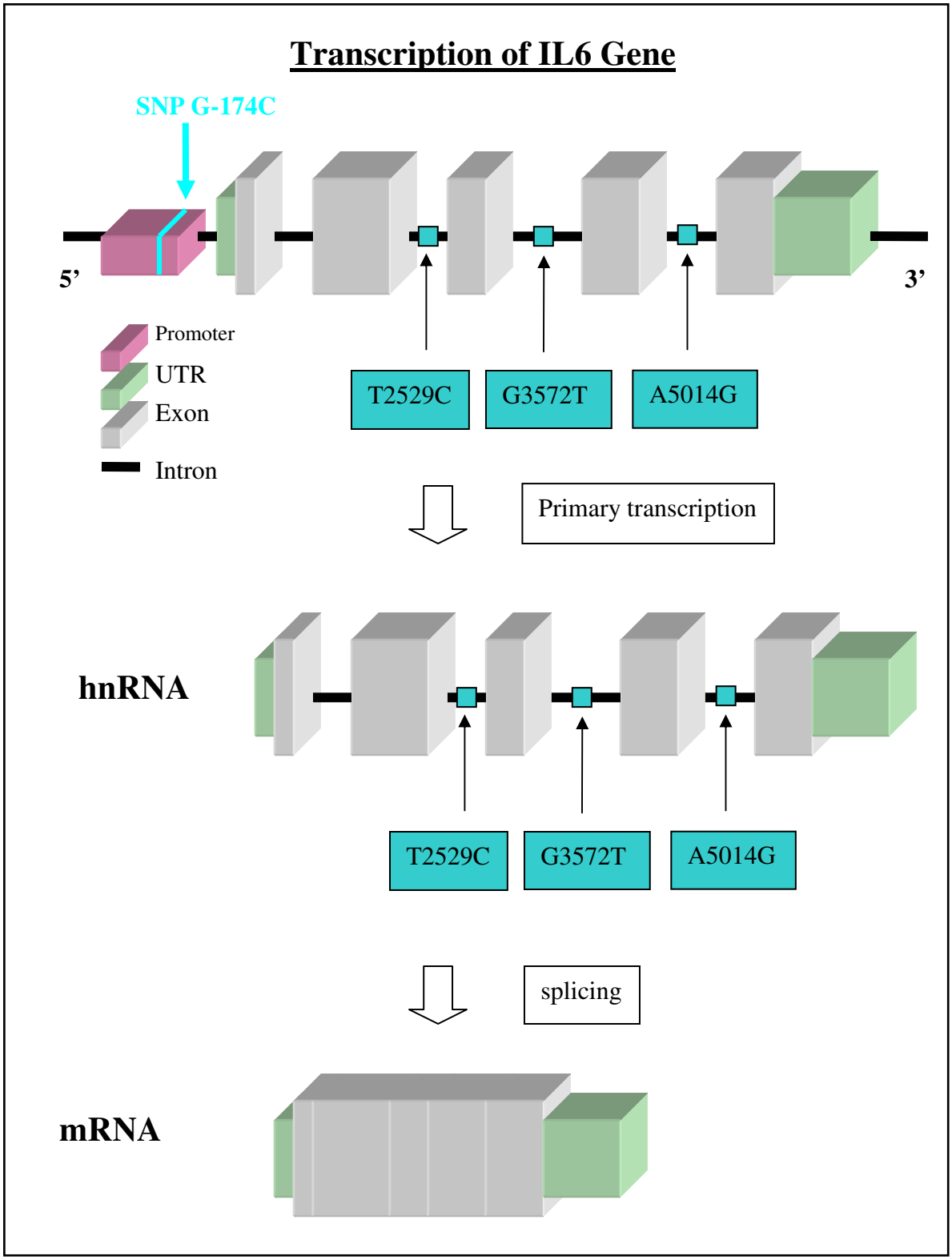


Figure 2-8: IL6 Transcription: Schematic representation of IL6 gene with promoter SNP G-174C and intron SNPs in light blue. Note that promoter region are not transcribed into hnRNA and intron regions are not transcribed into mRNA.

Table 2.2-1: Haplotypes for IL6 promoter SNP and intron SNPs.

SNP	G-174C	T2529G	G3572T	A5014G
Haplotype 1	G	T	G	A
Haplotype 2	C	C	T	G
Haplotype 3	G	T	T	G

SNP G-174C is in perfect linkage disequilibrium (LD) with SNP T2529G i.e. there are only two possible haplotypes GT and CC. SNP G3572T is in perfect LD with SNP A5014G. SNP G-174C is in complete LD with SNP G3572T and A5014G i.e. only three possible haplotypes exist: GGA, CTG and GTG. The ideal marker for the promoter SNP would therefore be T2529G. Markers G3572T and A5014G would also be useful, as the rarer haplotype GTTG could be identified and excluded.

2.2.8.3 DNA Extraction – Allelic Transcription Assay Design

Leukocyte DNA was extracted from blood from volunteers in the laboratory according to method previously described (see section 2.2.5) and used as control DNA, to allow design of the allelic transcription assay.

2.2.8.4 Genotyping SNP G-174C –Allelic Transcription Assay Design

Control DNA from volunteers was genotyped using LightTyper assay (see section 2.2.6)

2.2.8.5 RNA Extraction – Allelic Transcription Assay Design

Control RNA was extracted from the blood of volunteers in the laboratory, found to be heterozygous for SNP G-174C, according to the method previously described (see section 2.2.7.2). For all optimization steps, control RNA from leukocytes was used. To optimise DNase treatment one sample of vein cDNA (study sample) and placenta cDNA (RNA was extracted from placenta tissue samples available in the laboratory) was also used.

2.2.8.6 Assay Design for Intron SNPs

2.2.8.6.1 Primer Design

PCR primers were designed to amplify the region surrounding the intron SNP using Primer3 software http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi (see Sequences 2). To avoid cross homology with other regions of the genome, primers were 'BLASTed' using software provided by the national centre for biotechnology information (NCBI) http://www.ncbi.nlm.nih.gov/BLAST/ . Amplification products were 163bp, 249bp and 262bp in length for SNP 2529, 3572 and 5014 respectively.

Primer for SNP:	**Sequence of forward (F) and reverse (R) primers:**	
T2529C	F	5' - ctgaaaggcatgttcagcttc -3'
	R	5' – atctccccactttcaatgca – 3'
G3572T	F	5' - ggcagtttattcttggacagg – 3'
	R	5' – ataaaaggaggttccagccc -3'
G3572T new	F	5' – ggcagtttattcttggacagg – 3'
	R	5' –caatgtcccaaaacatgctg – 3'
A5014G	F	5' – ggtgggtctatggaaaggtg - 3
	R	5' – caaacaaataagatagtgatgctgg – 3'

Sequences 2: Sequence of primers used in amplification of intron regions surrounding SNPs. The forward and reverse primers in the right hand column anneal in areas adjacent to the SNP given in the left hand column, thus leading to amplification of the sequence surrounding the SNP.

The sequence required for this experiment was downloaded from GenBank, accession number: AF 372214. It became apparent during optimisation that restriction digest of the amplification product of the region surrounding SNP G3572T lead to restriction fragments of similar length (118 and 131 bp), thus difficult to distinguish on gel electrophoresis. New primers were designed for SNP G3572T to give an amplification product of 418bp (restriction fragment length 119 and 299 bp)

2.2.8.6.2 Restriction Enzymes

Restriction enzymes for the three intron SNPs were chosen using NEBcutter software.

Restriction enzymes were Mae I, Dde I, Msp I for SNP 2529, 3572, 5014 respectively. Optimum enzyme concentration was determined using 1.0, 2.0, 3.0, 4.0ul of enzyme in a 20ul reaction.

2.2.8.6.3 PCR Amplification and Restriction Digest of Intron Regions

PCR Amplification and Restriction Digest for SNP T2529C

2ul of genomic control DNA (concentration: 7-10ng/ul) was taken as a template for PCR and dried at 80ºC for 10 minutes on a 96 well plate before loading the PCR mixture. For the final experiment 3ul cDNA was used which was un-dried. Optimisation of the reaction had previously been performed using magnesium concentrations of 1.5, 2.0 and 2.5mM and a range of temperatures form 55ºC to 67ºC. The optimum conditions were 1.5mM magnesium and 57ºC for the annealing temperature. The concentration of the reagents used in the PCR reaction to amplify the intron regions are given in the box below:

PCR Reagents	**Amount**
10x PCR Buffer	2ul
5M Betaine	5.2ul
5mM dNTPs	0.5ul
25mM Mg^{2+}	1.2ul
Primers Forward	0.2ul
Reverse	0.2ul
Promega Taq	0.2ul
H_2O	10.5ul
Total PCR mix	**20ul**

The timing of the PCR program used for amplification of the intron regions are set out below:

PCR cycling program:	
Initial denaturation step	92ºC for 2 minutes
Denaturation step	92ºC for 30 seconds
Primer annealing step	57ºC for 30 seconds
Extension step	70ºC for 30 seconds
	go to step 2 and repeat cycles 29 times
Final extension step	70ºC for 2 minutes

10ul of PCR product was loaded onto a 96 well plate and 1unit of restriction enzyme MaeI was added. Samples were subjected to digestion at 45ºC for 16 hours.

3ul of PCR product was mixed with loading dye and loaded onto a 5% polyacrylamide MADGE (microplate array diagonal gel electrophoresis) gel, which had been pre-stained with ethidium bromide. Electrophoresis was performed at 150 volts(V), 2 amps for 14 minutes in a dry box.

PCR Amplification and Restriction Digest for SNP G3572T

Apart from the primers used in the PCR reaction (see Sequences 2), a magnesium concentration of 2.5mM and 62.2ºC annealing temperature, the PCR reaction for the amplification of the intron region surrounding SNP G3572T was performed as for SNP T2529C. The restriction digest for genotyping was performed as for SNP T2529C except for the use of restriction enzyme DdeI and a digest temperature of 37ºC.

PCR Amplification and Restriction Digest for SNP A5014G

Apart from the primers used in the PCR reaction (see Sequences 2), a magnesium concentration of 2.0mM and 62.2ºC annealing temperature, the PCR reaction for the amplification of the intron region surrounding SNP A5014G was performed as for SNP T2529C. The restriction digest for genotyping was performed as for SNP T2529C except for the use of restriction enzyme MspI and a digest temperature of 37ºC.

2.2.8.7 cDNA Synthesis

cDNA synthesis was performed by reverse transcription of RNA. The majority of IL6 RNA is mRNA, with hnRNA representing only a fraction of the total. The majority of cDNA therefore does not contain intron regions (see Figure 2-9). This can be used to confirm the presence of cDNA as a 'short product' following PCR, using primers spanning intron regions. The length of the product allows differentiation from

amplification of gDNA contamination, often present in RNA samples (see Figure 2-10). cDNA from hnRNA can not be differentiated from gDNA contamination in this way as both contain intron regions.

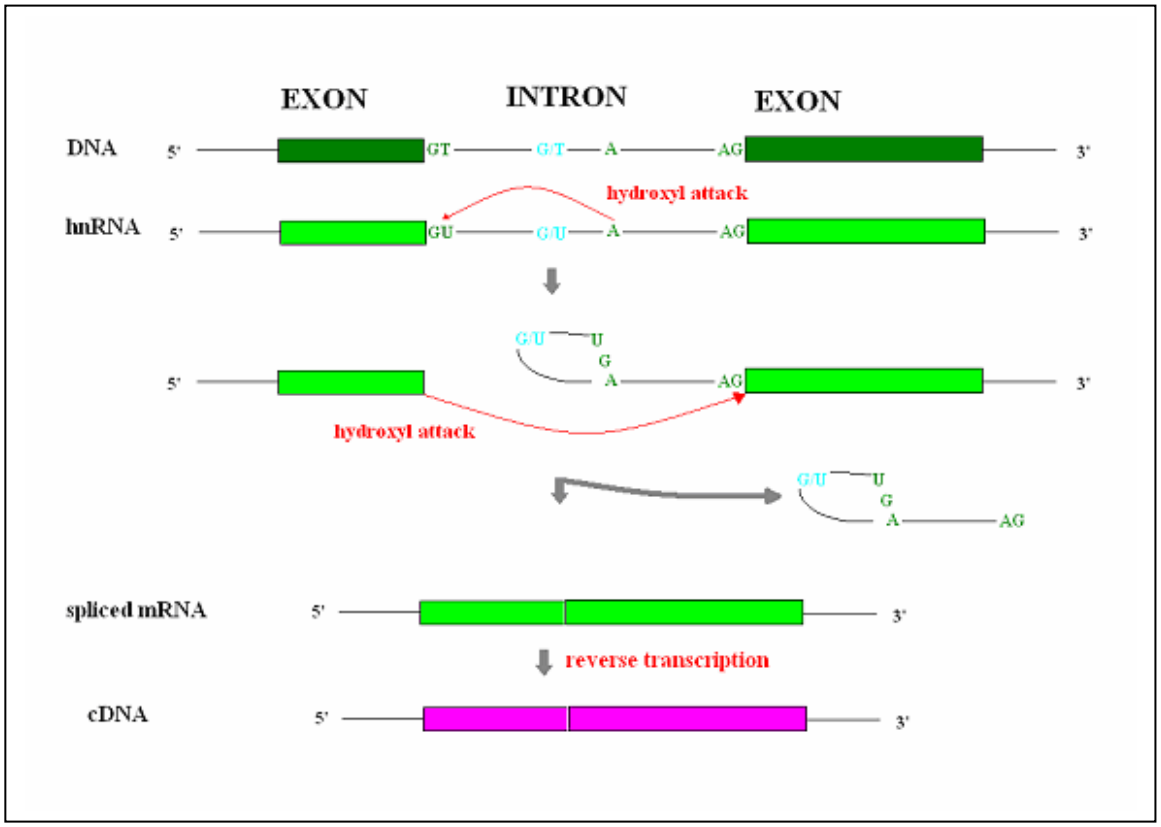


Figure 2-9: Transcription of gDNA into hnRNA, the splicing of hnRNA into mRNA and reverse transcription of mRNA into cDNA: The 5' end of the intron is autolytically cleaved to form a lariat. The lariat is an intermediate circular structure which is formed by connecting the 5' terminus (UG) to a base (A) within the intron. Subsequent cleavage of the 3' site releases the intron in lariat form. Concurrently the right exon is ligated (spliced) to the left exon resulting in mRNA. mRNA does not contain intron regions. cDNA transcribed from mRNA in turn does not contain intron regions.

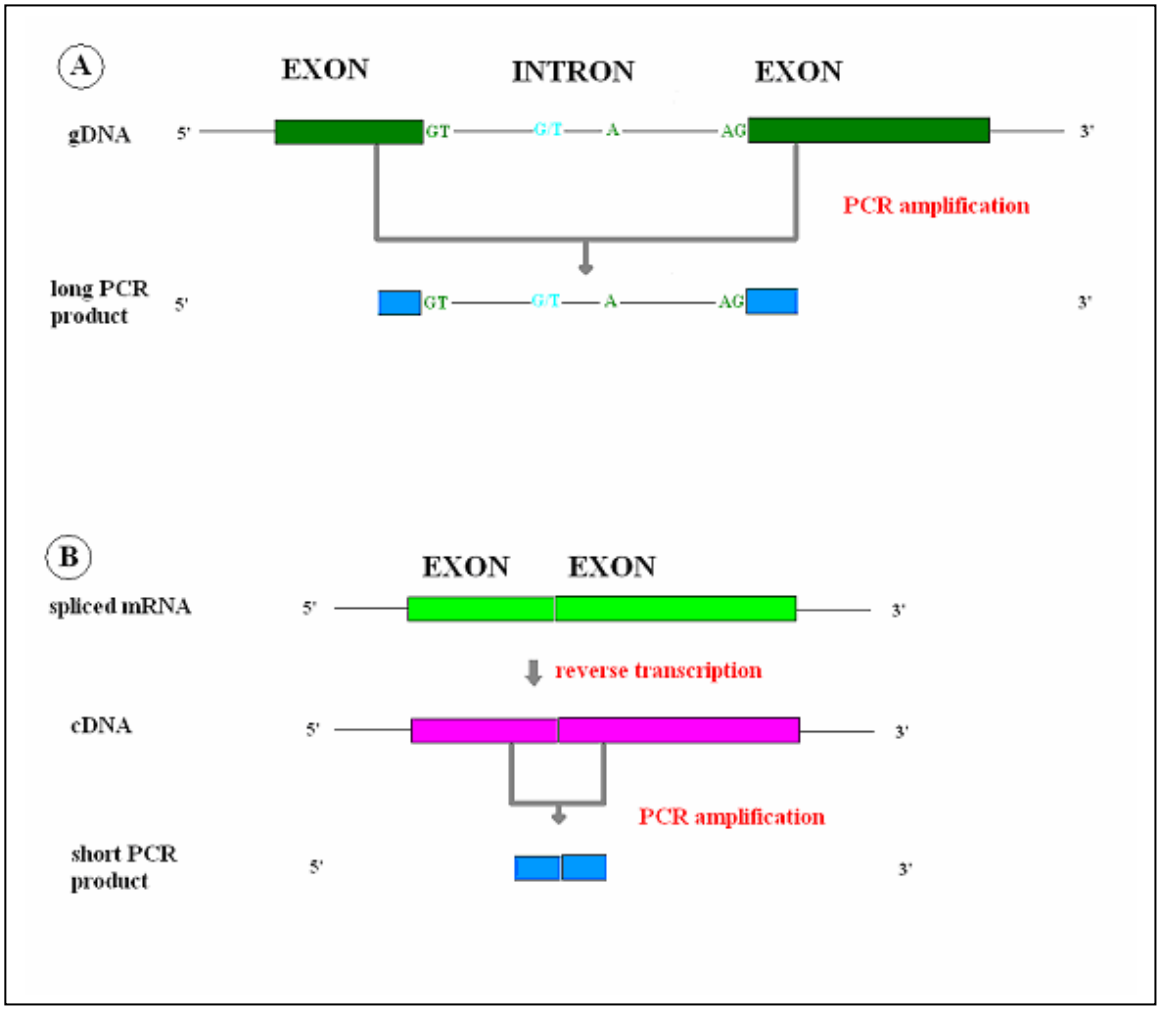


Figure 2-10: PCR products of a gDNA template (A) and cDNA template (B). The presence of intron regions (containing splice sites and SNP – in this case G/T) allows differentiation between the two products by length in gel electrophoresis.

In the optimisation phase oligo(dT) primer was used to synthesis cDNA. IL6 cDNA could be demonstrated on a gel, by showing a short product amplified using primers spanning intron 3 (Ex3/In3/Ex4 Primer see Sequences 3 & Sequences 4). However bands were faint and thus not useful for gel based quantitation. hnRNA exists before polyadenylation and it is not entirely clear, whether polyadenylation is complete before the splicing process begins, thus oligo(dT) primer may not be useful in amplifying hnRNA, therefore specific primers were used to improve signal strength (see Sequences 3 & Sequences 4).

Primer pair spanning intron 3:

Ex3/In3/Ex4	F	5′- gaaagcagcaaagaggcact – 3′
	R	5′ – agctctggcttgttcctcac -3′

Reverse primer for reverse transcription annealing in exon 4:

RevPrRtEx 4	5′ – tttctgcaggaactggatca – 3′

Sequences 3: Ex3/In3/Ex4 Primer used to detect presents of cDNA and Primers used for reverse transcription of RNA.

agtggtgtttgttttagggacacttaggtgataacaattctggtattctttcccagacat
gtaacaagagtaacatgtgtgaaagcaggcaaagaggcactggcagaaaacaacctgaacc
ttccaaagatggctgaaaaagatggatgcttccaatctggattcaatgaggtaccaactt
gtcgcactcacttttcactattccttaggcaaaacttctccctcttgcatgcagtgcctg
tatacatatagatccaggcagcaacaaaaagtgggtaaatgtaaagaatgttatgtaaat
ttcatgaggaggccaacttcaagctttttaaaggcagtttattcttggacaggtatggc
cagagatggtgccactgtggtgagattttaacaactgtcaaatgtttaaaactcccacag
gtttaattagttcatcctgggaaaggtactcggcagggccttttccctctctggctgcccc
tggcagggtccaggtctgccctccctccctgcccagctcattctccacagtgagataacc
tgcactgtcttctgattatttataaaaggaggttccagcccagcattaacaagggcaag
agtgcaggaagaacatcaaggggacaatcagagaaggatccccattgccacattctagc
atctgttgggctttggataaaactaattacatggggcctctgattgtccagttatttaaa
atggtgctgtccaatgtcccaaaacatgctgcctaagaggtacttgaagttctctagagg
agcagagggaaaagatgtcgaactgtggcaattttaacttttcaaattgattctatctcc
tggcgataaccaatttcccaccatctttcctcttaggagacttgcctggtgaaaatcat
cactggtcttttggagtttgaggtatacctagagtacctccagaacagatttgagagtag
tgaggaacaagccagagctgtgcagatgagtacaaaagtcctgatccagttcctgcagaa
aaaggtgggtgtgtcctcattccctcaacttggtgtgggggaagacaggctcaaagacag

Sequences 4: Sequence of exon 3 and 4 (highlighted in pink) and intron 3 containing SNP G3572T (green writing on green highlight): The primer for reverse transcription from exon 4 (grey) and the primer pair for amplification of short and long cDNA (light green - Ex3/In3/Ex4 Primer) transcription product can be seen.

For cDNA synthesis RNA was diluted in RNase free water to a concentration of 50ng/ul. 10ul of this dilution i.e. 500ng of RNA was used in the reverse transcription reaction. 500ng of primer was added to the RNA and both were heated in a microcentrifuge tube to 70°C for 5 minutes before cooling on ice.

The following was added in this order:

Mastermix for cDNA synthesis	
M-MLV RT 5x Reaction Buffer	5ul
dNTP 10nm	1.25ul
Moloney murine leukaemia virus reverse transcriptase RNase H- (M-MLV RT H-)	1ul (200 units)
Nuclease free water	6.75ul

The reaction was incubated at 53°C for 60 minutes and then inactivated for 15 minutes at 70°C.

2.2.8.8 Dealing with Genomic DNA contamination

RNA samples which have been extracted from tissue often contain a significant amount of genomic DNA contamination. Assays measuring mRNA expression can differentiate between cDNA and gDNA because the mRNA sequence differs from that of gDNA due to the loss of introns during the splicing process. However hnRNA, used as template in this experiment, is an exact copy of the genomic DNA i.e. it still contains the intron regions. Contaminating gDNA would therefore be co-amplified with hnRNA in any PCR, thus influencing the results of hnRNA quantification. gDNA content of the RNA samples therefore had to be kept to a minimum. In order to check for genomic DNA (gDNA) contamination an assay was used that amplified the promoter region of an unrelated gene *low density lipoprotein receptor (LDLR)*. Promoter regions are not transcribed into hnRNA and therefore allow differentiation between gDNA contamination and hnRNA. The LDLR assay was performed on cDNA from untreated RNA, RNA which had received one standard DNase treatment and from RNA treated twice with DNase (rigorous DNase treatment). Genomic DNA of known concentration was also subject to the same protocol to assess how much DNA the DNase process was able to digest.

2.2.8.8.1 Standard DNase Treatment

This treatment will remove up to 2ug of genomic DNA from total RNA in a 50ul reaction according to the manufacturer manual. This treatment was used on 20ul of RNA and 30ul of gDNA (7-10ng/ul). In the first step 1/10 of the total volume of 10x

DNase I buffer and 1ul of rDNase was added to the sample which was then incubated for 30 minutes at 37ºC. The rDNase was inactivated by adding 0.1 x volume of DNase inactivation reagent to the sample before incubation at room temperature for 2 minutes. The sample was centrifuged at 10,000 x g for 1.5 minutes and the supernatant (RNA) was carefully transfered to a fresh tube.

2.2.8.8.2 Rigorous DNase Treatment

This was used to treat 20ul of RNA and 30ul of gDNA (7-10ng/ul). In the first step 1ul of rDNase was added to the sample which was then incubated for 30 minutes at 37ºC. A further 1ul of rDNase was added and the sample incubated for a further 30 minutes at 37ºC. The rDNase was inactivated by adding 0.1 x volume of DNase Inactivation Reagent and incubating at room temperature for 2 minutes. The sample was then centrifuged at 10,000 x g for 1.5 minutes and the supernatant (RNA) was carefully transfer to a fresh tube. A second cycle of DNase treatment followed using 2ul of rDNase added to the reaction mix before incubation for 30 minutes at 37ºC. Inactivation of rDNase was carried out as before.

In the allelic transcription assay for vein, leukocytes and adipose tissue rigorous DNase treatment with DNA-free™ was performed on all samples prior to quantification by spectrophotometry.

2.2.8.8.3 LDLR Promoter Assay

Primers previously designed in the laboratory (by Xiao-he Chen) were used for amplification of the promoter region of the low density lipoprotein receptor gene (*LDLR*) (see Sequences 5). PCR conditions had previously been optimised.

Primers for amplification of promoter region of *LDLR*

LDLR-Promoter	F	5’-aggactggagtgggaatcagagc-3'
	R	5'- tgctgtgtcctagctggaaaccc -3

Sequences 5: Sequence of the forward (F) and reverse (R) primers used in amplification of the promoter region of the *LDLR* gene.

A 96 well plate was used. 3ul of cDNA was added to the well (not dried). Below are the reagents used in the PCR reaction amplifying the promoter region of the *LDLR* gene:

PCR Reagents	**20ul Reaction**
PCR Buffer	2ul
dNTP (25mM)	0.16ul
$MgCl_2$ (25mM) 2ul	2.5mM
Oligos (100pM/ul)	0.08ul + 0.08ul
Taq DNA Polymerase	0.04ul
H_2O	15.64ul

20ul of the PCR mixture was added to each well, the plate was sealed with a rubber mat and placed on a thermal cycler. The timings of the PCR program used are set out below:

PCR cycling program:	
Initial denaturation step	95ºC for 3 minutes
Denaturation step	94ºC for 1 minutes
Primer annealing step	68ºC for 40 seconds
Extension step	72ºC for 1 minutes
	go to step 2 and repeat cycles 35 times
Final extension step	72ºC for 10 minutes

3ul of PCR product was mixed with loading dye and loaded onto a 5% polyacrylamide MADGE gel. Electrophoresis was performed at 150V for 12 minutes in a dry box and the gel stained with vistra green. Imaging was performed using *Fluorimager*: Model 595, Molecular Dynamics, Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK and *TyphoonTRIO+*: variable Mode Imager, Amersham Biosciences UK Ltd.

2.2.8.9 Detection of Heteronuclear RNA using Gel based Method

2.2.8.9.1 Amplification of Intron Region

In order for the experiment to proceed the presence of IL6 hnRNA in the RNA samples had to be demonstrated. Concentration of hnRNA needed to be sufficient for

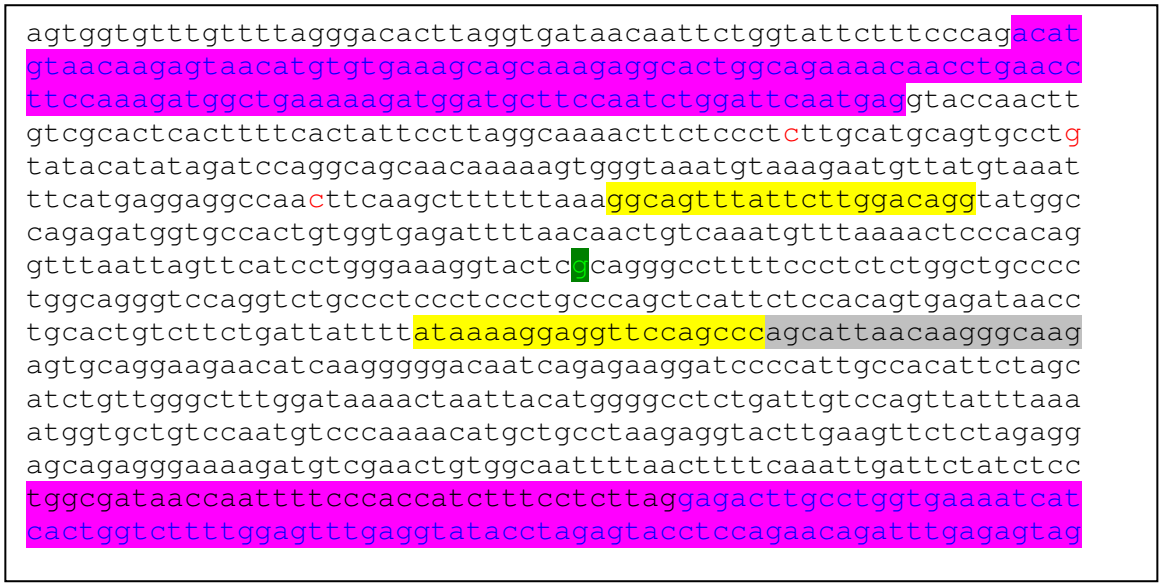
its PCR product to be visible on a gel. The control leukocyte RNA samples used, had been demonstrated to be free of gDNA contamination. Two types of cDNA were synthesised using specific primers, one annealing in intron 2 and one annealing in intron 3 (see Sequences 6).

Specific Primers for cDNA synthesis annealing in intron regions:	
Reverse Primer for RT Intron2	5’ - agtgtgggctcccaatctc - 3’
Reverse Primer for RT Intron3	5’- cttgcccttgttaatgct – 3’

Sequences 6: Specific primers for reverse transcription, annealing in intron regions.

Primers annealing in introns were used in order to ensure cDNA was transcribed from hnRNA only and not from mRNA. cDNA was used as a template for PCR for the region surrounding SNP T2529G in intron 2 and G3572T in intron 3 (see section 2.2.8.6, Sequences 7 and Sequences 8).

Sequences 7: Sequence for intron 2 of *IL6*: Exons - pink, SNP T2529G used as marker for promoter SNP G-174C – red on green, forward and reverse primer for PCR – yellow, reverse primer for cDNA synthesis - grey



Sequences 8: Sequence for intron 3 of *IL6*: Exons - pink, SNP G3572T used as marker for promoter SNP G-174C - green, forward and reverse primer for PCR – yellow, reverse primer for cDNA synthesis – grey.

2.2.8.10 Excluding Amplification from Un-degraded Splice Products

Intron splicing is followed by de-branching and degradation of the lariat (see above). It is unclear how quickly the splice product is degraded. It is therefore possible for un-degraded splice products to contaminate the reaction. To ensure PCR amplification arose from intact hnRNA and not from an un-degraded splice fragment, continuity of the amplified intron 3 region with adjacent exon regions had to be demonstrated. A further set of primers was therefore designed (see Sequences 9 & Sequences 10).

Primer for assay checking continuity of amplified intron template with adjacent exon:

Intron3/Exon3 continuity	Forward	5′ – gaaagcagcaaagaggcact - 3′
	Reverse	5′ – gcagccagagagggaaaag – 3′

Sequences 9: Sequence of a primer pair spanning the intron 3/exon 3 boundary. A PCR using this primer pair only amplifies templates with intact intron 3/exon 3 boundaries i.e. genomic DNA or hnRNA but not un-degraded splice products.

agtggtgtttgttttagggacacttaggtgataacaattctggtattctttcccagacat
gtaacaagagtaacatgtgtgaaagcagcaaagaggcactggcagaaaacaacctgaacc
ttccaaagatggctgaaaaagatggatgcttccaatctggattcaatgaggtaccaactt
gtcgcactcacttttcactattccttaggcaaaacttctccctcttgcatgcagtgcctg
tatacatatagatccaggcagcaacaaaaagtgggtaaatgtaaagaatgttatgtaaat
ttcatgaggaggccaacttcaagcttttttaaaggcagtttattcttggacaggtatggc
cagagatggtgccactgtggtgagattttaacaactgtcaaatgtttaaaactcccacag
gtttaattagttcatcctgggaaaggtactcgcagggcctttccctctctggctgcccc
tggcagggtccaggtctgccctccctccctgcccagctcattctccacagtgagataacc
tgcactgtcttctgattattttataaaaggaggttccagcccagcattaacaagggcaag
agtgcaggaagaacatcaaggggacaatcagagaaggatccccattgccacattctagc
atctgttgggctttggataaaactaattacatggggcctctgattgtccagttatttaaa
atggtgctgtccaatgtcccaaaacatgctgcctaagaggtacttgaagttctctagagg
agcagagggaaaagatgtcgaactgtggcaattttaacttttcaaattgattctatctcc
tggcgataaccaatttcccaccatctttcctcttaggagacttgcctggtgaaaatcat
cactggtcttttggagtttgaggtatacctagagtacctccagaacagatttgagagtag

Sequences 10: Sequence for intron 3 of *IL6*: Exons - pink, SNP G3572T used as marker for promoter SNP G-174C - green, primer for reverse transcribtion reaction - grey, forward and reverse primer for PCR - yellow

The forward primer was placed in exon 3 and the reverse primer just downstream of SNP G3572T i.e. amplicon spanned exon 3/intron 3 boundary. Optimisation of the

PCR reaction was performed using magnesium concentrations of 1.5, 2.0 and 2.5mM and a range of temperatures form 53ºC to 63ºC. Control DNA was used as a template in the optimisation.

3ul Leukocyte cDNA was used for PCR amplification. Apart from the primers Magnesium concentration of 2.0mM, 56ºC annealing temperature the PCR reaction conditions were as for T2529C.

2.2.8.11 Ensuring Amplification from Intact hnRNA

Findings of the above experiment (chapter 3) suggested, amplification could occur from un-degraded splice products. Design of the assay was therefore changed to ensure amplification occurred only from intact hnRNA. cDNA was synthesised using a reverse primer annealing in exon 4. A long PCR assay was designed for amplification of intron region three using a forward and a reverse primers placed in the adjacent exons 3 and 4 (spanning intron 3; product size=899bp - Ex3/In3/Ex4 primers) (Sequences 11).

Reverse Primer for cDNA synthesis

Reverse Primer for RT Exon4 5' - tttctgcaggaactggatca - 3'

Primer pair for long PCR amplifying region spanning intron 3

Ex3/In3/Ex4	Forward	5'- gaaagcagcaaagaggcact – 3'
	Reverse	5' – agctctggcttgttcctcac -3'

Sequences 11: Sequence of the reverse primer used in the cDNA synthesis from RNA. This reverse primer anneals in exon 4. Using the synthesised cDNA as a template in a subsequent PCR with the forward and reverse primer shown, allowed amplifying intron region 3.

Primers annealing in adjacent exons ensured amplification occurred from an intact sequence and not from an un-degraded splice product (see Sequences 12). This experiment allowed the demonstration of presence of mRNA derived cDNA as the amplification of this cDNA would result in a short product, distinguishable from the long amplicon of hnRNA. The former consists of transcript from the exon sequence only, whereas the later is transcribed from exon and intron regions.

This experiment was optimised using magnesium concentrations of 1.5mM, 2.0mM and 2.5mM and a range of temperatures form 54ºC to 64ºC.

agtggtgtttgttttagggacacttaggtgataacaattctggtattctttcccagacat
gtaacaagagtaacatgtgtgaaagcagcaaagaggcactggcagaaaacaacctgaacc
ttccaaagatggctgaaaaagatggatgcttccaatctggattcaatgaggtaccaactt
gtcgcactcacttttcactattccttaggcaaaacttctccctcttgcatgcagtgcctg
tatacatatagatccaggcagcaacaaaaagtgggtaaatgtaaagaatgttatgtaaat
ttcatgaggaggccaacttcaagctttttaaaggcagtttattcttggacaggtatggc
cagagatggtgccactgtggtgagattttaacaactgtcaaatgtttaaaactcccacag
gtttaattagttcatcctgggaaaggtactcgcagggccttttccctctctggctgcccc
tggcagggtccaggtctgccctccctccctgcccagctcattctccacagtgagataacc
tgcactgtcttctgattatttataaaaggaggttccagcccagcattaacaagggcaag
agtgcaggaagaacatcaaggggacaatcagagaaggatccccattgccacattctagc
atctgttgggctttggataaaactaattacatggggcctctgattgtccagttatttaaa
atggtgctgtccaatgtcccaaaacatgctgcctaagaggtacttgaagttctctagagg
agcagagggaaaagatgtcgaactgtggcaattttaacttttcaaattgattctatctcc
tggcgataaccaattttcccaccatctttcctcttaggagacttgcctggtgaaaatcat
cactggtcttttggagtttgaggtatacctagagtacctccagaacagatttgagagtag
tgaggaacaagccagagctgtgcagatgagtacaaaagtcctgatccagttcctgcagaa
aaaggtgggtgtgtcctcattccctcaacttggtgtgggggaagacaggctcaaagacag

Sequences 12: Sequence for intron 3 of IL6: Exons - pink, reverse primer for RT - grey, forward and reverse primer for amplification of intro 3 region -light green (Ex3/In3/Ex4 Primers). Amplification of intact hnRNA using the light green primers will result in an 899bp amplicon that can only arise from intact hnRNA or gDNA.

Primer optimisation for long PCR was performed using control DNA as the template. Amplification with long PCR was performed using 3ul cDNA un-dried on a 96 well plate to which 20ul of PCR master mix was added. Below are the reagents used in the PCR reaction:

PCR Reagents	**20ul Reaction**	**Final Concentration**
Long PCR Buffer	2ul	(1xPCR Buffer)
5M Betaine	5.2ul	(1.3M)
10mM dNTPs	0.5ul	
25mM Mg^{2+}	1.6ul	(2mM)
100pmol/ul Primers		
Forward	0.08ul	
Reverse	0.08ul	
Promega Taq	0.2ul	
1/250 PWO	0.4ul	
H_2O	9.94ul	

The timings of the PCR program used are set out below:

PCR cycling program:	
Initial denaturation step	94ºC for 2 minutes
Denaturation step	94ºC for 30 seconds
Primer annealing step	60ºC for 30 seconds for Ex3/In3/Ex4 primers
Extension step	68ºC for 1 minute
	go to step 2 and repeat cycles 35 times
Final extension step	68ºC for 20 minutes

The PCR product was loaded onto a 1% agarose gel which had been pre-stained with vistra green and run at 120 Volts in 1% 1xTBE buffer, with a vistra green concentration of 1:20000, for 30 minutes. Imaging was performed using *Fluorimager*: Model 595, Molecular Dynamics, Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK and *TyphoonTRIO+*: variable Mode Imager, Amersham Biosciences UK Ltd.

2.2.8.12 Nested PCR and Restriction Digest

Results from the previous experiment showed that hnRNA concentration was to low to give adequate bands for gel based quantification. PCR yield had to be improved and therefore a nested PCR was designed. Cycle numbers were optimised to ensure adequate band intensity. The first PCR used primer pair spanning intron 3, with the

forward primer annealing in exon 3 and the reverse primer annealing in exon 4 (Ex3/In3/Ex4 Primer pair Sequences 13 & Sequences 14).

Primer pair for long PCR amplifying region spanning intron 3		
Ex3/In3/Ex4	F	5'- gaaagcagcaaagaggcact – 3'
	R	5' – agctctggcttgttcctcac -3'

Sequences 13: Sequences of primer pair spanning intron 3.

agtggtgtttgttttagggacacttaggtgataacaattctggtattctttcccagacat
gtaacaagagtaacatgtgtgaaagcagcaaagaggcactggcagaaaacaacctgaacc
ttccaaagatggctgaaaaagatggatgcttccaatctggattcaatgaggtaccaactt
gtcgcactcacttttcactattccttaggcaaaacttctccctcttgcatgcagtgcctg
tatacatatagatccaggcagcaacaaaaagtgggtaaatgtaaagaatgttatgtaaat
ttcatgaggaggccaacttcaagctttttaaaggcagtttattcttggacaggtatggc
cagagatggtgccactgtggtgagattttaacaactgtcaaatgtttaaaactcccacag
gtttaattagttcatcctgggaaaggtactcgcagggccttttccctctctggctgcccc
tggcagggtccaggtctgccctccctccctgcccagctcattctccacagtgagataacc
tgcactgtcttctgattattttataaaaggaggttccagcccagcattaacaagggcaag
agtgcaggaagaacatcaaggggacaatcagagaaggatccccattgccacattctagc
atctgttgggctttggataaaactaattacatggggcctctgattgtccagttatttaaa
atggtgctgtccaatgtcccaaaacatgctgcctaagaggtacttgaagttctctagagg
agcagagggaaaagatgtcgaactgtggcaattttaacttttcaaattgattctatctcc
tggcgataaccaatttcccaccatctttcctcttaggagacttgcctggtgaaaatcat
cactggtcttttggagtttgaggtatacctagagtacctccagaacagatttgagagtag
tgaggaacaagccagagctgtgcagatgagtacaaaagtcctgatccagttcctgcagaa
aaaggtgggtgtgtcctcattccctcaacttggtgtgggggaagacaggctcaaagacag

Sequences 14: Sequence for intron 3 of IL6: Exons - pink, forward and reverse primer for amplification of intro 3 region -light green (Ex3/In3/Ex4 Primer pair). Amplification of intact hnRNA using the light green primers will result in an 899bp amplicon that can only arise from intact hnRNA or gDNA.

Amplification with long PCR was performed using 3ul cDNA un-dried on a 96well plate to which 20ul of PCR master mix was added. Below are the reagents used in the PCR reaction:

PCR Reagents	20ul Reaction
Long PCR Buffer	2ul
5M Betaine	5.2ul
10mM dNTPs	0.5ul
25mM Mg^{2+}	1.6ul
100pmol/ul Primers	
Forward	0.08ul
Reverse	0.08ul
Promega Taq	0.2ul
1/250 PWO	0.4ul
H_2O	9.94ul

The timings of the PCR program used are set out below:

PCR cycling program:	
Initial denaturation step	94ºC for 2 minutes
Denaturation step	94ºC for 30 seconds
Primer annealing step	60ºC for 30 seconds for Ex3/In3/Ex4 primer
Extension step	68ºC for 1 minute
	go to step 2 and repeat 30 cycles
Final extension step	68ºC for 20 minutes

The PCR product from this reaction was diluted 1 in 1000 (dilution was optimised using dilutions 1:100; 1:1000; 1:10.000; 1:100.000 and 1:1.000.000) and 2ul used as a template in the second PCR reaction in which the region surrounding SNP G3572T in intron 3 was amplified (Sequences 15 & Sequences 16).

Amplification of intron 3 region: Sequence of primers:		
G3572T new	Forward	5′ – ggcagtttattcttggacagg – 3′
	Reverse	5′ –caatgtcccaaaacatgctg – 3′

Sequences 15: Sequence of primer pair used in PCR reaction amplifying the region surrounding intron SNP G3572T.

agtggtgtttgttttagggacacttaggtgataacaattctggtattctttcccagacat
gtaacaagagtaacatgtgtgaaagcagcaaagaggcactggcagaaaacaacctgaacc
ttccaaagatggctgaaaaagatggatgcttccaatctggattcaatgaggtaccaactt
gtcgcactcacttttcactattccttaggcaaaacttctccctcttgcatgcagtgcctg
tatacatatagatccaggcagcaacaaaaagtgggtaaatgtaaagaatgttatgtaaat
ttcatgaggaggccaacttcaagctttttaaaggcagtttattcttggacaggtatggc
cagagatggtgccactgtggtgagattttaacaactgtcaaatgtttaaaactcccacag
gtttaattagttcatcctgggaaaggtactcgcagggccttttccctctctggctgcccc
tggcagggtccaggtctgccctccctccctgcccagctcattctccacagtgagataacc
tgcactgtcttctgattatttataaaaggaggttccagcccagcattaacaagggcaag
agtgcaggaagaacatcaaggggacaatcagagaaggatccccattgccacattctagc
atctgttgggctttggataaaactaattacatggggcctctgattgtccagttatttaaa
atggtgctgtccaatgtcccaaaacatgctgcctaagaggtacttgaagttctctagagg
agcagagggaaaagatgtcgaactgtggcaattttaacttttcaaattgattctatctcc
tggcgataaccaattttcccaccatctttcctcttaggagacttgcctggtgaaaatcat
cactggtcttttggagtttgaggtatacctagagtacctccagaacagatttgagagtag
tgaggaacaagccagagctgtgcagatgagtacaaaagtcctgatccagttcctgcagaa
aaaggtgggtgtgtcctcattccctcaacttggtgtgggggaagacaggctcaaagacag

Sequences 16: Sequence for intron 3 of IL6: Exons - pink, forward and reverse primer for amplification of intro 3 region -light green (Ex3/In3/Ex4 Primer pair). Amplification of intact hnRNA using the Ex3/In3/Ex4 primers will result in an 899bp amplicon that can only arise from intact hnRNA or gDNA. This is then amplified in a second PCR reaction using primer s annealing in intron 3 – red.

The template was loaded onto a 96well plate and 20ul of PCR master mix was added. The basic formula for the PCR mixture was:

PCR Reagents	
10x PCR Buffer	2.0ul
5M Betaine	5.2ul
5mM dNTPs	0.5ul
25mM Mg^{2+}	2.0ul
Primers	
Forward	0.2ul
Reverse	0.2ul
Promega Taq	0.2ul
H_2O	10.5ul

The timings of the PCR program used are set out below:

Initial denaturation step	92ºC for 2 minutes
Denaturation step	92ºC for 30 seconds
Primer annealing step	63ºC for 30 seconds
Extension step	70ºC for 30 seconds
	go to step 2 and repeat cycles 29 times
Final extension step	70ºC for 2 minutes
Final heating cycle	95ºC for 45 seconds followed by immediate cooling on ice

The rapid heating and cooling cycle at the end of the PCR ensured that heteroduplex formation occurred randomly without any new strand synthesis taking place that may have interfered with this process. Heteroduplexs can not be cut by the chosen restriction enzymes.

4ul of PCR product was loaded onto a 96 well plate and digested with 2 units of DdeI restriction enzyme for 16 hours at 37ºC in a 10ul reaction. (Restriction digest was optimised using known homozygous and heterozygous gDNA as a template in the nested PCR and using 3ul, 4ul and 5ul of nested PCR product and 1 unit, 2 units and 3 units of enzyme in a 10ul digest reaction.) Below are the reagents used in the restriction digest:

Restriction Digest Reagents	**10ul reaction**
PCR product	4.0ul
DdeI enzyme	0.2ul (2Unit)
DdeI Buffer	1.0ul
DD H2O	4.8ul

The timing of the restriction digest is set out below:

16hours at 37ºC

2.5ul of digest product was mixed with 4ul diluted loading dye and loaded onto a 5% polyacrylamide MADGE gel, which had been pre-stained with Ethidium bromide. Electrophoresis was performed at 150V for 14 minutes in a dry box. Imaging was

performed using *TyphoonTRIO+*: variable Mode Imager, Amersham Biosciences UK Ltd.

2.2.8.13 Standard Curve

Ratiometric analysis of the restriction digest product of the nested PCR required a standard curve. Control gDNA available in the laboratory, was genotyped for SNP G-174C using the Odyssey LightTyper assay and for SNP G3572T using restriction digest assay. One SNP G-174C homozygous CC and one homozygous GG DNA sample were selected. Concentration of control DNA stock solution was checked by two people at two separate time points using spectrophotometry. The stock solutions were diluted to a concentration of 10ng/ul by a laboratory technician. The two DNA samples at 10ng/ul each were diluted according to the ratios below (Table 2.2-3).

Table 2.2-3: Dilution ratios for standard curve for ratiometric analysis of allelic transcription assay

Standard	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
GG	1	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	0
CC	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0

A total of 30ng of each standard as well as two heterozygous gDNA samples were used as a template for a nested PCR (see section 2.2.8.12). Heterozygous gDNA samples were amplified on the same plate as the standard curve as quality control. The ratio of alleles in gDNA is 50:50. Thus heterozygous gDNA acts as a control for the accuracy of the standard curve.

The final digest product was diluted 1 in 50 before loading onto two ethidium bromide acrylamide gels to ensure the fluorescent signal from the digest product fell within the range of detection of the imager. Digest products were loaded twice onto the same gel and once onto a second gel. Gels were then de-stained twice. (Initially the gels were re-stained with vistra green following the de-staining step, however it became clear that the fluorescence measurement with vistra green gel produced inaccurate results due to the impurities within the gel. Therefore fluorescence measurements were taken as the average of the first measurement of the ethidium bromide gel and the subsequent two measurements of the de-stained gel.)

Gels were scanned using the *TyphoonTRIO+* variable Mode Imager after each step of staining and de-staining. This experiment was repeated 3 times to demonstrate extent of inter assay variance. A total of 36 gel images of the standard curve were analysed.

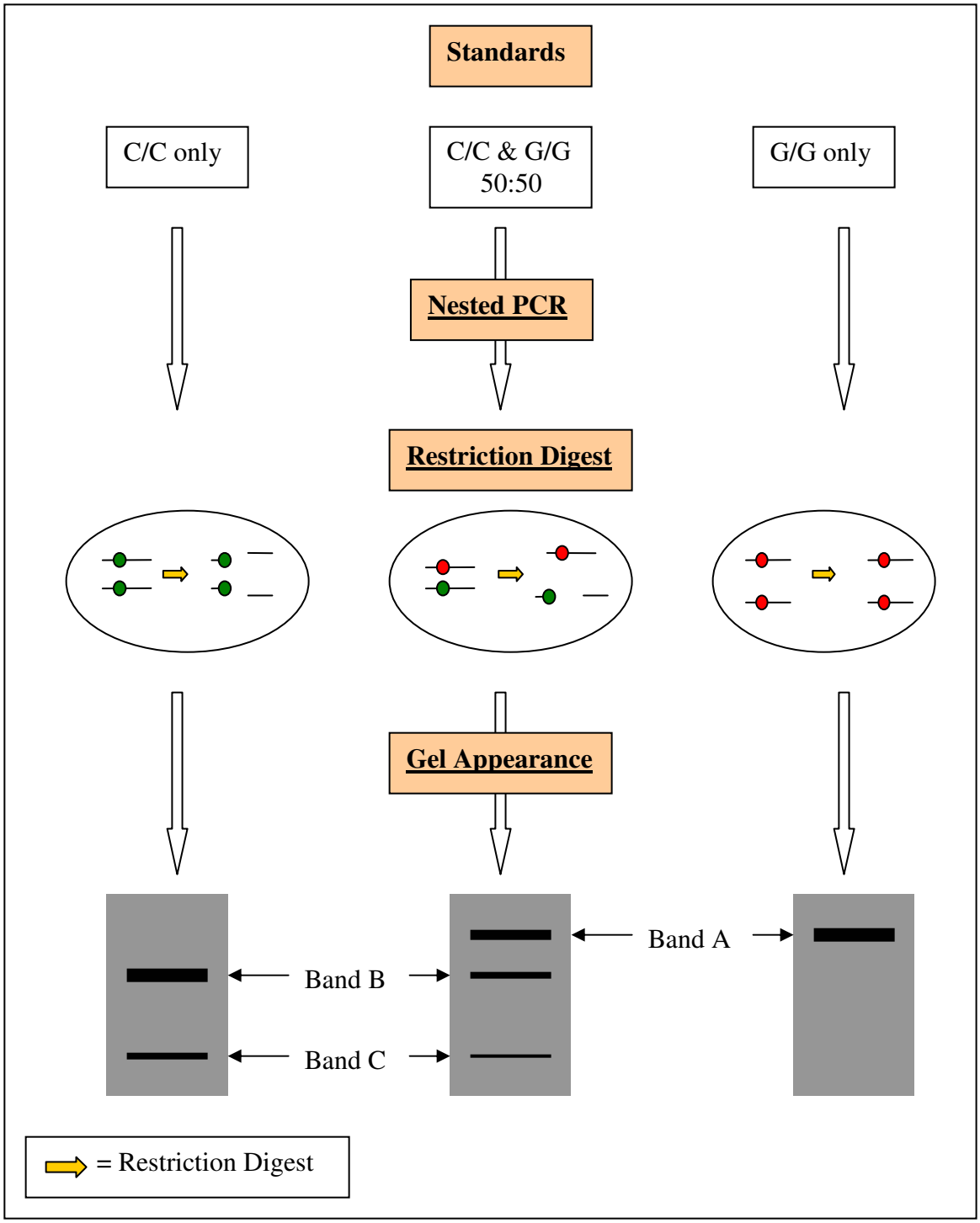


Figure 2-11: Gel appearances of digest products of PCR of different standard templates: Standard template containing only gDNA of genotype SNP-174 CC gives rise to a cut product (band B and band C visible on gel) following nested PCR and restriction digest; Standard template containing gDNA of both SNP-174 genotype GG and CC give rise to cut (band B and C) and uncut (band A) product; Standard template containing only gDNA of SNP-174 genotype GG gives rise to a single band A on the gel. Comparison of the fluorescence intensity of band A and band B allows calculation of the ratio of GG and CC template present in original PCR. Band C is not used in the calculation.

The gel pictures were analysed using Image Quant 6.0 software. Intensity of fluorescence of the uncut band A (see Figure 2-11), containing amplification product of the GG gDNA, was compared to intensity of the larger fragment B (see Figure 2-11) of the cut band (containing amplification product of the CC gDNA). The base pair difference between uncut product A (418bp) and fragment B (299bp) was 119bp. Therefore given a situation where exactly half the DNA strands are cut i.e. products of transcription of the G and the C allele were present in equal amounts, the measured fluorescence would not be equal between the two band. In order to account for this error, intensity of fluorescence for band A was multiplied by 299/418 (299/418xA=A*). To compare fluorescence between the bands, B was divided by the sum of A* and B (B/A*+B). These measurements were plotted on the Y-axis against the known concentrations of the standards measured on the X-axis.
Heteroduplex formation leads to non linearity of the curve. The highest number of heteroduplexes are formed when transcript from G and C allele are present in equal amounts. The number of heteroduplexes formed decreases as the concentration of the transcript product from one allele increases compared to the other. This would leads to a "sag" in the curve. To adjust for heteroduplex formation the known concentration of the standard q was squared i.e. $x=q^2$. Using Excel software a linear trend line was fitted. The resulting trend line equation was used to calculate the concentration ratio from the measured band intensity ratio of the heterozygous samples.

2.2.8.13.1 Statistical Analysis

The relationship between the known concentration ratio squared and the measured band intensity was investigated using linear regression analysis in SPSS for the three PCRs of the standards. The variance of the measured band intensity ratio was measured for each standard concentration, to assess the reproducibility of the assay.

2.2.8.14 Allelic Transcription Assay for Vein Samples cDNA

2.2.8.14.1 RNA Extraction from Vein

Using Odyssey LightTyper assay as described above 17 study patients were found to be heterozygous for SNP G-174C. RNA was extracted from their vein samples according to the method described above.

2.2.8.14.2 cDNA Synthesis - Vein

Heterozygous vein RNA samples were subjected to rigorous DNase treatment as described above. This RNA was used for cDNA synthesis using a specific primer complimentary to a sequence in exon 4. The cDNA was used in a PCR amplification of the LDLR promoter region to assess if samples were free of genomic DNA contamination. This was performed in duplicate. The PCR product was loaded onto an acrylamide gel, run on the electrophoresis and stained with vistra green.

2.2.8.14.3 Allelic Transcription Assay - Vein

Nested PCR amplification was performed as previously described using 3ul vein cDNA, 2ul of each of the 11 standards (see section 2.2.8.12) and two heterozygous gDNA samples as templates. Each sample was loaded three times on a 96 well PCR plate and nested PCR was performed as previously described (see section 2.2.8.12). The nested PCR product was then digested using restriction enzyme Dde I. (see Figure 2-12). In order to achieve equivalent intensity of fluorescence in the standard curve and the samples, sample digest products were diluted 1:2 and standard digest products were diluted 1:10. Each digest product was loaded onto a gel stained with ethidium bromide before electrophoresis at 150V for 14 minutes in a dry box. Band fluorescence intensity was measured using Typhoon Image Quant TL software. The gel was then de-stained twice and the band fluorescence intensity measured after each de-staining process.

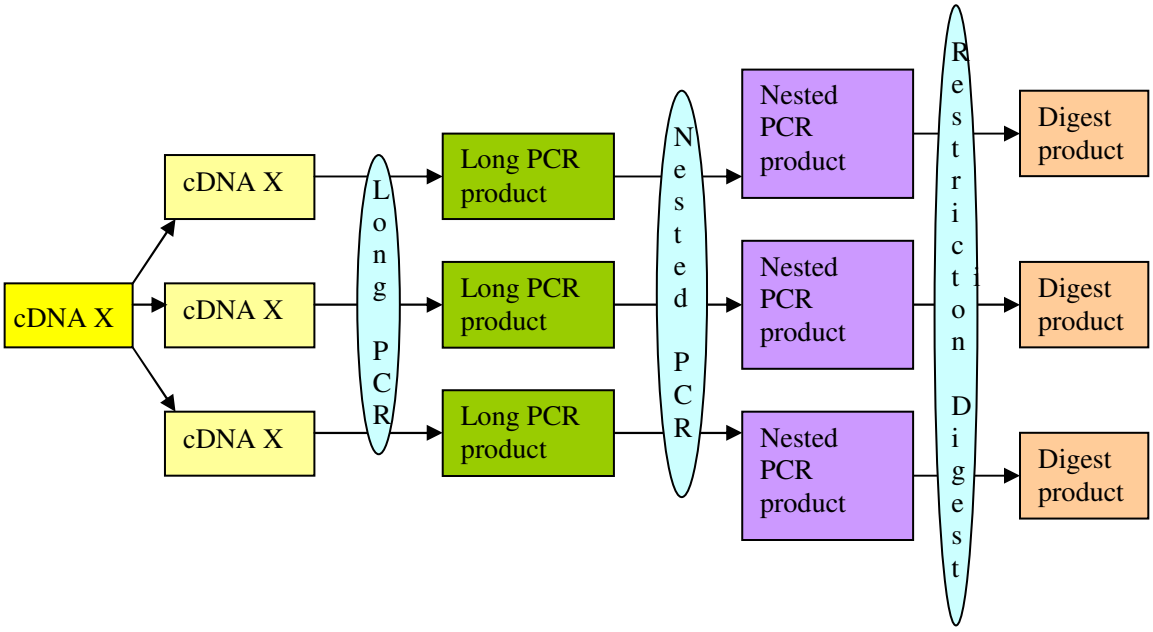


Figure 2-12: Work flow for individual cDNA sample during ratiometric analysis.

A standard curve was plotted and used to calculate the allelic transcription ratio (square root of calculated x-value) for the heterozygous gDNA samples and the individual vein samples. For the purpose of this study the allelic transcription ratio describes the ratio of RNA transcribed from the C-allele compared to total IL6 RNA transcribed.
Samples with at least one repeat measurement were included in the analysis. The data was not normally distributed. Therefore the Mann-Whitney test was used to test for difference between the results for gDNA and cDNA.

2.2.8.15 Allelic Transcription Assay for Control Leukocyte cDNA

The above experiment (see section 2.2.8.14) was repeated using control leukocyte RNA. It was hoped that leukocytes might express more IL6 than vein tissue, thus improving the accuracy of the assay.

2.2.8.15.1 RNA Extraction – Leukocytes

Eight blood samples were taken from a healthy heterozygous volunteer for RNA extraction according to the method described above (section 2.2.7.2).

2.2.8.15.2 cDNA Synthesis – Leukocytes

The eight heterozygous controls RNA samples were treated with rigorous DNase treatment. cDNA was synthesises using specific primers annealing in exon 4 as well as intron 4 (Sequences 17).

Reverse Primer for cDNA synthesis

Reverse Primer for RT Exon4 5' - tttctgcaggaactggatca - 3'

Reverse Primer for RT Intron4 5' – gaatgaggacacacccacct – 3'

Sequences 17: Sequences for two reverse primers used in cDNA synthesis from leukocyte RNA. Different reverse primers were used in an attempt to increase cDNA yield.

The intron 4 reverse primer was chosen to test if the yield of cDNA derived from hnRNA could be improved by placing the reverse primer in the intron region ensuring cDNA synthesis occurred only from hnRNA. Genomic DNA contamination was

checked by PCR amplification of the LDLR promoter region in triplicate, loading of the PCR product onto a vistra green acrylamide gel, electrophoresis and image fluorescence detection as previously described.

2.2.8.15.3 Allelic Transcription Assay - Leukocytes

Nested PCR was performed for leukocyte control cDNA samples in triplicate using 3ul of leukocyte cDNA and 2ul of heterozygous gDNA and standards as a template as previously described. Each PCR product was digested by Dde I restriction digest. In order to achieve equivalent intensity of fluorescence in the standard curve and the samples, sample digest products were diluted 1:2 and standard digest products were diluted 1:10, before loading onto an ethidium bromide gel and electrophoresis at 150V for 14 minutes in a dry box. Band intensity on the gel was measured using Typhoon ImageQuant TL software. The gel was then de-stained twice and the band fluorescence intensity measured after each de-staining process.

A standard curve was plotted and used to calculate the allelic transcription ratio for two heterozygous gDNA samples and the individual leukocyte cDNA samples (square root of calculated x-value).
The Mann Whitney test was used to look for a difference between the mean allelic transcription ration for vein cDNA and heterozygous gDNA.

The original study plan had been to examine allelic transcription in all tissue types. Because of results obtained in the vein and leukocyte assay, muscle and adipose tissue were not examined at this stage. The study proceeded to examine relative IL6 mRNA expression in different tissues in individuals.

2.2.9 Ex Vivo IL6 Expression in Different Tissues

Raised *circulating* levels of IL6 have been linked to aspects of vascular disease (see section 1.5 & 1.6). IL6 is expressed in a variety of cells including monocytes/macrophages, adipocytes, endothelial cells, fibroblasts and myocytes. The *circulating* levels of IL6 are therefore the result of its secretion from all these tissues types. However the relative contribution individual tissues make to the plasma IL6 level is not known.

This study aimed to gain further insight into the relative RNA expression of IL6 in different tissues by comparing relative *IL6* transcription in a variety of tissue types in a single individual. Real Time PCR analysis (RT-PCR) was used to measure relative IL6 expression in different tissues. Results could provide clues to the likely in vivo contribution of individual tissues to circulating IL6 in healthy individuals.

2.2.9.1 Subjects

33 patients were selected from the patients recruited to the study. Selection criteria were: donation of all four tissue samples (vein, adipose tissue, muscle and blood=complete complement of tissues). All were healthy patients free of cardiovascular disease (ASA grades I or II). None of the patients suffered with diabetes. One patient was found to be asthmatic, with acute symptoms on the day of operation subsequent to tissue collection. He had taken inhaled steroids prior to sample collection and was therefore excluded from the study leaving 32 subjects.

2.2.9.2 RNA Extraction

RNA was extracted from vein, blood, muscle and peripheral adipose tissue of all selected 32 patients. RNA was extracted from vein, muscle and adipose tissue using the modified TRI Reagent® (Sigma-Aldrich) method as described in section 2.2.7.1. Leukocyte RNA was extracted using the PAXgene^{TM} Kit as described in section 2.2.7.2. RNA was quantified using spectrophotometry.

2.2.9.3 cDNA Synthesis

In order to establish which cDNA synthesis method had the best result in terms of best cDNA yield, random hexamer primers as well as oligo (dT) primers were used. There was no obvious difference between primers used. For reverse transcription 500ng of RNA was transcribed into cDNA using random hexamer primers (PrimerDesign Ltd) and M-MLV reverse transcriptase (PrimerDesign Ltd).

Annealing Step:

For each RNA sample the following was added to a 500µl tube:

RNA	10.0 ul
Hexomer primer/ dNTP mix	2.5 ul
Final volume	**12.5ul**

A lid was applied and the sample heated at 65°C for 5 minutes on a heated block before transferring onto ice.

Extension step

Mastermix was made up according to the protocol:

MMLV 5Xbuffer	5.0 ul
RNA/DNase-free water	6.5 ul
MMLV enzyme	1.0 ul
Final volume	**12.5 ul**

12.5ul of this Mastermix was added to each of the samples on ice. Samples were mixed and incubated on a heated Block according to the protocol below:

37ºC	for 15 minutes
42ºC	for 45 minutes

The synthesised cDNA was used in real-time PCR analysis.

2.2.9.4 Real-Time PCR Analysis

2.2.9.4.1 Real-Time PCR Principle

Real time PCR uses a fluorescent reporter probe method. This experiment used a sequence specific probe with a fluorescent reporter at one end and a quencher at the opposite end. During PCR amplification the annealed probe is broken down by the 5' to 3' exonuclease activity of the Taq (thermus aquaticus) polymerase leading to separation of the reporter and the quencher and thus to a detectable increase in fluorescence. The increase in fluorescence is proportional to the increase in target of the reporter probe during each PCR cycle.

According to the basic PCR equation:

$N=N_0 \times E^{Cp}$

where: N=generated copy number at a certain cycle

N_0= initial copy number

E=PCR efficiency

C_p=Cycle number at which PCR amplification begins its exponential phase

C_p is considered the point most reliably proportional to the initial concentration. Where different primers or probes are used the efficiency of the PCR may vary. The LightCycler® 480 Real Time PCR System allows the use of standard curves in the experiment. These are used to perform an efficiency correction calculation to increase the accuracy of the result.

Relative quantification compares the ratio of a target sequence to a reference sequence in an unknown sample. Reference genes are housekeeping genes. The principle of relative quantification is based on the assumption that the reference gene is equally expressed across all samples.

The LightCycler® 480 Real Time PCR System uses a calibrator sample to normalise samples within one run and provides a constant calibration point between several LightCycler system runs. The result is expressed as a normalised ratio:

$$\text{Normalised ratio} = \frac{(\text{concentration.target/concentration.ref})_{\text{sample}}}{(\text{concentration.target/concentration.ref})_{\text{calibrator}}}$$

2.2.9.4.2 Real-Time PCR: Optimization

All four tissue types showed IL6 expression in the optimisation phase. Cp values were 29.2 to 36.3 depending on cDNA concentration and tissue type. In order to

achieve the lowest Cp values, but taking into account limited amount of RNA available, it was decided to use 1.5ul of cDNA in the RT-PCR reaction.

2.2.9.4.3 Real-Time PCR: Experimental Set-Up

Real Time PCR analysis was performed to measure levels of IL6 mRNA expression in 128 samples using the LightCycler® 480 Real Time PCR System.
The IL6 assay (AppliedBiosystems) consisted of pre-designed primers and a hydrolysis probe labelled with fluorescein and a quencher. Different concentrations of cDNA (0.5ul; 1.0ul; 2.0ul) in a 20ul RT PCR reaction were used for all different tissue types, to establish expression in these tissues and the optimum level of cDNA template. Two patients' samples (vein, leukocytes, muscle and adipose tissue) were used for optimisation. Reaction volume was optimised to 10 ul and IL6 assay concentration was optimised to the minimum level required in order to save costs.
A variety of housekeeping genes were used in the optimisation phase. The first assay used was already available in the laboratory. It consisted of a multiplex reaction for ubiquitin C (UBC) and glyceraldehyde 3-phosphate dehydrogenase GAPDH. The UBC assay worked consistently for control samples; however the GAPDH assay failed repeatedly. It was assumed that this failure was either the result of a problem with the multiplex reaction or a problem relating to the GAPDH cDNA in the study samples. After discussion with the company it became clear that this particular assay had been withdrawn from the market, as other researchers had had similar problems. The likely explanation for the GAPDH failure was the competitive nature of multiplex PCR reaction leading to preferential amplification of a more abundant transcript, not a degradation of GAPDH cDNA. To test this, a simplex RT PCR reaction for GAPDH was performed. This showed consistent expression of GAPDH in the study samples. It was therefore decided to use a GAPDH simplex reaction in all future reactions. This was cost effective as the assay had been supplied free of charge as part of a promotional offer.
Final analysis was performed as relative quantification with reference to the housekeeping gene GAPDH. The assay for GAPDH used pre-designed primers and a probe labelled with fluorescein (FAM) at the 5' end and with a dark quencher dye near the 3'-end. Both target and housekeeping assay were cDNA specific. Assays were run on the ROCHE LC480® using a FAM filter. Relative quantification data was analysed using ROCHE LC480® Relative Quantification Software.

RT-PCR for each tissue type was performed on one plate which contained a standard curve and a calibrator sample as well as negative template controls. The assay was performed as a simplex reaction. All reactions were performed in duplicate. Failed samples were re-run in duplicate.

IL6 assay:

The reagents for the mastermix for the IL6 RT PCR are listed below:

Universal mastermix x 2	5ul
IL6 20x TaqMan expression assay	0.25ul
RNase free H2O	3.25ul
total	**8.5ul**

GAPDH Assay:

The GAPDH Primer Probe Mix (x10) for the GAPDH RT PCR assay was made up according to the following formula:

Primer R (100pmol/ul)	0.05ul
Primer F (100pmol/ul)	0.05ul
Probe No. 60 (10pmol/ul)	0.1 ul
Water (PCR grade)	0.8 ul
total	**1.0 ul**

The primer probe mix was heated to 95°C for 1 minute.

The mastermix for the GAPDH RT PCR assay was made up according to the following formula:

Universal Mastermix x 2	5ul
GAPDH Primer Probe Mix x 10	1.0ul
RNase free Water	2.5ul
Total	**8.5ul**

The cDNA template (1.5ul) and the Mastermix (8.5ul) were loaded onto a plate which was sealed with an optical adhesive cover, before centrifugation to prevent bubble formation. The plate was placed into the ROCHE LC480®. Thermal cycler conditions were:

Taq activation:	95°C	10 minutes
Amplification:	95°C	15 seconds
	60°C	1 minute – one acquisition per cycle

The average cycle number was 45. Samples were designated as zero expression if both duplicates of the reference amplification were positive and both IL6 amplifications were negative. Samples which gave a zero result were repeated to ensure the result was not due to a technical error. Results were included in the analysis as zero expression if both the initial result and the repeat analysis gave a value of zero. Values for samples included in the analysis were normalised to the calibrator sample to allow comparison between individual PCR runs.

2.2.9.4.4 Standard Curves

Standard curves were used to calculate efficiency of the RT PCR reaction. cDNA was synthesised from control RNA extracted from leukocytes (see section 2.2.7.2). This cDNA was used to obtain standard cDNA concentrations. The cDNA was diluted according to the dilution factors:

x1.0	x1.25	x1.5	x1.75	x2.0	x4.0	x8.0	x16.0

Thus the relative concentration of the standard samples was known and could be used to calculate the efficiency of the RT-PCR reaction, which in turn was used in the relative quantification.

2.2.9.4.5 Statistical Analysis

Statistical analysis was performed using SPSS software. The normalised concentration ratios were grouped together according to tissue type. The normalised concentration ratio was not normally distributed in any of the tissues. Therefore to test if there was a significant difference in expression between tissue types, the

Mann-Whitney U test was applied. To ensure any significant difference shown by the Mann-Whitney U test was not related to different variance between the groups, the data was then Log transformed and reanalysed using the independent t-test with equal variance not assumed.

To ensure that the analysis was not affected by extreme outliers, normalised concentration values in the four tissues were paired for each subject. Variation of IL6 expression between tissues was then assessed for the individual patient by Wilcox signed rank test.

Data for adipose tissue were analysed for correlation between body mass index and IL6 RNA expression, using the Spearman correlation.

2.2.10 Relative Expression of IL6 in Different Tissues with Regards to SNP G-174C Genotype

Expression of IL6 may be influenced by tissue type as well as G-174C genotype or by the interaction of genotype with tissue type. To try and assess the effects of SNP G-174C in a variety of tissues, levels of IL6 RNA in adipose tissue, vein, muscle and leukocytes were measured and analysed according to G-174C genotype.
As this analysis consisted of an inter-individual comparison, potential confounding variables were also examined. It has previously been found that plasma levels of IL6 vary with age; and smoking has previously been found to affect IL6 expression (140;272). The association of IL6 expression with age and smoking was therefore also investigated in the individual tissues.

2.2.10.1 Subjects and Samples

Of the 68 patients taking part in the study, 43 patients were included in the analysis of tissue specific IL6 expression according to SNP G-174C genotype. 25 patients were not included as they could not be genotyped due to loss of access to the Odyssey LightTyper. All included 43 patients donated blood for genotype analysis, vein and adipose tissue for RNA extraction to the study. Of these 40 patients also agreed to a muscle biopsy and 34 also had blood taken for RNA extraction (Table 2.2-4).

Table 2.2-4: Numbers of samples processed for each tissue type

Tissue type	**Number of samples**
Adipose tissue	43
Leukocytes	34
Vein tissue	43
Muscle tissue	40
Total number of samples	**160**

2.2.10.2 DNA Extraction and Genotyping

Leukocyte DNA was extracted from whole blood for genotype analysis as previously described. Samples were genotyped using the LightTyper assay described in section 2.2.8.4.

2.2.10.3 RNA Extraction and cDNA Synthesis

RNA was extracted from all the collected tissues including leukocytes according to the methods previously described. 500ng of RNA was used for reverse transcription with random hexamer primers into cDNA.

2.2.10.4 Real-Time PCR Analysis

Real Time PCR analysis was performed to measure levels of IL6 RNA expression in 160 samples using ROCHE LC480 as described in section 2.2.9.4. Analysis was performed as relative quantification with reference to the housekeeping gene GAPDH. Relative quantification was performed using ROCHE relative quantification software. RT-PCR analysis for each tissue type was performed on one plate which also contained a standard curve and a calibrator sample. All samples were performed in duplicate. Only samples with valid duplicate results were included in the analysis. Samples were designated as zero expression if both duplicates of the reference amplification were positive and both IL6 amplifications were negative. Samples were called invalid if either one of the reference or one of the IL6 amplifications was negative. Samples with zero expression for IL6 and high Cp-values for GAPDH outside the range of the standard curve, indicating very low overall RNA levels leading to poor amplification, were designated as invalid. Samples which gave a zero result or an invalid result were repeated to ensure the result was not due to a technical error or low initial RNA content. Values for samples included in the analysis were normalised to the calibrator sample to allow comparison between individual PCR runs.

2.2.10.5 Statistical Analysis

Statistical analysis was performed using SPSS software except for the Fisher Exact test which used a web based resource http://www.matforsk.no/ola/fisher.htm . To look for difference in IL6 expression depending on genotype all valid results were included. Tissues were analysed individually using the Kruskal Wallis test (see 6Appendix C:c:).

One-way ANOVA was employed to test if there was a significant difference in age distribution in the different genotype groups.

To test for an association between age and IL6 expression, tissues were analysed for all genotype groups together. A linear regression model was applied. As the data was non-normally distributed the Spearman correlation coefficient was used to test for a linear relationship.

To test if the genotype groups differed significantly with regards to smoking status the Fisher exact test was applied. The Mann Whitney U test was applied to test if there was a significant difference between smokers and non smokers with regards to IL6 expression.

2.2.11 Allelic Transcription Assay for Adipose Tissue Samples cDNA

Results from the allelic transcription experiments for vein and leukocytes and the ex vivo expression of IL6 mRNA in different tissues experiment, suggested investigating allelic transcription in adipose tissue might provide further insight into the effect of promoter SNP G-174C and allow refinement of the allelic transcription assay (see section 3.8 & 3.9). The allelic transcription experiment was therefore performed using adipose tissue RNA.

2.2.11.1 Subjects and Samples

Seventeen adipose tissue samples from SNP G-174C heterozygous patients were used in this experiment.

2.2.11.2 RNA Extraction and cDNA Synthesis

RNA was extracted from the adipose tissue samples according to the method described above. All RNA samples were subjected to rigorous DNase treatment prior to quantitation using spectrophotometry. This RNA was used in cDNA synthesis, using specific primers complimentary to a sequence in exon 4 (cDNA Ex4) and intron 4 (cDNA In4) (see Sequences 18). The reverse primer annealing in an intron region was chosen in order to investigate whether the yield from hnRNA could be improved. Reverse transcription can only occur from hnRNA not mRNA when the primer anneals in an intron. This eliminates competitive transcription from mRNAs.

Reverse primers for cDNA synthesis from adipose tissue hnRNA	
Reverse Primer for RT Exon4	5' - tttctgcaggaactggatca - 3'
Reverse Primer for RT Intron4	5' – gaatgaggacacacccacct -3'

Sequences 18: Sequences of reverse primers used in cDNA synthesis from adipose tissue RNA.

The final experiment used cDNA In4 as template. The cDNA was used in a PCR amplification of the LDLR promoter region to investigate gDNA contamination according to the method described in section 2.2.8.8.3. This was performed in triplicate. The PCR product was loaded onto an acrylamide gel, run on the electrophoresis and stained with vistra green.

cDNA Ex4 was used as template for PCR for amplification of intron 3 and adjacent exon/intron boundaries in order to confirm the presence of IL6 mRNA in the samples. The PCR amplification product was loaded onto an acrylamide gel and stained with vistra green. As the PCR primers (Ex3/In3/Ex4) spanned intron 3, the presents of IL6 mRNA in the tissue samples could be confirmed by demonstrating a short product on the gel. A long product represented either gDNA contamination or amplification from hnRNA.

2.2.11.3 Allelic Transcription Assay – Adipose Tissue

Nested PCR amplification was performed as previously described (see section 2.2.8.12), using adipose tissue cDNA, 11 standards and three heterozygous gDNA samples as template. 3ul of template was loaded onto the PCR plate before adding the PCR Mix. Each sample was loaded three times on a 96 well PCR plate.
The nested PCR product was then digested using restriction enzyme Dde I. In order to achieve equivalent intensity of fluorescence in the standard curve and the samples, sample digest products were diluted 1:2 and standard digest products were diluted 1:10.
Each digest product was loaded on a gel stained with ethidium bromide and band fluorescence intensity measured using Image Quant TL. The gel was then de-stained twice and the band fluorescence intensity measured after each de-staining process.

A standard curve was plotted and this curve used to calculate the allelic transcription ratio for the heterozygous gDNA samples and the individual adipose tissue samples. The mean of the results from the three PCRs was taken for each individual sample to calculate the allelic transcription ratio. The Mann Whitney test was used to test if the mean allelic transcription ratio for vein cDNA differed significantly from the ratio measured for gDNA.
Genotyping for SNP G3572T was performed on all 17 heterozygous samples, using the restriction digest method previously described in section 2.2.8.6.3 to identify rare haplotype SNP G-174C/SNP G3572T: GC/TT, i.e. homozygous in the intron SNP despite being heterozygous in the promoter SNP

3 Results

3.1 Patient Recruitment/Sample Collection

3.1.1 Patient Recruitment

108 patients were approached about the study in the pre-assessment unit or by post asking them to take part in the study. Recruitment was ineffective when patients were recruited from the pre-assessment unit because for many of the patients the operating date had not been set and it was unclear which surgeon would be operating on these patients. Not all surgeons took part in the sample collecting process so a number of patients who had agreed to take part were lost to the study. The method of recruitment was therefore changed to recruiting only patients who had been allocated an operating date and these patients were recruited by letter and telephone. This improved the numbers successfully recruited; however some patients were still lost, as operating dates were frequently changed for patients at the last minute. Of the 108 patients approached about the study 95 agreed to take part. Thirteen of the patients approached about the study declined to take part i.e. uptake was 88%. 68 patients donated tissue samples to the study; of these one patient was subsequently found to have taken steroids on the day of surgery and was thus excluded from the study (see Table 3.1-1 for demographic data of study participants). 25 patients had agreed to take part but did not donate samples. These patients were lost because they were operated on by a surgeon not taking part in the study or because the investigator was not available to process the tissue samples. Two patients were found not to be suitable for the study on the day of the operation. One suffered from bleeding tendency and one did not require a groin dissection (see Figure 2-2).

Table 3.1-1: Demographic data of the study participants.

	Number of Patients
Total	67
Gender	
male	24
female	43
Risk Factors for atherosclerosis	
hypertension	8
hypercholesterolaemia	1
diabetes mellitus	0
history of MI or Stroke	0
Smoking	
non-smokers	47
smokers	14
gave up smoking within 2-4 weeks	2
no smoking history available	3
Age	**Mean age (age range)**
	48 years (22-70 years)

Of the 67 study patients recruited 32 were un-medicated and 35 were medicated (for medication details see Table 3.1-2).

Table 3.1-2: Number of subjects taking a particular drug. Note that out of 67 patients 32 were un-medicated. The total in the table is more than the medicated 35 patients as some patients were taking more that one drug.

Medical Therapy	Number of Patients taking medication
Antihypertensive	8
Anti-depressant	6
Diuretic	4
Contraceptive agent/HRT/Raloxifene	8
Aspirin	2
Adcal/ Alendronic acid	1
Omeprazole	1
Simvastatin	1
Allopurinol	1

3.1.2 Sample Collection

Although the majority of patients agreed to donate all four tissue types to the study, one patient had concerns regarding the muscle biopsy and only donated fat, vein and blood. In three cases collection of muscle sample was technically impossible because access to the muscle could not be gained without extending the field of dissection, as these patients had short saphenous vein surgery only.

In the initial stages of patient recruitment there was no system available that would allow extraction of RNA from blood. Therefore the first seven patients did not have blood taken for RNA extraction.

3.2 Patient Follow-Up

19 patients were contacted for follow up, four of which had been recruited in the pre-assessment unit and 15 had been recruited by letter ± telephone call. 12 were 'very satisfied' with the way in which they were recruited and seven were 'satisfied'. All four patients recruited in the pre-assessment clinic were 'very satisfied'. All 19 patients would consider taking part in a clinical trial again. None reported

complications associated with bleeding such as active bleeding, groin haematoma or excessive bruising. However two patients had groin infections. One was a minor infection which responded to oral antibiotics the other required surgical drainage of abscess. There was no haematoma associated with the abscess. The average time scale for return to normal daily activity was 1-2 weeks. Average pain scores (scale: 1-10) on the first and second postoperative day were 3.9 and 3.6 respectively. None of the patients felt that they had suffered any ill effect through taking part in the study.

3.3 DNA Extraction from Leukocytes

DNA yield from 9ml of whole blood varied between 44.5ug and 391.0ug.

3.4 Genotyping for G-174C

The Odyssey LightTyper assay was used for genotyping. Below are examples of the Odyssey LightTyper output curves (Figure 3-1, Figure 3-2 & Figure 3-3). Single melt curve peaks are seen for homozygous samples. Peaks occur at lower temperatures for the imprecisely bound probe. Homozygous samples show a double melt curve peak indicating presence of both alleles. A total of 42 out of 68 patients were genotyped for SNP G-174C. The remaining 26 could not be genotyped as access to the Odyssey LightTyper was lost due to changes within the laboratory staffing structure. Of the 42 DNA samples genotyped for G-174C, 7 were homozygous CC, 18 were homozygous GG and 17 were heterozygous GC. (see Table 3.4-1; for individual results of genotyping see Table_Apx C-33).

Table 3.4-1: Genotype of subjects

Genotype	**Number of subjects**
GG	18
GC	17
CC	7
Total	**42**

The genotype distribution was in Hardy Weinberg Equilibrium (CHI-squared value = 0.72, not significant with one degree of freedom)

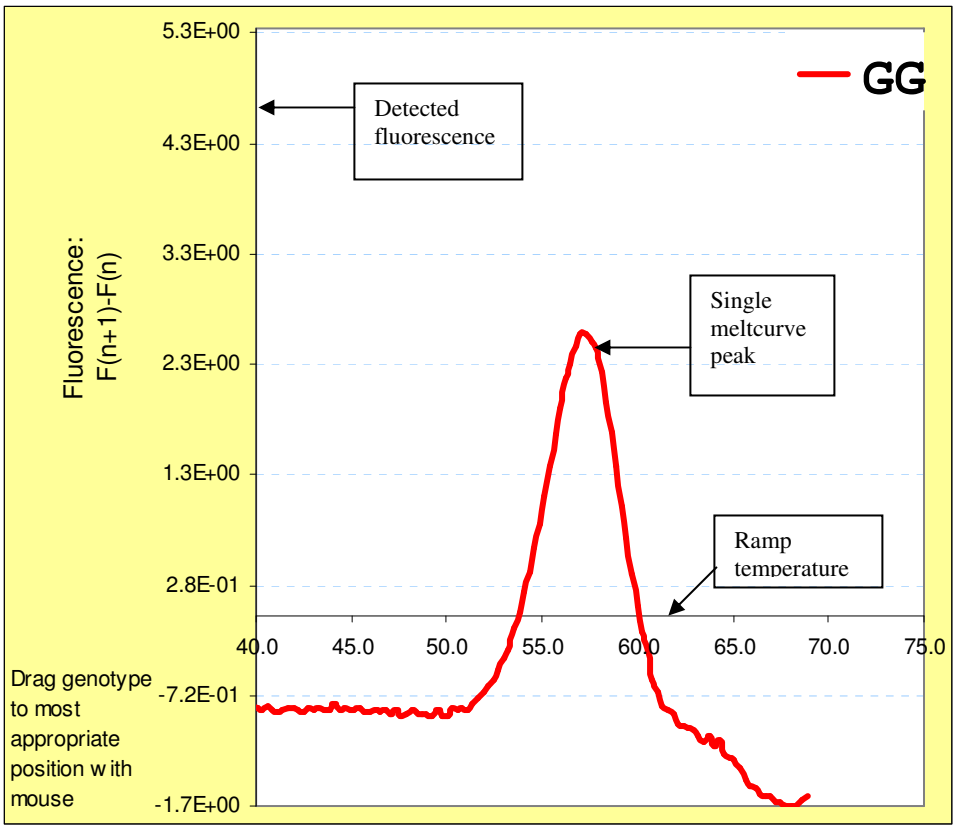


Figure 3-1: Example of output from Odyssey LightTyper showing a melt curve for a homozygote (common allele) individual. Y-axis represents detected fluorescence and x-axis represents ramp temperature. This curve was obtained by PCR amplification of genomic DNA. Post PCR the product was subjected to thermal ramping leading to separation of a fluorescent probe from the PCR amplicon and quencher probe. The resulting increase in fluorescence was detected by the Odyssey LightTyper and is represented by the peak of the melt curve. The temperature at which separation of the probe occurs is higher when the probe is precisely bound to the G-allele than when it is imprecisely bound to the C-allele. A single peak at a relatively high temperature (57 degree centigrade in this instance) indicates that only G-alleles are present.

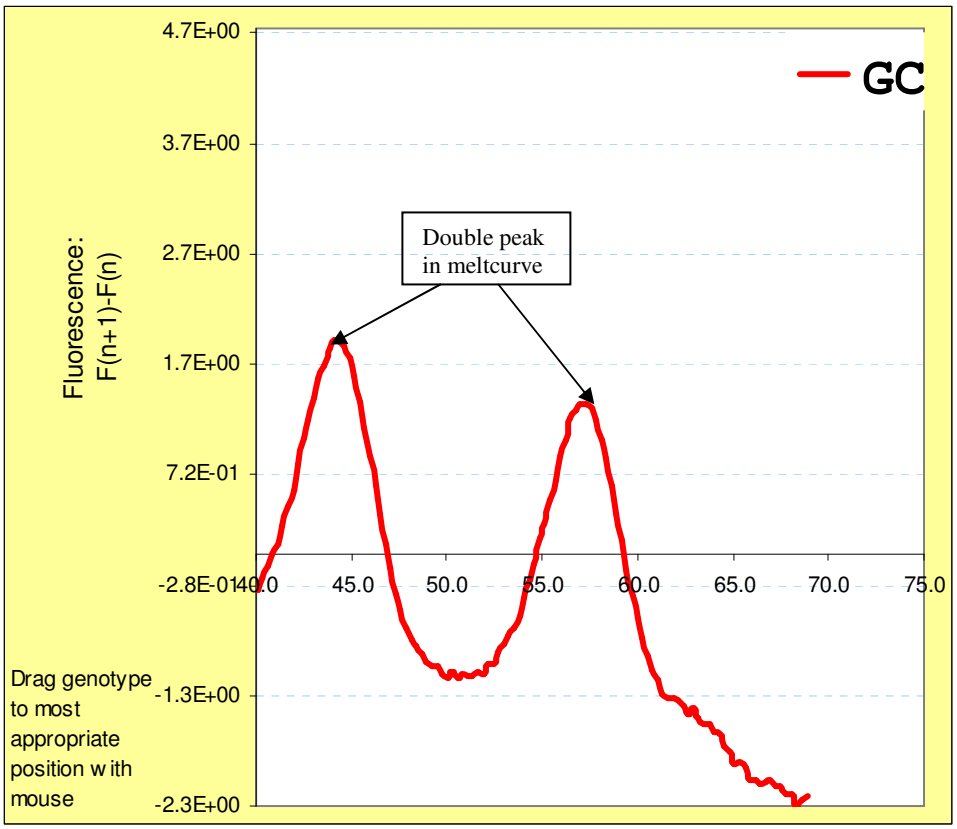


Figure 3-2: Odyssey LightTyper output for heterozygote individual. There are two peaks in the melt curve one for the common allele G (high temperature 57 degree centigrade) and one for the rare allele C (low temperature 44.5 degree centigrade).

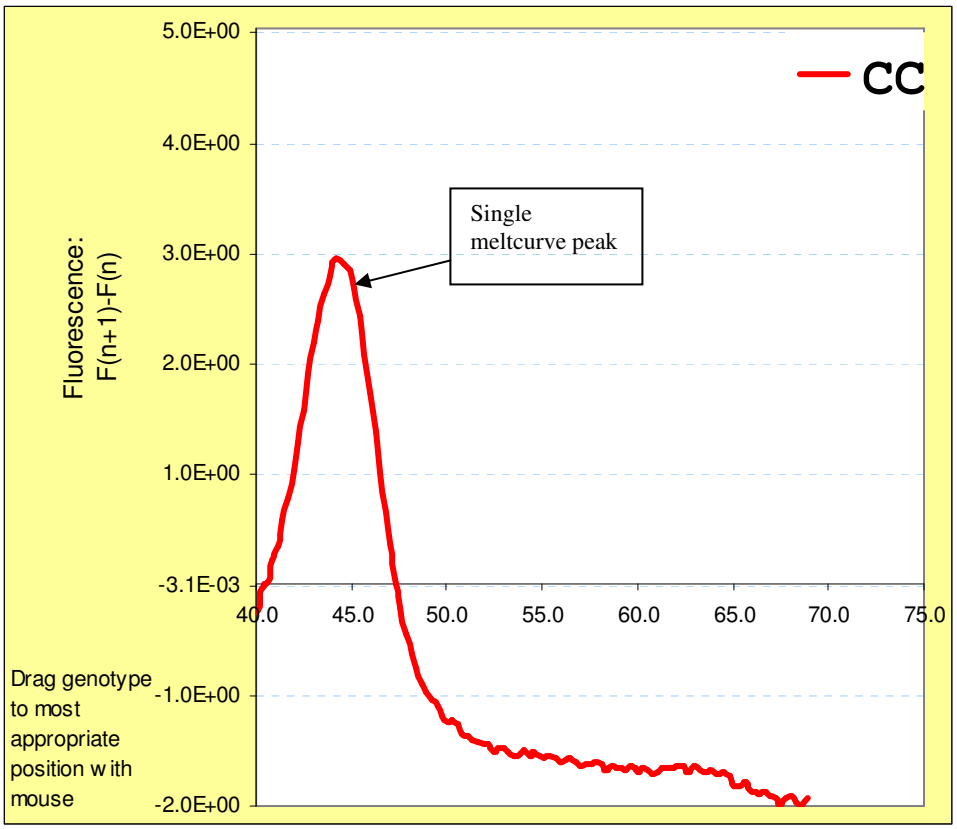


Figure 3-3: Odyssey LightTyper output for a homozygote (rare C allele) individual. There is one peak in the melt curve at a low temperature (44.5 degree centigrade).

3.5 RNA Extraction and Quantification

RNA was extracted from 45 vein samples, 42 muscle samples, 45 fat samples and 40 blood samples. Vein and muscle tissue are high in fibrous content making homogenisation difficult. An automatic homogeniser of adequate size was not available in the laboratory and tissues was therefore homogenised by hand. The laborious nature of this process allowed a maximum of six samples to be processed per day. RNA yield was poor and repeat RNA extraction from tissue samples was necessary for individual experiments. Initially 100mg of tissue per sample was used on average for homogenisation in 1ml TRI Reagent® for vein, muscle and adipose tissue as per manufacturer's protocol. RNA yield using this method was low, in particular for adipose tissue. The method was therefore modified (see section 2.2.7.1) to using smaller amounts of tissue in a greater volume of TRI Reagent®. Leukocyte RNA was extracted from 2mls of blood with an average RNA yield of 10.54ug (see Table 3.5-1).

Table 3.5-1: Average RNA yield in different tissues

	mean vein RNA yield in ug	mean muscle RNA yield in ug	mean adipose tissue RNA yield in ug	mean leukocyte RNA yield in ug
initial method	2.89	1.92	1.63	
final method	8.41	10.89	3.18	10.54

3.6 RNA Purity

Purity of RNA as assessed by A260:A280 ratio varied between tissue samples (see Table 3.6-1). Leukocyte RNA had the lowest protein contamination. Protein contamination in RNA from tissue samples was high. In particular fat samples had substantial protein contamination.

Table 3.6-1: Average A260/A280 values for different tissues

Average A260:A280 ratio	vein	muscle	fat	Leukocytes
In tris HCL Buffer	1.73	1.74	1.33	1.91

3.7 RNA Quality

RNA samples are easily degraded by RNases, nucleases that act as catalysts in the hydrolytic cleavage of RNA into smaller components. RNases are common in the environment. Thus it was necessary to ensure collection and storage conditions of the study samples did not lead to degradation of RNA. The results of electrophoresis of RNA samples on a denaturing urea acrylamide gel, suggest that RNA extracted in this experiment was of equal quality to RNA, used successfully in previous experiments. The gel showed clear 28s and 18s bands with little or no degradation (see Figure 3-4).

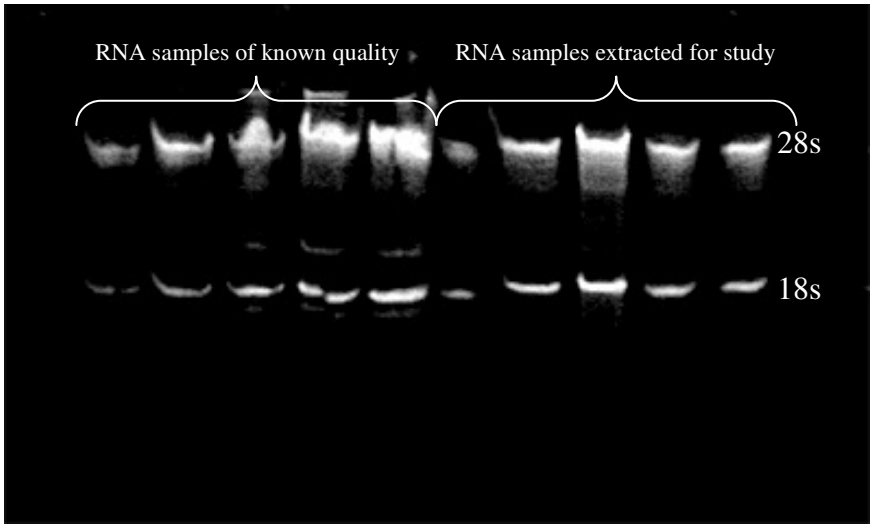


Figure 3-4: A denaturing urea acrylamide gel showing 28s and 18s RNA bands: Comparison of RNA of known quality with control RNA extracted and stored for the purpose of this study. Clear 28S and 18S bands indicate little or no degradation of RNA.

3.8 Preferential Allelic Transcription Assay

3.8.1 gDNA Contamination

PCR using genomic DNA from leukocytes, treated with the standard DNase treatment, as a template was able to amplify the LDLR promoter region in all three samples, indicating incomplete digestion (see Figure 3-5). PCR from genomic DNA treated with rigorous DNase treatment showed no amplification indicating complete digestion. cDNA samples from standard DNased RNA from vein, placenta and leukocytes showed promoter amplification; however the same RNA treated with rigorous DNase treatment showed no amplification from cDNA indication complete digestion of genomic DNA contamination.

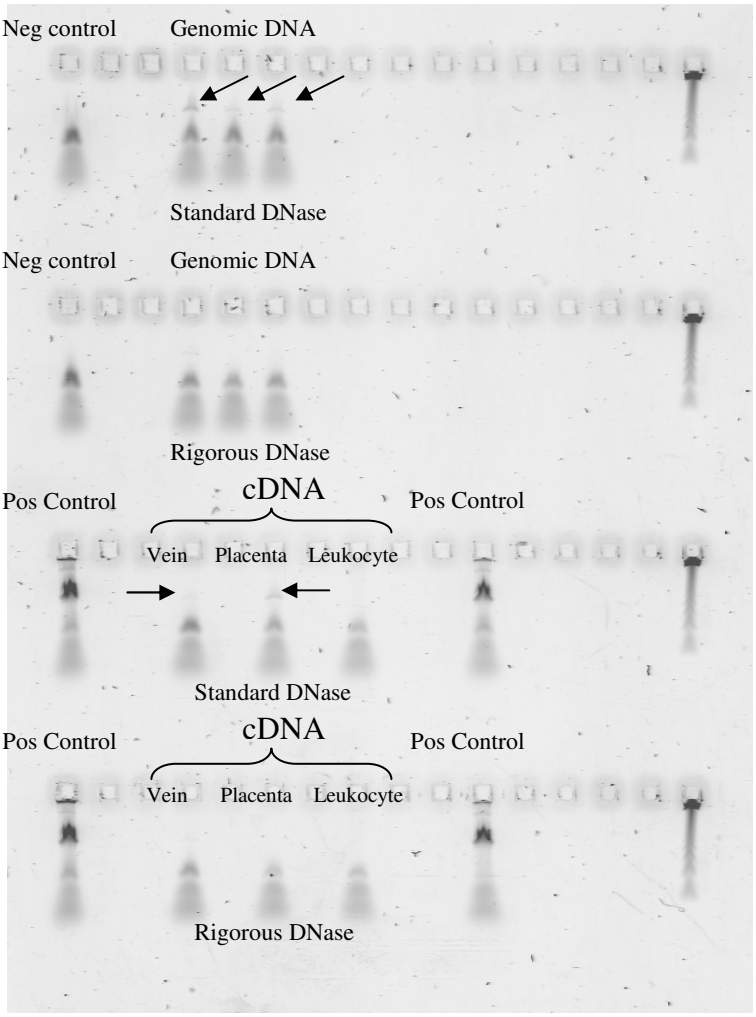


Figure 3-5: Acrylamide gel 5% stained with vistra green: Result of PCR amplification of promoter region of *LDL-R* (35 PCR cycles). All samples treated with rigorous DNase protocol failed to show amplification indicating complete digestion of genomic DNA/contamination. Samples treated with standard DNase protocol show residual amplification, indicating some residual gDNA/contamination is present (arrows).

Rigorous DNase treatment was enough to digest at least 300ng of gDNA and appeared to be sufficient to rid control RNA samples of gDNA contamination. Therefore all samples for the experiment were treated with rigorous DNase treatment.

3.8.2 Detection of hnRNA Using Gel Based Method

3.8.2.1 Amplification of Intron Region

Using cDNA synthesised from RNA free of gDNA contamination, intron region 2 and intron region 3 were amplified using PCR. The correct sized product could be

demonstrated following PCR for intron 3 but not for intron 2. Despite designing another primer pair for intron 2, this region failed to amplify.

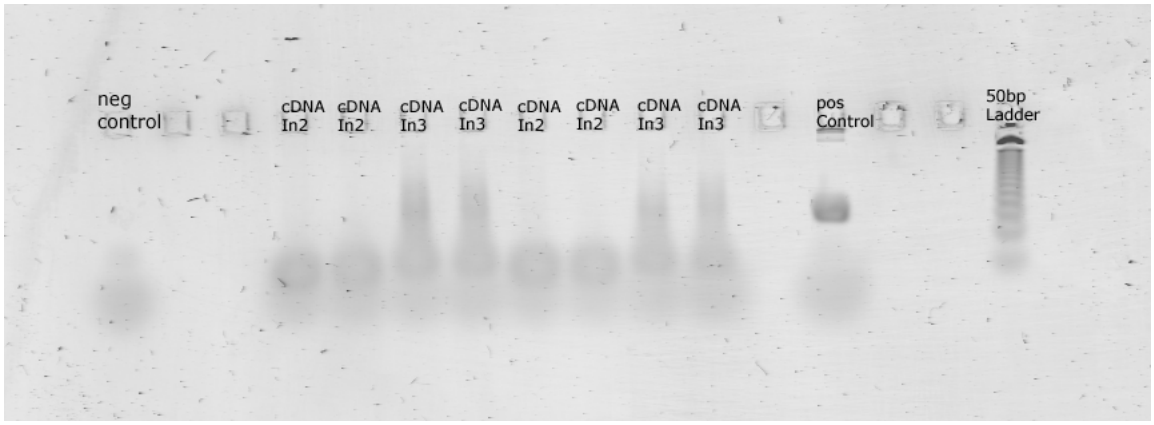


Figure 3-6: Acrylamide gel 5% with vistra green stain: PCR amplification of intron 2 region of *IL6* from cDNA-In2 and cDNA-In3 templates. Amplification could not be demonstrated.

Figure 3-6 shows PCR amplification of the region surrounding SNP T2529C in intron two. Different cDNAs had been used as template. Some had been synthesised using a reverse primer annealing in intron 2 (cDNA in2) other using a primer annealing in intro 3 (cDNA in3). No obvious bands were seen.

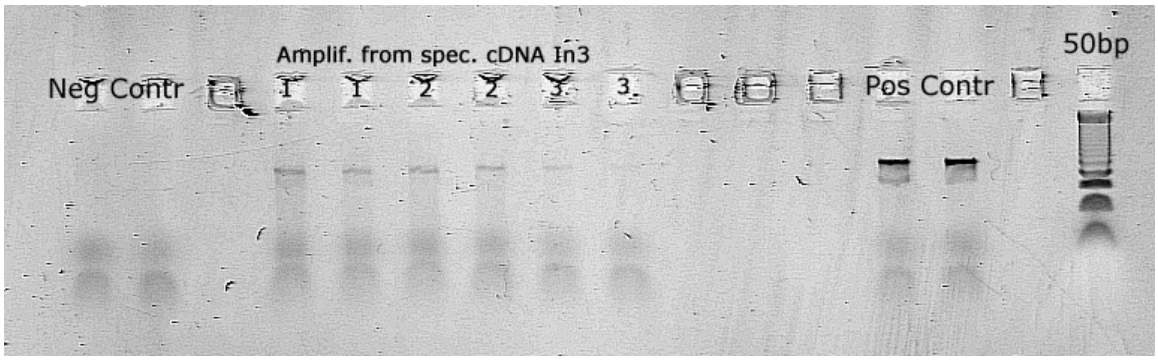


Figure 3-7: Acrylamide gel 5% with vistra green stain: PCR amplification of intron 3 region of *IL6* from cDNA-In3 templates. Bands are visible for all samples demonstrating amplification.

Figure 3-7 shows PCR amplification of the region surrounding SNP G3572T in intron three. The cDNA used as template had been synthesised using a reverse primer annealing in intron 3 (cDNA in3). Bands of the correct size were demonstrated suggesting possible presence of hnRNA.

3.8.3 Excluding Amplification from Un-degraded Splice Product

Results of section 3.8.2.1 suggested that hnRNA was present, as intron regions could be amplified. To test if amplification may have occurred from an un-degraded splice product (spliced but un-degraded intron sequence) amplification was attempted of the exon 3/intron 3 boundary. It was not possible to demonstrate a PCR product for this reaction, suggesting amplification could have occurred from splice product (see Figure 3-8).

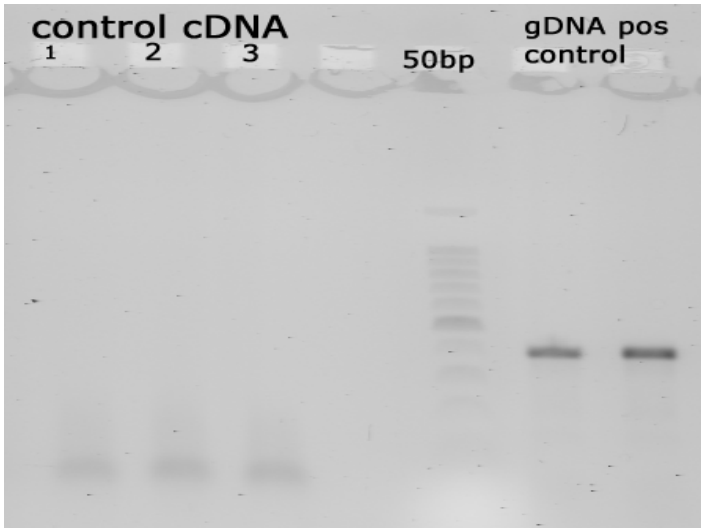


Figure 3-8: Agarose Gel 1.5% EB stain: PCR amplification of control leukocyte cDNA template of Exon/Intron 3 boundary - no product was visible for cDNA. Positive gDNA controls both showed bands of the correct size. Repeat staining of the gel with Vistra Green also failed to demonstrate bands for cDNA.

3.8.4 Ensuring Amplification from Intact hnRNA

To ensure amplification of intron3 could not occur from a splice product, intron 3 was amplified including the adjacent exon/intron boundaries. Both a short product (mRNA derived) and a long product (hnRNA or gDNA) could be demonstrated. LDLR promoter amplification was unsuccessful indicating freedom from genomic DNA contamination Figure 3-9. Assuming freedom of gDNA contamination, this experiment showed the presence of IL6 mRNA in control leukocyte RNA (short product) and hnRNA (long product). Therefore hnRNA cDNA PCR product was concluded to be a suitable candidate for nested PCR template.

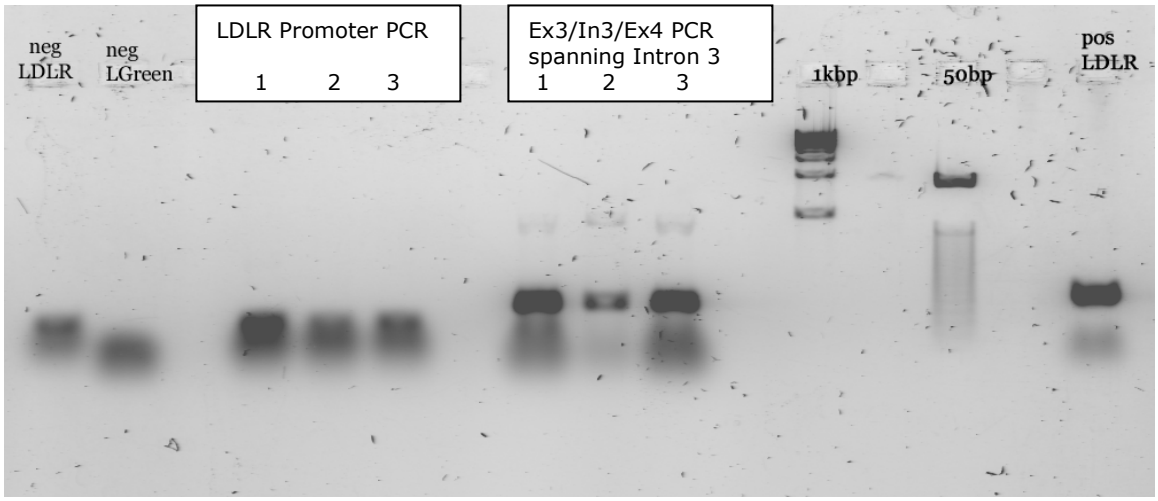


Figure 3-9: Agarose Gel 1.5% stained with vistra green loaded with PCR amplification product of control leukocyte cDNA (samples 1, 2 & 3) and negative water control (neg): There is no amplification in the LDLR promoter assay, indicating lack of gDNA contamination. Amplification of intron 3 from control leukocyte cDNA template using primer Ex3/In3/Ex4, shows a faint band for sample 1, 2 and 3 of about 899 bp (potential amplification product from hnRNA) and a short product from cDNA template.

3.8.5 Nested PCR and Restriction Digest

Using the nested PCR allelic transcription assay the region surrounding SNP G3572T in intron 3 could be amplified sufficiently to produce clearly visible bands on an ethidium bromide gel (Figure 3-10). Following digestion the bands were of sufficient fluorescence intensity to allow fluorescence quantification. In a nested PCR the first product is diluted to reduce potential contaminants. Different dilution factors were tested to determine optimal signal strength. In the final experiment a dilution factor 1:1000 was used.

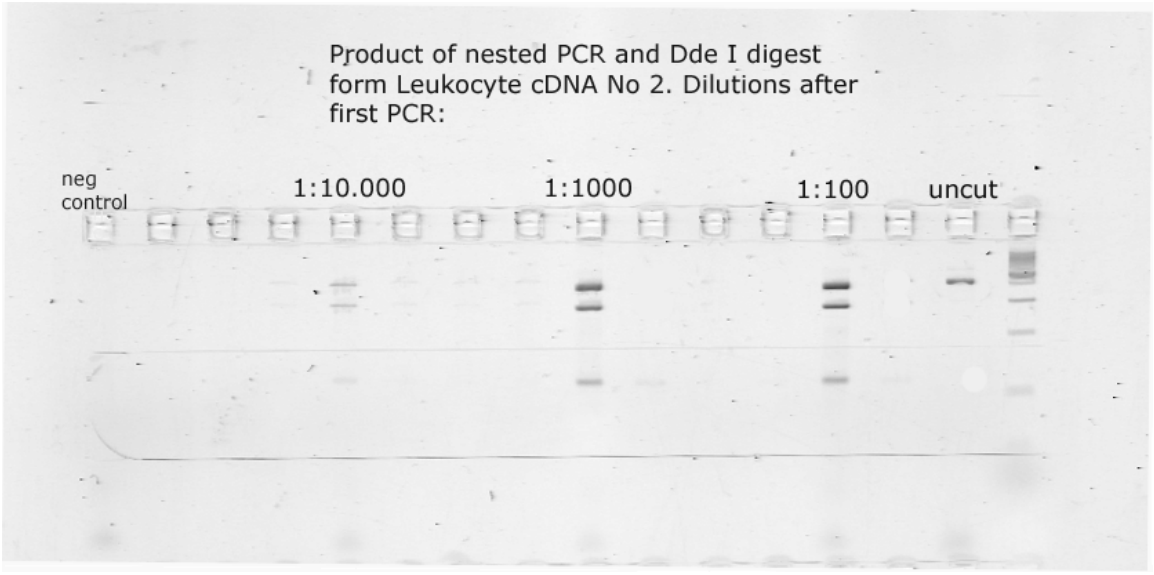


Figure 3-10: Acrylamide gel 5% with ethidium bromide stain: Product of the nested PCR of control leukocyte cDNA for amplification of intron region 3 and restriction digest Dde I. The amplification product of the first PCR was diluted. A dilution of 1:1000 results in clearly visible bands following second PCR and restriction digest. Bands are not clearly visible at a dilution of 1:10 000.

3.8.6 Standard Curve

Nested PCR was performed using a mixture of two types of homozygous gDNA at known ratios (standards) (see section 2.2.8.13) as a template. The PCR product was digested and loaded onto a gel allowing measurement of ratio between alleles as previously described. Below are examples of the gel appearances of the standard samples (see Figure 3-11 & Figure 3-12).

Figure 3-11: Acrylamide gel 5% with ethidium bromide stain: Amplification product of the nested PCR and restriction digest using gDNA standards (gDNA of varying ratio between two samples, each homozygous for SNP G-174C; one GG the other CC) as a template,

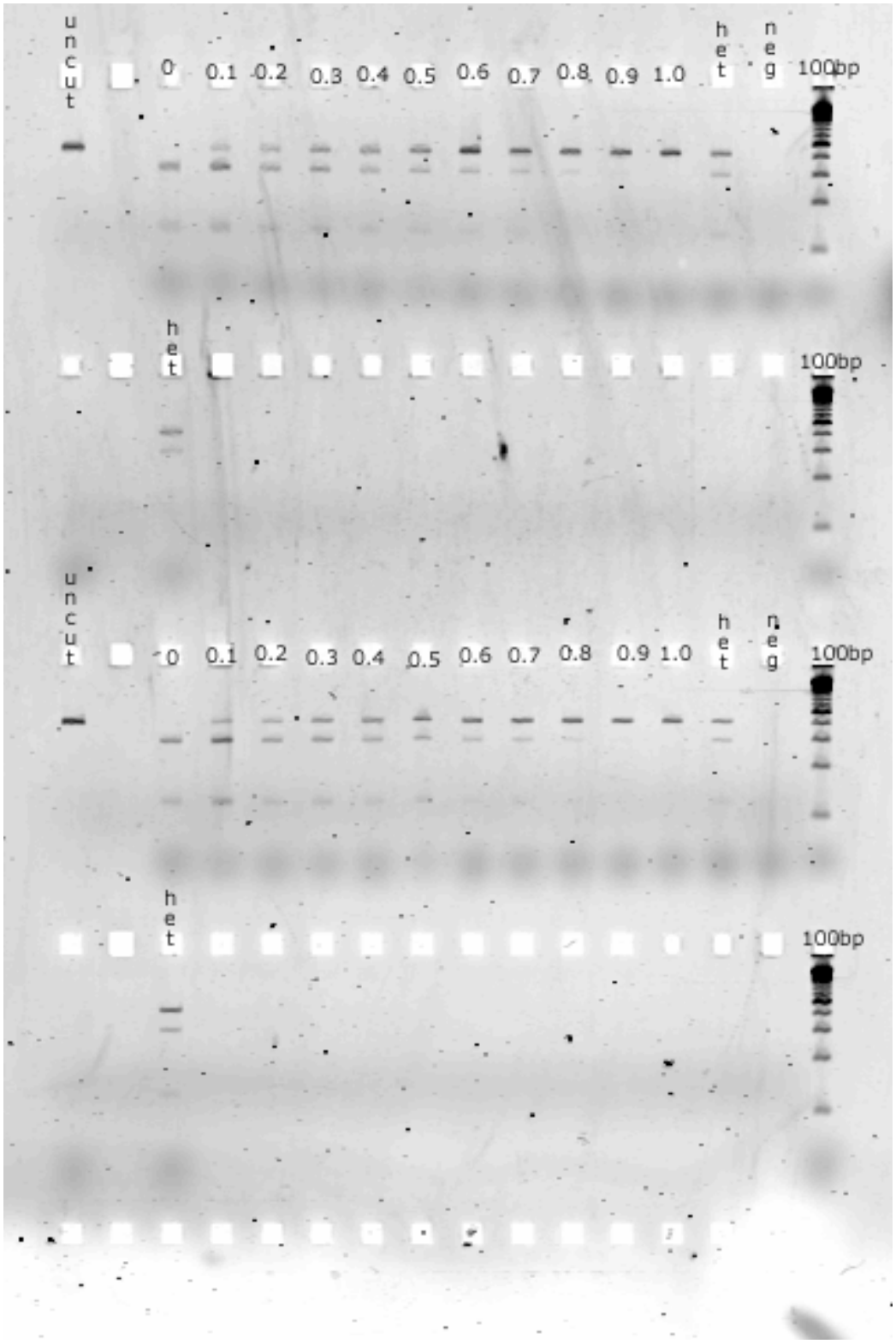


Figure 3-12: Acrylamide gel 5% with ethidium bromide stain: Nested PCR and restriction digest product of standards (ratios 0-1.0), heterozygous gDNA samples (het) and negative water control (neg) as well as an uncut nested PCR product (uncut) loaded twice onto the gel.

Below are the standard curves achieved by plotting the measured fluorescence intensity ratios against the known concentration ratios of the standard samples (squared) and fitting a trendline (see Figure 3-13) as previously described (see section 2.2.8.13; for data see Table_Apx C-38, Table_Apx C-39, Table_Apx C-40).

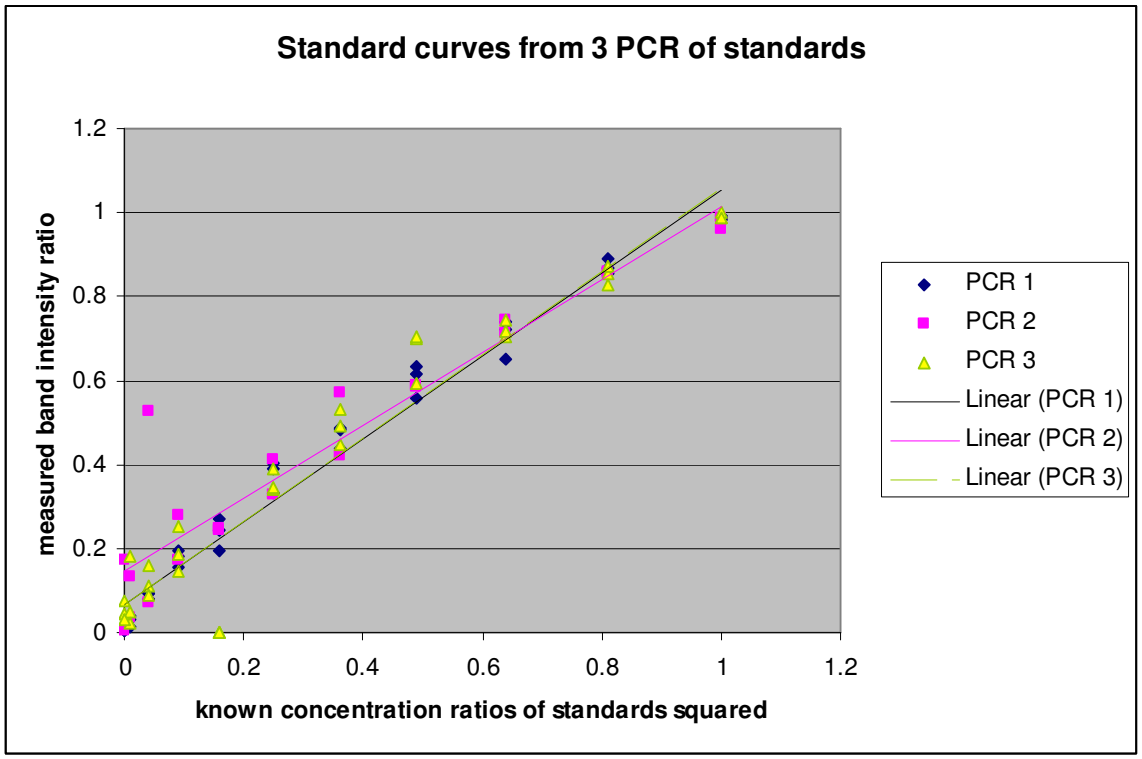


Figure 3-13: Standard curves: Standard samples (varying ratios of two gDNA samples homozygous for SNP G-174C; one GG the other CC) were used as templates for the nested PCR reaction and restriction digest. Fluorescence measurement ratios of the two larger bands were plotted on a graph against the known ratios of the standard samples squared. This was performed in triplicate using the same standards as a template. To create the standard curve a trendline was fitted. Note that the trendline from PCR 1 and PCR 3 are super-imposed indicating good reproducibility of the assay for the standard samples.

Linear regression analysis indicated a very strong linear relationship between the variables. R-squared for PCR 1, 2, 3 was 0.98, 0.90, 0.97 respectively with a significance value $p<0.001$. (for SPSS linear regression analyses see Table_Apx C-16, Table_Apx C-17, Table_Apx C-18).

The variance in measured band intensity for each standard concentration was close to zero (Table 3.8-1)

Table 3.8-1: Variance of measured band intensity ratio for each standard concentration.

Standard concentration ratio	10:0	9:1	8:2	7:3	6:4	5:5	4:6	3:7	2:8	1:9	0:10
Variance	0.003	0.004	0.024	0.002	0.001	0.001	0.003	0.003	0.001	0.000	0.000

Each nested PCR contained two samples of heterozygous gDNA as a control. The measured band intensity ratios for the two samples of heterozygous gDNA were plotted on the standard curves (see Figure 3-14). Calculated allelic concentration ratio was 0.51 (expected=0.50)

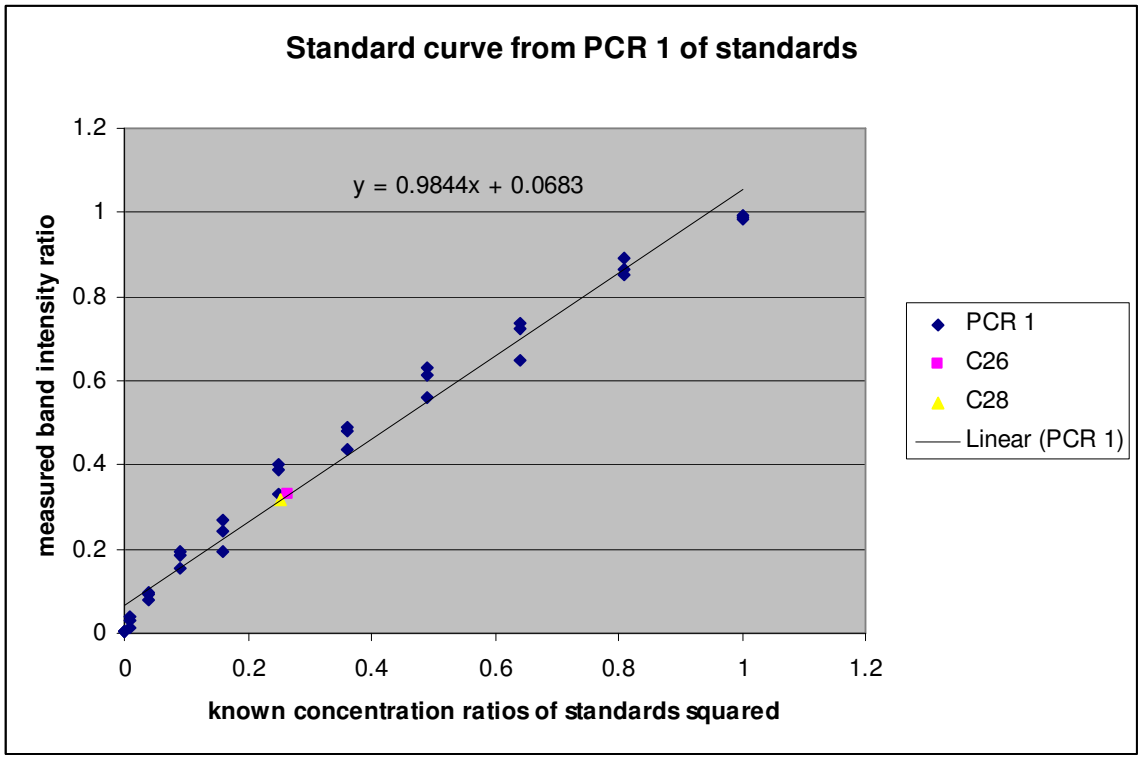


Figure 3-14: Standard curve from PCR 1 using heterozygous gDNA (control gDNA 26 and 28 = C26 and C28) as well as standard samples as a template. The measured band fluorescence intensity ratio of the heterozygous gDNA samples is plotted on the standard curve. The values for heterozygous gDNA are marked in yellow and pink. The calculated concentration ratio squared for heterozygous gDNA was 0.26 i.e. allelic ratio was 0.51 (expected=0.50)

3.8.7 Allelic Transcription Assay for Vein Sample cDNA

Following design and validation of the allelic transcription assay, study vein sample cDNA were examined for gDNA contamination. Despite rigorous DNase treatment 4 samples out of 17 showed gDNA contamination in one of the PCRs (see Figure 3-15).

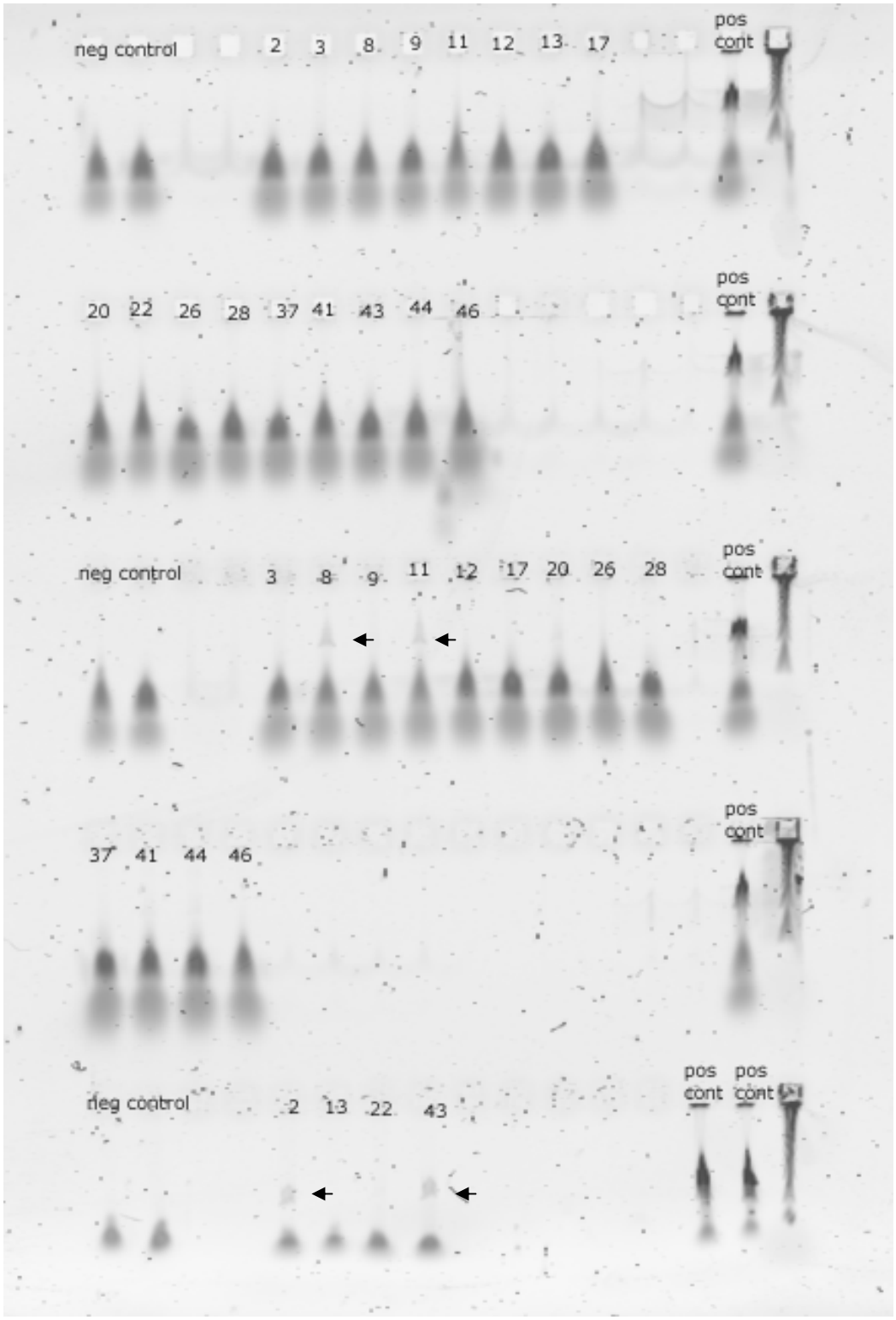


Figure 3-15: Acrylamide Gel 5% stained with vistra green: Results from PCR amplification of LDLR promoter region for vein samples (numbered samples); negative water (neg control) and positive gDNA (pos control) controls are included. Bands indicating gDNA contamination are seen in samples 2, 8, 11 and 43.

Nested PCR and restriction digest was performed in triplicate on all samples including those contaminated with gDNA. Out of 17 vein samples 13 samples had measurements for all three PCRs, for 3 vein samples only two out of three PCRs showed amplification. Samples with at least one duplicate measurement were included in the analysis i.e. one samples was excluded from the analysis (see Figure 3-16:).

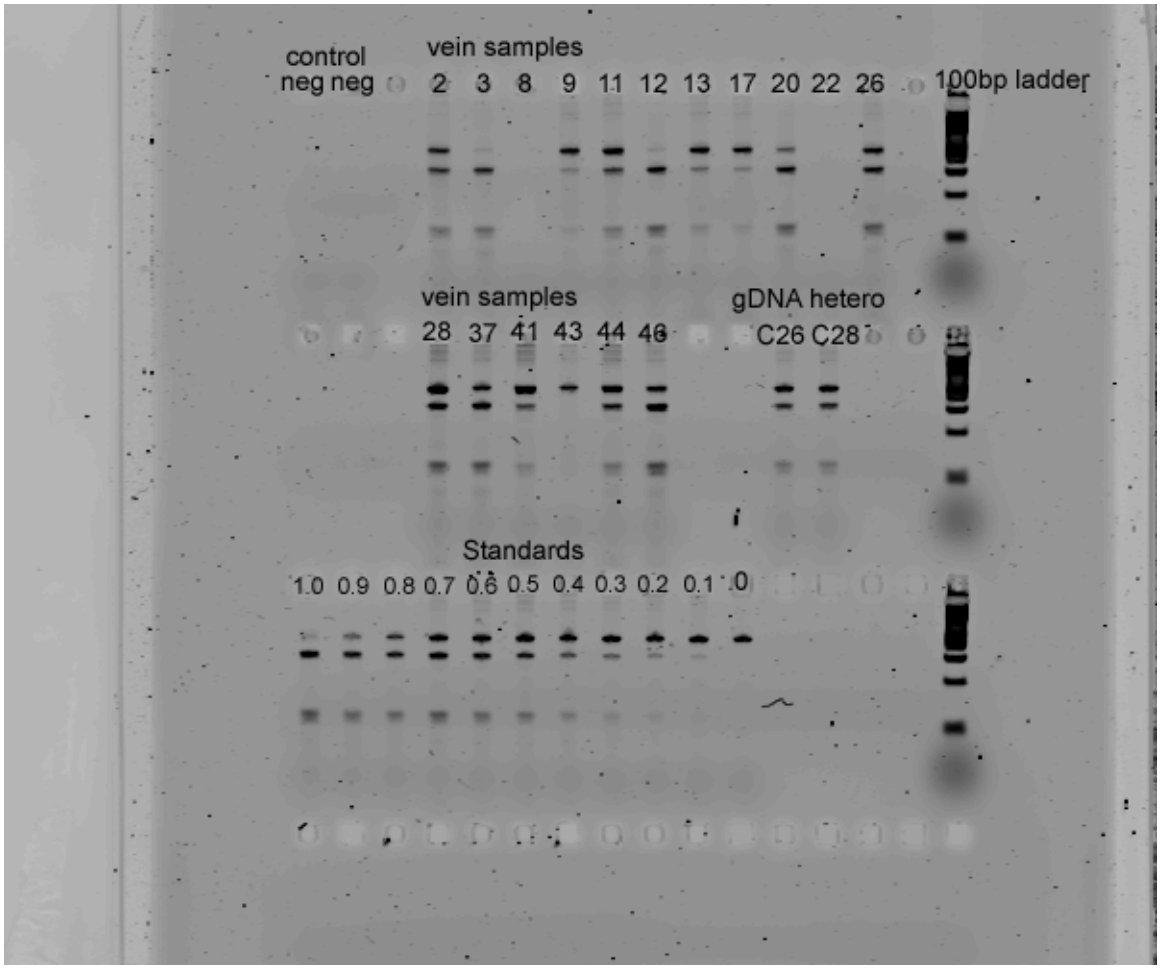


Figure 3-16: Acrylamide gel 5% with ethidium bromide stain: Example of one of the repeat nested PCR/restriction digest products for vein cDNA. Negative (neg) controls and heterozygous (hetero) gDNA samples are also included. Numbers 1.0-0 represent the standard homozygous ratio.

All values for the standard curve samples were plotted on a graph and a linear trend line was fitted using Excel software. The average values of band fluorescence intensity for the two genomic DNA samples were then plotted on the graph using the calculated trend line equation. The mean calculated allelic ratio for the gDNA

samples was 0.53 (see Figure 3-17). (Data see Table_Apx C-43 and Table_Apx C-44)

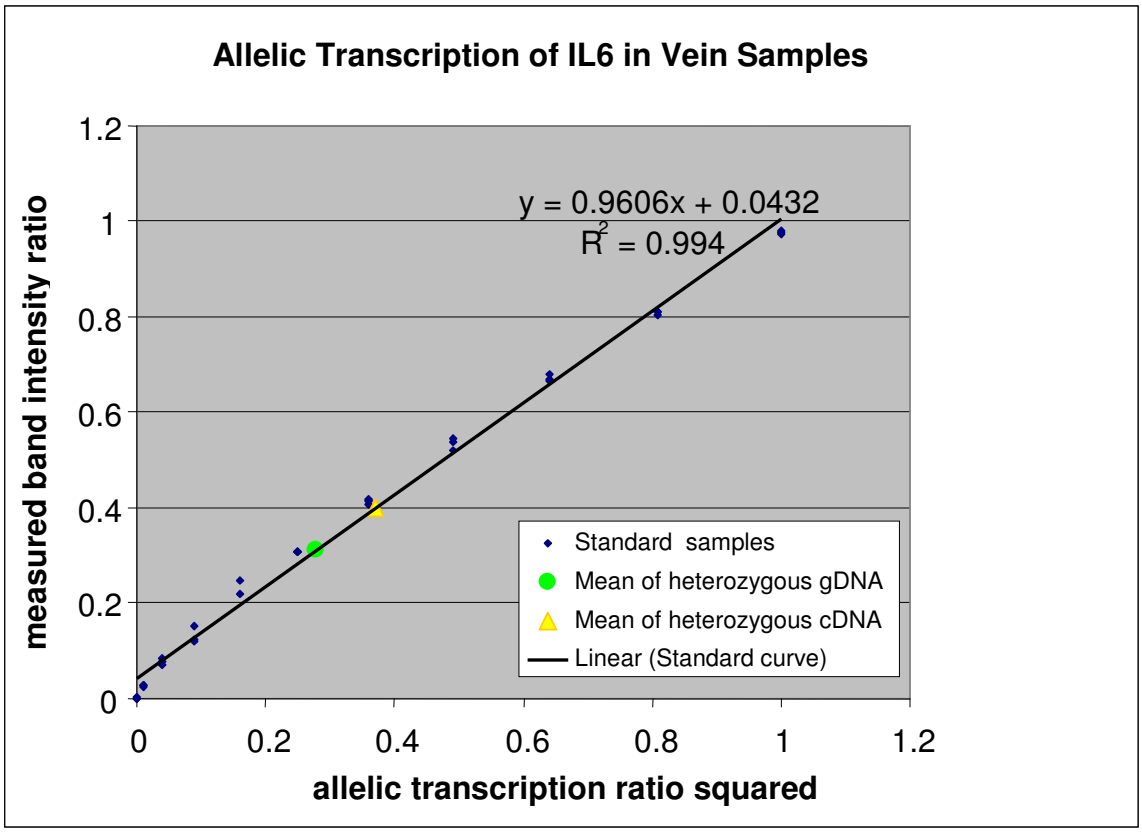


Figure 3-17: Graph showing standard curve and mean result for two heterozygous gDNA samples as well as the mean value for vein samples. The mean of the vein samples falls to the right of the mean for gDNA indicating that the C-allele was preferentially transcribed.

The mean allelic transcription ratio for all the vein samples was 0.54 (SEM±0.05). This was not significantly different from the calculated allelic ratio for the genomic DNA samples 0.53 (Mann Whitney U test p=0.78) (for statistical analysis see Table_Apx C-1).

When individual tissue samples are plotted on this curve, the results were widely spread (see Figure 3-18).

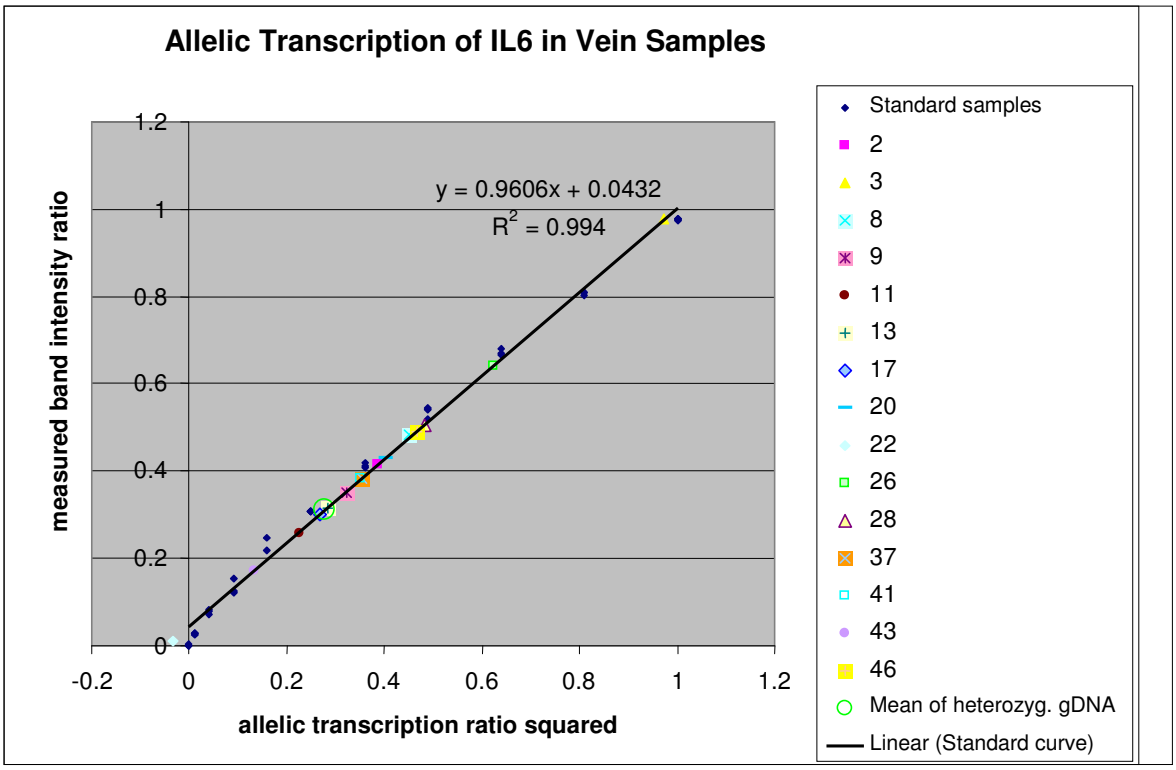


Figure 3-18: Graph showing standard curve and result for the mean of two heterozygous gDNA samples as well as individual values for the vein samples. The vein samples cluster to the right of the value for gDNA indicating that in the majority of samples the C-allele was preferentially transcribed.

To assess the reproducibility of the measurements for the individual samples, the mean of the 3 PCRs was plotted and the 95% confidence intervals were calculated (see Figure 3-19).

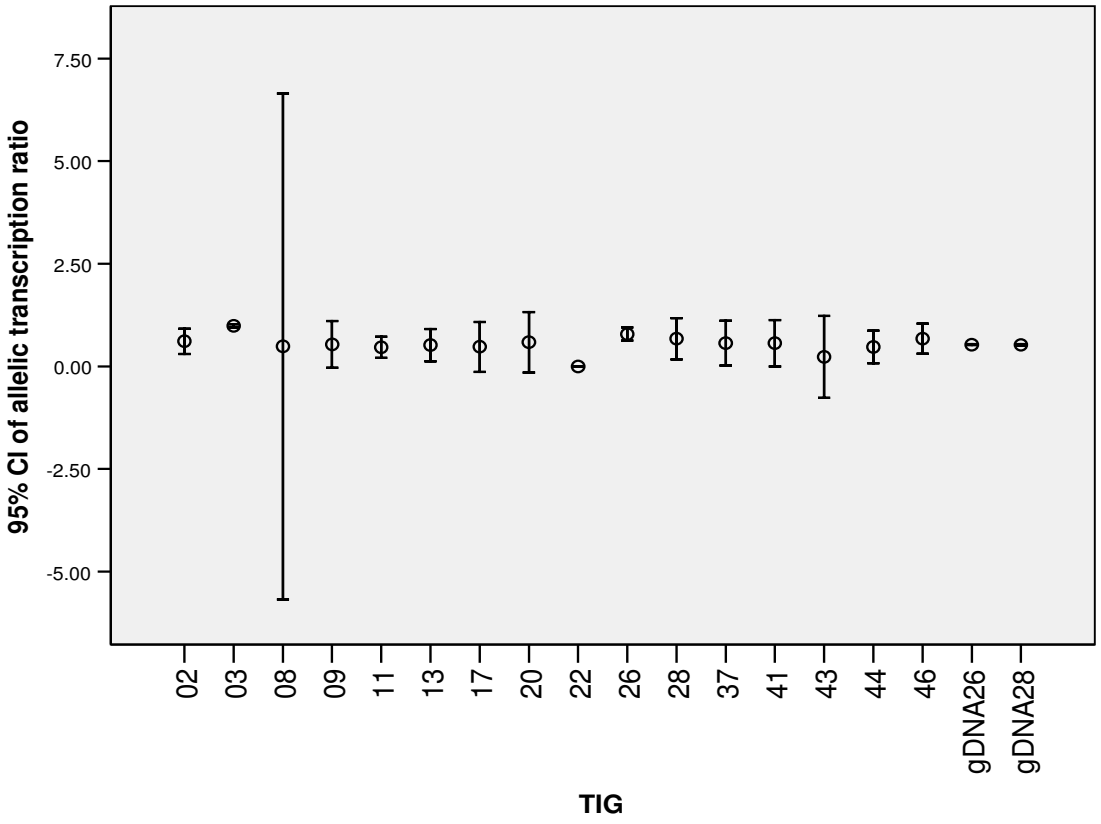


Figure 3-19: Mean allelic transcription ratio for individual vein samples (TIG sample numbers) and results for two heterozygous gDNA samples. The error bars indicate the 95% confidence interval (CI). CIs for gDNA are narrow indicating good reproducibility with minimal variation. CIs in the majority of cDNA samples are wide indicating poor reproducibility of results.

CI for gDNA were narrow consistent with the reproducibly results observed for gDNA. Though some showed good reproducibility CI for the majority of cDNA samples were wide, demonstrating the poor reproducibility of individual measurements. The mean variance for individual samples was 0.07.

3.8.8 Allelic Transcription Assay - Leukocytes

All eight RNA samples appeared clear of cDNA contamination, with no visible amplification in LDLR promoter PCR (see Figure 3-20).

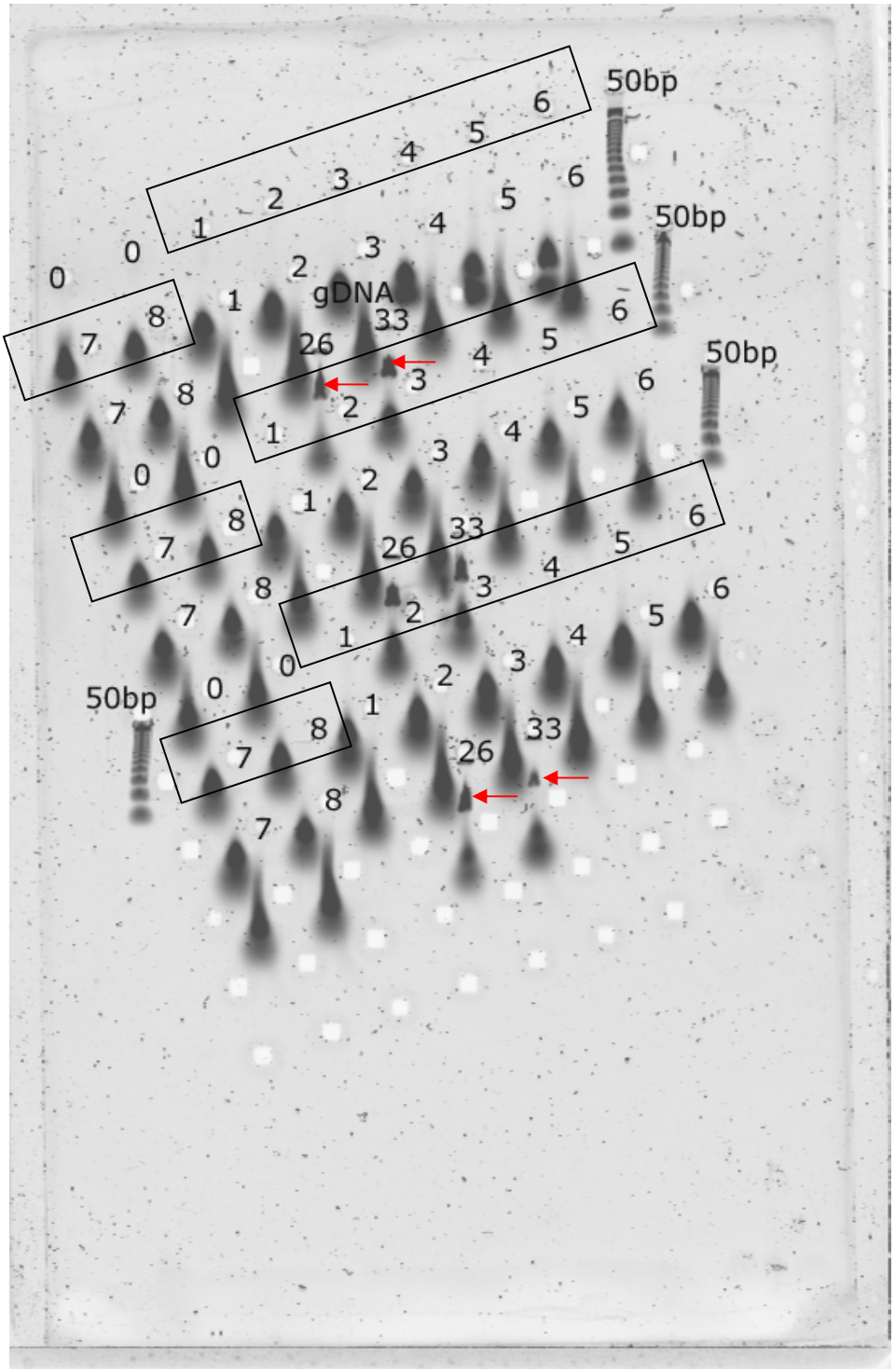


Figure 3-20: Result of LDL-R promoter PCR amplification on an acrylamide Gel (5%) stained with vistra green stain: 0=negative control, 1-8 = leukocyte, 1-8 = leukocyte, 26 and 33 = genomic DNA (positive control). A 50bp marker is included on the gel. Bands are seen only in the positive control (arrows) indicating cDNA samples are free of gDNA contamination.

The result of the nested PCR and digest are shown on the gel below (see Figure 3-21). (Results of ratiometric analysis see Table_Apx C-42)

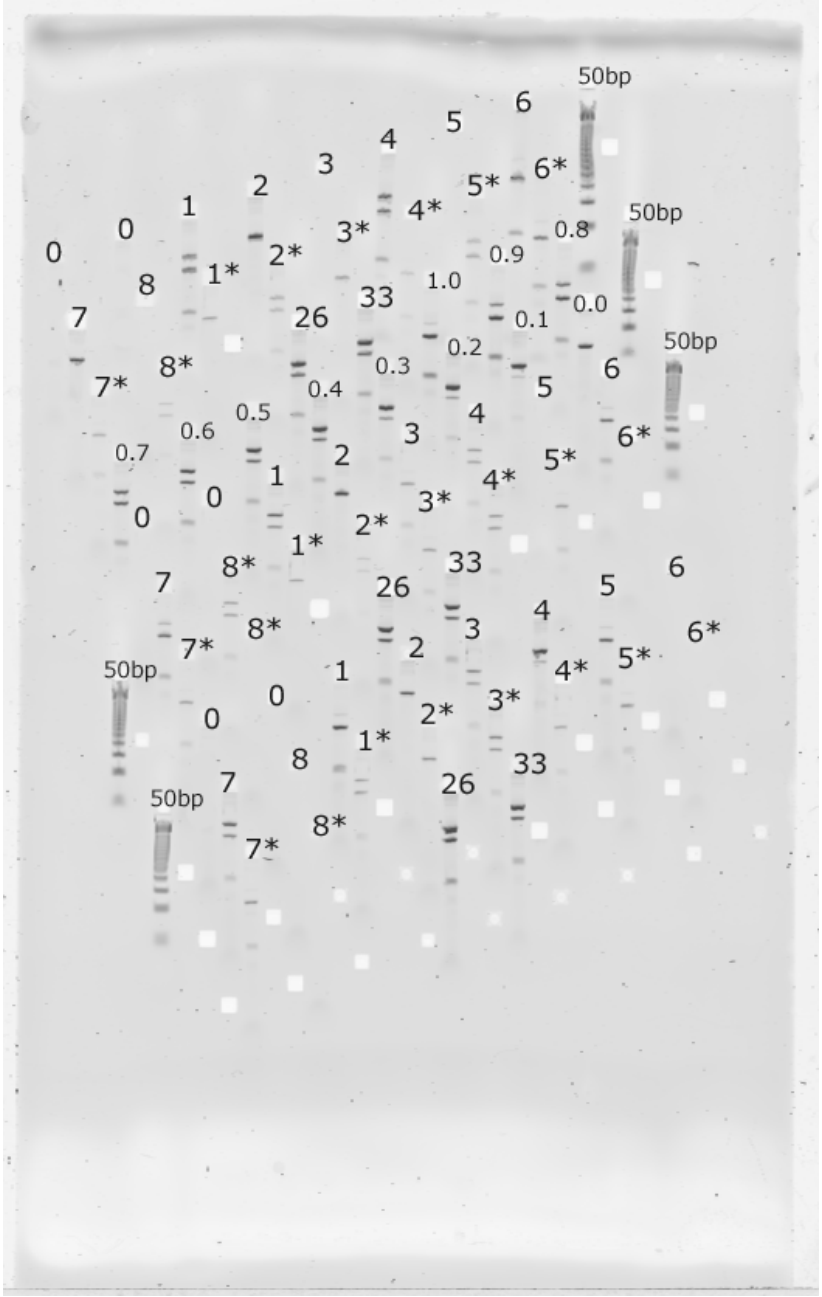


Figure 3-21: Result of leukocyte cDNA amplification with nested PCR and digest described in section 2.2.8.12: Samples (1-8) = cDNA Ex4, samples (1*-8*) = cDNA In4, heterozygous genomic DNA samples 26 and 33 as well as standards (1.0-0.0) and negative control=0 are included on the gel. Two different types of cDNA were used as template to see if the reproducibility of the assay could be improved.

Out of the 16 samples all three PCRs gave a valid result in 10 cases, two PCRs worked for two samples and four samples only showed amplification in one PCR. The mean allelic transcription value of all cDNA samples was 0.65 (SEM±0.08). The mean calculated allelic ratio for gDNA was 0.56 (see Figure 3-22). This suggests that the C-allele was preferentially transcribed.

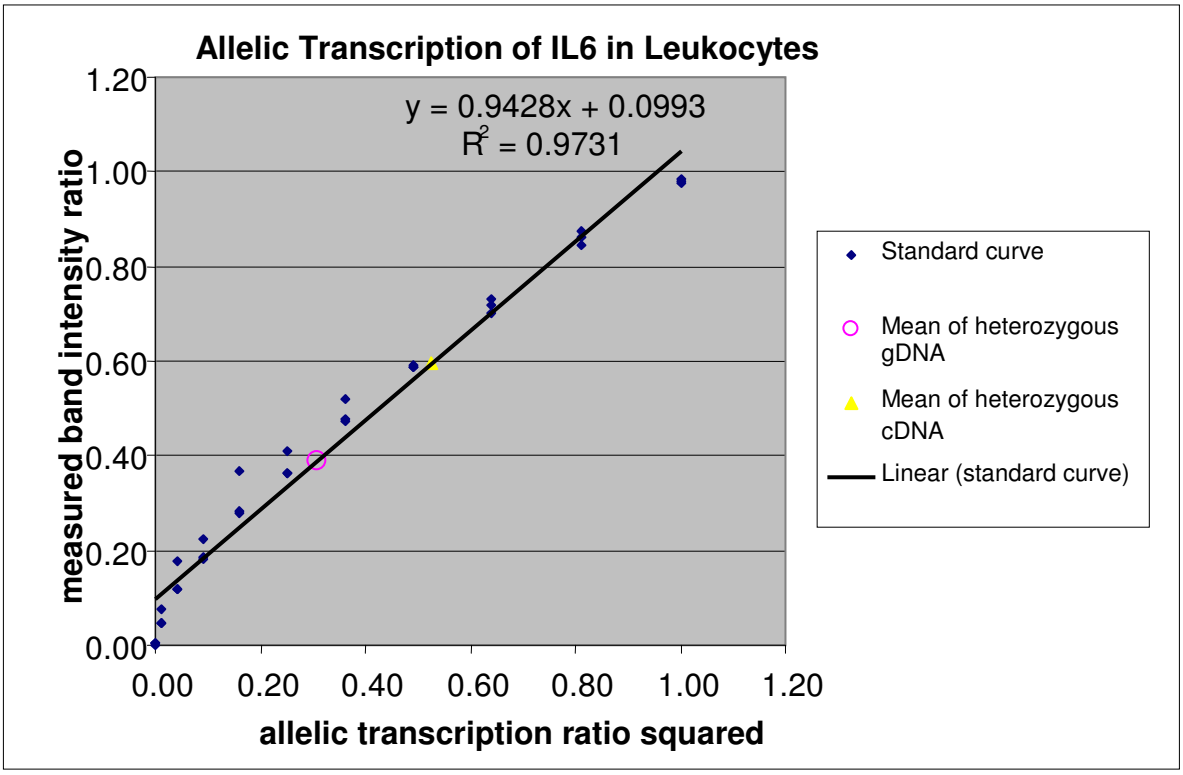


Figure 3-22:Graph showing standard curve and mean result for the heterozygous gDNA samples as well as the mean value for the leukocyte cDNA samples.

When plotted independently on the standard curve, mean values of the three PCRs predominantly fell to the right of the values for gDNA i.e. indicating greater transcription from the C-allele compared to the G-allele in the majority of cases (see Figure 3-23:).

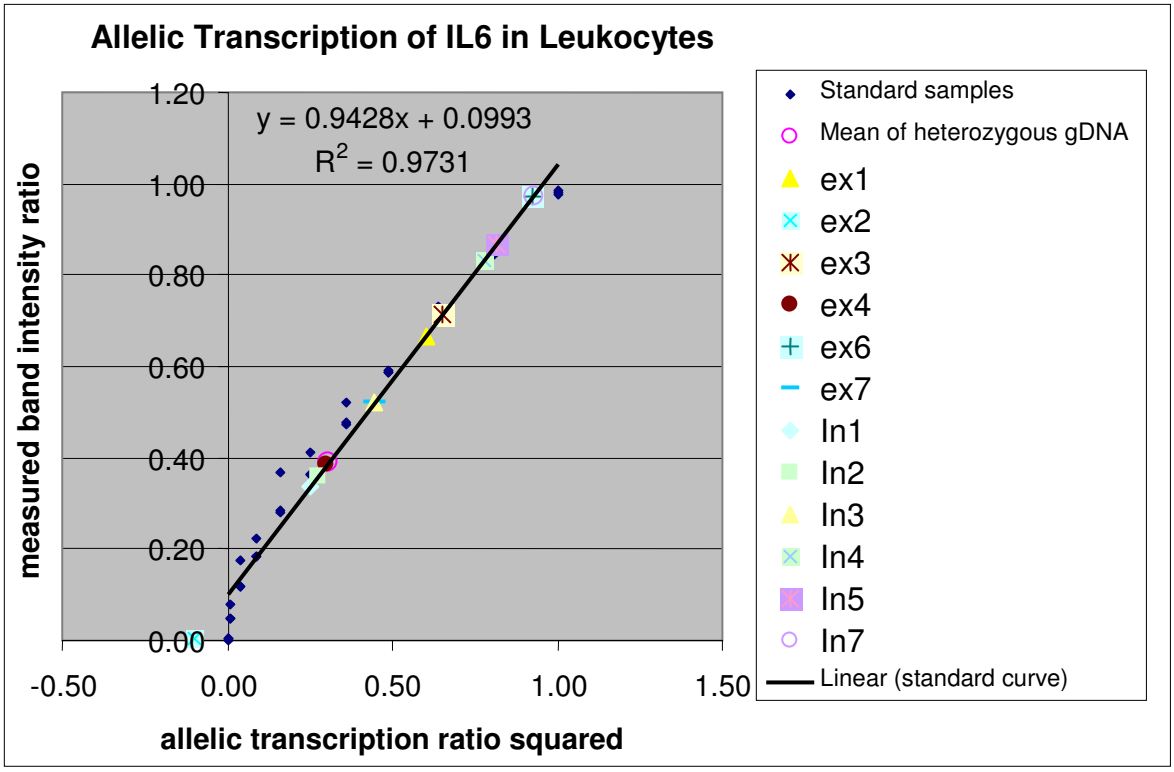


Figure 3-23:Graph showing standard curve and mean result for heterozygous gDNA as well as individual values for the leukocyte cDNA samples.

The Mann Whitney test showed that the mean calculated allelic transcription ratio for the cDNA samples did not differed significantly form the value obtained for genomic DNA $p<0.47$ (see Table_Apx C-2).

To assess variation between the three PCRs carried out for one cDNA sample, the 95% CI was calculated for individual cDNA samples, and plotted on a graph (Only samples with at least two valid PCRs included) (see Figure 3-24).

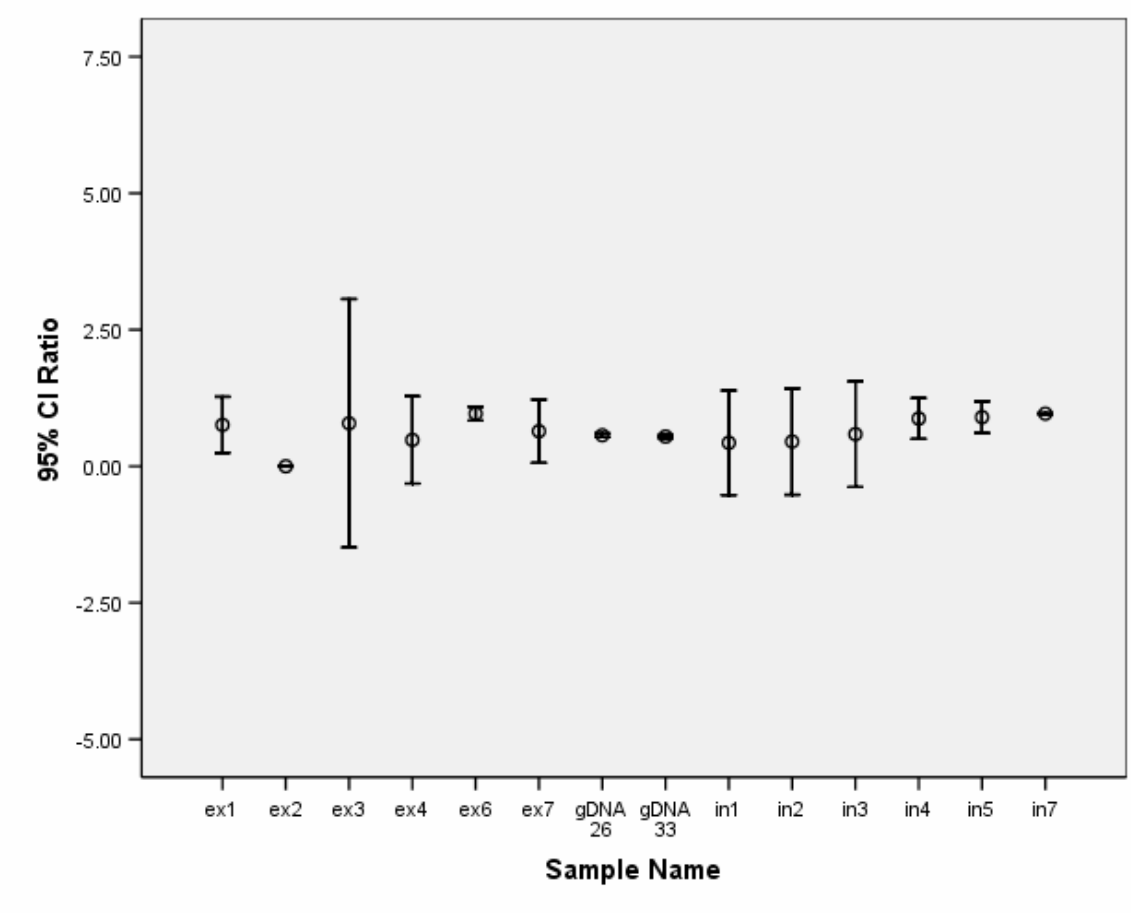


Figure 3-24: Graph showing the allelic transcription ratio for individual leukocyte samples as well as results for heterozygous gDNA samples. The bars indicate the 95% confidence interval

Low inter-assay variability was demonstrated for gDNA samples, with narrow 95% CI. The CI for leukocyte cDNA samples remained wide, indicating high inter-assay variability. Mean variance for cDNA Ex4 and cDNA In4 were 0.04 and 0.08 respectively, i.e. assay reproducibility was not improved by annealing the reverse primer in an intron region for cDNA synthesis. The between sample variance was 0.08.

3.9 Ex Vivo IL6 Expression in Different Tissues

3.9.1 Subjects

All patients were of white British origin. In 11 out of the 32 patients (128 tissue samples) at least one of the tissues failed to give real time quantification data, despite repeated attempts. Normalised concentration ratios were available for all four tissues (complete tissue complement) in 21 patients. In order to avoid inter-individual confounding variables distorting the final result, only patients with relative real time quantification data for all four tissue types were include in the analysis. The mean age of included patients was 48 years (range 26-69 years) and the mean body mass index was 26.7kg/m^2 (range 22-33kg/m^2). Demographic data of included patients is listed in Table 3.9-1.

Table 3.9-1: Demographic data for the 21 subjects with complete tissue complements.

	Number
Subjects	21
Smoking Status	
non-Smoker	14
smoker	7
Gender	
male	9
Female	12
Medication	
nil	10
Oral Contraceptive	2
Amitriptyline	2
Ca Channel Blocker	1
Diuretics	1
Aspirin	1
Ventolin	1
Omeprazole	1
Celest	1
Allopurinol	1

3.9.2 Standard Curves

Standard curves for GAPDH showed good replicates for all dilutions (see Figure 3-25).

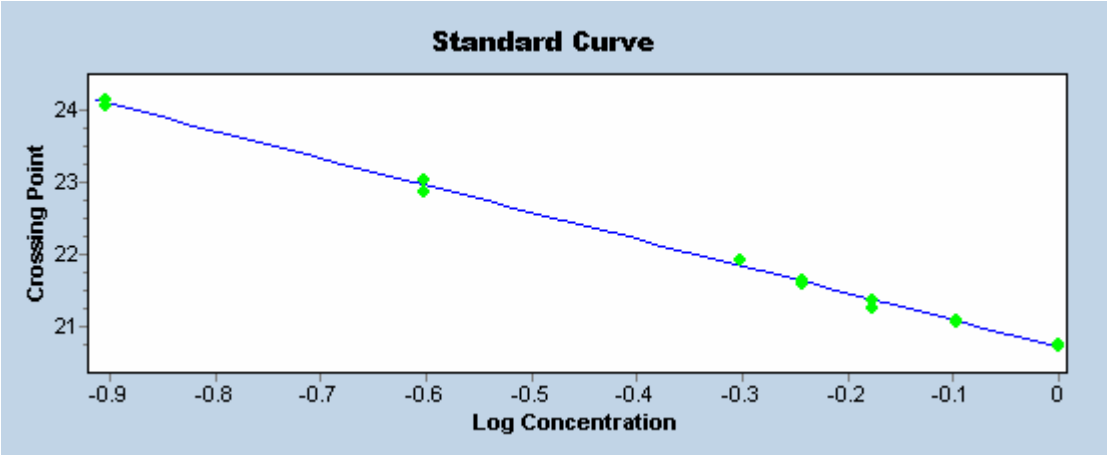


Figure 3-25: GAPDH standard curve for Leukocyte cDNA shows good reproducibility of the assay with replicate measurement for different dilutions showing minimal variability.

The IL6 standard curves showed greater variability in the repeats than the GAPDH. Efficiency of the IL6 assay also tended to be less then the efficiency of the GAPDH assay, as shown by the steeper gradient of the GAPDH standard curve (see Figure 3-26).

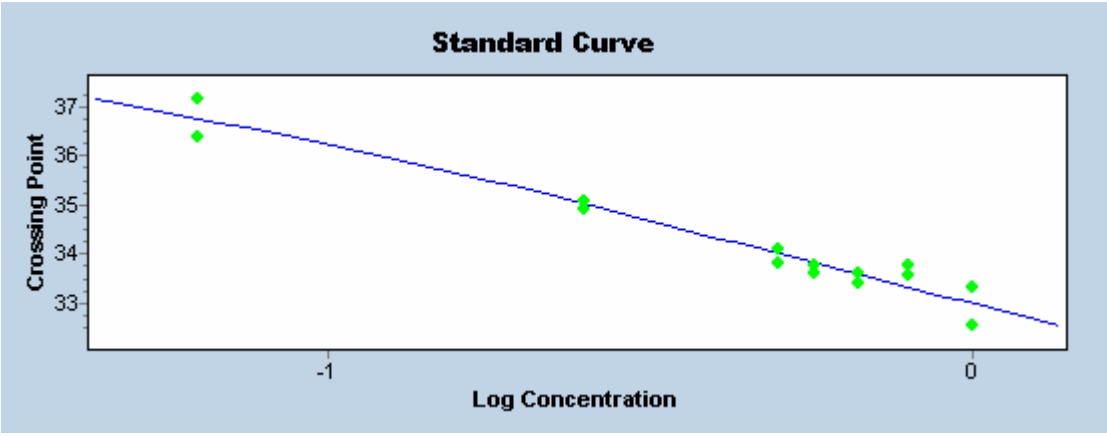


Figure 3-26: IL6 standard curve from Leukocyte cDNA.

3.9.3 RT-PCR Amplification Curves

The RT-PCR amplification curves of individual tissues are shown below (see Figure 3-27, Figure 3-28, Figure 3-29 & Figure 3-30). These graphs show the change in fluorescence i.e. product amplified per PCR cycle. In the figures the top graph shows the IL6 assay amplification curves. The bottom graph shows the reference amplification curve for the same samples. The majority of reactions in the IL6 assay enter the exponential phase of amplification around cycle 32 - 34 (C_p-value); however several samples have C_p values as low as 26 in the adipose tissue assay. The GPDH assay shows mean $C_p$values of 28 with values as low as 19 for some samples.

Both GAPDH and IL6 assay appeared to work reliably for the majority of samples. C_p-values for GAPDH tended to be lower than for IL6, indicating lower expression of IL6 compared to the housekeeping gene.

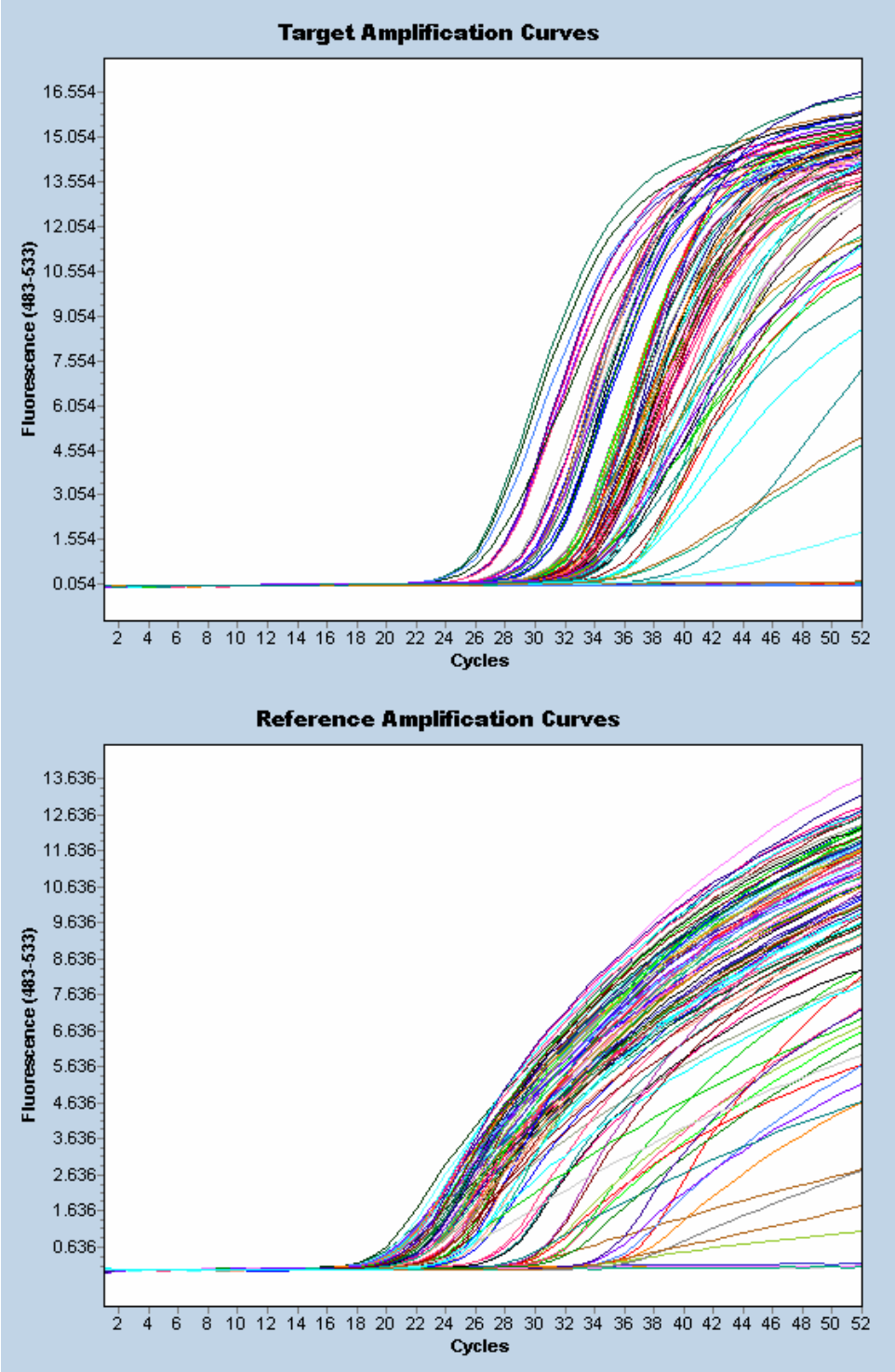


Figure 3-27: These graphs show the change in fluorescence i.e. product amplified per PCR cycle. The top figure shows the IL6 assay amplification curves of adipose tissue. The bottom figure shows the reference amplification curve for the same samples. The majority of reactions in the IL6 assay enter the exponential phase of amplification around cycle 32 (C_p-value), however several sample have C_p values as low as 26. The GPDH assay shows mean $C_p$values of 28 with values as low as 19 for some samples.

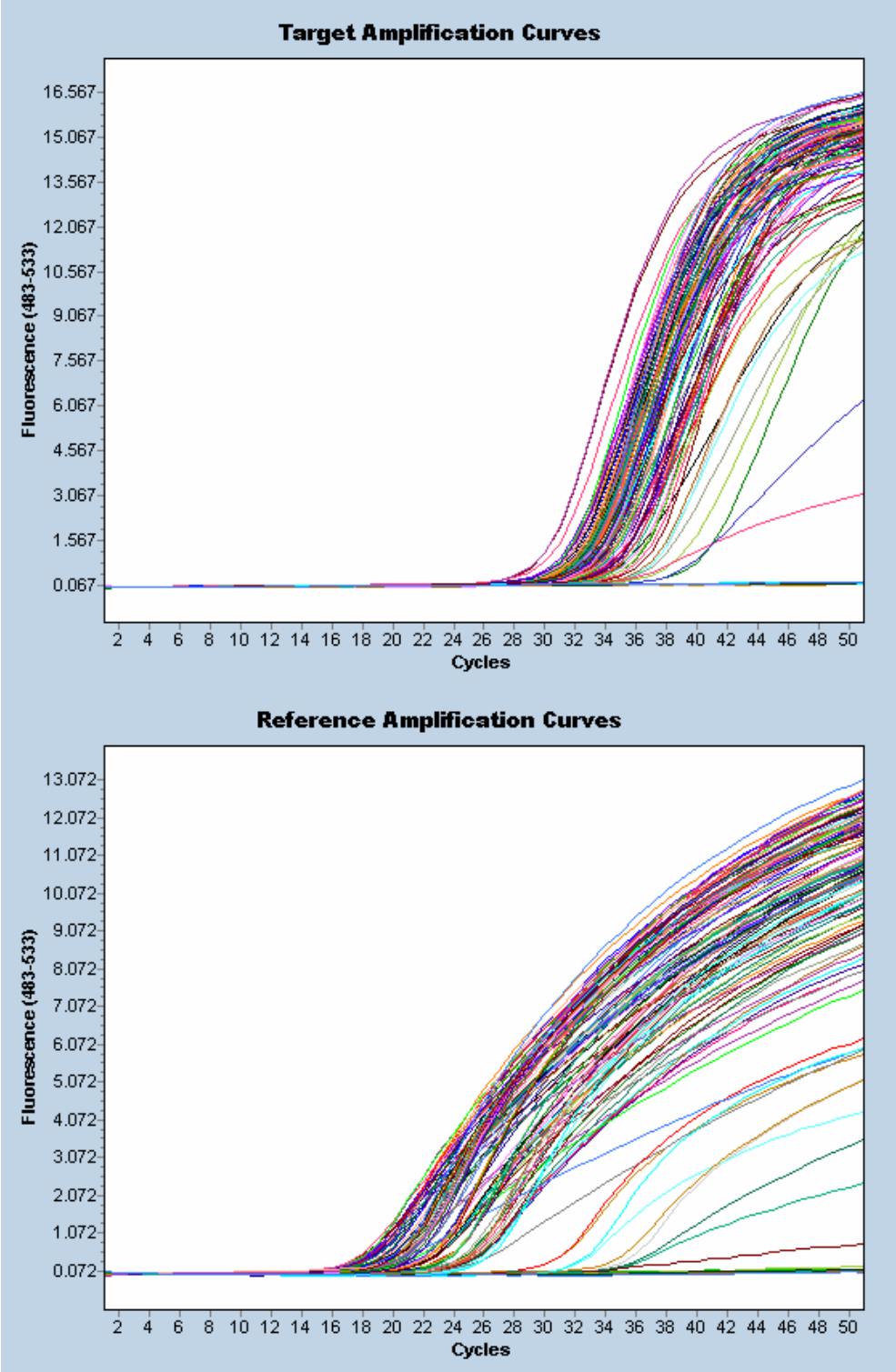


Figure 3-28: RT PCR curves for GAPDH (reference gene) and IL6 (target gene) for vein samples: The graphs show the change in fluorescence i.e. product amplified per PCR cycle. The top figure shows the IL6 assay amplification curves. The bottom figure shows the reference amplification curve.

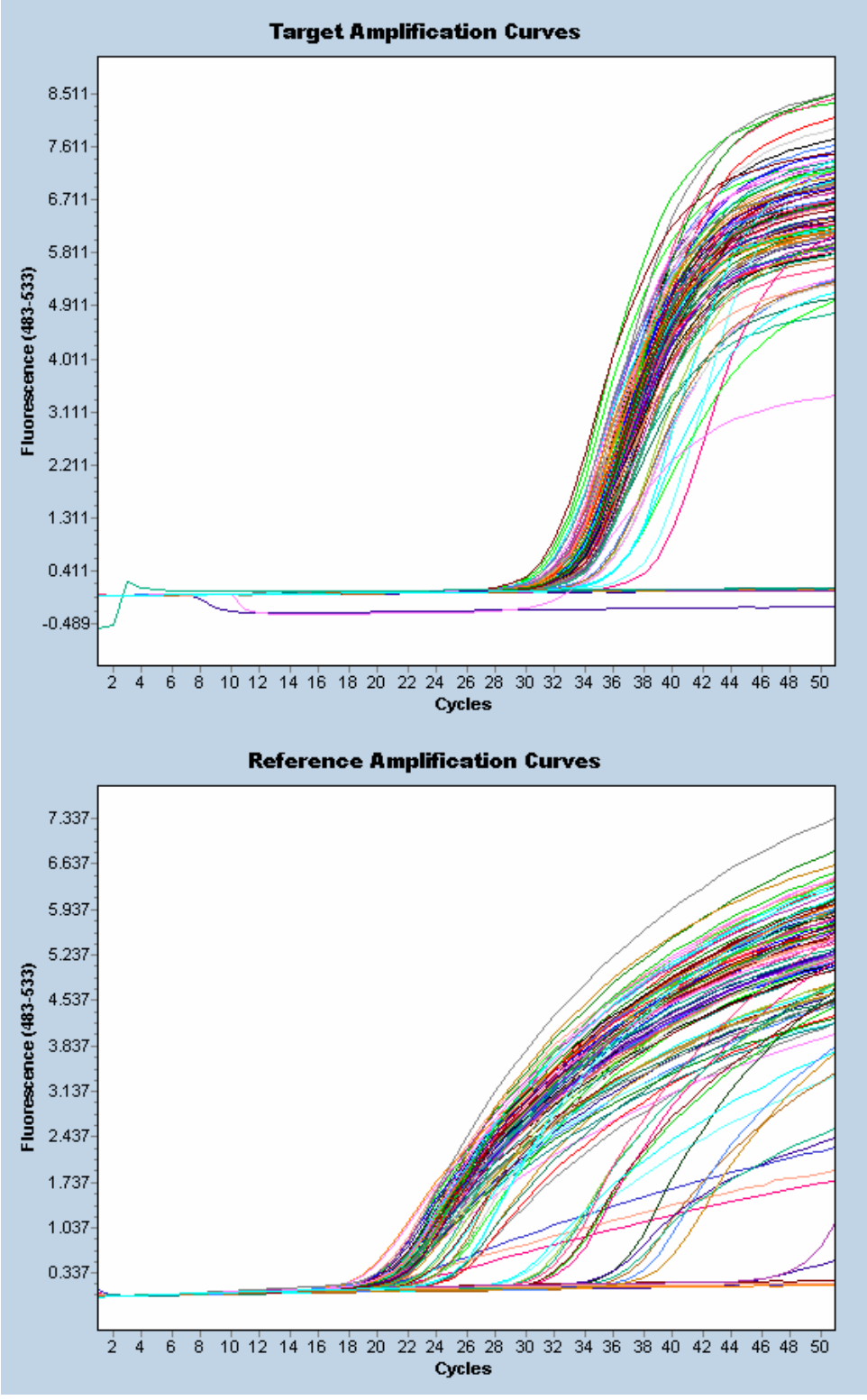


Figure 3-29: RT PCR curves for GAPDH (reference gene) and IL6 (target gene) for leukocyte samples. The graphs show the change in fluorescence i.e. product amplified per PCR cycle. The top figure shows the IL6 assay amplification curves. The bottom figure shows the reference amplification curve.

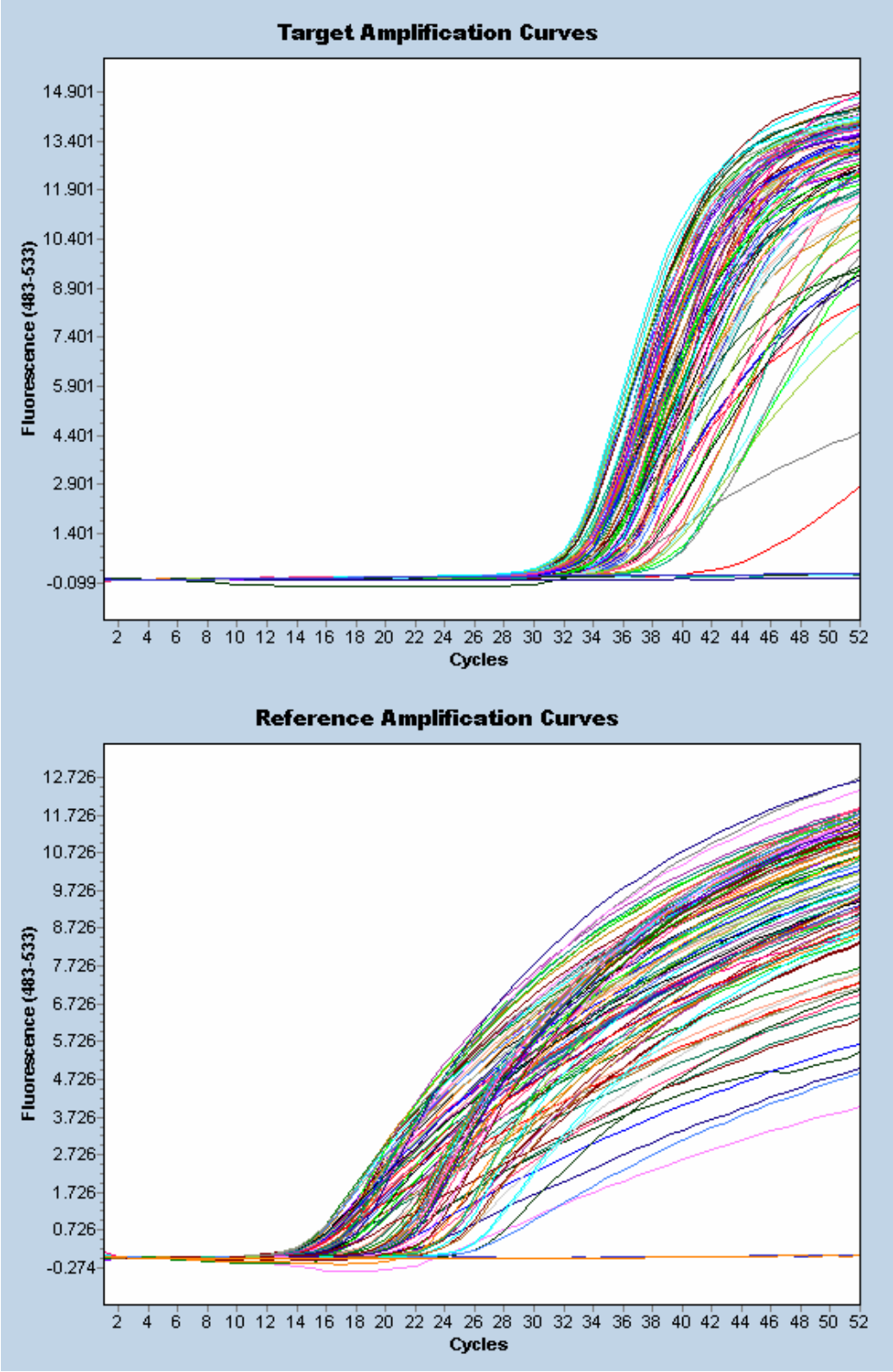


Figure 3-30: RT PCR curves for GAPDH (reference gene) and IL6 (target gene) for skeletal muscle samples. The graphs show the change in fluorescence i.e. product amplified per PCR cycle. The top figure shows the IL6 assay amplification curves. The bottom figure shows the reference amplification curve.

3.9.4 Relative Quantification of IL6 mRNA Expression

The measured relative IL6 mRNA expression in adipose tissue, leukocytes, vein tissue and muscle tissue is given below (see Table 3.9-2). Values were normalised to a calibrator sample which was included in each LightCycler system run. Individual runs may vary in efficiency leading to erroneous results. The calibrator sample provides a constant calibration point between individual LightCycler system runs thereby reducing this error.

Table 3.9-2: Normalised concentration ratio of IL6 mRNA in four different tissues in 21 patients (TIG X).

	normalised concentration ratio by tissue type			
Patient identification number	**adipose tissue**	**leukocytes**	**vein**	**muscle**
TIG 010	14.9	1.49	0.4	0.01
TIG 014	2.49	0.76	0.24	1.42E-04
TIG 015	1.08E+02	1.81	7.56	0.03
TIG 017	98.16	0.72	0.82	0.02
TIG 018	3.03E+02	6.11	15.52	0.72
TIG 019	13.23	0.4	5.21	0.09
TIG 021	2.77E+02	10.35	3.66	0.72
TIG 024	38.42	0.47	0.38	0.04
TIG 025	6.17	1.37	1.63	0.04
TIG 028	1.74E+02	0.46	0.66	0.02
TIG 029	27.61	0.83	0.24	0.04
TIG 030	21.85	1.52	4.34	0.08
TIG 031	2.38	0.6	1.28	0.02
TIG 034	11.16	1.53	0.22	0
TIG 035	5.8	0.9	1.65	4.84E-03
TIG 036	16.63	0.66	0.64	0.04
TIG 037	4.6	0.64	0.16	7.89E-03
TIG 038	57.38	0.24	0	0.04
TIG 041	15.07	0.35	0.23	0.03
TIG 043	2.48	0.85	0.41	0.05
TIG 046	0	0.43	0.9	0.04

Mean normalised concentration ratio of IL6 mRNA compared to the housekeeping gene GAPDH was 57.2 (SEM $\pm$ 19.4) [median=15.1] for adipose tissue; 1.6 (SEM $\pm$ 0.5) [median=0.8] for leukocytes; 2.2 (SEM $\pm$ 0.8) [median=0.7] for vein and 0.1 (SEM $\pm$ 0.05) [median=0.04] for muscle.

Median and mean relative expression of IL6 mRNA was significantly higher in adipose tissue than in all other tissues (Mann Whitney U: (two-tailed) p< 0.001; independent t-Test: (two-tailed) p≤ 0.001) (see Figure 3-31). Expression in vein and leukocytes was also significantly higher than in muscle tissue (Mann Whitney U: (two-tailed) p< 0.001; independent t-Test: (two-tailed) p≤ 0.001). There was no significant difference between vein tissue and leukocytes (Mann Whitney U: (two-tailed) p=0.55; independent t-Test: (two-tailed) p=0.93). (For statistically analysis please see Table_Apx C-20 to Table_Apx C-31.)

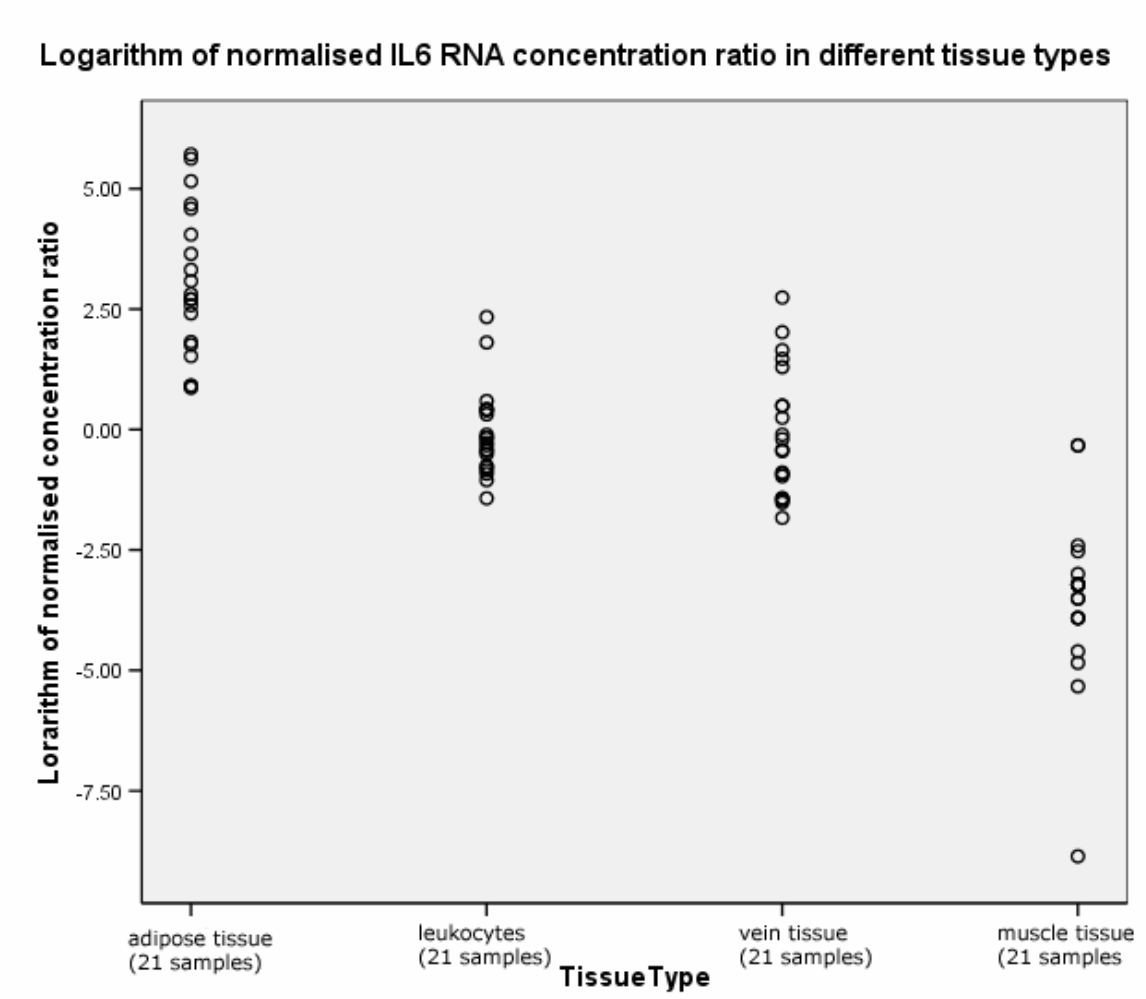


Figure 3-31: Individual values for logarithm of normalised concentration ratio of IL6 RNA expression in different tissues. There are 21 samples represented in each tissue type. IL6 expression data were logarithmically transformed for this graphic representation to allow better visualisation of individual values.

These findings were the result of comparing the mean IL6 RNA expression between tissues in a small sample number. Such a comparison is susceptible to skew from outlying values in some patients in one or more tissue types. An intra-individual comparison between tissue types is less likely to be skewed by outlying values (see Figure 3-32). The relative expression in different tissues in one individual was therefore examined using the Wilcox signed rank test. Figure 3-2 shows that in all individuals adipose tissue has the highest IL6 RNA expression of all four tissue types. Fat expression was on average 55.5 [median=25.2]; 47.5 [median=22.8] and 2178.3 [median=542.7] times higher than IL6 expression in leukocytes, vein and muscle respectively in individuals. Leukocytes and vein expressed 304.6 [median=19.9] and 136.5 [median=27.8] times more IL6 mRNA than muscle in individuals.

The difference in IL6 mRNA expression was statistically significant in fat when compared to all other tissues examined in that subject. (Wilcox signed rank test $p<0.001$). Vein and leukocytes also expressed significantly more IL6 mRNA than muscle (Wilcox signed rank test $p<0.001$). There was no significant difference between the expression in vein and leukocytes (Wilcox signed rank test $p=0.72$) Table_Apx C-32.

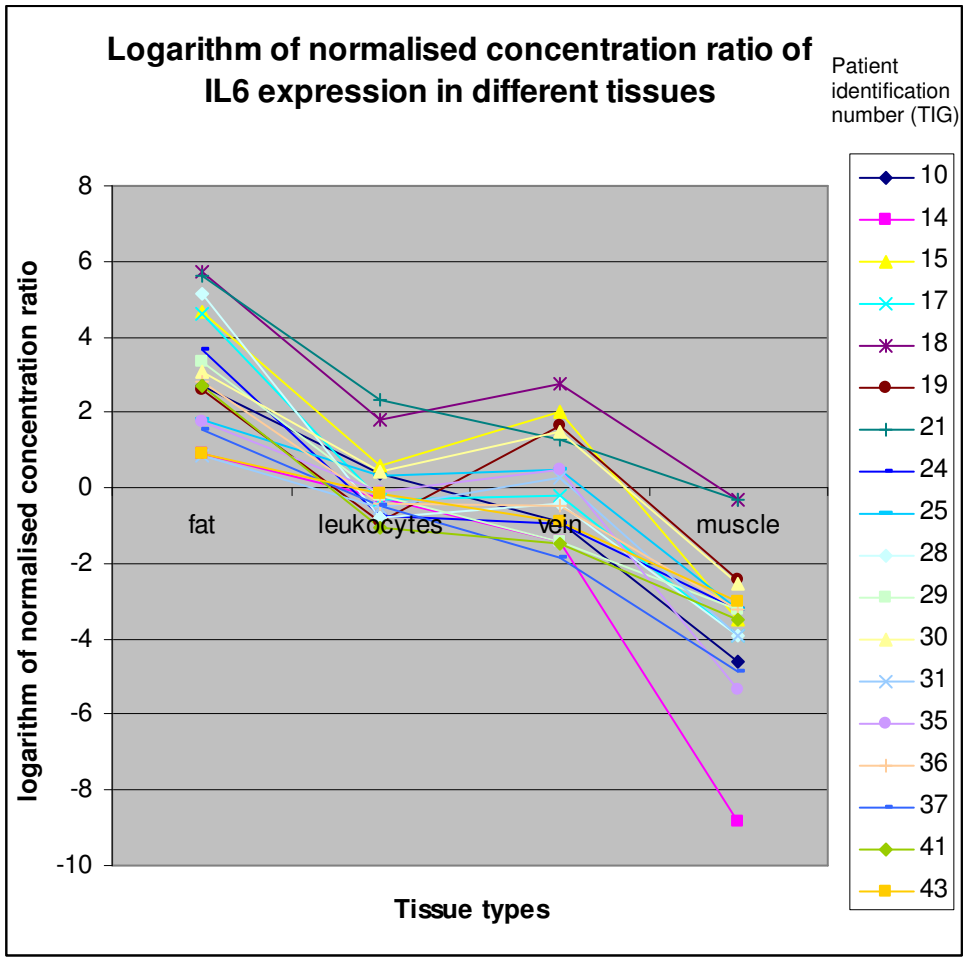


Figure 3-32: Logarithm of normalised concentration ratio of IL6 mRNA for individual patients in all four tissue types. Data were logarithmically transformed for this graphic representation to allow better visualisation.

3.9.4.1 Correlation between BMI and relative IL6 RNA Expression in Adipose Tissue

There was no significant correlation between patients BMI and IL6 mRNA expression (p=0.12) in adipose tissue (see Figure 3-33).

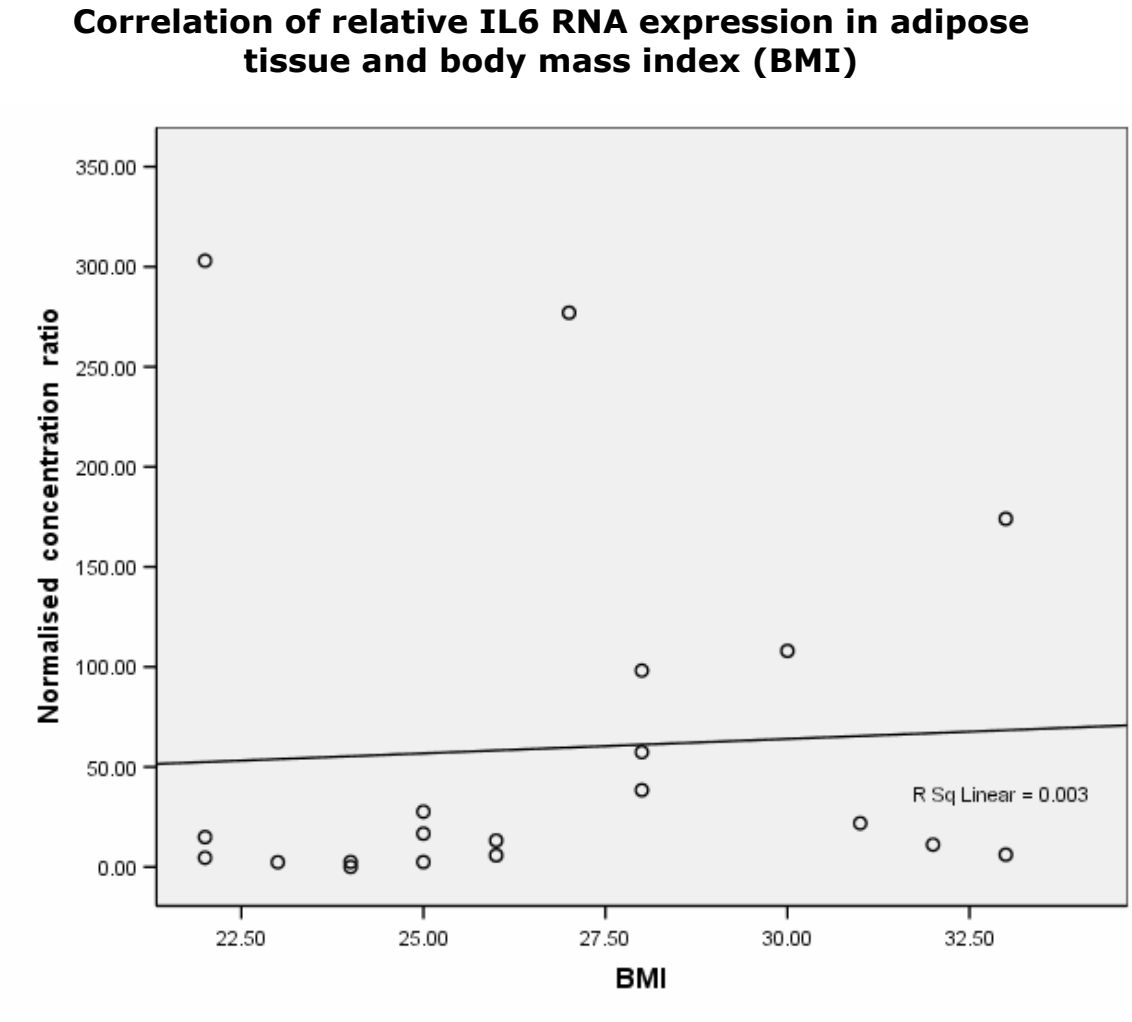


Figure 3-33: This graph shows the correlation between measured relative IL6 mRNA expression in adipose tissue (normalised concentration ratio) and BMI of the patient. No significant correlation is shown.

3.10 Relative Expression of IL6 in Different Tissues with Regards to SNP G-174C Genotype

3.10.1 Results for Adipose Tissue

Of the 42 samples examined 10 gave an invalid result, i.e. 32 samples were analysed. The genotype distribution of the 32 samples is given in Table 3.10-1.

Table 3.10-1: genotype distribution in the adipose tissue samples

Adipose tissue:		
Genotype	**Frequency**	**Percent**
C/C	6	18.8
G/C	11	34.4
G/G	15	46.9
Total	**32**	**100.0**

The mean expression of IL6 mRNA was highest in the CC genotype group and lowest in the GG group (see Table 3.10-2). The mean normalised relative IL6 mRNA concentration ratio was 173.71 (SEM±109.92) for the CC genotype, 43.39 (SEM±18.37) for the GC genotype and 28.40 (SEM±35.44) for the GG group. Large inter-individual variation was seen in the expression of IL6 mRNA, relative expression ranging between 3.91 and 671.00 for the CC genotype, zero to 174.00 for the GC genotype and 1.12 to 108.00 for the GG genotype (see Figure 3-34). The CC group expressed on average 4.0 times as much IL6 mRNA as the GC genotype and 6.1 times as much as the GG genotype (see Table 3.10-2).

Table 3.10-2: IL6 mRNA normalised concentration ratio in different genotypes in adipose tissue.

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
CC	6	173.7117	269.24805	109.92006	-108.8468	456.2702	3.91	671.00
GC	11	43.3864	60.95622	18.37899	2.4354	84.3373	.00	174.00
GG	15	28.3993	35.43978	9.15051	8.7734	48.0252	1.12	108.00
Total	32	60.7972	128.61417	22.73599	14.4268	107.1675	.00	671.00

The normalised concentration ratio was non-normally distributed (Kolmogorov Smirnov Z=1.81, asympt. sign. Two tailed 0.003), therefore non-parametric tests were employed to assess differences in expression.

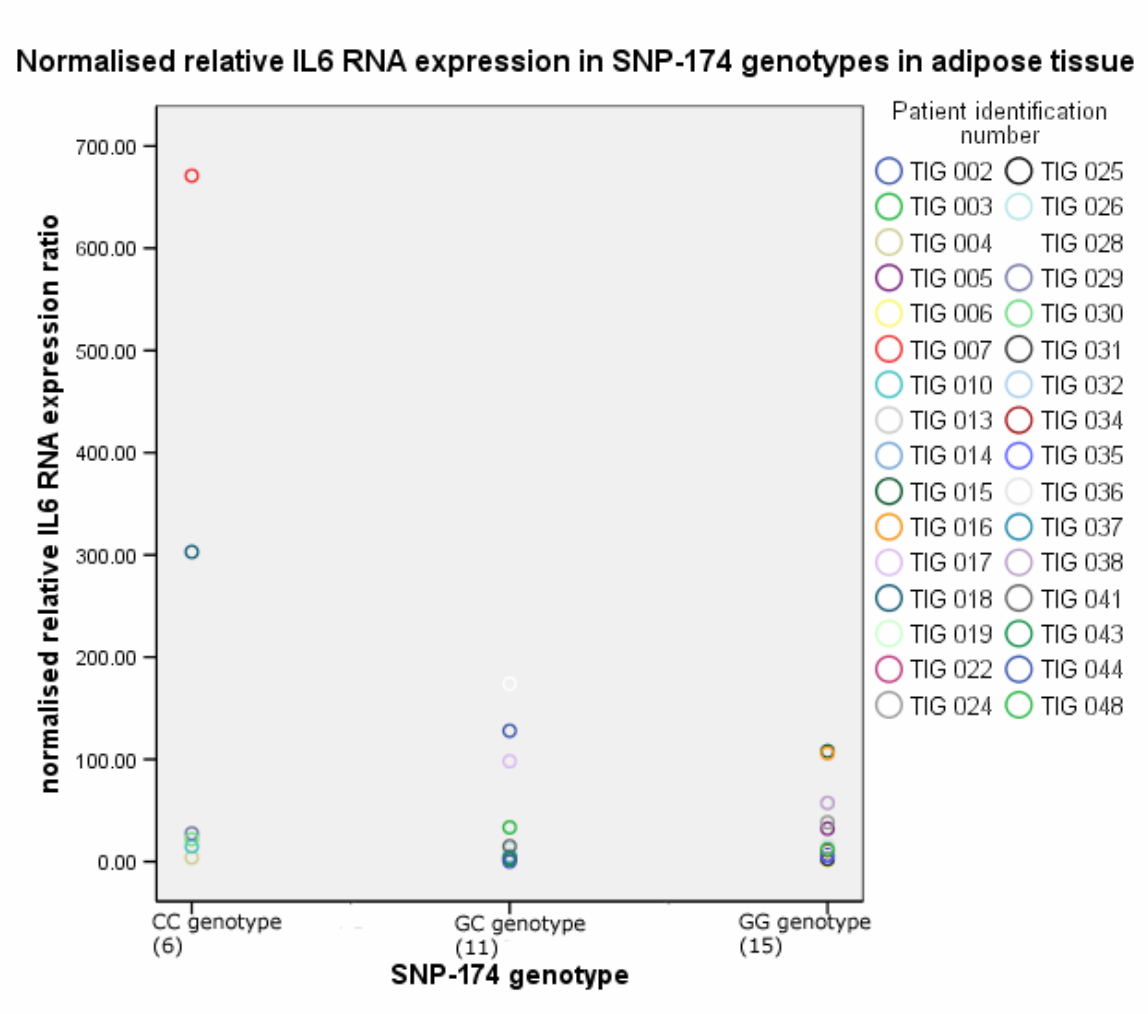


Figure 3-34: The graph shows the distribution of normalised concentration ratio of IL6 RNA expression for the three different genotypes in adipose tissue. The individual coloured circles represent one individual with the patient identification number given in the legend.

The Kruskal Wallis test showed that the differences in IL6 expression between genotype groups in adipose tissue were not significant (p=0.375) (Table_Apx C-3).

3.10.2 Results for Leukocytes

Of the 34 samples included in the study all gave valid results following RT-PCR. See Table 3.10-3 for genotype distribution.

Table 3.10-3: Genotype distribution in Leukocytes.

Leukocytes:		
Genotype	**Frequency**	**Percent**
C/C	5	14.7
G/C	14	41.2
G/G	15	44.1
Total	**34**	**100.0**

The normalised relative IL6 mRNA expression ratio was 2.38 (SEM±0.95) for the CC genotype, 1.29 (SEM±0.58) for the GC genotype and 0.77 (SEM±0.12) for the GG genotype. The range of the IL6 mRNA expression ratio was 0.83 to 6.11 for the CC group, 0.35 to 8.78 for the GC group and 0.24 to 1.81 for the GG group (Table 3.10-4).

Table 3.10-4: IL6 mRNA normalised concentration ratio in different genotypes in leukocytes.

					95% Confidence Interval for Mean			
	N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Minimum	Maximum
CC	5	2.3800	2.12321	.94953	-.2563	5.0163	.83	6.11
GC	14	1.2936	2.17053	.58010	.0403	2.5468	.35	8.78
GG	15	.7653	.48077	.12413	.4991	1.0316	.24	1.81
Total	34	1.2203	1.67349	.28700	.6364	1.8042	.24	8.78

Normalised concentration ratio was non-normally distributed (Kolmogorov Smirnov Z=1.80, asympt. sign. Two tailed 0.003), and non-parametric tests were employed to assess differences in expression.

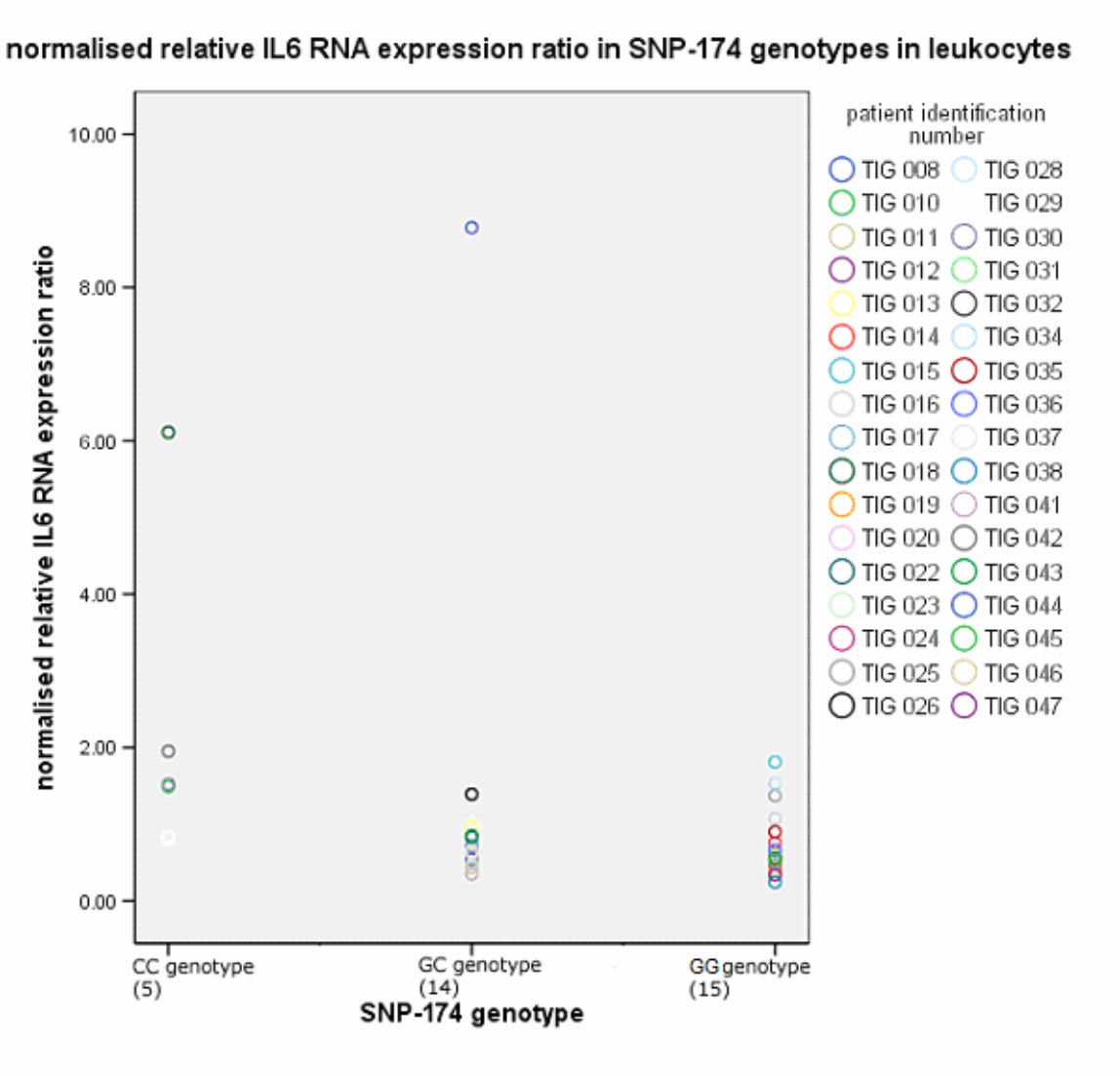


Figure 3-35: Distribution of normalised concentration ratio for the three different genotypes in leukocytes. The individually coloured circle represent one individual of a certain genotype and the patient identification number is given in the legend.

Again mean expression was highest in the CC genotype group and lowest in the GG group (Figure 3-35). The CC genotype expressed 1.8 times more than the GC genotype and 3.1 times more than the GG genotype. The GC genotype group expressed on average 1.7 times more IL6 mRNA than the GG group. The difference between genotype groups was statistically significant (p=0.028) (see Table_Apx C-4).

3.10.3 Results for Vein Tissue

42 samples underwent RT PCR. Four of these had invalid results, leaving 38 samples to be included in the analysis. The genotype distribution in this group is listed in Table 3.10-5.

Table 3.10-5: Genotype distribution in vein samples.

Vein Tissue:		
Genotype	**Frequency**	**Percent**
C/C	7	18.4
G/C	16	42.1
G/G	15	39.5
Total	**38**	**100.0**

The mean normalised relative IL6 mRNA expression ratio was 4.06 (SEM±2.04) for the CC genotype, 0.65 (SEM±0.27) for the GC genotype and 1.53 (SEM±0.54) for the GG genotype. The range of relative IL6 mRNA expression ratio was 0.24 to 15.52 for the CC genotype, zero to 4.54 for the GC genotype and zero to 7.56 for the GG genotype (Table 3.10-6).

Table 3.10-6: IL6 normalised concentration ratio in different genotypes in vein samples.

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
CC	7	4.0614	5.39343	2.03852	-.9267	9.0495	.24	15.52
GC	16	.6463	1.07651	.26913	.0726	1.2199	.00	4.54
GG	15	1.5300	2.08484	.53830	.3755	2.6845	.00	7.56
Total	38	1.6242	2.89354	.46939	.6731	2.5753	.00	15.52

The IL6 expression ratio was non-normally distributed (Kolmogorov Smirnov Z=1.93, asympt. sign. Two tailed 0.001). Non-parametric tests were therefore used to test for significant difference.

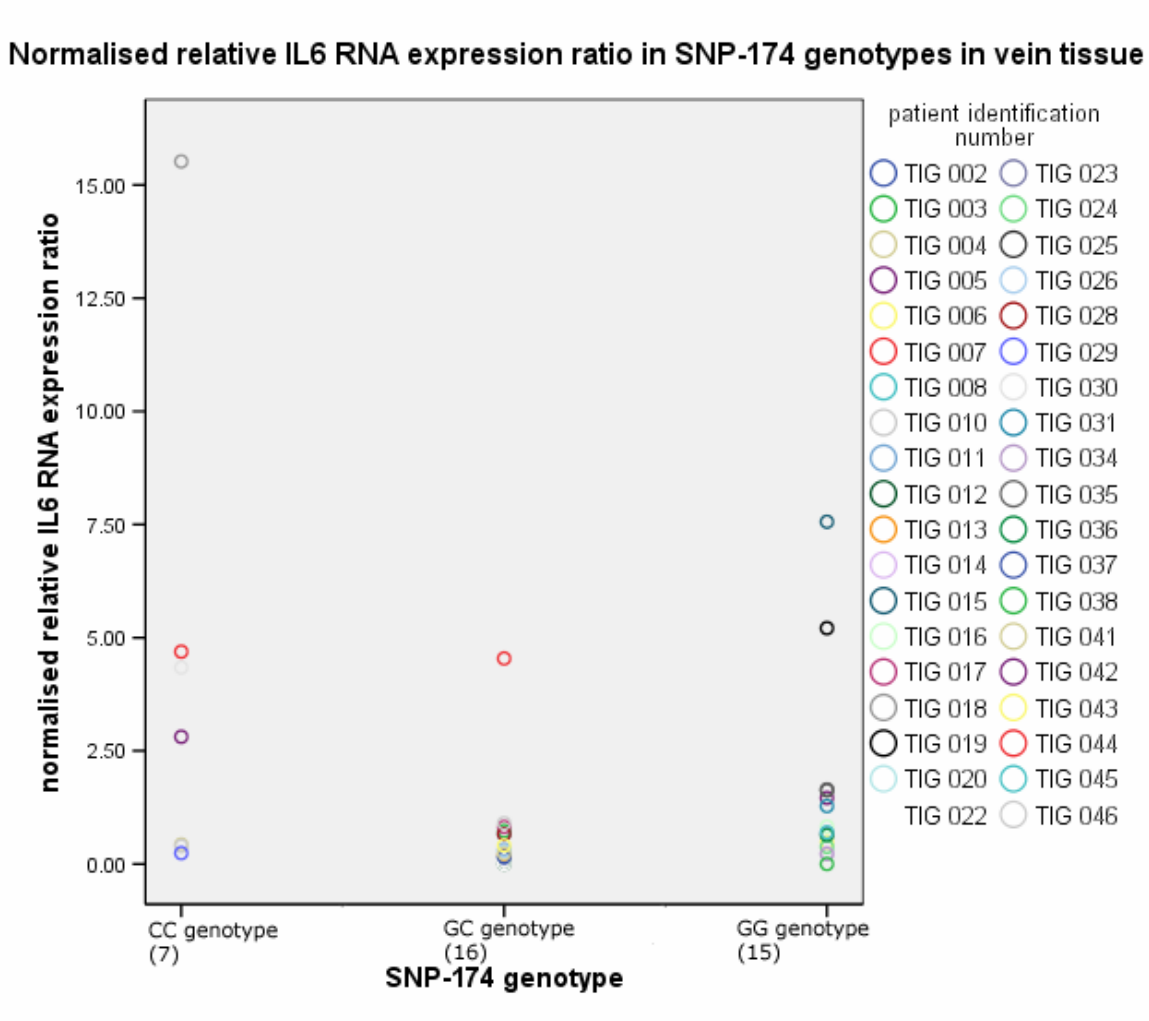


Figure 3-36: Distribution of IL6 normalised concentration ratio in vein tissue in different genotypes. The individual coloured circle represent one individual of a certain genotype with the patient identification number given in the legend.

Relative IL6 RNA expression was highest in the CC genotype group. The expression in the CC genotype was 6.3 times higher than in the GC genotype. Expression in the CC group was also 2.7 times higher than in the GG genotype. The GG genotype expressed 2.4 times more IL6 than the GC genotype (Figure 3-36). Differences in expression were borderline significant (p= 0.041) Table_Apx C-5.

3.10.4 Results for Muscle Tissue

40 muscle RNA cDNA samples were used as template in RT-PCR. Of these three did not have genotype information. Three returned an invalid result i.e. six muscle

samples could not be included in the analysis. 34 samples were included in the final analysis. The genotype distribution of the 34 samples is shown in Table 3.10-7.

Table 3.10-7: Genotype distribution for muscle tissue

Muscle Tissue:		
Genotype	**Frequency**	**Percent**
C/C	6	17.6
G/C	14	41.2
G/G	14	41.2
Total	**34**	**100.0**

The mean normalised relative IL6 mRNA expression ratio was 0.16 (SEM±0.11) for the CC genotype, 0.038 (SEM±0.01) for the GC genotype and 0.03 (SEM±0.01) for the CC genotype. The range was 0.01 to 0.72 for the CC group, 0.01 to 0.16 for the GC group and zero to 0.72 for the GG group (Table 3.10-8). The data was non-normally distributed (Kolmogorov Smirnov Z=2.33, asympt. sign. Two tailed <0.001).

Table 3.10-8: IL6 normalised concentration ratio in different genotypes in muscle.

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
CC	6	.1550	.27776	.11339	-.1365	.4465	.01	.72
GC	14	.0375	.03856	.01030	.0152	.0598	.01	.16
GG	14	.0296	.02390	.00639	.0158	.0434	.00	.09
Total	34	.0550	.12133	.02081	.0127	.0973	.00	.72

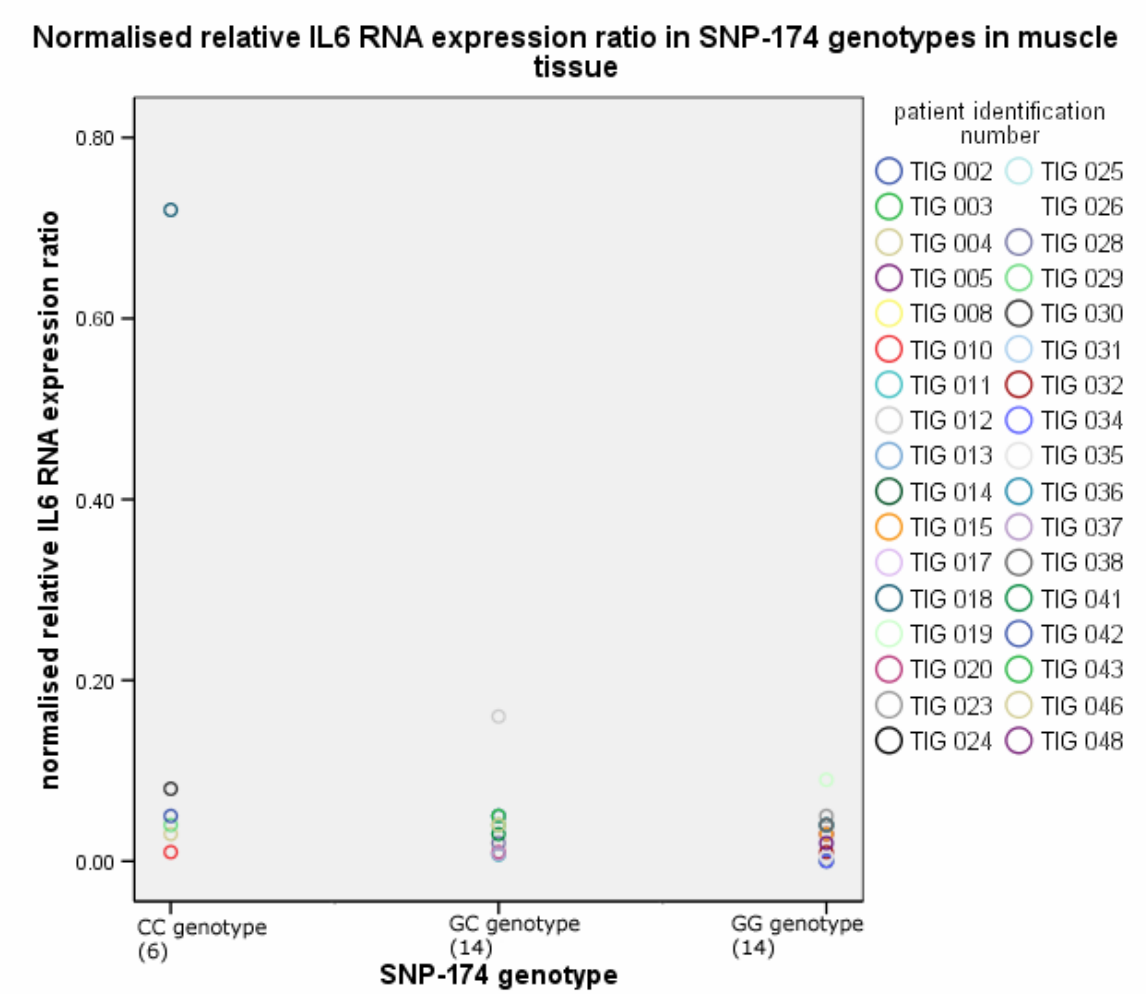


Figure 3-37: Distribution of IL6 normalised concentration ratio in muscle tissue in different genotypes. The individual coloured circles represent one individual of a certain genotype and the patient identification number is given in the legend.

Similar to the findings in the other tissues, the CC genotype expressed on average 4.1 times more IL6 than the GC genotype and 5.2 times more than the GG genotype. The lowest expresser overall was the GG genotype (Figure 3-37). None of these differences were statistically significant (p=0.28) (Table_Apx C-6).

3.10.5 Age Dependence

The distribution of age between genotype groups was tested as not all patients had donated all four tissues and therefore differences in age distribution between genotype groups could have affected the result (Table 3.10-9). There was no

significant difference in age distribution between the genotype groups for any of the tissues. For statistical analysis please see Table_Apx C-7, Table_Apx C-8, Table_Apx C-9, Table_Apx C-10).

Table 3.10-9: Mean age in different genotype groups in different tissues

	CC genotype Mean age (range) n=numbers	**GC genotype** Mean age (range) n=numbers	**GG genotype** Mean age (range) n=numbers
Fat tissue	49.5 (30-65) n=6	43.6 (23-66) n=11	45.9 (25-66) n=15
Leukocytes	50.0 (30-61) n=5	46.7 (23-69) n=14	45.7 (27-66) n=15
Vein tissue	50.9 (30-65) n=7	46.1 (23-69) n=16	47.1 (27-66) n=15
Muscle tissue	48.5 (30-61) n=6	48.2 (24-69) n=14	44.6 (25-66) n=14

The Spearman correlation coefficient was used to test for a significant linear relationship between age and IL6 mRNA expression, as the data was not normally distributed. No significant linear correlation was seen between age and IL6 expression in leukocytes and in muscle (Spearman correlation coefficient=-0.17 and 0.30 and p-value 0.35 and 0.08 respectively) (see Figure 3-39 and Figure 3-41). Fat and vein showed significant linear correlation (see Figure 3-38 and Figure 3-40). For fat there was a moderate linear correlation between age and IL6 mRNA expression (Spearman correlation coefficient=0.45, p-value=0.010). For vein a moderate linear relationship was also demonstrates (Spearman correlation coefficient =0.42, p-value =0.008) (for statistical analysis see Table_Apx C-11, Table_Apx C-12, Table_Apx C-13, Table_Apx C-14).

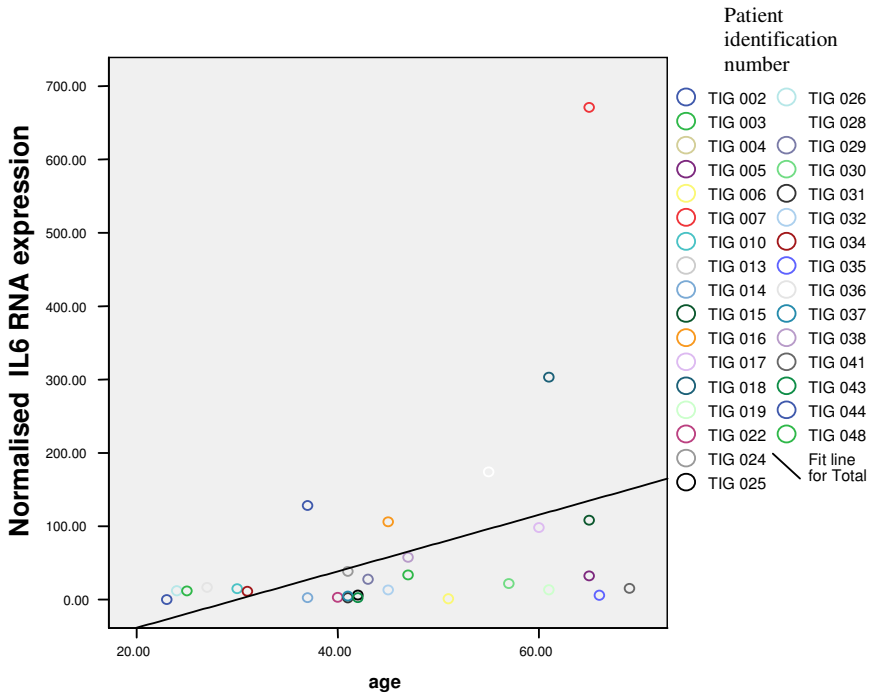


Figure 3-38: Linear regression for age/IL6 expression in adipose tissue. The coloured circles represent individual patients and the patient identification number is given in the legend. The 'fit line' shows the linear relationship between age and IL6 mRNA expression ratio. The graph shows a moderate linear relationship which is significant.

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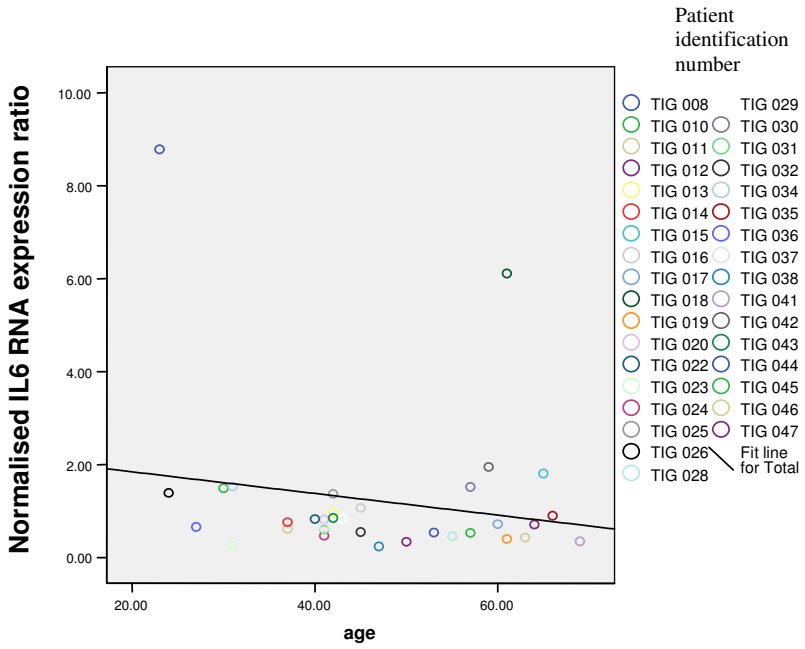

Figure 3-39: Linear regression for age/IL6 expression in leukocytes. The coloured circles represent individual patients and the patient identification number is given in the legend. The 'fit line' shows the relationship between age and IL6 mRNA expression ratio. The graph shows no significant linear relationship between age and IL6 mRNA expression ratio in leukocytes.

Correlation of IL6 RNA expression with age in vein tissue

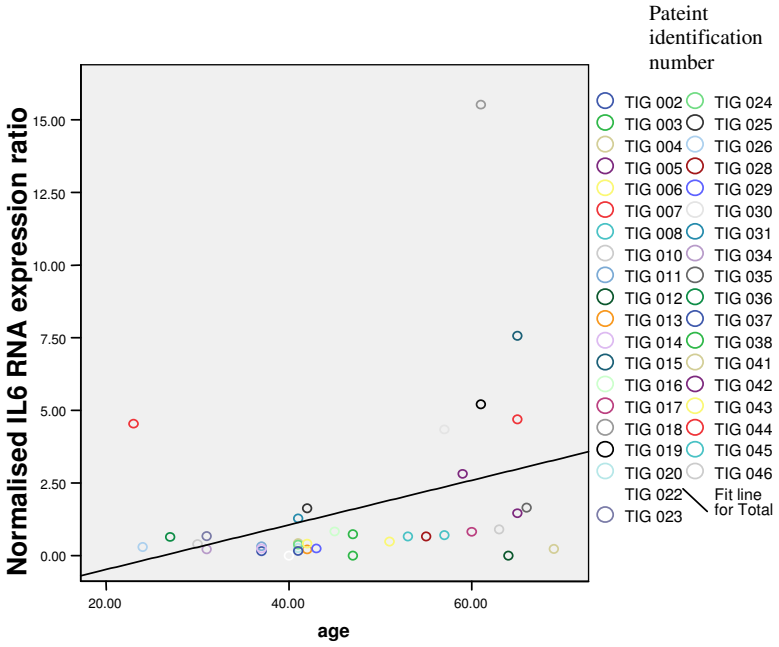

Figure 3-40: Linear regression for age/IL6 expression in vein tissue. The coloured circles represent individual patients and the patient identification number is given in the legend. The 'fit line' shows the linear relationship between age and IL6 mRNA expression ratio. The graph shows a moderate linear relationship which is significant.

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Correlation of IL6 RNA expression with age in muscle tissue

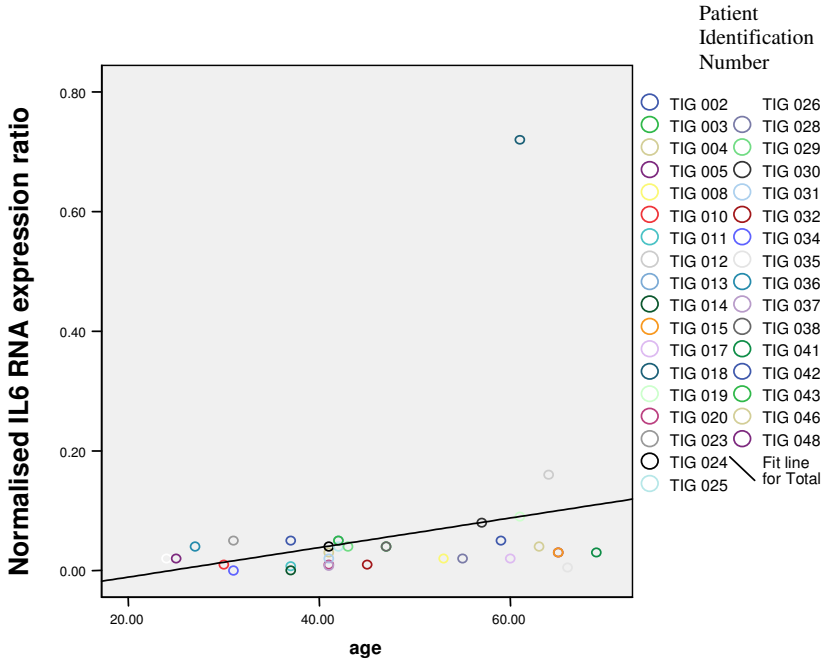

Figure 3-41: Linear regression for age/IL6 expression in muscle tissue. The coloured circles represent individual patients and the patient identification number is given in the legend. The 'fit line' shows the relationship between age and IL6 mRNA expression ratio. The graph shows no significant linear relationship between age and IL6 mRNA expression ratio in muscle tissue.

3.10.6 Association with Smoking Status

There was a difference in the distribution of smokers and non-smokers between the genotype groups (see Table 3.10-10). This was of borderline statistical significance (p-value=0.05) (for statistical analysis see Table_Apx C-15). In all tissues smokers expressed more IL6 than non-smokers; however the result did not reach statistical significance in any of the tissues (see Table 3.10-11).

Table 3.10-10: Number of smokers and non smokers in each genotype group.

			Smoking Status		
			Non-smokers	Smokers	Total
genotype	C/C	Count	15	9	24
		% within genotype	62.5%	37.5%	100.0%
	G/C	Count	34	14	48
		% within genotype	70.8%	29.2%	100.0%
	G/G	Count	48	8	56
		% within genotype	85.7%	14.3%	100.0%
Total		Count	97	31	128
		% within genotype	75.8%	24.2%	100.0%

Table 3.10-11: Median expression ratio in different tissues.

Tissue type	Median IL6 expression ratio in non smokers	Median IL6 expression ratio in smokers	Mann Whitney U test P value (asympt. two tailed)
Adipose tissue	12.5 n=22	33.4 n=7	0.067
Leukocytes	0.64 n=25	0.82 n=7	0.210
Vein	0.42 n=26	0.82 n=9	0.059
Muscle	0.03 n=24	0.05 n=8	0.079

3.11 Allelic Transcription Assay for Adipose Tissue Samples

The previously designed allelic transcription assay showed good reproducibility for genomic DNA but proved unreliable when vein or leukocyte hnRNA was measured (see section 3.8). It was felt that the most likely cause was low copy numbers in the primary template. Adipose tissue had been shown to expresses 40 times as much IL6 mRNA as leukocytes; therefore it was thought that using adipose tissue hnRNA as a template in the allelic transcription assay may lead to improved accuracy of the assay. Thus the allelic transcription assay was performed using adipose tissue RNA as a template

3.11.1 gDNA Contamination

cDNA samples were checked for gDNA contamination. Despite rigorous DNA treatment, 11 adipose tissue samples out of 17, showed gDNA contamination (see Figure 3-42). In the majority of cases contamination was only shown in one out of three PCRs suggesting low levels of contamination, however in two cases contamination was seen in all three PCRs. In particular sample number 44 showed strong bands in all three PCRs suggesting significant gDNA contamination.

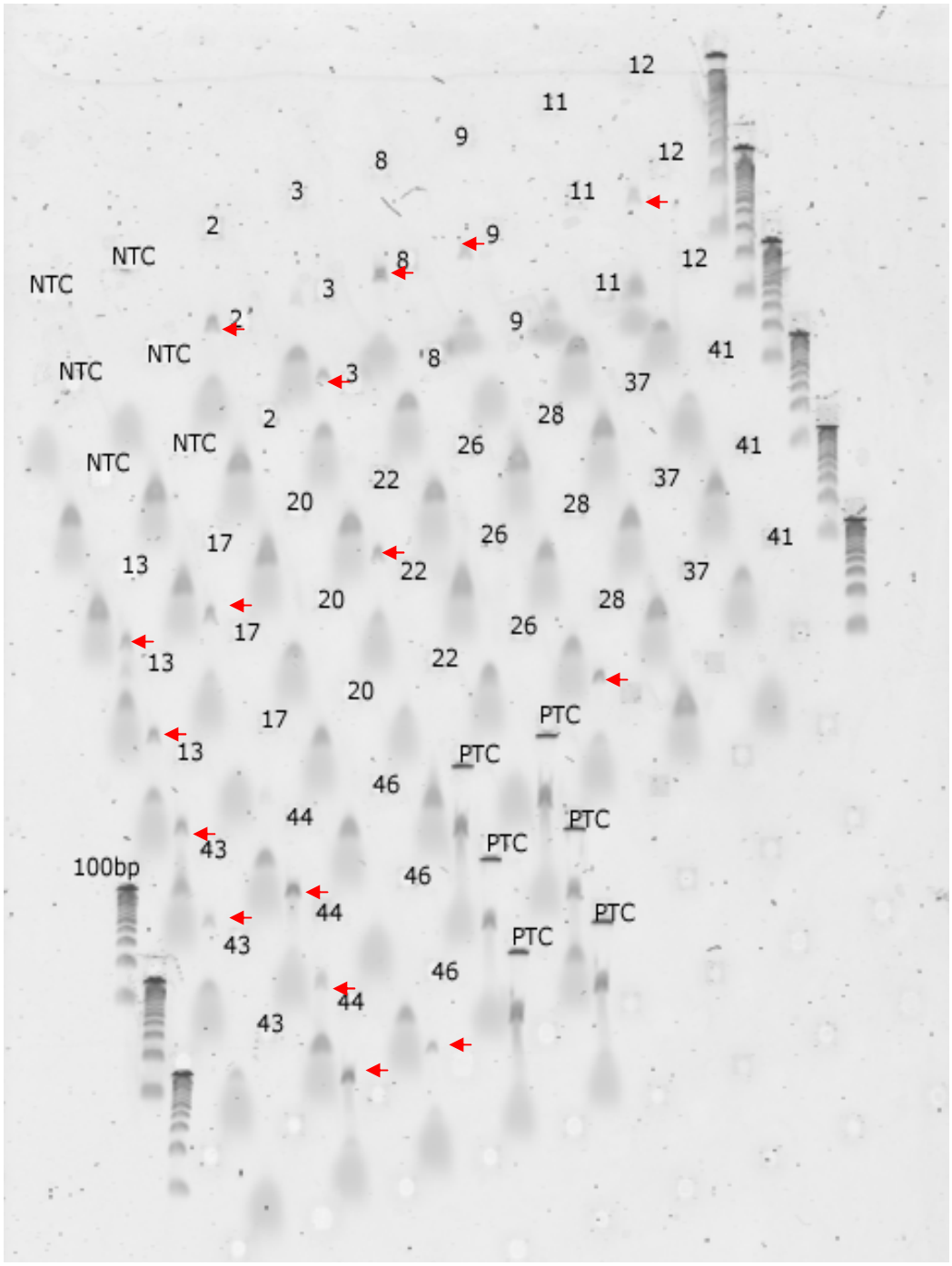

Figure 3-42: Results for LDLR promoter assay for adipose tissue samples (numbered samples): Acrylamide Gel 5% stained with vistra green: NTC=negative template control; PTC=positive template control (template=gDNA). Bands indicating gDNA contamination are seen in samples 2, 3, 8, 9, 12, 13, 17, 22, 28, 43, 44 and 46 (red arrows).

When cDNA Ex4 was used in a PCR for amplification of intron 3 and adjacent intron exon boundaries, the following results were seen:

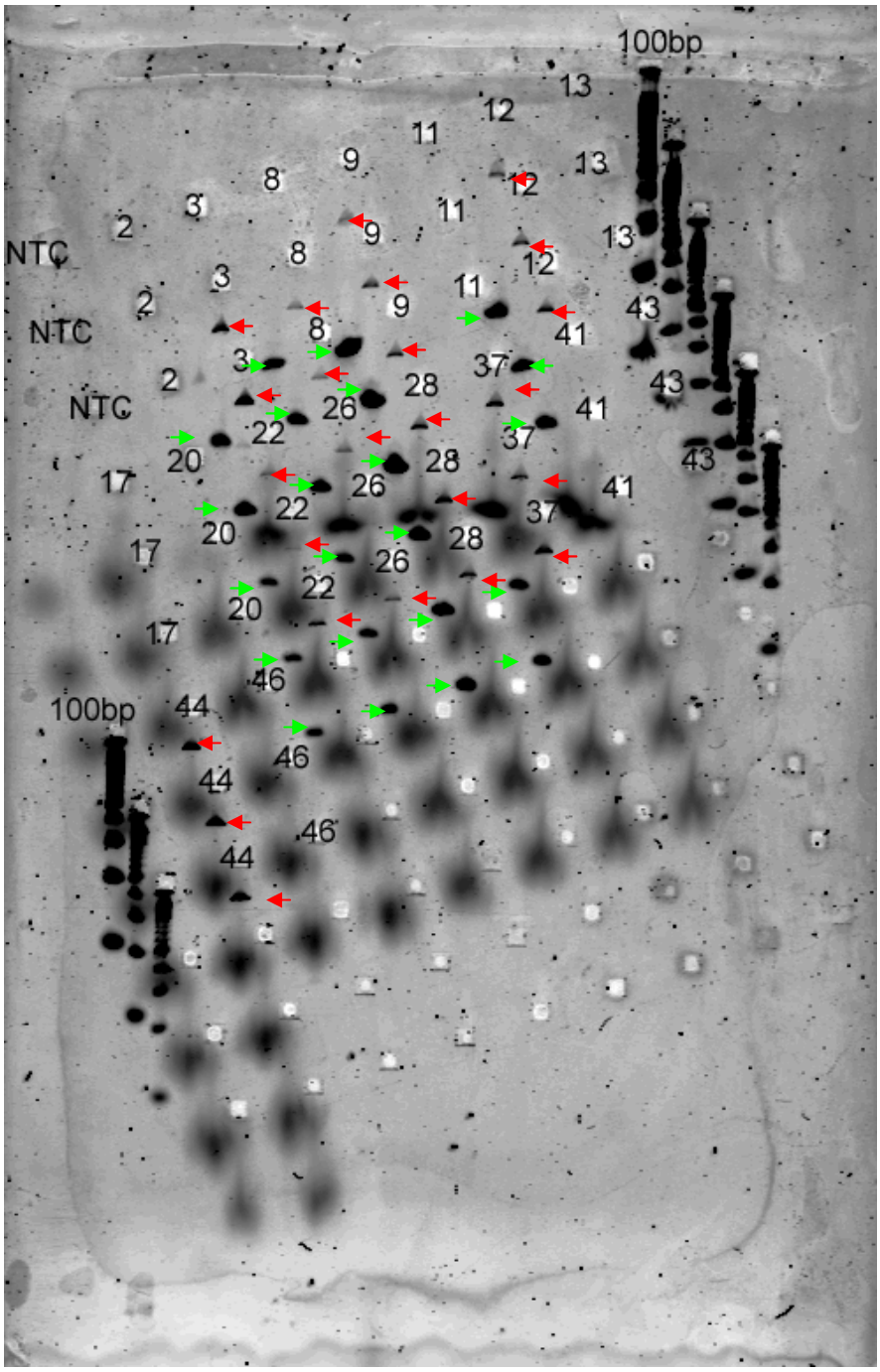

Figure 3-43: This gel shows the result of the PCR amplifying IL6 intron 3 region and adjacent intron/exon boundaries. Samples 2, 11, 13, 17, 20, 41, 43 and 46 failed to amplify. Samples 3, 8, 9, 12, 22, 26, 28 and 37 show a short (green arrows) and a long (red arrows) product indicating presence of cDNA (green arrows) and hnRNA/gDNA (red arrows), sample 44 only shows a long product i.e. there gDNA contamination but no cDNA and therefore no hnRNA.

Figure 3-43 shows the result of the PCR amplifying IL6 intron 3 region and adjacent intron/exon boundaries. Eight samples failed to amplify. Eight samples showed a short and a long product indicating presence of cDNA and hnRNA/gDNA, sample 44 showed a long product only i.e. there is gDNA contamination but no cDNA and therefore most probably no hnRNA. Sample 44 was therefore excluded form the final analysis.

3.11.2 Genotyping for Intron SNP G3572T

Sample number (TIG) 12 showed amplification from the C-allele only in repeat runs of the adipose tissue allelic transcription assay. The lack of variability in the results appeared different form the rest of the samples. This raised the possibility the Subject 12 was a rare haplotype SNP G-174C/SNP G3572T: GC/TT. Genotyping for SNP G3572T was therefore performed on study gDNA samples. Results are shown in Figure 3-44.

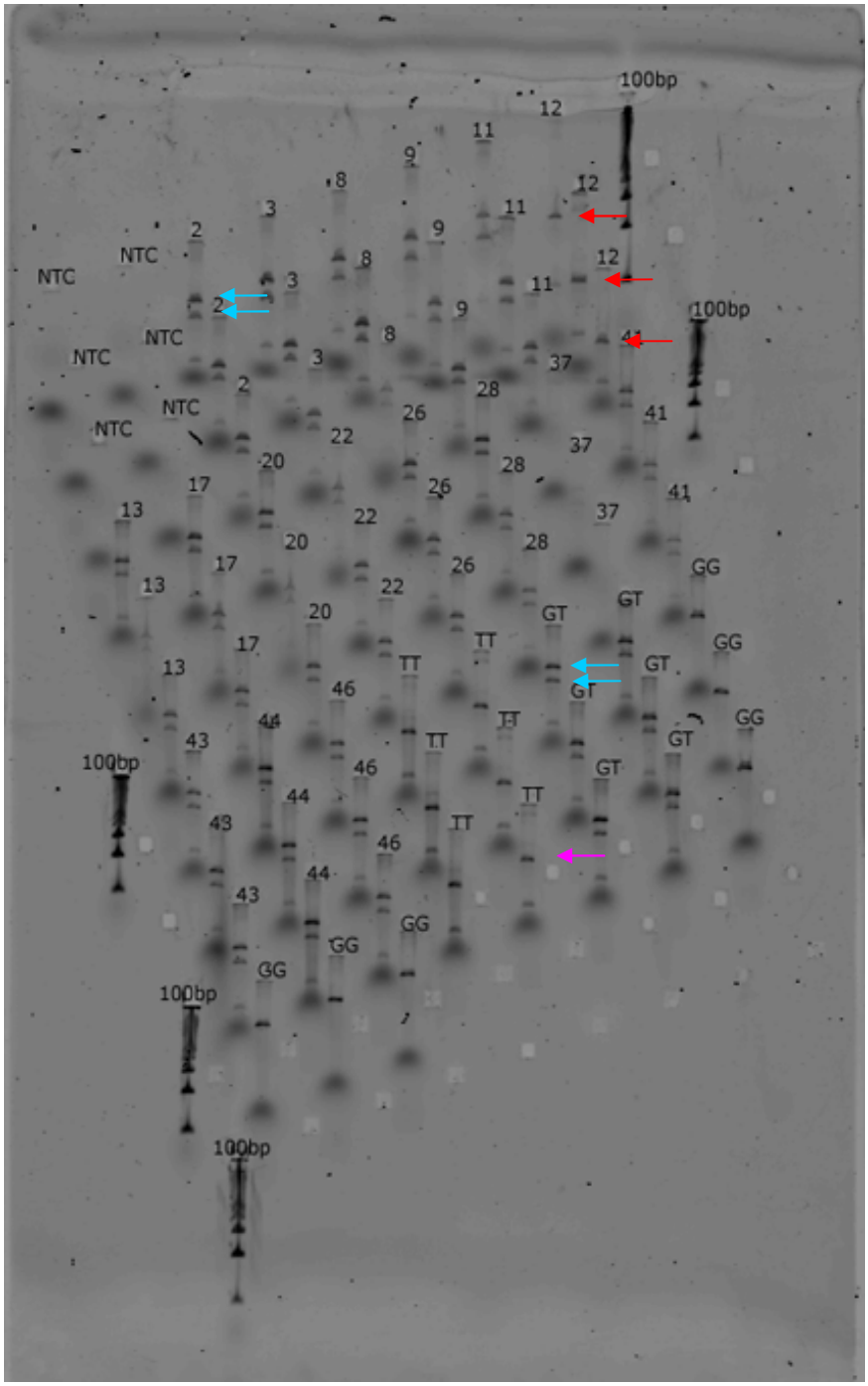

Figure 3-44: Genotyping for intron SNP G3572T in heterozygotes for promoter SNP G-174C using restriction digest: Acrylamide Gel 5% stained with ethidium bromide. NTC=negative control; TT or GG=positive gDNA control homozygous for SNP G3572T; GT=positive gDNA control heterozygous for SNP G3572T. Samples are numbered with patient identification number. Majority of samples are heterozygous with both uncut and cut band visible (blue arrows). Exception is sample No. 12 which only shows the cut band (red arrows) identical to the pattern seen in the known homozygous TT gDNA control (pink arrow)

All samples, except one, were heterozygous with both uncut and cut band visible (blue arrows) Figure 3-44. The exception was sample number (TIG) 12 which only showed the cut band (red arrows) identical to the pattern seen in the known homozygous TT gDNA control (pink arrow). Sample number 12 was therefore concluded to be rare haplotype GC/TT. This explained the apparent consistent amplification from only the C-allele in the allelic transcription assay. Sample number 12 was therefore excluded from the analysis.

3.11.3 Results of Allelic Transcription Assay in adipose tissue

In repeat experiments template cDNA In4 showed better reproducibility of results (mean variance for individual samples = 0.007) than cDNA Ex4 (mean variance for individual samples = 0.1). The final experiment was therefore performed using cDNA In4 as a template. The results for the allelic transcription assay for adipose tissues are displayed in Figure 3-45. Samples with at least one duplicate measurement were included in the analysis. The acrylamide gel shows nested PCR/restriction digest products for tissue samples as well as negative template control, heterozygous gDNA samples and standard curve samples.

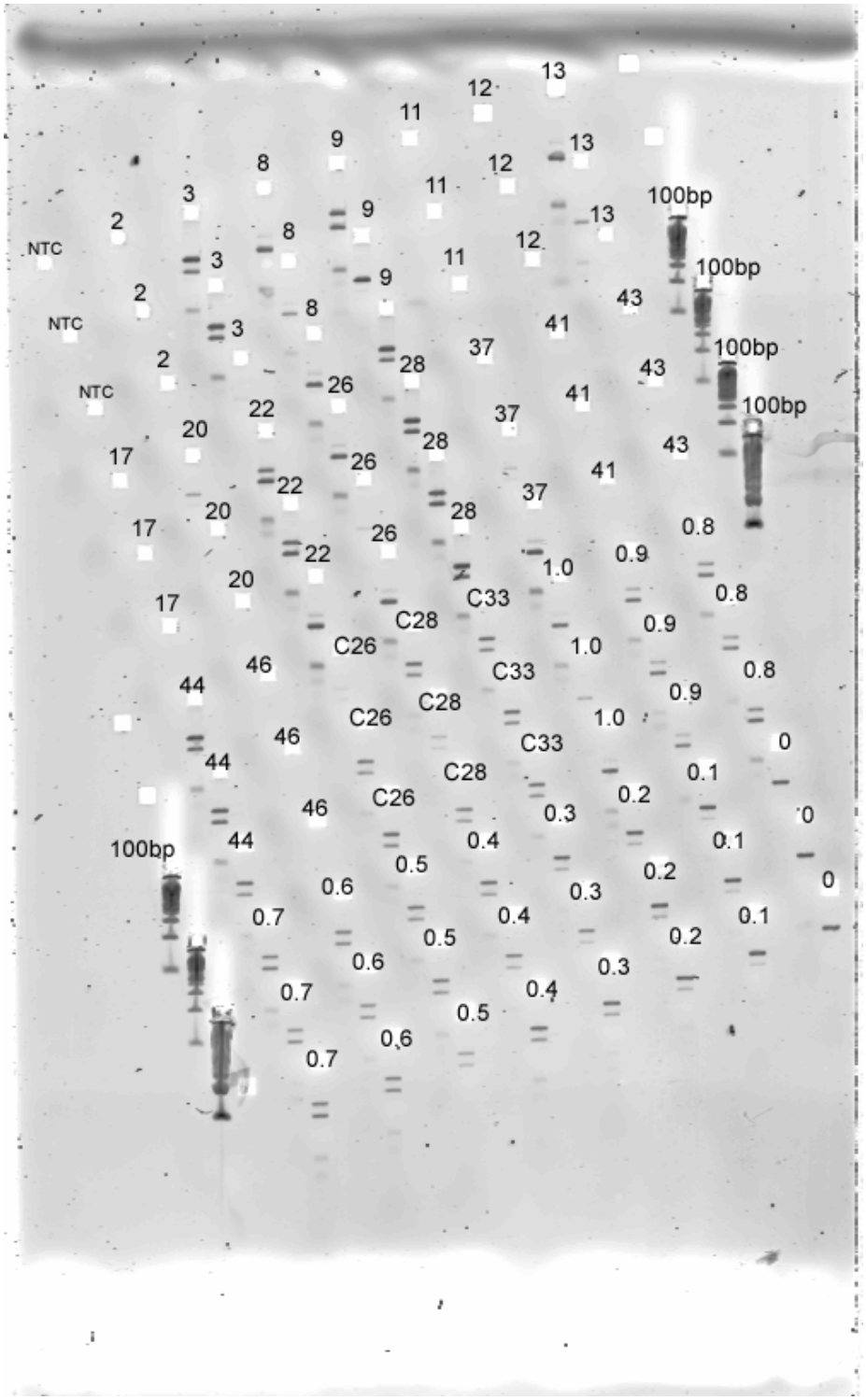

Figure 3-45: Acrylamide gel 5% stained with ethidium bromide: Result of the allelic transcription assay for adipose tissue. NTC=negative template control, C26, C28, C33 = heterozygous gDNA samples, 0-1.0 = standards, samples labelled: 2,3,8,9,11,12,13,17,20,22,26,28,37,41,43,44,46 = patient samples (TIG number).

Following fluorescence intensity measurement, values for the standard curve samples were plotted on a graph and a linear trend line was fitted using Excel software. The average values of band fluorescence intensity for the two genomic DNA samples were then plotted on the graph using the calculated trend line equation. The mean calculated allelic ratio for the gDNA samples was 0.56. The mean values of all measurements for the adipose tissue samples were also plotted (see Figure 3-46:). (Data see Table_Apx C-45)

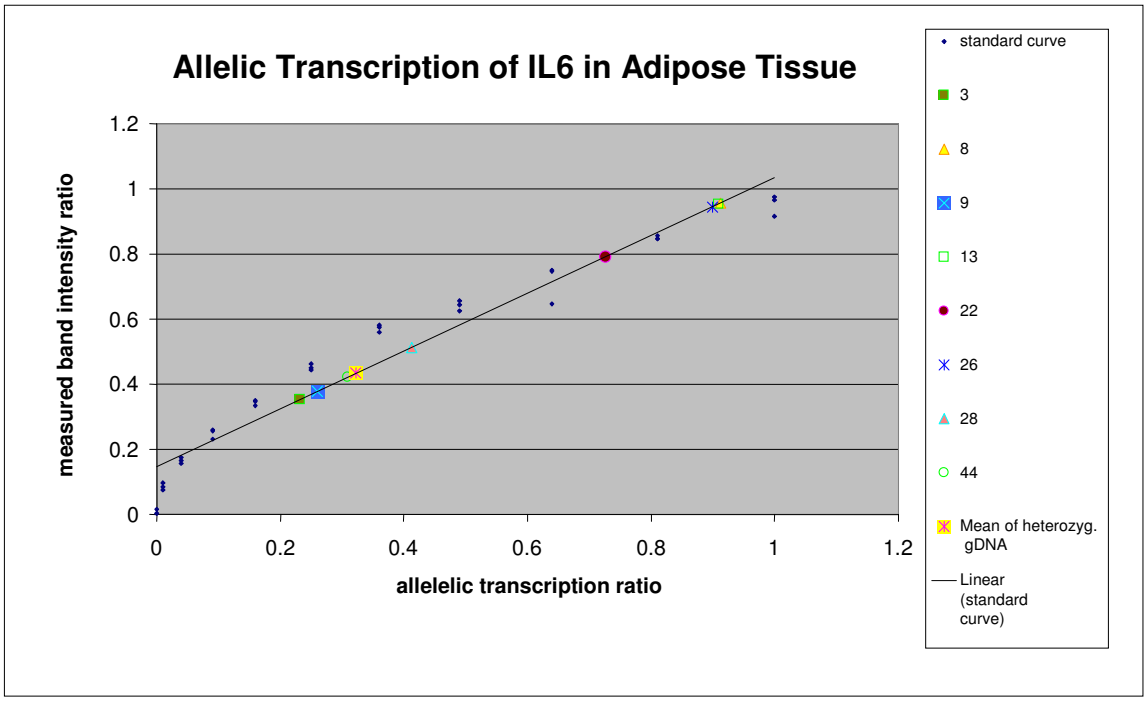

Figure 3-46: Graph showing results for the allelic transcription assay for adipose tissue, using cDNA In4: The standard curve and mean result for two heterozygous gDNA samples, as well as values for adipose tissue samples are shown. Note the proximity of sample 44 (known to have gDNA contamination present but no cDNA) to the value for gDNA.

In line with previous results in this thesis the C-allele appeared to express more IL6 hnRNA than the G-allele. The mean allelic transcription ratio for all the adipose tissue samples was 0.76 (SEM±0.08) indicating that on average 76% of IL6 hnRNA was transcribed form the C-allele. Statistically this was not significantly different from the calculated allelic ratio for the genomic DNA samples 0.56 (Mann Whitney test p=0.38) (for statistical analysis see Table_Apx C-19)

As in the previous allelic transcription assay for vein there was wide inter-individual variation in the allelic transcription ratio.

To assess the reproducibility of the measurements for the individual samples, the mean of the 3 PCRs was plotted and the 95% confidence intervals were calculated (see Figure 3-47).

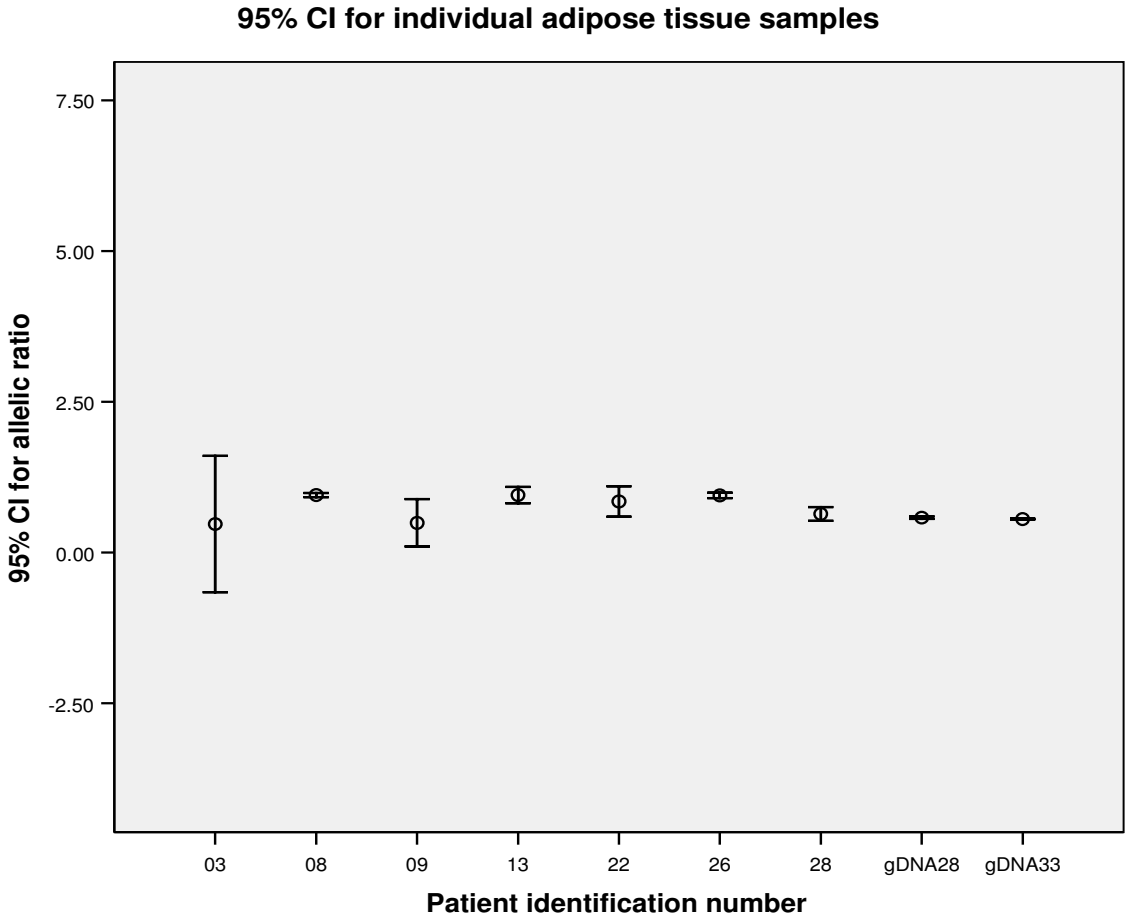

Figure 3-47: Graph showing the allelic transcription ratio for individual samples and results for two heterozygous gDNA samples. The error bars indicate the 95% confidence interval. Compared to the previous allelic transcription assay for vein and leukocytes the 95% CI are narrower indicating improved reproducibility of the results.

Confidence intervals were narrow compared to previous allelic transcription assays in vein and leukocytes suggesting improvement in the reproducibility of the results. Mean variance of the allelic transcription ratio for the individual adipose tissue samples was 0.008 compared to a variance of 0.05 and 0.06 for vein and leukocyte respective.

4 Discussion

4.1 Patient Recruitment

Participation in the study required patients to donate tissue in addition to samples routinely removed during varicose vein surgery. This represented an invasive procedure. Despite this, the majority a patients agreed to take part in the study, with an uptake of 88%. The fact that samples were collected during anaesthesia and no extra hospital attendances were required could have contributed to the high uptake. Of the 95 patients who agreed to take part in the study, 25 did not donate tissue samples. This was due to the difficulty encountered in sample collection in remote hospitals, frequent cancellation of operations and the generic booking system in which patients were not assigned to a named consultant. This resulted in recruited patients being operated on by consultants not taking part in the study or on an operating list which could not be attended by the researcher collecting samples. These difficulties contributed to fewer patients donating tissue samples than anticipated in the conception of this study. However, sample numbers achieved, were sufficient to attempt allelic assay design/validation and study expression in different tissues.

The patient follow-up was designed as a safety control to ensure there was no increase in bleeding complications from the additional muscle biopsy and to assess the level of patient satisfaction with the study process. No detailed conclusions can be drawn from the follow-up, as no control group was questioned. However, there were no bleeding complications reported in the follow up. Two patients developed groin infections. This rate is higher than some rates reported in the literature, but the infection rate following 'clean surgery' is often grossly underestimated (277;278). In studies with a surveillance period of greater than 30 days, suitable definitions and wound infection scores such as the ASEPSIS scoring system (279;280), the reported infection rate is 15% or more (278). Thus the infection rate of 10.5% in this study appears to be within normal limits and it seems that muscle biopsy was safe in this unit. The average return to normal daily activity was 1-2 weeks. This compares favourably with a study showing a median time period of return to work following varicose vein surgery at 2 weeks for self employed subjects and 4 weeks for employees (281). Pain scores recorded on the first and second post-operative day are lower than scores previously reported (282). The additional

procedures necessary to perform this study did not therefore appear to have had adverse effects on the patients.
The method of recruitment both in the pre-assessment and by letter+/-telephone call was entirely acceptable to patients and all patients were satisfied with the study process. However, the results on patient satisfaction and acceptability of the recruitment process may have been biased by the lack of anonymity, as these were assessed by telephone interview. Lack of anonymity has previously been shown to skew results from questionnaires regarding patient satisfaction (283).

The original aim was to recruit healthy individuals, free from significant cardiovascular disease and un-medicated to the study. As recruitment occurred in the Day Surgery Unit, patients with significant cardiovascular disease were automatically excluded from the study. It soon became apparent that restricting the subjects to un-medicated patients would be impractical, as it would significantly reduce the number able to join the study. Thus patients of ASA grade I and II (I=normal healthy individual, II=mild systemic disease that does not limit activity) were included even if medicated. Patients currently taking steroids or suffering from acute inflammation/infection or untreated carcinoma were excluded from the analysis. Of the 69 recruited patients 32 were un-medicated. The most common medications taken by participants were antihypertensive therapy and hormone therapy. Previous studies have shown that treating heart failure patients with beta blockers lowers IL6 levels (284). Calcium channel blockers also appear to exercise an effect on IL6 expression, however both positive and negative effects have been reported in the literature (285-287). The effects of angiotensin-converting enzyme inhibitors and angiotensin II receptor antagonists are likely to be one of IL6 suppression, more specifically the effect of these agents may depend on G-174C genotype (288).
Hormone treatment may also decrease IL6 levels as estradiol inhibits IL6 expression. It is thought that lack of 17 beta-oestradiol following the menopause is partly responsible for the increase in circulating IL6 and it has been shown that HRT can decrease IL6 expression (289). Antidepressants have been found to suppress pro-inflammatory cytokine production (290). Though interest has mainly focused on INF-gamma/IL10 ratio as well as IL2, TNFα and IL1β, it is likely that antidepressant agents also affect IL6 as both IL1 β and TNFα up regulate expression of IL6.
Diuretics such as frusemide have been found to inhibit the production and release of IL6 from peripheral mononuclear cells and there is a suggestion that thiazide

diuretics may decrease circulating IL6 levels (291;292). Aspirin is thought to decrease IL6 expression through NF-kappa B antagonism and cyclo-oxygenase inhibition (293).
Therefore it seems likely that participant's medication may have affected IL6 RNA expression in individuals, but its precise effect is difficult to define. The effect of medication appears to be largely one of IL6 suppression; however this effect may be counterbalanced by the IL6 induction resulting from the treated condition, e.g. hypertension. This study was designed to eliminate the influence of confounding variables, such as medication, on the result, by performing intra-individual comparisons in the allelic transcription assay and by investigating different tissues in the same individual. Thus the results of the intra-individual comparisons should be largely unaffected by participant's medication.

4.2 RNA Extraction

RNA extraction from fibrous tissue (skeletal muscle and vein tissue) and lipid rich tissue such as peripheral adipose tissue, required optimisation of the technique. RNA extraction was a laborious process, preventing a high throughput of samples. The quality of the RNA extracted for our study appeared satisfactory for the control samples tested with minimal or no degradation. Quality was not assessed for the study samples as there was only a limited amount of RNA available. However as handling and storage for the study samples was the same as for the control samples, equal quality was assumed for the study samples as for the control samples.
For vein and muscle A260:A280 ratios were below the ideal target of 1.9 and 2.1 indicating that some protein contamination was present in the samples. The purity of adipose RNA remained particularly low with mean A260:A280 ratios of 1.3.
A260:A280 ratios were above 1.9 for leukocytes. Protein contamination can affect downstream enzymatic reactions e.g. may interfere with cDNA synthesis (276). This may in part account for the lack of amplification in RT PCR analysis of 9% of vein, 8% of muscle and 23% of adipose tissue samples. In comparison, all leukocyte samples amplified in the real time PCR assay.

4.3 Genotyping

The CC genotype was rarer than all other genotypes in the study population. The reported allele frequency varies widely in different ethnic groups. Even within

European populations the reported allele frequency varies considerably, so there is no consensus as to which allele is the more common. However the finding of this study that the C-allele is the less common allele has been replicated in other studies conducted in European populations (240). The fact that genotypes were in Hardy-Weinberg equilibrium also validates the genotyping in this study.

4.4 Preferential Allelic Transcription Assay Design

4.4.1 Experimental design

4.4.1.1 Amplification of Heteronuclear RNA

The lack of suitable markers for the promoter SNP G-174C in exon regions of the gene led to a variety of difficulties. The design of the new allelic IL6 transcription assay relied on the amplification of RNA intron transcripts containing markers in LD with SNP G-174C. These were only present in hnRNA. A previous experiment in murine cell culture showed that IL6 hnRNA levels correlate well with IL6 mRNA levels suggesting that hnRNA can be used to assess gene expression (294;295) and therefore hnRNA was used as a template in this study. Beside the practical need to use hnRNA as a template there is an advantage in measuring primary transcript. The promoter SNP G-174C most likely regulates primary transcription and thus level of the primary transcription product (hnRNA). Therefore measuring hnRNA rather than mRNA may reflect the effect of a promoter SNP more accurately than conventional mRNA assays.

However, one disadvantages of using hnRNA as a template rather than mRNA lies in the inability to differentiate between hnRNA and gDNA contamination. The usual way of differentiating between cDNA and genomic DNA in quantification assays is for one of the primers or the amplicon itself to span an intron exon boundary. As both hnRNA and genomic DNA have the same sequence this method could not be used. It was therefore important to ensure minimum contamination with genomic DNA and to find a different way of checking for genomic DNA contaminations in the study samples. Promoter regions are not transcribed into RNA and can therefore be used to assess gDNA contamination. Amplification of the promoter region of an unrelated gene (LDLR) was used. This method was able to provide information about the presence of gDNA contamination in the study samples. However, it did not eliminate the effect of gDNA contamination on the study results.

IL6 expression is low in un-stimulated tissues and some authors claim that IL6 is not constitutively expressed (24). This study demonstrated that IL6 expression does occur in un-stimulated ex vivo tissues (short product in amplification of region spanning intron 3). However it showed that IL6 RNA is not abundant in un-stimulated tissue, as shown by the high Cp values in the RT PCR experiments (see section 4.5 & 4.6). Because hnRNA make up only a fraction of total IL6 RNA, the study was relying on amplification of a fraction of an already low abundance

template. It therefore had to be demonstrated, that intron regions of hnRNA could be amplified and visualised on a gel. Therefore amplification of intron region 2 and intron region 3 was attempted. Only intron region 3 could be amplified. This could be explained by a difference in efficiency between the assays for intron 2 and 3. High reaction efficiency may be required when dealing with very low template numbers. Another possible reason for the lack of intron 2 amplification is a more efficient splice process affecting intron 2. Intron 2 may be removed earlier in the splicing process than intron 3 leading to a less abundant template. A further possible explanation for the successful amplification of intron 3 but not intron 2 would be the presence of alternative splice products in which intron retention had occurred. No alternative splice product retaining intron 3 has been described to date. However, intron retention in general has been less well studied than other forms of alternative splicing. This is due to the difficulty that arises from differentiating between true alternative splice forms and un-spliced or partially spliced pre-mRNAs (29). It is therefore possible such an alternative splice product could be found in the future, however at present no such evidence exists.

Amplification of intron 3 from hnRNA seemed possible and further experiments were therefore focused on intron region 3 rather than intron region 2. During the splice process the intron is released as a free lariat and degraded in the nucleus. However it is unclear how long intron fragments exist before degradation. Therefore a splice fragment which had not yet been degraded was a further possible template for intron 3 amplification. To investigate this, attempts were made to amplify exon3/intron3 boundary from cDNA. This was unsuccessful. Failure to amplify exon/intron 3 boundary may have been due to inefficiency of the reaction. However an alternative explanation would be that the previously demonstrated amplicon of intron region 3 was the product of a free floating fragment of spliced intron 3 and not hnRNA. Therefore the assay design had to be changed to ensure amplification would occur from intact hnRNA only. This was achieved by basing the assay on amplification of a region spanning intron 3 and the adjacent intron/exon boundaries. This experimental set up raised further problems as it used specific primer annealing in exon 4 for reverse transcription. Therefore, there was no preferential reverse transcription of hnRNA and the majority of cDNA arose from mRNA (as indicated by the relatively stronger band in Figure 3-9), resulting in minimal amplification of the long product arising from hnRNA, with a correspondingly weak band on the gel (see Figure 3-9). The planned gel based analysis of allelic transcription involved

measurement of fluorescence intensity of this band. The signal from this band would have been too weak to perform reliable measurements therefore a nested PCR was designed. The nested PCR demonstrated adequate PCR product from intron 3 amplification, to perform analysis of preferential allelic transcription. This was the first time the feasibility of a gel based allelic transcription assay for IL6 using hnRNA as a template had been demonstrated.

4.4.1.2 Standard Curve

In order for the experiment to proceed a standard curve had to be designed and validated. There was a strong linear relationship between the known concentration ratios and the measured band intensity ratio for the standard gDNA samples. Variance of the measured band intensity ratios for the individual standard concentrations was close to zero. Standard curves produced by three separate experiments were very similar, showing good reproducibility of the results.
When band fluorescence intensity ratios for heterozygote gDNA samples were plotted on the standard curve the estimated concentration ratio was marginally higher than expected at 0.51 (expected ratio: 0.50 or q^2=0.25). This may be due to inaccuracy in the preparation of the standards. This should not have affected the results as heterozygous gDNA samples were included in each experimental run. Point of equal expression from both alleles could therefore be accurately determined. It was concluded that the standards prepared and the assay design gave a reliable, linear standard curve, which could be used in the ratiometric measurement of allelic transcription.

4.4.2 Allelic Transcription Assay for Vein Samples

Genomic DNA contamination was still visibly present in four vein samples following rigorous DNase treatment. This is not surprising as the nucleic acid content of RNA samples from tissues may vary widely, ranging from nearly pure RNA to containing mostly DNA (296). Genomic DNA contamination is most likely the result of insufficient homogenisation during RNA extraction. Collagen rich tissues, such as vein are difficult to homogenise fully and often give rise to low yield and poor quality RNA (297).
The LDLR promoter assay which was used to check for gDNA contamination provided a rough guide only as to the degree of contamination. Because different assays were used to amplify the LDLR promoter region and IL6 hnRNA, no direct comparison could be made as to the ratio of gDNA and hnRNA in the samples. It is therefore

unclear to what degree gDNA contamination affected the results for the preferential allelic transcription assay. One can assume that gDNA contamination skewed the results for hnRNA towards the ratio found for gDNA samples in the experiment. Thus any effect of promoter SNP G-174C on preferential transcription would be underestimated by the assay.

The results of the allelic transcription assay for vein samples did showed slightly more expression from the SNP G-174C C-allele then from the G-allele though this was not significant. The mean results for individuals were widely spread on the standard curve. Potentially this was the result of true inter-individual variation in allelic expression or alternatively the result of poor reproducibility of the assay. The later may be more likely as reproducibility of the individual results in different PCR reactions was poor. Variance in repeat PCR of individual samples was more than ten times the variance seen for standard curve. It is likely that this variance rendered the result inaccurate. This type of assay, using gel based analysis, has been successfully used previously for allelic transcription analysis of the angiotensin II type 1 receptor gene (AGTR 1) in placenta RNA (298). However that study investigated a non-coding SNP in the 3' UTR and an encoding SNP, thus was able to use mRNA as a template. This study however used hnRNA; therefore the initial copy number would have been substantially lower than in the AGTR 1 study. In an ideal situation every cDNA molecule present will be amplified during each cycle of the exponential phase of the PCR. However in reality this is rarely the case. With an abundant template this does not lead to significant alteration of the ratio of different alleles. However if the copy number is very low the lack of amplification of just one molecule in the early phases of PCR could significantly affect the final allele ratio due to the exponential nature of PCR amplification, thus leading to variation in repeat PCRs. It could be argued that amplification of individual molecules occurs at random, therefore if enough repeat measurements are taken the average should tend towards the correct ratio. However, tissues such as those examined in this study have a low RNA yield making a substantial number of repeat experiments obsolete.

4.4.3 Allelic Transcription Assay for Leukocytes

Unlike the RNA samples extracted form tissues, which demonstrated gDNA contamination in some samples, gDNA contamination was not demonstrated in the leukocyte RNA samples. This is likely to relate to sample type. Tissues are more difficult to homogenize than cell suspensions for mechanical reasons. gDNA

contamination increases with reduced homogenization, therefore RNA extracted form tissue is more likely to contain gDNA contamination than cell suspensions (299;300). This experiment differed from the vein assay in as much as all RNA samples were taken from one individual. Therefore the results of the samples were expected to be similar. However, results varied widely between individual samples (between sample variance of 0.08).

This could be related to poor reproducibility of the assay or variation in RNA and cDNA preparation. Variation in RNA or cDNA preparation is unlikely to influence allelic transcription ratio as both alleles were subject to the same preparation and should be equally affected. Therefore the most likely explanation for the spread of the results is poor reproducibility of the assay. Variation between the three repeat PCRs for individual sample was also substantial as indicated by the wide confidence intervals and the measured variance (> x10 the variance seen for standard curve), which was similar to the variance seen in vein samples. The experimental setup allows good reproducibility for gDNA amplification as indicated by the narrow confidence intervals for the two genomic DNA samples. Therefore it is likely that the wide spread of the results is related to a problem with the template, most likely low copy numbers, leading to stochastic amplification in the initial PCR. An attempt to increase yield of cDNA synthesised from hnRNA by using a reverse primer annealing in intron 4 did not improved variability. Mean variance for individual samples was 0.04 and 0.08 for cDNA Ex4 and cDNA In4 respectively.

If one argues that stochastic amplification in low copy number samples can be over come by increasing repeat measurements (see section 4.4.2), this experiment may provide a true reflection of the allelic transcription pattern. This experiment showed a not statistically significant trend towards higher expression of the C-allele compared to the G-allele. However it would be difficult to argue that such a result could be relied upon.

The designed allelic assay is unlikely to be useful in assessing allelic transcription in tissues with low IL6 expression. It may be more successfully employed for samples with a higher baseline expression or in stimulated tissues or cells. It may also be useful in cell culture experiments with a higher RNA yield.

4.5 Ex Vivo IL6 Expression in Different Tissues

IL6 was expressed in adipose, vein and muscle tissue as well as leukocytes. Overall expression was low, as indicated by the relatively high Cp-values measured when compared to Cp values of the reference gene. These findings are not surprising, as it has previously been stated that IL6 is not constitutively expressed (24) and that adult vascular endothelial cells incubated without stimulus released scant IL6 activity (301), thus low expression values had been expected.

To date, comparative data on IL6 expression has been available in the form of the gene expression atlas (302). The gene expression atlas has generated gene expression data from a set of human and mouse tissue samples using high throughput gene expression profiling techniques. The tissue samples and cell lines were analysed. The tissue samples were predominantly from a normal physiological state. The gene expression atlas data was based on commercially obtained tissues which originated from different individuals. There is large variation in IL6 expression in individuals, partially related to variables such as smoking, medication and age. Such confounding variables are likely to have affected the results of the gene expression atlas experiment (140;272;288). In contrast, this study uses paired tissue samples from one individual, taken at the same time point thereby reducing the effect of confounding variables, giving a more accurate insight into the effect of tissue type on IL6 gene expression. In addition vein, adipose and muscle tissue, are not represented in the atlas, though all have been shown to express IL6. IL6 RNA expression in adipose tissue is of particular interest, as obesity is a known risk factor for arterial disease. For a long time adipose tissue was regarded as a passive organ designed for energy storage. However it has recently become apparent that adipose tissue has an important endocrine function and is able to release a variety of active mediators into the circulation including IL6 (159). Obesity has seen a rapid increase in prevalence in recent years (303) and is likely to result in a further rise in the occurrence of atherosclerosis (158). Therefore the above results provide important additional data to the gene expression atlas data. Another advantage of this study was the ex vivo nature of the samples, which are likely to reflect the in vivo expression better than cell culture experiments.

The presented data showed that adipose tissue expressed significantly more IL6 mRNA than the other tissues examined, suggesting that increased adiposity might influence circulating IL6 levels and thus potentially vascular risk and treatment outcome. These data are consistent with a 23% fall in plasma IL6 levels associated with 33% weight loss in the very obese (304). Indeed, arterio-venous differences in plasma IL6 levels across an abdominal subcutaneous tissue bed suggest that up to 30% of plasma IL6 may be derived form adipose tissue depending on BMI (305). Adipose tissue thus appears to be an important source of circulating IL6. Increase in adiposity will therefore lead to an increase in IL6 due to a volume increase in the production site of IL6. This study tested for a correlation between the BMI of the study subject and adipose IL6 mRNA expression in this subject. This may appear to be the wrong approach because an equal amount of total RNA was used in each examined sample. Differences in adipose tissue volume between individual should therefore not be able to influence the results. However, one may still expect to see a correlation, as increased adiposity may lead to a change in the cell composition of adipose tissue with a relative increase in those cells that are mainly responsible for the expression of IL6. Only 10% of IL6 derived from adipose tissue cell culture arises from adipocytes (167). Other sources of IL6 in adipose tissue are stromal-vascular cells and macrophages, with macrophages thought to be the main contributors (306;307). Adipose tissue macrophage infiltration is increased in obese subjects (308) and decreases during weight loss (309). Therefore the relative IL6 expression in adipose tissue may increase with adiposity beyond a pure volume effect. This study does not support this view, as there was no significant correlation between BMI and IL6 expression in adipose tissue. It is possible that variables such as age, smoking or medication masked any effect of cell composition on adipose IL6 expression. Further studies may provide better insight.

It is not possible to deduce the exact percentage adipose tissue contributes to the circulating IL6 from relative IL6 mRNA levels measured in this study. Various steps exist that exert post transcriptional control, i.e. not all mRNA will be translated into protein, and these post-transcriptional changes may vary between tissue types. In addition, protein production within the cell may not translate into equivalent secretion of the protein, as an intracellular signalling pathway for IL6 is thought to exist (21). It is therefore possible that a proportion of IL6 will never leave the cell. In addition, some of the IL6 secreted by adipose tissue may act in a paracrine manner and therefore remain within the tissue rather than enter the circulation, though release of IL6 has been demonstrated from peripheral fat (160). Despite

these reservations, it is thought that the circulating levels of IL6 are mainly regulated at the level of gene expression (20), making it likely that the difference in mRNA expression will be reflected in tissue contribution to IL6 plasma levels.
It is known that visceral obesity constitutes a greater risk factor for atherosclerosis than peripheral adiposity (310). Studies looking at site of production of circulating IL6 suggest a greater contribution from visceral fat than from peripheral adipose tissue supporting the view that IL6 may be the link between obesity and arterial disease (167;168). This study examined subcutaneous fat samples only. It is likely that the relative contribution of visceral fat to systemic IL6 is even higher than the figures presented here, emphasising the importance of weight loss in decreasing circulating levels of IL6.
Adipose tissue expressed 35 times as much IL6 mRNA as leukocytes in this study. However the percentage contribution of adipose tissue to plasma IL6 levels depends on levels of adiposity, thus its' relative importance is decreased in lean subjects. In addition IL6 secreted by circulating leukocytes will immediately contribute to IL6 plasma levels unlike adipose derived IL6 which may remain within the tissue itself. Thus leukocyte derived IL6 may play a more important role in plasma levels then the figures for mRNA expression suggest.
In this study the mean mRNA expression in adipose tissue, leukocytes and vein tissue was significantly greater than the expression in muscle. The transcription of IL6 in muscle as well as net release from muscle has been shown to increase following exercise (311;312). Although exercise also appears to increase IL6 expression in adipose tissue, the increase is relatively smaller than the one observed in muscle. One would expect exercise to lead to a relatively higher increase in contribution from muscle to circulating IL6 levels (313). In addition, it has been shown that IL6 release from muscle increases from the fasting level following a high fat meal. In this study patients presented to hospital following overnight rest and overnight fast. The relative level of IL6 expression in muscle in more active subjects and subjects in the postprandial phase may therefore be higher than demonstrated here.

Another factor to consider is that in this study IL6 expression was calculated relative to a housekeeping gene. Relative quantification is advantageous as it avoids a variety of errors that can affect absolute quantification: difference in the quality and the amount of the starting material as well as differences in the RNA preparation and reverse transcription steps. These errors are avoided as the housekeeping gene is

exposed to the same preparation stages as the target gene (314). However relative quantification carries its own drawbacks: Housekeeping genes are thought to be constantly expressed in all tissues relative to total mRNA examined as they are necessary for basis cell survival. This forms the basis of relative Quantification. However it has been shown that levels may vary under certain experimental conditions (297;314). GAPDH, the housekeeping gene used in this experiment, is a key enzyme in the control of glycolysis and as such is expected to be present in all cells and exhibit minimal variation (314). Gene expression atlas data shows stable expression of GAPDH across a variety of different tissues and a number of authors have verified the suitability of GAPDH as a reference gene (315-317).

To date GAPDH has been commonly used in experiments measuring IL6 expression (318-320). However, other authors have shown that certain experimental conditions and level of cell differentiation may affect expression of GAPDH (321-323). All cell types examined in this experiment were obtained from adult tissue and are therefore likely to show the same amount of differentiation. Tissue was collected from human volunteers without stimulation of cells or induction of hypoxia known to affect GAPDH (323;324). Therefore experimental conditions should not have affected GAPDH expression, but some variation in expression affecting the final results can not be excluded.

A number of patients had considerably higher IL6 expression compared to other subjects in some tissues. Possible reasons include unidentified genetic variables or variables such age, smoking, medication or undiagnosed infection at the time of sample collection. The design of the study aimed to reduce the influence of such variables on the outcome measure of relative tissue expression by performing *intra*-individual comparison. However, it can not be excluded that some variables may affect individual tissues differently. Investigating potential causes for higher expression in some patients requires an *inter*-individual comparison which may be difficult to interpret in a relatively small sample set. Such a comparison was therefore not performed.

4.6 Relative Expression of IL6 in Different Tissues with Regards to SNP G-174C Genotype

In all tissues the CC group expressed more IL6 than the other genotypes, results consistent with the trend seen in the allelic transcription assay. The trend towards higher expression in the CC genotype was statistically significant in leukocytes and vein tissue. In leukocytes the CC genotype expressed significantly more than the GC and the GG genotype. Expression was highest in the CC and lowest in the GG group in keeping with an allele effect on expression. In vein there was a significant difference in expression between the genotype groups. The CC group expressed more than the GC genotype and the CC genotype expressed on average more than the GG genotype. In addition the GG group expressed more IL6 than the GC group. These findings cannot be explained by a simple allele effect. One possible explanation is the effect of haplotype. A cell culture study demonstrated that haplotype G-597A, G-572C, -373$A_nT_{n,}$ G-174C may affect gene transcription, with higher expression for haplotype GG9/11G and lower expression for AG8/12G(24). It may therefore be that the high expressing haplotype was more prevalent in the SNP G-174C GG genotype group in the vein sample. This can not be verified as haplotype was not examined in this study. Equally, higher expression in the GG genotype group compared to the CC genotype could also be explained by confounding variables affecting the results.

Studying haplotypes may have added value to our study but further subdivision of an already small sample group would have made interpretation difficult.

The effect of *IL6* promoter SNP G-174C may vary according to the tissue type in which the gene is expressed (24). In this study some tissues showed significant difference in IL6 mRNA expression between genotypes, while others did not. It is difficult to draw conclusions from this with regards to the interaction of genotype and tissue type. Overall there was a trend towards higher expression in the CC genotype in all tissues. The fact that this was not significant in some tissues may relate to sample size but could equally represent tissue specific variation of expression.

The observed trend of increase expression in the CC genotype in all tissue types, is contrary to the effect of promoter SNP G-174C originally observed in cell culture, which showed higher expression in the G-allele (238). The reason for this discrepant observation may be the effect of cell culture on gene expression. Cytokine gene transcription has been shown to be different in vitro from in vivo when comparing

genotypes in other studies (182;325). In this study the samples were taken from human subjects and RNA extracted without intermediate cell culture or stimulation. The in vivo RNA profile was preserved by immediate submersion of samples in RNA stabilising solution and subsequent storage at -80°C. The above experiment is therefore likely to represent a more accurate model of the in vivo effect of the promoter SNP then tissue or cell culture.
However, the results of this experiment have to be interpreted cautiously. This experiment was an inter-individual comparison and this, in addition to the small sample size, renders it susceptible to the effects of confounding variables such as smoking. The distribution of smokers between the genotype groups, with the highest percentage of smokers in the CC genotype group, was borderline significantly different. Smoking itself was not significantly associated with raised levels of IL6 RNA in any of the tissues. However the mean expression was higher in smokers than in non-smokers in all tissues. IL6 is increased in the serum of smokers (140;148), it is therefore possible that smoking increased IL6 expression in the tissues examined. It is possible that a significant effect of smoking on IL6 mRNA expression could not be shown due to low sample number and therefore the borderline difference in distribution of smokers could have affected the results.
Statistically there was no significant difference in the age of the subjects in each genotype group. Thus age should not have influenced the results. A significant correlation between age and IL6 expression was demonstrated for fat and vein tissue. In both tissues, IL6 expression increases with age. This correlates with previous findings showing that IL6 plasma levels are elevated after the menopause or andropause (326). As fat tissue may contribute significantly to circulating levels of IL6 the increased production in adipose tissue in older subjects may explain some of the age related change in plasma levels.

Interpretation of the results of this experiment was difficult due to the small sample numbers in each genotype group entered into the study. Sample number was further reduced by invalid results, particularly in adipose tissue were 23.8% of the samples were invalid. This is most likely related to the poor quality of the fat RNA samples, which may have interfered with cDNA synthesis in some cases. RNA extracted from adipose tissue is known to be of lesser quality and be more likely to contain partially degraded RNA subfractions (297) which can affect down stream applications.
Due to the laborious nature of RNA studies it would have been difficult to achieve larger numbers in this study.

There appeared to be large inter-individual variation in IL6 RNA expression within genotype and within tissue type. Confounding variables may have had a far greater effect on gene expression than SNP G-174C, thus masking its effect. Hence the need for an assay which is well controlled, thus minimising effect of confounding variable, and does therefore not require large sample numbers. The allelic transcription assay previously developed appeared to be such an assay; however reliability was poor for leukocytes and vein tissue. One of the most likely causes for the poor reproducibility was low hnRNA copy numbers. It had been shown that adipose tissue expresses significantly more IL6 RNA than the other tissues examined. An attempt was therefore made to use the allelic transcription assay on adipose tissue samples.

4.7 Allelic Transcription Assay for Adipose Tissue

A large proportion (11 out of 17) of samples showed gDNA contamination despite rigorous DNase treatment. One sample showed particularly strong bands in repeat LDLR assays suggesting substantial gDNA contamination. No cDNA could be detected in this sample. The sample was therefore excluded from final analysis but included in the study to act as a further positive control with an expected IL6 RNA transcription ratio identical to gDNA. The calculated transcription ratio (0.55) for this sample was close to the expected ratio (0.56) (calculated ratio of heterozygous gDNA controls) (Table_Apx C-45). The small variation between the two ratios may be due to inaccuracies in the measurement. Alternatively, a small amount of cDNA could have been present in the sample but could not be demonstrated on a gel with out nested PCR. A common cause for gDNA contamination is inadequate homogenisation. However, adipose tissue was easier to homogenise than the vein samples. As vein had a smaller proportion of contaminated samples than adipose tissue, inadequate homogenisation is unlikely to be the sole cause of the residual gDNA contamination. It has been recognised that the unique nature of adipose tissue (high lipid content) requires specific extraction methods to gain high quality RNA samples (327). This study used the same RNA extraction method for vein, muscle and adipose tissue. This was preferable due to the added cost of using a tissue specific extraction method; however the use of tissue specific RNA isolation methods may have improved RNA quality and yield increasing the number of usable samples. gDNA contamination reduces any measurement of real difference in allelic transcription between the alleles. It is not possible to assess the exact influence gDNA had on results, as contribution of hnRNA and gDNA to the final result can not be differentiated, however it is likely that the demonstrated difference in allelic transcription underestimates the true value.

Genotyping for SNP G3572T was performed to exclude any rare SNP G-174C/SNP G3572T GT haplotypes. Inclusion of rare SNP G-174C/SNP G3572T GT haplotypes would lead to bias towards higher expression in the C-allele. Only one out of 17 samples was found to contain the rare haplotype. In the SeattleSNPs Haplotype Data (http://pga.gs.washington.edu/data/il6/ilkn6.phase.out) population the ratio of haplotype SNP G-174C/SNP G3572T GG to GT was about 2:1. The rare haplotype is less common in this study population. This could be explained by the SeattleSNPs

Haplotype Data being derived from a European/Afro American panel, were as subjects used in the allelic transcription assay were only Europeans.
The reproducibility of the allelic transcription assay was improved compared to previous experiments by utilising adipose tissue RNA as a template. Mean variance for repeat assays for individual adipose tissue samples was 0.008 compared to 0.004 for gDNA (standard curve) and 0.05 and 0.06 for vein and leukocyte respectively. Accuracy of the assay was likely to have been improved by an increase in template due to higher IL6 expression in adipose tissue. cDNA yield was further improved by placing the start site for reverse transcription into an intron region. This eliminated competition from mRNA templates maximising transcription from hnRNA.

There was no statistically significant difference in allelic transcription between the G and the C allele. However, expression from the C-allele was three times higher than from the G-allele. This correlates with findings from the RT-PCR analysis showing the CC genotype to have the highest levels of RNA expression in all tissues. Possibly the lack of significance in the allelic transcription assay is related to the low number of samples included and the effect of gDNA contamination (328). Further work in improving elimination of gDNA contamination and using a greater number of samples may be valuable. Results form this study have suggested that, for tissues with a relatively high IL6 hnRNA expression, the allelic transcription assay may become a suitable assay to assess the effect of promoter SNP G-174C on IL6 RNA expression. The advantage of comparing allelic transcription in heterozygote individuals is the exclusion of confounding variables, which may lead to variation in gene expression when comparing expression between different individuals. Intra-individual comparison thus has clear advantages. Further work to improve this assay appears justified.

5 Summary and Conclusion

This study aimed to investigate the effect of promoter SNP G-174C and the influence of tissue type on IL6 expression in humans. This was to be investigated at the mRNA and hnRNA level in human tissue. The aim was ambitious.

Subject recruitment and sample collection were logistically difficult and hindered by changes in the NHS health care provision. However, the majority of patients undergoing varicose vein surgery in the study centres were willing to donate tissue for scientific research and thus a sufficient number of tissue samples were obtained to proceed with the study. Those patients, who were followed up, were satisfied with their recruitment and did not feel adversely affected by the additional tissue sampling during their procedure. Their average return to normal daily activity and pain scores following tissue sampling compared favourably with those previously reported for varicose vein surgery. All those questioned were willing to participate in future studies and as thus it would appear that the patient experience in this study was satisfactory. The tissues collected are a valuable resource of a variety of ex vivo tissues from adult individuals, which can be utilised in comparative gene expression studies in the future.

Interleukin 6 plays an important role in the development and the progression of arterial disease. Greater understanding of the mechanisms controlling *IL6* gene expression may be useful in the development of treatment strategies. Results in this study indicated that promoter SNP G–174C may have an effect on *IL6* gene transcription. A significant difference in IL6 mRNA expression between genotypes was shown in vein tissue and in leukocytes, with the highest expression in the CC genotype. No statistically significant difference in IL6 mRNA expression was demonstrated in adipose tissue or muscle, though again mean expression was highest in the CC genotype. Thus IL6 expression may in part be determined by SNP G-174C genotype in some tissues, with a trend towards higher expression from the CC genotype.

This experiment was valuable as it used tissue sampled from human volunteers rather than cell culture, thus giving a more accurate picture of the in vivo expression profile of *IL6*. The study measured IL6 mRNA expression rather than circulating protein levels. Measuring circulating protein levels is technically easier, as subjects are only required to donate blood samples and protein assays allowing high through put of samples are commercially available. Thus a greater sample number can be

achieved in protein studies. However, promoter polymorphisms are likely to act at the level of primary gene transcription and a variety of steps exist, influencing IL6 protein expression after primary transcription occurs (see chapter 1.3). Therefore RNA expression studies are more likely to show true effects of promoter polymorphisms on gene expression. Conversely RNA studies are laborious, leading to difficulties in achieving high sample numbers as was demonstrated in this study.

This study suggests that IL6 mRNA expression may not be influenced by a simple SNP G-174C allele effect in some tissues. All tissues, with the exception of vein, showed a trend towards higher expression of IL6 mRNA associated with numbers of C-alleles. However, in vein tissue the CC genotype was associated with the highest IL6 mRNA expression, but the GG genotype expressed more than the GC genotype. Further studies are necessary to investigate this result.
It is difficult to draw conclusions as to the effect the interaction of tissue type with genotype. Though no significant difference between genotype was found in some tissues but significant differences in others, there was a trend towards higher expression in the CC genotypes in all tissues. It is possible that the effect of the promoter SNP was too small to become statistically significant in the relatively small sample number in some tissues. Thus a larger study is required to investigate the effect of interaction of genotype and tissue type on gene expression.

A significant correlation was found between increasing age and IL6 mRNA expression in adipose tissue and vein tissue. An association between IL6 plasma levels and age has been shown in a previous study (326). As adipose tissue may contribute significantly to circulating levels of IL6, the increased production of IL6 in adipose tissue in older subjects may explain some of the age related changes in plasma levels.

Studies with low sample numbers such as this study are susceptible to the influence of confounding variables, therefore this study attempted to design an allelic transcription assay to minimise the effect of confounding variables, allowing conclusions from smaller sample numbers. This is the first study to use a gel based assay to assess the effect of promoter SNP G-174C on IL6 hnRNA expression in heterozygotes. Assessing allelic transcription in heterozygous individuals is advantageous as confounding variables act on both alleles and therefore should not influence results. The experiment showed a trend for higher expression in the C-

allele in vein tissue, though this was not statistically significant. The accuracy of the assay was reduced by gDNA contamination and high variance in repeat samples. Variance was thought to relate to low copy number of IL6 hnRNA in the samples. Leukocytes also showed a trend towards higher expression in the C-allele, again the difference was not statistically significant. In both the vein and the leukocyte assay the variance in repeat samples was greater than the variance seen for gDNA samples/standard curve. Thus, the assay may remain inaccurate for vein and leukocytes.

In the adipose tissue allelic transcription assay the mean hnRNA expression from the C–allele was greater than from the G-allele. Again the difference was not statistically significant. gDNA contamination was again seen in the adipose tissue samples, however variance of repeats was improved and was similar to that seen for gDNA/standard curve samples suggesting that higher levels of IL6 RNA improved assay precision. Employing adipose tissue specific extraction methods to improve quality and purity of RNA samples, thus further minimising gDNA contamination, may further improve the precision of the assay and allow it to become a more accurate tool in future studies.

This experiment established for the first time that it is possible to demonstrate IL6 hnRNA expression using a gel based method. Though its use is likely to remain limited to tissues with high IL6 expression, the advantages of the intra–individual comparison and the likely proximity of hnRNA expression to promoter effect justify further development of this technique.

Risk of atherosclerosis is likely to be influenced by circulating levels of IL6. IL6 is expressed in a variety of tissues. These contribute to the circulating levels of IL6, however the relative contribution individual tissues make to the plasma IL6 level is unknown. This study investigated the relative mRNA expression of IL6 in different tissues. It was demonstrated that the relative IL6 mRNA expression was significantly greater in adipose tissue then in vein tissue, skeletal muscle tissue and leukocytes within individuals. This is the first study to demonstrate this difference in IL6 mRNA expression in individuals. This may have implications when considering the role of obesity in atherosclerotic disease development. It was further demonstrated that the relative RNA expression in adipose tissue, leukocytes and vein tissue is significantly greater than in muscle. This is the first study using a variety of tissues from one individual to compare in vivo RNA expression profiles for IL6.

The study also showed that there is a high inter-individual variation of IL6 RNA expression, regardless of genotype. This may be the result of genetic variables not examined in this study or confounding variables such as age, smoking and medication etc. In this context the advantage of intra-individual studies of IL6 expression is apparent. Using tissue samples from one individual minimises the effects of confounding variables on study outcome as all tissues are exposed to the same variables. Equally studying allelic transcription minimises the effect of confounding variables. Thus refinement of the techniques established in this study may yield valuable results in the future.

6 Future work

The aim of this study was to identify the effect of promoter SNP G-174C on IL6 RNA expression, thus defining a marker that could be used in Mendelian randomisation studies allowing the nature of the IL6/vascular disease association to be explored. Though SNP G-174C may affect gene expression the idea that one single polymorphism significantly affects circulating protein levels and ultimately disease process may be an over-simplification. A promoter SNP may affect RNA expression, however beyond this, multiple levels of regulation exist which may influence protein levels. Equally other polymorphisms within the IL6 gene may influence expression and haplotype studies are likely to be more relevant then the isolated study of one polymorphism. Protein levels are also regulated through a variety of positive and negative feed back loops involving other mediators. Levels of these mediators may in turn be determined by polymorphisms within their genes. Thus IL6 circulating levels may be regulated by a number of polymorphisms in a variety of genes. The isolated effect of a single promoter SNP on gene expression would have to be substantial to show an effect on disease development. This study showed that although SNP G-174C may affect RNA expression to an extent, the effect is unlikely to be of this magnitude

The results of this study therefore have to be used in conjunction with findings from other experiments, e.g. studies investigating IL6 haplotype (24). Besides further studies into the regulation of IL6 expression through polymorphisms within the *IL6* gene, the delineation of the pathophysiological pathways of atherosclerosis will require further investigation of all mediators involved in vascular disease development including those with known effect on IL6 expression. Mediators affecting IL6 expression such as TNFα, IL1β, platelet derived growth factor, transforming growth factor β (TGFβ), IL13 and IL4 have already been extensively investigated and further research into their regulatory mechanisms will help clarify the pathophysiological pathways involved in vascular disease development (12;24;25). Furthermore recent studies have shown that interleukin 6 receptor (IL6R) polymorphisms associate with IL6 plasma levels and the mechanism of this association will require further study (329-331). Soluble IL6R (sIL6R) possibly affect IL6 expression via negative feed back loops or plasma levels of IL6 could be decreased through increased binding and internalisation of IL6/sIL6R complexes

(332;333). Soluble IL6R (sIL6R) is generated by proteolytic cleavage (shedding) of cognate IL6R or by alternative splicing of the IL6R mRNA (40;45;334). A *IL6R* SNP known to affect IL6 levels (SNP rs 8192284) occurs at a proteolytic cleavage site of IL6R and it has been suggested that variation at this point may affect circulating levels of sIL6R (330;335). Further studies examining the effect of IL6R polymorphisms on sIL6R levels may help to clarify IL6 regulation.

The biological activity of IL6 may also be affected through modulation of IL6 signalling pathways. Recently it has been proposed that a member of the family of LIM containing proteins, LIM only 4 (LMO4) can modulate IL6 signalling, possibly by stabilising the gp130 complex i.e. LMO4 may act as a positive regulator in IL6 signalling (336). Proteins containing LIM domains have been previously suggested to play a role in the development of arterial disease (337-339). It is possible that factors leading to over expression of LMO4 may contribute to increasing IL6 signalling with a potential role in arterial disease development. Delineation of LMO4 regulatory processes will be of relevance to the understanding of vascular disease.

The list of mediators with potential to modulate arterial disease development is extensive and includes many not mentioned in this thesis. The ongoing study of individual genetic variants of these mediators and their effect on gene expression will continue to advance our understanding. However, in terms of association studies (phenotype/genotype association) individual polymorphisms are likely to be less relevant unless the isolated effect of the individual variant is substantial. This is demonstrated by the discrepant observations of association studies of SNP G-174C and atherosclerosis.

In this context, the recent introduction of genome wide association studies (GWAS) offers a more rational approach towards identifying disease associated genomic variants (340). Given the complexity of biological pathways, the non-hypothesis driven approach of GWA studies, investigating disease association with genome wide variations, is likely to identify relevant genes more rapidly than has previously been possible. The development of micro array technology, allowing detection of a large number of genetic variants in a short space of time and the recent completion of the Human Genome Project as well as the International HapMap Project have been pivotal to the introduction of GWA studies. Locus 9p21.3 has been named as the most replicated locus associated with coronary artery disease in GWA studies. It contains the sequence of two cyclin-dependent kinase inhibitors, CDKN2A and CDKN2B (341). These may affect pathogenesis of atherosclerosis as part of the TGFβ-induced growth inhibition pathway (342;343). In addition, a GWAS has

identified loci near the IL6R gene which associate with CRP levels, an independent risk factor for myocardial infarction and stroke (344). GWA studies offer an exciting opportunity for the future and are likely to accelerate the understanding of the pathogenic pathways of arterial disease.

Appendix

Appendix A: Materials

a: Materials for PCR

DNA Samples

Control DNA:

DNA was extracted from blood donated by volunteers working in the Human Genetics Division. Following extraction, the DNA samples were diluted to roughly equal concentrations of 7-10ng/ul. These samples were used as templates in the optimisation of experiments.

DNA Samples:

Nine millilitre K-EDTA venous blood was taken from patients participating in the study. DNA was extracted from these samples and quantified using Pico Green assay.

RNA Samples

Control RNA:

Control RNA was obtained from blood donated by volunteers in the laboratory. RNA was extracted using the PAXgene^{TM} Kit.

Primers

Primers were designed using 'primer 3' software. All primers were supplied by MWG-Biotech (Ebersberg, Germany) except for In/Ex Primer and Exon Primer which was supplied by Sigma-Aldrich. (See Appendix 1 for list of primers)

Enzymes

Taq DNA polymerase (5U/ul): Purchased from Promega Company; Madison, Wi, USA, Catalogue number: M1665

Pwo DNA polymerase: purchased from Roche Diagnostics, Lewes, UK.

Msp I: Restriction endonuclease, purchased from New England BioLabs Inc., http://www.neb.com , Cat No: R0106S

Dde I: Restriction endonuclease, purchased from New England BioLabs Inc., http://www.neb.com , Cat No: R0175S

Mae I: Restriction endonuclease, purchased from Roche Diagnostics GmbH, Roche Applied Science, Nonnenweg 2, 82372 Penzberg, Germany, Cat No: 10 822 221 001

PCR Reagents

10x PCR Buffer: Provided with Taq DNA polymerase by Promega, Madison, WI, USA

10x Long PCR buffer: homemade reagent:

- 500mM tris-HCL, pH 8.9
- 140mM ammonium sulphate

Betaine: Purchased from Sigma Chemicals Co. U.S.A. B-2629, [107-43-7], EC NO 203-490-6

Desoxynucleotide Triphosphates (dNTPs) 100nM, pH: 7.5: Purchased from Promega, Madison, WI, USA, Catalogue Number: U1240

Magnesium Chloride 25mM: Provided with Taq DNA polymerase by Promega, Madison, WI, USA

Primers 100pmo/ul: purchased from MWG-Biotech, Ebersberg, Germany (www.mwgdna.com)

b: Chemicals for Gels

Acrylamide (Acrylamide-bis-acrylamide) solution (ration 19:1): from Severn Biotech Ltd. UK. Catalogue Number 003661

10x TBE: made on site from:

Tribase, tris (hydroxymethyl) methylamine: from Fisher Scientific UK Ltd, Product Code: t/3710/60

Boric Acid: from Fisher Scientific UK Ltd, UK. Code B/3800/60

EDTA (Ethylenediamietetra-acetic acid sodium salt): from BDH Lab Supplies, Poole, UK. Product Number: 10315

Ammonium Persulphate: from Fisher Scientific International Co. UK. Ltd. Product Number: 6160/53

TEMED (N,N,N',N'-Tetramethylethylendediamine): from Sigma Chemical Co., U.S.A. EC No203-744-6, Product Code: T-8133

Agarose: purchased from Life Technologies, Paisley. Scotland. 15510-019

Chemicals for staining Gels

Ethidium Bromide 10mg/ml: from Sigma Chemical Co., MO, USA. EEC No214-984-6, Product-Code: E-1510

Vistra Green™ 10000x concentration: Purchased from Amersham Biosciences UK Ltd., Product Code: RPN5786

c: Instruments

Fluorimager: Model 595, Molecular Dynamics, Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK

TyphoonTRIO+: variable Mode Imager, Amersham Biosciences UK Ltd.

Thermal Cycler: DNA Engine Tetrad, MJ Research Inc., USA

Centrifuge: IEC Centra MP4, International Equipment Company Inc. USA

Microcentrifuge: IEC Micromax, International Equipment Company Inc. USA

Centrifuge with swing out rotor: Multifuge® 3L-R, Heraeus, Kendro Laboratory Products GmbH, Post-box 1563, Hanau, Germany

Output power supply: 200-220 V, 50-60HZ, Bio-Rad Laboratories Inc., USA

d: Commonly used Materials and Chemicals

Pipettes: Pipetman (2ul, 20ul,200ul, 1000ul), Gilson S.A. Villiers-le-Bel, France

Disposable Lab Tips: 0.5-10ul Elkay Laboratory Products, Unit E Lutyen Industrial Centre, Bilton Road, Kingsland Buisness Park, Basingstoke, Hants RG24 8LJ Prod Code RESUPIP 010; 2-200ul Code: 94300120 and 101-1000ul Code; 94300220 from Thermoscientific www.thermofisher.com

Multiple Channel Finnpipette®: Labsystem, Helsinki, Finland

Filter Tips 10,20,200,1000: Greiner Labortechnik GmbH, A-4550 Kremsmünster, Bad Hallerstr. 32, Austria. Product No: 771288, 774288, 739288, 740288

Microtubes (0.5ml and 1.5ml): Purchased from Alpha Laboratories Ltd, Catalogue Number: LW2372 & LW 2375, Hampshire, UK

Microtubes (200µl): 8 strip tubes, Alpha Laboratorie Ltd, 40 Parham Drive, Eastleigh, Hants SO50 4NU, UK, www.alphlabs.co.uk, Cat No: LW 2510

96(8x12) well "industry-standard" plates: purchased from Thermo-Fast®, Abgene House UK, (www.abgene.com), catalogue-no: AB-0800

384-Well plate: skirted, polypropylene, white, Greinerbio-one, Maybachstr. 2, 72636 Frichenhausen, Germany www.gbo.com/bioscience , Part No: 785207

Microseal™ 'A' Film: For sealing 384 Well plates for PCR MJ Research, www.mjr.com Cat No:MSA 5001

Screw Cap MCT: 1.5ml skirted Thermoscientific www.thermofisher.com Code: 02-1415-4500

Screw Cap for MCT: Thermoscientific www.thermofisher.com Code: 02-1480-0500

Glass plates: were cut from standard glass to 110mmx170mm size; purchased from local supplier.

MADGE former: purchased from MADGE BIOLtd., Nottinghan, UK.

50bp DNA Step Ladder: Purchased from Promega, Madison, WI, USA, Catalogue#G4521

100bp DNA Step Ladder: Purchased from Promega, Madison, WI, USA, Catalogue#G2101

Formamide dye mix: home made from 98% deionised formamide (Sigma Chemicals Co, USA) 49ml, 10mM EDTA (pH 8.0, 0.5M) 1ml, Xylene Cyanol FF, Bromophenol blue (Sigma Chemical Co, USA)

Chill-Out™ 14: Liquid Wax: MJ Research, www.mjr.com

Magnesium Sulfate (1.00M): purchased from Sigma Chemical Co (www.sigmaaldrich.com), PO Box 14508, St. Louis Mo 63178 USA 314-771-5750; Cat-No: M-3409

e: Software

Primer3: http://www.genmewi.mit.du/cgi-bin/priemr/primer3_www.cgi

and http://www.ncbi.nlm.nih.gov/BLAST/

ImageQuant TL: Image Analysis Software V2003, Amersham Biosciences for use with Typhoon

ImageQuant Version 5.0: Image Analysis Software, Molecular® Dynamics for use with Fluorimager

NEBcutter: http://tools.neb.com/NEBcutter2/index.php used to find restriction enzymes

SPSS 14: Statistics software package

Microsoft Exel: Microsoft Office Excel 2003

RT-PCR quantitation Software: Roche Applied Science LightCycler® 480 Software Version 1.0

f: Buffers and Solutions

10xTBE (ph 8.3): This buffer was prepared using 108g Tribase, 55g Orthoboric acid, NA-EDTA 9.3g made up to 1liter with distilled water. The buffer was stored at room temperature.

Long PCR Buffer: This was made using 140mM ammonium acetate (Sigma-Aldrich Company Ltd, Poole, UK).

Betaine (5M): This solution was made from 58.55g Betaine ($C_5H_{11}NO_2$) dissolved in distilled and autoclaved water to make 100ml. Betaine (5M) was stored at 4ºC for a maximum of 2 weeks.

Sticky Silane: This was prepared using 99ml pure ethanol, 500ul glacial acetic acid, 500ul γ-methylacryloxypropyltrimetoxy silane. Sticky silane was applied to the glass surface before it was placed on the gel former.

Ethidium Bromide Staining Solution: This was prepared using 20ul ethidium bromide, 20ml 10xTBE and 180ml distilled water. The solution was kept in a light excluding container. Gels were stained in this solution for 25 minutes prior to electrophoresis.

Vistra Green staining Solution: This was prepared using 10ul Vistra Green, 10ml 10xTBE and 90ml distilled water. The solution was kept in a light excluding container up to a maximum of one week. For every gel stained, a further 5ul Vistra Green was added to the solution. Gels were stained for 20 minutes after electrophoresis.

Ammonium Persulfate (APS) 25%: This was prepared using 2.5g Ammonium Persulfate dissolved in distilled water to make 10mls of solution. APS 25% was stored at 4ºC for a maximum of 6 weeks.

g: Gels

Polyacrylamide Gel (5%): This was made using 36.7ml distilled water, 8.3ml 30% Polyacrylamide, 5ml 10xTBE, 150ul TEMED and 150ul APS. Immediately after mixing the solution was poured into a MADGE former. A glass plate (one side previously coated with sticky silane) was placed silane side down onto the gel former and the gel was left to set.

Agarose Gel (1%): 1g of Agarose was added to 100ml 1xTBE and heated in the microwave for 2 minutes on high power. The evaporated water was replaced and ethidium bromide was added to the gel (5ul Ethidium bromide per 100ml of Gel), before pouring the mixture into the gel former.

Denaturing Urea Acrylamid Gel: 23ml DEPC Water, 3.8ml 30% Acrylamide and 14.4g Urea were mixed until the Urea had resolved. Then 3ml of 10x TBE, 32ul TEMED and 240ul of 25% APS was added to the mixture, which was then poured between two Glass plates with a comb fitted into the top of the gel.

h: Materials used for DNA extraction

ELB (Erythrocyte lysis bufferx10): This buffer was made up using 5.05g potassium hydrogen carbonate, 41g ammonium chloride, 1ml 0.5M EDTA ph 8.0 made up to 500ml double distilled sterile water. This was diluted 1 in 10 for use and autoclaved for 15 minutes at 121°C. The solution was placed in the fridge overnight prior to use.

NLB (Nucleic lysis buffer): This buffer was made up using 5.0ml 1.0M tris pH 8.0, 40ml 5.0M sodium chloride, 2.0ml 0.5M EDTA pH 8.0 made up to 500ml distilled sterile water.

10% SDS (sodium dodecyle sulphate): 10g SDS dissolved in distilled sterile water, made up to 100ml.

Protease 40g/ml: from Sigma-Aldrich Co, Code: P6911, 800mg of Protease was made up to 20ml with distilled sterile water.

Saturated sodium chloride (6M): 35g NaCl in 100ml distilled sterile water.

70% Ethanol: 350ml Ethanol was mixed with distilled sterile water.

10mM tris/1.0mM EDTA buffer pH 7.5: 5ml 1.0M tris pH 7.5 and 1ml 0.5M EDTA pH 8.0 was made up to 500ml with distilled sterile water. It was then autoclaved for 15 minutes at 121°C.

PicoGreen: Molecular Probe Cambridge Bioscience, Cambridge, UK, Code: P 7581

Absolute ethanol: Fisher Scientific, Code: E/0650DF/25 www.fisher.co.uk

50ml conical tubes: Greiner bio-one, Maybachstr.2, 72636 Frickenhausen, Germany www.gbo.com/bioscience Part-No: 227261

15ml conical tubes: Greiner bio-one, Maybachstr.2, 72636 Frickenhausen, Germany www.gbo.com/bioscience Part-No: 227261

Sorvall RT 7: centrifuge with swing out rotor and buckets, Sorvall, Newtown, Ct, USA

Roller SRT 2: roller mixer, Stuart Scientific Holmethorpe Ave. Holmethorpe Industrial Estate Redhill Surrey RH1 2NB UK, Tel: +44 1737 766431

G24 Environmental Incubator Shaker: New Brunswick Scientific Co. Inc., Edison, N.J., USA

i: Materials used for RNA extraction

Disposable Scalpel: Size 21 Ref 0207, Swann Morton, Sheffield, England

Disposable Syringe: 5ml Ref: SS05S, Terumo Europe N.V., 3001 Leuven, Belgium

Disposable Needle: 18G Ref: NN1838R and 21G Ref: 2138R, Terumo Europe N.V., 3001 Leuven, Belgium

Vacutainer Systems: Safety-LokTM Blood Collection System, Becton Dickinson Vacutainer Systems, 1 Becton Drive, Franklin Lakes, NJ,USA

Microtubes 1.5ml: Alpha Laboratories Ltd, 40 Paxham Drive, Eastleigh, Hants, SO50 4NU, UK, Prod-No: LW2375

RNAlater™: RNA Stabilization Solution for Tissue, Sigma (www.sigmaaldrich.com), 3050 Spruce Street, Saint Louis, Missouri 63103, USA; Product number: R 0901

TRI REAGENT™: Purchased from SIGMA (www.sigmaaldrich.com), 3050 Spruce Street, Saint Louis, Missouri 63103, USA; Product number: T 9424

Homogenisation pestle (1.5ml): Purchased from Bioquote limited, The Royal Centre, James St.York, YO10 3DW, UK; catalogue number: PMT - 15

Chloroform: Purchased from SIGMA (www.sigmaaldrich.com), 3050 Spruce Street, Saint Louis, Missouri 63103, USA; Product number: C 2432

Isopropanol: Purchased from SIGMA (www.sigmaaldrich.com), 3050 Spruce Street, Saint Louis, Missouri 63103, USA; Product number: I 9516

DNA-free™: Purchased from Ambion (www.ambion.com); Catalogue number: 1906< contains: DNase I, RNase free (2units/ul), 10x DNase Buffer, DNase Inactivating reagent, Nuclease-free Water/0.1 mM EDTA

RNA storage solution: Purchased from Ambion (www.ambion.com), pH = 6.4; Catalogue number: 7001

PAXgene™ *Blood RNA Kit*: purchased from Qiagen, PreAnalytiX, Qiagen House Fleming Way, Crawley, West Sussex, RH10 9NQ Product No: 762174

PAXgene™ *Blood RNA Tubes:* purchased from Qiagen, PreAnalytiX, Qiagen House Fleming Way, Crawley, West Sussex, RH10 9NQ Product No: 761125

Urea Polyacrylamide Gel Sample Buffer: 10mg sucrose, 20mg Bromophenol Blue, 20mg Xylene Cyanol, 90ml deionised Formamide, brought up to a final volume of 100ml with water.

Fridge Centrifuge: Biofuge fresco, Heraeus, Kendro Laboratory Products, Germany

Sorvall RT 7: centrifuge with swing out rotor and buckets, Sorvall, Newtown, Ct, USA

G24 Environmental Incubator Shaker: New Brunswick Scientific Co. Inc., Edison, N.J., USA

DU®7500 Spectrophotometer: BeckmanCoulter, Buckinghamshire, UK

NanoDrop: Spectrophotometer ND-1000, Labtech International, www.Labtech.co.uk

Hotbox Oven with fan size 2(Baker): Sanyo Gallenkamp plc. Loughborough, UK

RNaseZap®: Ambion® (www.ambion.com), catalogue number: 9780.9782

Diethylpyrocarbonate (DEPC): Sigma Chemicals Co (www.sigmaaldrich.com), PO Box 14508, Saint Louis, Mo 63178, USA; Product number: D-5758

DEPC-treated Water: Ambion® (www.ambion.com), catalogue number: 9906

Dri-Block DB-1 (Heat Block): Tecam® (Cambridge) Ltd., Duxford, Cambridge, England

j: Materials used for cDNA Synthesis

M-MLV-RT: Moloney Murine Leukemia Virus Reverse Reverse Transcriptase, Promega, www.promega.com, Part-No: M368B

M-MLV RT 5x Reaction Buffer: Promega, www.promega.com supplied with reverse transcriptase

Oligo (dT)15 Primer: Promega, www.promega.com, Cat No: C1101

Specific Primers: MWG-Biotech (Ebersberg, Germany)

RT kit for 50 reactions with random hexamer primer: RT-Hex, PrimerDesign Ltd, Building 27, Southampton University, Highfield Campus, Southampton, Hants, UK, SO17 1BJ. *(*www.primerdesign.co.uk*)*

dNTPs: Desoxynucleotide Triphosphates, Promega, www.promega.com, Cat No: U1240

k: Materials used for TaqMan assay

Mastermix-R: Realtime qPCR Mastermix, PrimerDesign Ltd, Building 27, Southampton University, Highfield Campus, Southampton, Hants, UK, SO17 1BJ. (www.primerdesign.co.uk)

IL6 RT-PCR assay: Cat-No: Hs00174131_m1, Applied Biosystems, Division Headquarters, 850 Lincoln Centre Drive, Foster City, CA 94404, U.S.A. (*www.appliedbiosystems.com*)

GAPDH RT PCR assay: Roche Universal ProbeLibrary Probe #60, Roche Diagnostics GmbH, Roche Applied Science, 68298 Mannheim, Germany

Plates: LightCycler® 480 Multiwell Plates 384, Roche, 68298 Mannheim, Germany, #04 729 749 001

Plate sealers: LightCycler® 480 Sealing Foil, Roche, 68298 Mannheim, Germany, #04 729 757 001

ROCHE LightCycler LC480: Roche Instrument Centre AG, Forrenstrasse, CH-6343 Rotkreuz, Switzerland

RT-PCR quantitation Software: Roche Applied Science LightCycler® 480 Software Version 1.0

Appendix B: Primer Sequences

a: PCR Primers

LDLR-Promoter	F	5'-aggactggagtgggaatcagagc-3'
	R	5'- tgctgtgtcctagctggaaaccc -3'
G-174C	F	5'- cgctagcctcaatgacga - 3'
	R	5' – tctttgttggagggtgagg - 3'
Ex3/In3/Ex4 Primer pair	F	5'- gaaagcagcaaagaggcact – 3'
	R	5' – agctctggcttgttcctcac -3'
T2529C	F	5' - ctgaaaggcatgttcagcttc -3'
	R	5' – atctccccactttcaatgca – 3'
G3572T	F	5' - ggcagtttattcttggacagg – 3'
	R	5' – ataaaaggaggttccagccc -3'

G3572T new	F	5′ – ggcagtttattcttggacagg – 3′
	R	5′ –caatgtcccaaaacatgctg – 3′
A5014G	F	5′ – ggtgggtctatggaaaggtg - 3
	R	5′ – caaacaaataagatagtgatgctgg – 3′
Intron3/Exon3 continuity	F	5′ – gaaagcagcaaagaggcact - 3′
	R	5′ – gcagccagagagggaaaag – 3′

b: Probes

SNP G-174C Assay:

FITC Probe:	5′-ctttagcatCgcaagacac- 3′
DABCYL Probe:	5′-aaccttattaagattgtgcaatgtgacg- 3′

c: Primers used in cDNA Synthesis

Reverse Primer for RT Intron3 5′- cttgcccttgttaatgct – 3′

Reverse Primer for RT Intron2 5′ - agtgtgggctcccaatctc - 3′

Reverse Primer for RT Exon4 5′ - tttctgcaggaactggatca - 3′

Reverse Primer for RT Intron4 5′ – gaatgaggacacacccacct – 3′

Appendix C: Statistical analysis

a: Allelic transcription assay for vein tissue

Ranks

	sample_type	N	Mean Rank	Sum of Ranks
allelic_ratio	cDNA	16	9.63	154.00
	gDNA	2	8.50	17.00
	Total	18		

Test Statistics(b)

	allelic_ratio
Mann-Whitney U	14.000
Wilcoxon W	17.000
Z	-.281
Asymp. Sig. (2-tailed)	.779
Exact Sig. [2*(1-tailed Sig.)]	.837(a)

a Not corrected for ties.
b Grouping Variable: sample_type

Table_Apx C-1: Mann Whitney test for vein ratiometric analysis, testing for difference between the mean allelic transcription ratio for cDNA samples and heterozygous gDNA samples.

b: Allelic Transcription Assay for Leukocytes

Ranks

	sample_type	N	Mean Rank	Sum of Ranks
allelic_ratio	cDNA	12	7.83	94.00
	gDNA	2	5.50	11.00
	Total	14		

Test Statistics(b)

	allelic_ratio
Mann-Whitney U	8.000
Wilcoxon W	11.000
Z	-.730
Asymp. Sig. (2-tailed)	.465
Exact Sig. [2*(1-tailed Sig.)]	.549(a)

a Not corrected for ties.
b Grouping Variable: sample_type

Table_Apx C-2: Mann Whitney test for leukocyte ratiometric analysis testing for difference between the mean allelic transcription ratio for cDNA samples and heterozygous gDNA samples.

c: Kruskal Wallis for difference in IL6 mRNA expression in different SNP G-174C genotypes in different tissues

Test Statistics[a,b,c]

	normConc Ratio
Chi-Square	1.961
df	2
Asymp. Sig.	.375

a. Kruskal Wallis Test
b. Grouping Variable: Numeric_Genotype
c. numertissue = Fat

Table_Apx C-3: Kruskal Wallis test for difference in IL6 RNA expression in different genotypes in adipose tissue

Test Statistics[a,b,c]

	normConc Ratio
Chi-Square	7.469
df	2
Asymp. Sig.	.024

a. Kruskal Wallis Test
b. Grouping Variable: Numeric_Genotype
c. numertissue = Leukocytes

Table_Apx C-4: Kruskal Wallis test for difference in IL6 RNA expression in different genotypes in leukocytes.

Test Statistics[a,b,c]

	normConc Ratio
Chi-Square	6.367
df	2
Asymp. Sig.	.041

a. Kruskal Wallis Test

b. Grouping Variable: Numeric_Genotype

c. numertissue = Vein

Table_Apx C-5: Kruskal Wallis test for difference in IL6 RNA expression in different genotypes in vein tissue

Test Statistics[a,b,c]

	normConc Ratio
Chi-Square	2.521
df	2
Asymp. Sig.	.284

a. Kruskal Wallis Test

b. Grouping Variable: Numeric_Genotype

c. numertissue = Muscles

Table_Apx C-6: Kruskal Wallis test for difference in IL6 RNA expression in different genotypes in skeletal muscle tissue

d: ANOVA for age distribution in different genotype groups

ANOVA for fat tissue

age

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	133.896	2	66.948	.359	.701
Within Groups	5404.979	29	186.379		
Total	5538.875	31			

a tissueType = fat

Table_Apx C-7: ANOVA for age in adipose tissue.

ANOVA for leukocytes

age

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	68.327	2	34.164	.194	.825
Within Groups	5455.790	31	175.993		
Total	5524.118	33			

a tissueType = leukocytes

Table_Apx C-8: ANOVA for age in leukocytes

ANOVA for vein tissue

age

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	110.738	2	55.369	.313	.733
Within Groups	6196.340	35	177.038		
Total	6307.079	37			

a tissueType = vein

Table_Apx C-9: ANOVA for age in vein tissue

ANOVA for muscle tissue

age

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	114.832	2	57.416	.317	.731
Within Groups	5615.286	31	181.138		
Total	5730.118	33			

a tissueType = muscle

Table_Apx C-10: ANOVA for age in muscle tissue

e: Spearman Correlation Coefficient

Testing for linear relationship between age and IL6 expression in different tissues.

Correlations[a]

			age	normConc Ratio
Spearman's rho	age	Correlation Coefficient	1.000	.447*
		Sig. (2-tailed)	.	.010
		N	32	32
	normConcRatio	Correlation Coefficient	.447*	1.000
		Sig. (2-tailed)	.010	.
		N	32	32

*. Correlation is significant at the 0.05 level (2-tailed).

a. numertissue = Fat

Table_Apx C-11: Spearman correlation coefficient for relationship between age and IL6 expression in adipose tissue

Correlations[a]

			age	normConc Ratio
Spearman's rho	age	Correlation Coefficient	1.000	-.165
		Sig. (2-tailed)	.	.352
		N	34	34
	normConcRatio	Correlation Coefficient	-.165	1.000
		Sig. (2-tailed)	.352	.
		N	34	34

a. numertissue = Leukocytes

Table_Apx C-12: Spearman correlation coefficient for relationship between age and IL6 expression in leukocytes

Correlations[a]

			age	normConc Ratio
Spearman's rho	age	Correlation Coefficient	1.000	.424**
		Sig. (2-tailed)	.	.008
		N	38	38
	normConcRatio	Correlation Coefficient	.424**	1.000
		Sig. (2-tailed)	.008	.
		N	38	38

**. Correlation is significant at the 0.01 level (2-tailed).

a. numertissue = Vein

Table_Apx C-13: Spearman correlation coefficient for relationship between age and IL6 expression in vein tissue

Correlations[a]

			age	normConc Ratio
Spearman's rho	age	Correlation Coefficient	1.000	.301
		Sig. (2-tailed)	.	.084
		N	34	34
	normConcRatio	Correlation Coefficient	.301	1.000
		Sig. (2-tailed)	.084	.
		N	34	34

a. numertissue = Muscles

Table_Apx C-14: Spearman correlation coefficient for relationship between age and IL6 expression in muscle tissue

f: Testing for difference in distribution of smokers

Crosstab

			smoke		
			Non-smokers	Smokers	Total
genotype	C/C	Count	15	9	24
		% within genotype	62.5%	37.5%	100.0%
	G/C	Count	34	14	48
		% within genotype	70.8%	29.2%	100.0%
	G/G	Count	48	8	56
		% within genotype	85.7%	14.3%	100.0%
Total		Count	97	31	128
		% within genotype	75.8%	24.2%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)
Pearson Chi-Square	5.957[a]	2	.051	.048
Likelihood Ratio	6.081	2	.048	.052
Fisher's Exact Test	6.091			.048
N of Valid Cases	128			

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 5.81.

Table_Apx C-15: Testing for difference in distribution of smokers

g: Linear regression

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.989(a)	.978	.977	.04997	.978	1380.299	1	31	.000

a Predictors: (Constant), ConcSquared

Table_Apx C-16: Linear regression for standard curve: Result of PCR 1 for ratio metric analysis.

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.948(a)	.900	.895	.09992	.900	179.168	1	20	.000

a Predictors: (Constant), ConcSquared

Table_Apx C-17: Linear regression for standard curve: Result of PCR 2 for ratio metric analysis.

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.985(a)	.969	.968	.05934	.969	882.362	1	28	.000

a Predictors: (Constant), ConcSquared

Table_Apx C-18: Linear regression for standard curve: Result of PCR 3 for ratio metric analysis.

h: Allelic Transcription Assay for Adipose tissue

Ranks

	sample	N	Mean Rank	Sum of Ranks
allelic_ratio	TIG sample	7	5.43	38.00
	control genomic DNA	2	3.50	7.00
	Total	9		

Test Statistics(b)

	allelic_ratio
Mann-Whitney U	4.000
Wilcoxon W	7.000
Z	-.878
Asymp. Sig. (2-tailed)	.380
Exact Sig. [2*(1-tailed Sig.)]	.500(a)

a Not corrected for ties.
b Grouping Variable: sample

Table_Apx C-19: Mann Whitney test for adipose tissue: Testing for difference between the mean allelic transcription ratio for cDNA samples and heterozygous gDNA samples.

i: RT-PCR experiment in paired tissue samples

Test Statistics(a)

	NormConRatio
Mann-Whitney U	32.000
Wilcoxon W	263.000
Z	-4.742
Asymp. Sig. (2-tailed)	.000

a Grouping Variable: TissueType

Table_Apx C-20: Mann Whitney U test for adipose tissue and leukocytes

Test Statistics(a)

	NormConRatio
Mann-Whitney U	46.500
Wilcoxon W	277.500
Z	-4.377
Asymp. Sig. (2-tailed)	.000

a Grouping Variable: TissueType

Table_Apx C-21: Mann Whitney U test for adipose tissue and vein tissue

Test Statistics(a)

	NormConRatio
Mann-Whitney U	20.500
Wilcoxon W	251.500
Z	-5.040
Asymp. Sig. (2-tailed)	.000

a Grouping Variable: TissueType

Table_Apx C-22: Mann Whitney U test for adipose tissue and muscle tissue

Test Statistics(a)

	NormConRatio
Mann-Whitney U	197.000
Wilcoxon W	428.000
Z	-.591
Asymp. Sig. (2-tailed)	.554

a Grouping Variable: TissueType

Table_Apx C-23: Mann Whitney U test for leukocytes and vein tissue

Test Statistics(a)

	NormConRatio
Mann-Whitney U	19.000
Wilcoxon W	250.000
Z	-5.078
Asymp. Sig. (2-tailed)	.000

a Grouping Variable: TissueType

Table_Apx C-24: Mann Whitney U test for leukocytes and muscle tissue

Test Statistics(a)

	NormConRatio
Mann-Whitney U	40.500
Wilcoxon W	271.500
Z	-4.536
Asymp. Sig. (2-tailed)	.000

a Grouping Variable: TissueType

Table_Apx C-25: Mann Whitney U test for vein and muscle tissue

j: Independent sample t-test

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
LN_norm_conc_ratio	Equal variances assumed	6.557	.014	8.005	39	.000	3.13439	.39156	2.34238	3.92639
	Equal variances not assumed			7.908	30.390	.000	3.13439	.39637	2.32532	3.94345

Table_Apx C-26: Independent sample t-test for adipose tissue and leukocytes

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
LN_norm_conc_ratio	Equal variances assumed	.399	.531	6.828	38	.000	3.10361	.45451	2.18350	4.02371
	Equal variances not assumed			6.828	37.210	.000	3.10361	.45451	2.18286	4.02435

Table_Apx C-27: Independent sample t-test for adipose tissue and vein tissue

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
LN_norm_conc_ratio	Equal variances assumed	.222	.640	12.562	38	.000	6.55821	.52205	5.50138	7.61504
	Equal variances not assumed			12.562	37.354	.000	6.55821	.52205	5.50078	7.61564

Table_Apx C-28: Independent sample t-test for adipose tissue and muscle tissue

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
LN_norm_conc_ratio	Equal variances assumed	4.352	.044	-.087	39	.931	-.03078	.35311	-.74501	.68345
	Equal variances not assumed			-.086	33.264	.932	-.03078	.35638	-.75563	.69406

Table_Apx C-29: Independent sample t-test for vein tissue and leukocytes

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
LN_norm_conc_ratio	Equal variances assumed	1.636	.208	7.909	39	.000	3.42382	.43292	2.54816	4.29949
	Equal variances not assumed			7.794	28.070	.000	3.42382	.43928	2.52411	4.32354

Table_Apx C-30: Independent sample t-test for leukocytes and muscle

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
LN_norm_conc_ratio	Equal variances assumed	.001	.972	7.016	38	.000	3.45461	.49237	2.45785	4.45136
	Equal variances not assumed			7.016	35.381	.000	3.45461	.49237	2.45542	4.45379

Table_Apx C-31: Independent sample t-test for vein tissue and muscle

k: Wilcoxon Signed Ranks Test

Test Statistics(c)

	leuko - fat	vein - fat	muscle - fat	vein - leuko	muscle - leuko	muscle - vein
Z	-3.980(a)	-3.980(a)	-3.980(a)	-.365(b)	-4.015(a)	-3.980(a)
Asymp. Sig. (2-tailed)	.000	.000	.000	.715	.000	.000

a Based on positive ranks.
b Based on negative ranks.
c Wilcoxon Signed Ranks Test

Table_Apx C-32: Wilcox signed rank test for paired tissue samples IL6 expression patterns

I: Genotyping Data G-174C:

	AN 001	AN001 1	AN001 2	AN 001 4	Array num
?T21	*G/C*	*G/C*	*G/C*	G/C	*AN001H05*
?T27	*Unk*	*G/C*	*G/C*	G/C	*AN001H04*
blank	Unk	Unk	Unk	Unk	AN001A01
blank	Unk	Unk	Unk	Unk	AN001B01
blank	Unk	Unk	Unk	Unk	AN001C05
blank	Unk	Unk	Unk	Unk	AN001D05
blank	Unk	Unk	Unk	Unk	AN001F07
blank	Unk	Unk	Unk	Unk	AN001G07
blank	Unk	Unk	Unk	Unk	AN001H07
C24	G/G	G/G	Unk	G/G	AN001C07
C26	G/C	Unk	G/C	G/C	AN001D07
C27	C/C	Unk	C/C	Unk	AN001E07
neg control	Unk	Unk	Unk	Unk	AN001A07
neg control	Unk	Unk	Unk	Unk	AN001B07
T02	Unk	G/C	G/C	G/C	AN001H06
T03	G/C	Unk	G/C	G/C	AN001F01
T04	Unk	Unk	C/C	C/C	AN001A05
T05	G/G	G/G	G/G	G/G	AN001B02
T06	G/G	G/G	G/G	Unk	AN001F05
T07	C/C	C/C	C/C	C/C	AN001D06
T08	Unk	G/C	G/C	G/C	AN001G01
T09	G/C	G/C	G/C	G/C	AN001B05
T10	Unk	C/C	C/C	C/C	AN001A02
T11	Unk	G/C	Unk	G/C	AN001C04
T12	G/C	G/C	G/C	Unk	AN001C03
T13	G/C	Unk	G/C	G/C	AN001A03
T14	G/G	Unk	G/G	G/G	AN001E06
T15	G/G	G/G	Unk	G/G	AN001D04
T16	G/G	G/G	G/G	G/G	AN001C06
T17	G/C	G/C	G/C	Unk	AN001A06
T18	Unk	C/C	C/C	C/C	AN001A04
T19	G/G	G/G	G/G	G/G	AN001E01
T20	G/C	G/C	G/C	G/C	AN001E02
T22	Unk	Unk	G/C	G/C	AN001D03
T23	G/G	G/G	G/G	G/G	AN001B06
T24	G/G	Unk	G/G	G/G	AN001B04
T25	G/G	G/G	Unk	G/G	AN001E04
T26	G/C	Unk	G/C	Unk	AN001F06
T28	G/C	G/C	G/C	G/C	AN001G05
T29	C/C	C/C	C/C	C/C	AN001H03
T30	C/C	C/C	C/C	C/C	AN001E05
T31	G/G	G/G	G/G	G/G	AN001F02
T32	Unk	G/G	G/G	Unk	AN001G06
T34	G/G	G/G	G/G	G/C	AN001H02
T35	G/G	G/G	G/G	G/G	AN001C01
T36	G/G	G/G	G/G	G/G	AN001G04

T37	G/C	G/C	G/C	G/C	AN001D01
T38	G/G	G/G	G/G	G/G	AN001F03
T41	G/C	G/C	G/C	G/C	AN001G02
T42	C/C	C/C	C/C	C/C	AN001F04
T43	G/C	G/C	G/C	G/C	AN001H01
T44	G/C	G/C	G/C	G/C	AN001B03
T45	G/G	G/G	G/G	G/G	AN001E03
T46	G/C	G/C	G/C	G/C	AN001C02
T47	G/G	G/G	G/G	G/G	AN001D02
T48	G/G	G/G	G/G	G/G	AN001G03

Table_Apx C-33: Genotyping data for SNP G-174C

m: Results of Real Time PCR

Fat	Cp Median	Concentration Ratio	Concentration Ratio STD	Normalized Ratio	Normalized Ratio STD	male=1/ female=2	non smoker=1 smoker=2	Genotype
TIG 002	27.55	48.61	1.44	1.28E+02	40.41	2	1	G/C
TIG 003	32.96	12.73	1.36	33.44	11.13	2	2	G/C
TIG 004	37.23	1.49	0.12	3.91	1.27	2	1	C/C
TIG 005	30.07	12.27	0.56	32.24	10.26	2	?	G/G
TIG 006	33.8	0.43	0.02	1.12	0.36	2	1	G/G
TIG 007	26.14	2.55E+02	31.92	6.71E+02	2.28E+02	1	2	C/C
TIG 008		Invalid	Invalid	Invalid	Invalid	1	1	G/C
TIG 009	27.63	1.27E+03	44.31	3.35E+03	1.06E+03	2	2	G/C
TIG 010	29.89	5.67	0.43	14.9	4.83	2	1	C/C
TIG 011		Invalid	Invalid	Invalid	Invalid	2	1	G/C
TIG 012		Invalid	Invalid	Invalid	Invalid	1	1	G/C
TIG 013	34.74	2.47	1.1	6.5	3.58	2	2	G/C
TIG 014	32.57	0.95	0.11	2.49	0.84	2	1	G/G
TIG 015	26.37	41.13	15.03	1.08E+02	52.46	2	1	G/G
TIG 016	29.27	40.46	4.96	1.06E+02	35.98	2	1	G/G
TIG 017	29.16	37.35	6.55	98.16	35.47	2	2	G/C
TIG 018	30.45	1.15E+02	21.43	3.03E+02	1.11E+02	1	2	C/C
TIG 019	32.06	5.03	0.35	13.23	4.27	1	2	G/G
TIG 020		Invalid	Invalid	Invalid	Invalid	2	2	G/C
TIG 021	31.6	1.05E+02	56.31	2.77E+02	1.73E+02	2	1	
TIG 022	35.22	1.15	0.21	3.03	1.11	2	1	G/C
TIG 024	30.27	14.62	1.18	38.42	12.5	1	1	G/G
TIG 025	33.81	2.35	0.05	6.17	1.95	2	1	G/G
TIG 026	33.62	4.56	1.13	11.97	4.82	2	1	G/C
TIG 028	30.85	66.15	4.03	1.74E+02	55.75	1	1	G/C
TIG 029	32.59	10.51	0.43	27.61	8.77	1	1	C/C
TIG 030	32.63	8.31	1.82	21.85	8.41	2	1	C/C
TIG 031	34.83	0.91	0.38	2.38	1.25	2	1	G/G
TIG 032	33.39	4.99	1.19	13.13	5.2	2	1	G/G
TIG 034	32.5	4.25	0.55	11.16	3.81	1	1	G/G
TIG 035	33.47	2.21	0.53	5.8	2.3	1	1	G/G
TIG 036	31.36	6.33	2.73	16.63	8.94	1	2	G/G
TIG 037	34.09	1.75	0.14	4.6	1.5	2	1	G/C
TIG 038	34.2	21.84	10.83	57.38	33.97	2	1	G/G
TIG 041	32.36	5.73	1.82	15.07	6.77	1	1	G/C
TIG 042		Invalid	Invalid	Invalid	Invalid	2	2	C/C
TIG 043	36.61	0.95	0.24	2.48	1	2	1	G/C
TIG 044		0	0	0	0	1	1	G/C
TIG 045		Invalid	Invalid	Invalid	Invalid	1	1	G/G
TIG 046		0	0	0	0	2	1	G/C
TIG 047		0	0	0	0	1	1	G/G

TIG 048	33.45	4.5	1.94	11.84	6.37	2	1	G/G
TIG 057		Invalid	Invalid	Invalid	Invalid	1	2	
TIG 061		Invalid	Invalid	Invalid	Invalid	2	2	

Table_Apx C-34: Results of real time PCR for fat tissue

Leukocytes	Cp Median	Concentration Ratio	Concentration Ratio STD	Normalized Ratio	Normalized Ratio STD	male=1/ female=2	non smoker=1 smoker=2	Genotype
TIG 008	34.24	0.39	0.01	0.54	0.05	1	1	G/C
TIG 009	31.79	9.64	0.23	13.42	1.22	2	2	G/C
TIG 010	33.07	1.07	0.09	1.49	0.18	2	1	C/C
TIG 011	33.42	0.44	0.08	0.62	0.11	2	1	G/C
TIG 012	32.93	[0.51	0.02	0.71	0.07	1	1	G/C
TIG 013		Invalid	Invalid	Invalid	Invalid	2	2	G/C
TIG 014	33.7	0.55	0.12	0.76	0.16	2	1	G/G
TIG 015	32.53	1.3	0.28	1.81	0.39	2	1	G/G
TIG 016	33.52	0.77	0.00673	1.07	0.09	2	1	G/G
TIG 017	32.86	0.52	0.1	0.72	0.14	2	2	G/C
TIG 018	34.61	4.39	0.09	6.11	0.55	1	2	C/C
TIG 019	34.03	0.29	0.05	0.4	0.07	1	2	G/G
TIG 020	33.49	0.59	0.11	0.82	0.15	2	2	G/C
TIG 021	33.15	7.43	0.31	10.35	1	2	1	
TIG 022	33.51	0.6	0.1	0.83	0.15	2	1	G/C
TIG 023		Invalid	Invalid	Invalid	Invalid	2	1	G/G
TIG 024	33.08	0.33	0.09	0.47	0.13	1	1	G/G
TIG 025	33.77	0.99	0.07	1.37	0.15	2	1	G/G
TIG 026		Invalid	Invalid	Invalid	Invalid	2	1	G/C
TIG 027		Invalid	Invalid	Invalid	Invalid	2	1	
TIG 028	34.48	0.33	6.64E-03	0.46	0.04	1	1	G/C
TIG 029	32.93	0.59	0.07	0.83	0.11	1	1	C/C
TIG 030	33	1.09	0.02	1.52	0.14	2	1	C/C
TIG 031	34	0.43	0.01	0.6	0.05	2	1	G/G
TIG 032	32.97	0.39	0.06	0.55	0.08	2	1	G/G
TIG 033	35.1	0.22	0.24	0.31	0.3	2	1	
TIG 034	32.69	1.1	0.1	1.53	0.19	1	1	G/G
TIG 035	33.04	0.64	0.17	0.9	0.22	1	1	G/G
TIG 036	33.61	0.48	0.01	0.66	0.06	1	2	G/G
TIG 037	33.54	0.46	0.09	0.64	0.13	2	1	G/C
TIG 038	35.22	0.17	0.18	0.24	0.22	2	1	G/G
TIG 039	33.49	0.68	0.01	0.95	0.09	2	1	
TIG 040	33.36	1.03	0.11	1.43	0.18	2	1	
TIG 041	33.92	0.25	0.05	0.35	0.07	1	1	G/C
TIG 042	32.91	1.4	0.08	1.95	0.2	2	2	C/C
TIG 043	33.54	0.61	0.01	0.85	0.08	2	1	G/C
TIG 044	31.45	6.31	0.58	8.78	1.07	1	1	G/C
TIG 045	34.66	0.38	0.06	0.53	0.09	1	1	G/G
TIG 046	32.16	0.31	0.02	0.43	0.05	2	1	G/C
TIG 047		Invalid	Invalid	Invalid	Invalid	1	1	G/G

Table_Apx C-35: Results of real time PCR for leukocytes

vein	Cp Median	Concentration Ratio	Concentration Ratio STD	Normalized Ratio	Normalized Ratio STD	male=1/ female=2	non smoker=1 smoker=2	Genotype
TIG 002	33.58	0.15	0.26	0.15	0.29	2	1	G/C
TIG 003	33.54	0.73	0.03	0.73	0.3	2	2	G/C
TIG 004	37.52	0.43	0.15	0.43	0.37	2	1	C/C
TIG 005	32.31	1.46	0.47	1.46	0.77	2	?	G/G
TIG 006	33.88	0.48	0.36	0.48	0.43	2	1	G/G
TIG 007	32.7	4.69	0.49	4.69	1.97	1	2	C/C
TIG 008	34.83	0.66	0.15	0.66	0.31	1	1	G/C
TIG 009	33.74	8.86	1.06	8.87	3.75	2	2	G/C
TIG 010	33.51	0.4	0.08	0.4	0.18	2	1	C/C
TIG 011	34.75	0.32	0.18	0.32	0.23	2	1	G/C
TIG 012		0	0	0	0	1	1	G/C
TIG 013	34.26	0.21	0.34	0.21	0.37	2	2	G/C
TIG 014	32.53	0.24	0.2	0.24	0.24	2	1	G/G
TIG 015	34.08	7.56	1.14	7.56	3.29	2	1	G/G
TIG 016	35.79	0.83	0.23	0.83	0.42	2	1	G/G
TIG 017	32.9	0.82	0.17	0.82	0.38	2	2	G/C
TIG 018	33.06	15.52	17.88	15.52	20.28	1	2	C/C
TIG 019	30.18	5.21	0.28	5.21	2.12	1	2	G/G
TIG 020	33.32	0.26	0.06	0.26	0.12	2	2	G/C
TIG 021	34.73	3.66	1.87	3.66	2.5	2	1	
TIG 022		0	0	0	0	2	1	G/C
TIG 023	34.39	0.67	0.21	0.67	0.35	2	1	G/G
TIG 024	33	0.38	0.03	0.38	0.16	1	1	G/G
TIG 025	31.47	1.63	1.09	1.63	1.35	2	1	G/G
TIG 026	34.34	0.29	0.05	0.29	0.13	2	1	G/C
TIG 028	31.62	0.66	0.01	0.66	0.27	1	1	G/C
TIG 029	33.43	0.23	0.41	0.24	0.45	1	1	C/C
TIG 030	32.71	4.34	0.23	4.34	1.77	2	1	C/C
TIG 031	36.96	1.28	0.12	1.28	0.53	2	1	G/G
TIG 032		Invalid	Invalid	Invalid	Invalid	2	1	G/G
TIG 034	33.57	0.22	0.32	0.22	0.35	1	1	G/G
TIG 035	39.18	1.65	5.57	1.65	6.12	1	1	G/G
TIG 036	32.4	0.64	0.07	0.64	0.27	1	2	G/G
TIG 037	33.09	0.16	0.07	0.16	0.1	2	1	G/C
TIG 038		0	0	0	0	2	1	G/G
TIG 041	33.41	0.23	0.05	0.23	0.11	1	1	G/C
TIG 042	33.16	2.81	1.13	2.81	1.67	2	2	C/C
TIG 043	33.23	0.41	0.04	0.41	0.17	2	1	G/C
TIG 044	32.23	4.53	1.14	4.54	2.21	1	1	G/C
TIG 045	36.11	0.7	0.21	0.7	0.37	1	1	G/G
TIG 046	31.53	0.9	0.15	0.9	0.4	2	1	G/C
TIG 047		0	0	0	0	1	1	G/G
TIG 048		Invalid	Invalid	Invalid	Invalid	2	1	G/G

TIG 057		Invalid	Invalid	Invalid	Invalid	1	2	
TIG 061		0	0	0	0	2	2	

Table_Apx C-36: Results of real time PCR for vein tissue

muscle	Cp Median	Concentration Ratio	Concentration Ratio STD	Normalized Ratio	Normalized Ratio STD	male=1/ female=2	non smoker=1 smoker=2	Genotype
TIG 002	32.88	0.03	0.02	0.05	0.03	2	1	G/C
TIG 003	35.64	0.02	6.83E-03	0.04	0.02	2	2	G/C
TIG 004	34.02	0.02	0.01	0.03	0.02	2	1	C/C
TIG 005	33.44	0.02	1.96E-03	0.03	0.01	2	?	G/G
TIG 008	38.24	0.01	1.01E-03	0.02	5.32E-03	1	1	G/C
TIG 009	33.51	0.27	0.09	0.41	0.19	2	2	G/C
TIG 010	36.02	7.60E-03	4.03E-03	0.01	7.35E-03	2	1	C/C
TIG 011	35.17	4.62E-03	1.28E-03	7.11E-03	3.07E-03	2	1	G/C
TIG 012	35.81	0.11	0.03	0.16	0.07	1	1	G/C
TIG 013	33.74	0.03	6.23E-04	0.05	0.02	2	2	G/C
TIG 014	39.45	9.23E-05	5.47E-03	1.42E-04	8.57E-03	2	1	G/G
TIG 015	35.76	0.02	6.37E-03	0.03	0.01	2	1	G/G
TIG 016		Invalid	Invalid	Invalid	Invalid	2	1	G/G
TIG 017	35.39	0.01	2.13E-03	0.02	6.59E-03	2	2	G/C
TIG 018	35.27	0.47	0.13	0.72	0.31	1	2	C/C
TIG 019	32.69	0.06	7.09E-03	0.09	0.03	1	2	G/G
TIG 020	35.71	8.91E-03	4.02E-03	0.01	7.69E-03	2	2	G/C
TIG 021	36.76	0.47	0.06	0.72	0.25	2	1	
TIG 022	28.46	37.12	2.72E+04	57.07	4.27E+04	2	1	G/C
TIG 023	33.71	0.03	2.86E-03	0.05	0.02	2	1	G/G
TIG 024	32.77	0.03	3.52E-03	0.04	0.01	1	1	G/G
TIG 025	35.09	0.02	5.48E-03	0.04	0.01	2	1	G/G
TIG 026	35.53	0.01	0.01	0.02	0.02	2	1	G/C
TIG 028	34.5	0.01	4.65E-04	0.02	7.41E-03	1	1	G/C
TIG 029	33.68	0.02	1.61E-03	0.04	0.01	1	1	C/C
TIG 030	34.44	0.05	0.01	0.08	0.03	2	1	C/C
TIG 031	34.67	0.01	6.01E-03	0.02	0.01	2	1	G/G
TIG 032	34.24	9.48E-03	7.18E-04	0.01	4.90E-03	2	1	G/G
TIG 034		0	0	0	0	1	1	G/G
TIG 035	38.63	3.15E-03	8.88E-03	4.84E-03	0.01	1	1	G/G
TIG 036	35.57	0.03	1.67E-03	0.04	0.01	1	2	G/G
TIG 037	34.9	5.13E-03	7.10E-04	7.89E-03	2.81E-03	2	1	G/C
TIG 038	35.77	0.03	3.01E-03	0.04	0.01	2	1	G/G
TIG 041	34.32	0.02	4.98E-03	0.03	0.01	1	1	G/C
TIG 042	39.23	0.03	0.05	0.05	0.09	2	2	C/C
TIG 043	34.48	0.03	6.30E-03	0.05	0.02	2	1	G/C
TIG 045	36.15	1.2	0.62	1.85	1.14	1	1	G/G
TIG 046	32.71	0.03	2.23E-03	0.04	0.01	2	1	G/C
TIG 047		Invalid	Invalid	Invalid	Invalid	1	1	G/G
TIG 048	38.01	0.01	3.51E-03	0.02	8.82E-03	2	1	G/G
TIG 057		0	0	0	0	1	2	
TIG 061	38.55	8.47E-04	1.94E-03	1.30E-03	3.06E-03	2	2	

Table_Apx C-37: Results of real time PCR for muscle tissue

n: Allelic Transcription Assay

Standard curve PCR 1:

PCR 1		Band A	Band B	B/(299/418*A+B)	(known conc.ratio)^2	mean of EB,dest1,dest2
first loading on gel:						
ethidium bromide	Lane 2	2039.23	77265.87	0.98		
de-stained once	Lane 2	1309.48	76103.36	0.99		
de-stained twice	Lane 2	316.29	32311.19	0.99	1	0.99
ethidium bromide	Lane 3	26840.84	106200.85	0.85		
de-stained once	Lane 3	24460.38	107722.42	0.86		
de-stained twice	Lane 3	8974.27	52404.77	0.89	0.81	0.87
ethidium bromide	Lane 4	29449.31	43569.3	0.67		
de-stained once	Lane 4	30627.02	54617.34	0.71		
ethidium bromide	Lane 4	11702.03	29059.66	0.78	0.64	0.72
de-stained once	Lane 5	60774.75	54289.95	0.56		
de-stained twice	Lane 5	59405.07	58857.77	0.58		
ethidium bromide	Lane 5	26739.63	46051.37	0.71	0.49	0.61
de-stained once	Lane 6	57959.81	34669.09	0.46		
de-stained twice	Lane 6	62154.1	32861.46	0.43		
ethidium bromide	Lane 6	29483.83	29979.32	0.59	0.36	0.49
de-stained once	Lane 7	101804.39	37501.19	0.34		
ethidium bromide	Lane 7	102687.41	39602.1	0.35		
de-stained once	Lane 7	56904.16	37325.69	0.48	0.25	0.39
de-stained twice	Lane 8	91038.18	20503.09	0.24		
ethidium bromide	Lane 8	101953.92	22704.15	0.24		
de-stained once	Lane 8	64020.28	22747.94	0.33	0.16	0.27
de-stained twice	Lane 9	124815.57	19089.61	0.18		
ethidium bromide	Lane 9	133791.96	18181.14	0.16		
de-stained once	Lane 9	88195.23	20781.16	0.25	0.09	0.19
ethidium bromide	Lane 10	102398.45	5493.37	0.07		
de-stained once	Lane 10	110426.45	7172.98	0.08		
de-stained twice	Lane 10	69778.17	7578.57	0.13	0.04	0.09
ethidium bromide	Lane 11	115782.33	1698.9	0.02		
de-stained once	Lane 11	121769.75	1823.19	0.02		
de-stained twice	Lane 11	75570.33	4602.81	0.08	0.01	0.04
ethidium bromide	Lane 12	103060.94	0	0.00		
de-stained once	Lane 12	119541.1	495.36	0.01		
de-stained twice	Lane 12	66753.7	373.59	0.01	0	0.00
second loading on gel:						
ethidium bromide	Lane 2	853.09	59730.38	0.99		
de-stained once	Lane 2	978.29	68732.07	0.99		
de-stained twice	Lane 2	472.53	56281.61	0.99	1	0.99
ethidium bromide	Lane 3	19790.45	86299.99	0.86		
de-stained once	Lane 3	18868.45	91972.66	0.87		
de-stained twice	Lane 3	6773.92	71294.63	0.94	0.81	0.89
ethidium bromide	Lane 4	29980.47	41495.61	0.66		
de-stained once	Lane 4	22088.63	42657.52	0.73		
de-stained twice	Lane 4	7876.87	26878.38	0.83	0.64	0.74
ethidium bromide	Lane 5	49352.56	37440.65	0.51		
de-stained once	Lane 5	29257.03	40880.85	0.66		

de-stained twice	Lane 5	12077.11	22310.29	0.72	0.49	0.63
ethidium bromide	Lane 6	55778.07	28574.45	0.42		
de-stained once	Lane 6	37844.67	29593.05	0.52		
de-stained twice	Lane 6	14848.89	10929.49	0.51	0.36	0.48
ethidium bromide	Lane 7	65951.52	24510.37	0.34		
de-stained once	Lane 7	42963.76	28466.78	0.48		
de-stained twice	Lane 7	18956.66	8566.64	0.39	0.25	0.40
ethidium bromide	Lane 8	153510.65	19054.09	0.15		
de-stained once	Lane 8	119455.2	17939.68	0.17		
de-stained twice	Lane 8	70265.19	18221.44	0.27	0.16	0.20
ethidium bromide	Lane 9	93433.98	12004.17	0.15		
de-stained once	Lane 9	53951.64	7058.5	0.15		
de-stained twice	Lane 9	22843.81	3173.5	0.16	0.09	0.16
ethidium bromide	Lane 10	98360.57	6581.19	0.09		
de-stained once	Lane 10	55418.19	4700.13	0.11		
de-stained twice	Lane 10	24197.51	1673.17	0.09	0.04	0.09
ethidium bromide	Lane 11	109613.21	1129.66	0.01		
de-stained once	Lane 11	56073.09	1396.17	0.03		
de-stained twice	Lane 11	25231.28	836.17	0.04	0.01	0.03
ethidium bromide	Lane 12	121147.88	32.07	0.00		
de-stained once	Lane 12	65477.08	168.24	0.00		
de-stained twice	Lane 12	26933.68	102.92	0.01	0	0.00
third loading on gel:						
ethidium bromide	Lane 16	1316.01	55129.15	0.98		
de-stained once	Lane 16	499.25	62645.53	0.99		
de-stained twice	Lane 16	2372.32	63191.61	0.97	1	0.98
ethidium bromide	Lane 17	22684.98	95684.59	0.86		
de-stained once	Lane 17	25615.66	112014.51	0.86		
de-stained twice	Lane 17	29253.65	112868.59	0.84	0.81	0.85
ethidium bromide	Lane 18	24601.79	38122.46	0.68		
de-stained once	Lane 18	37856.49	41700.8	0.61		
de-stained twice	Lane 18	29221.01	39812.76	0.66	0.64	0.65
ethidium bromide	Lane 19	45095.29	42356.85	0.57		
de-stained once	Lane 19	52354.62	45619.35	0.55		
de-stained twice	Lane 19	52133.6	47736.17	0.56	0.49	0.56
ethidium bromide	Lane 20	48688.31	26621.55	0.43		
de-stained once	Lane 20	54234.93	29057.39	0.43		
de-stained twice	Lane 20	55557.71	32166.93	0.45	0.36	0.44
ethidium bromide	Lane 21	59432.22	21969.59	0.34		
de-stained once	Lane 21	68541.4	24470.38	0.33		
de-stained twice	Lane 21	68053.38	23197.95	0.32	0.25	0.33
ethidium bromide	Lane 22	68087.14	15949.96	0.25		
de-stained once	Lane 22	75783.93	16437.58	0.23		
de-stained twice	Lane 22	80370.08	18934.77	0.25	0.16	0.24
ethidium bromide	Lane 23	76500.49	11858.55	0.18		
de-stained once	Lane 23	89272.39	15346.69	0.19		
de-stained twice	Lane 23	93069.53	14382.95	0.18	0.09	0.18
ethidium bromide	Lane 24	79924.13	6922.65	0.11		
de-stained once	Lane 24	95172.2	4315.83	0.06		
de-stained twice	Lane 24	101257.17	5391.12	0.07	0.04	0.08
ethidium bromide	Lane 25	74755.39	558.26	0.01		

de-stained once	Lane 25	88353.62	1039.06	0.02		
de-stained twice	Lane 25	95743.37	1226.82	0.02	0.01	0.01
ethidium bromide	Lane 26	79397.81	815.69	0.01		
de-stained once	Lane 26	92457.01	0	0.00		
de-stained twice	Lane 26	95885.41	0	0.00	0	0.00

Table_Apx C-38: Results for standard curve PCR 1 - ratiometric analysis

Standard Curve PCR 2

PCR 2		Band A	Band B	B/(299/418*A+B)	(known conc.ratio)^2	mean of EB,dest1,dest2
first loading on gel:						
ethidium bromide	Lane 2	911.85	21986.43	0.97		
de-stained once	Lane 2	309.05	13134.68	0.98		
de-stained twice	Lane 2	598.53	5667.54	0.93	1	0.96
ethidium bromide	Lane 3	6413.89	30871.11	0.87		
de-stained once	Lane 3	1870.77	13025.28	0.91		
de-stained twice	Lane 3	668.47	1702.64	0.78	0.81	0.85
ethidium bromide	Lane 4	7790.02	13690.05	0.71		
de-stained once	Lane 4	1658.45	7236.03	0.86		
de-stained twice	Lane 4	800.46	1133.57	0.66	0.64	0.74
ethidium bromide	Lane 5	11421.7	6378.88	0.44		
de-stained once	Lane 5	2959.63	6103.38	0.74		
de-stained twice	Lane 5	856.77	853.49	0.58	0.49	0.59
ethidium bromide	Lane 6	16452.34	8590.28	0.42		
de-stained once	Lane 6	3838.33	6092.77	0.69		
de-stained twice	Lane 6	895.76	971.37	0.60	0.36	0.57
ethidium bromide	Lane 7	49562.47	17044.8	0.32		
de-stained once	Lane 7	12279.07	11044.25	0.56		
de-stained twice	Lane 7	3841.23	1486.64	0.35	0.25	0.41
ethidium bromide	Lane 8	27377.93	4978.66	0.20		
de-stained once	Lane 8	6579.27	3307.46	0.41		
de-stained twice	Lane 8	1924.93	212.59	0.13	0.16	0.25
ethidium bromide	Lane 9	41395.98	5010.03	0.14		
de-stained once	Lane 9	12105.66	3840.98	0.31		
de-stained twice	Lane 9	2219.44	1003.79	0.39	0.09	0.28
ethidium bromide	Lane 10	41153.1	30132.76	0.51		
de-stained once	Lane 10	12204.9	44566.74	0.84		
de-stained twice	Lane 10	2064.59	469.97	0.24	0.04	0.53
ethidium bromide	Lane 11	36141.24	785.9	0.03		
de-stained once	Lane 11	14547.93	1190.28	0.10		
de-stained twice	Lane 11	2895.67	768.18	0.27	0.01	0.13
ethidium bromide	Lane 12	67726.28	44.35	0.00		
de-stained once	Lane 12	16616.39	3761.15	0.24		
de-stained twice	Lane 12	2699.95	725.49	0.27	0	0.17
second loading on gel:						
ethidium bromide	Lane 16	236.34	33810.8	1.00		
de-stained once	Lane 16	199.68	38825.99	1.00		

de-stained twice	Lane 16	626.8	14385.79	0.97	1	0.99
ethidium bromide	Lane 17	10674.18	45825.14	0.86		
de-stained once	Lane 17	11268.83	49854.73	0.86		
de-stained twice	Lane 17	4032.55	17293.26	0.86	0.81	0.86
ethidium bromide	Lane 18	9965.25	20788.58	0.74		
de-stained once	Lane 18	11520.21	19552.93	0.70		
de-stained twice	Lane 18	4070.93	6584.38	0.69	0.64	0.71
ethidium bromide	Lane 19	13657.6	14668.97	0.60		
de-stained once	Lane 19	15865.87	18301.47	0.62		
de-stained twice	Lane 19	5773.5	4885.85	0.54	0.49	0.59
ethidium bromide	Lane 20	21933.63	11690.69	0.43		
de-stained once	Lane 20	24433.54	13306.76	0.43		
de-stained twice	Lane 20	7261.87	3415.41	0.40	0.36	0.42
ethidium bromide	Lane 21	59140.24	20723.19	0.33		
de-stained once	Lane 21	69776.46	25316.13	0.34		
de-stained twice	Lane 21	24382.52	8100.69	0.32	0.25	0.33
ethidium bromide	Lane 22	23213.7	5182.27	0.24		
de-stained once	Lane 22	31264.29	6025.65	0.21		
de-stained twice	Lane 22	13105.16	3723.11	0.28	0.16	0.24
ethidium bromide	Lane 23	59435.87	8739.24	0.17		
de-stained once	Lane 23	66949.2	9724.34	0.17		
de-stained twice	Lane 23	24753.37	3710.71	0.17	0.09	0.17
ethidium bromide	Lane 24	50458.19	3272.07	0.08		
de-stained once	Lane 24	55886.86	3033.58	0.07		
de-stained twice	Lane 24	23718.39	1177.8	0.06	0.04	0.07
ethidium bromide	Lane 25	47338.25	797.61	0.02		
de-stained once	Lane 25	52210.23	1582.68	0.04		
de-stained twice	Lane 25	20707.04	765.46	0.05	0.01	0.04
ethidium bromide	Lane 26	49628.57	90.08	0.00		
de-stained once	Lane 26	55784.98	416.54	0.01		
de-stained twice	Lane 26	20982.37	0	0.00	0	0.00

Table_Apx C-39: Results for standard curve PCR 2 - ratiometric analysis

Standard Curve PCR 3

PCR 3A		Band A	Band B	B/(299/418*A+B)	(known conc.ratio)^2	mean of EB,dest1,dest2
first loading on gel:						
ethidium bromide	Lane 2	97.53	27879.61	1.00		
de-stained once	Lane 2	35.15	4705.66	0.99		
de-stained twice	Lane 2	90.58	2560.49	0.98	1	0.99
ethidium bromide	Lane 3	8403.45	38072.74	0.86		
de-stained once	Lane 3	1337.46	6241.01	0.87		
de-stained twice	Lane 3	883.35	3242.21	0.84	0.81	0.86
ethidium bromide	Lane 4	3317.82	7324.52	0.76		
de-stained once	Lane 4	400.02	678.62	0.70		
de-stained twice	Lane 4	298.81	400.76	0.65	0.64	0.70
ethidium bromide	Lane 5	3496.94	4740.78	0.65		

de-stained once	Lane 5	364.26	779.71	0.75		
de-stained twice	Lane 5	407.64	649.55	0.69	0.49	0.70
ethidium bromide	Lane 6	37329.76	23764.25	0.47		
de-stained once	Lane 6	5820.6	4343.36	0.51		
de-stained twice	Lane 6	3550.88	2477.85	0.49	0.36	0.49
ethidium bromide	Lane 7	37108.35	13492.46	0.34		
de-stained once	Lane 7	5909.37	2188.87	0.34		
de-stained twice	Lane 7	3411.16	1301.84	0.35	0.25	0.34
ethidium bromide	Lane 8					
de-stained once	Lane 8					
de-stained twice	Lane 8				0.16	
ethidium bromide	Lane 9	49720.39	5813.29	0.14		
de-stained once	Lane 9	8094.35	1091.43	0.16		
de-stained twice	Lane 9	4358.86	1069.85	0.26	0.09	0.18
ethidium bromide	Lane 10	57733.08	3006.47	0.07		
de-stained once	Lane 10	9631.57	1204.35	0.15		
de-stained twice	Lane 10	5425.11	493.76	0.11	0.04	0.11
ethidium bromide	Lane 11	32774.06	1079.44	0.04		
de-stained once	Lane 11	6312.4	1224.29	0.21		
de-stained twice	Lane 11	3494.13	980.55	0.28	0.01	0.18
ethidium bromide	Lane 12	37780.67	0	0.00		
de-stained once	Lane 12	5964.01	170.66	0.04		
de-stained twice	Lane 12	3484.98	245.15	0.09	0	0.04
second loading on gel:						
ethidium bromide	Lane 2	0	6957.65	1.00		
de-stained once	Lane 2	0	6608.78	1.00		
de-stained twice	Lane 2	0	4485.14	1.00	1	1.00
ethidium bromide	Lane 3	222.68	1424.54	0.90		
de-stained once	Lane 3	34.3	1019.88	0.98		
de-stained twice	Lane 3	261.74	553.32	0.75	0.81	0.87
ethidium bromide	Lane 4	782.7	1638.24	0.75		
de-stained once	Lane 4					
de-stained twice	Lane 4				0.64	0.75
ethidium bromide	Lane 5	1747.97	1937.36	0.61		
de-stained once	Lane 5	350.04	1000.2	0.80		
de-stained twice	Lane 5				0.49	0.70
ethidium bromide	Lane 6	8444.5	5572.87	0.48		
de-stained once	Lane 6	2268.33	2746	0.63		
de-stained twice	Lane 6	1179.14	778.93	0.48	0.36	0.53
ethidium bromide	Lane 7	10995.7	3479.66	0.31		
de-stained once	Lane 7	2373.09	1371.91	0.45		
de-stained twice	Lane 7	890.18	459.49	0.42	0.25	0.39
ethidium bromide	Lane 8					
de-stained once	Lane 8					
de-stained twice	Lane 8				0.16	
ethidium bromide	Lane 9	14579.75	1460.95	0.12		
de-stained once	Lane 9	4009.85	1694.83	0.37		
de-stained twice	Lane 9	1400.97	353.45	0.26	0.09	0.25
ethidium bromide	Lane 10	16969.15	869.03	0.07		
de-stained once	Lane 10	4422.72	652.27	0.17		
de-stained twice	Lane 10	2216.34	519.97	0.25	0.04	0.16

ethidium bromide	Lane 11	12832.31	120.27	0.01		
de-stained once	Lane 11	2504.79	158.86	0.08		
de-stained twice	Lane 11				0.01	0.05
ethidium bromide	Lane 12	13814.44	5.24	0.00		
de-stained once	Lane 12	4464.15	60.26	0.02		
de-stained twice	Lane 12	3225.47	598.57	0.21	0	0.08
third loading on gel:						
ethidium bromide	Lane 16	175.26	9882.17	0.99		
de-stained once	Lane 16	258.76	13053.29	0.99		
de-stained twice	Lane 16	184.31	12149.62	0.99	1	0.99
ethidium bromide	Lane 17	2542.1	9076.21	0.83		
de-stained once	Lane 17	3721.13	11984.1	0.82		
de-stained twice	Lane 17	3274.03	12064.4	0.84	0.81	0.83
ethidium bromide	Lane 18					
de-stained once	Lane 18	1782.4	3206.08	0.72		
de-stained twice	Lane 18	1705.16	3129.88	0.72	0.64	0.72
ethidium bromide	Lane 19	1126.62	1177.61	0.59		
de-stained once	Lane 19					
de-stained twice	Lane 19				0.49	0.59
ethidium bromide	Lane 20	8536.52	5192.96	0.46		
de-stained once	Lane 20	11616.95	6734.27	0.45		
de-stained twice	Lane 20	11303.71	6346.12	0.44	0.36	0.45
ethidium bromide	Lane 21	9555.11	3485.52	0.34		
de-stained once	Lane 21	13013.02	4919.07	0.35		
de-stained twice	Lane 21	12978.91	4993.96	0.35	0.25	0.34
ethidium bromide	Lane 22					
de-stained once	Lane 22					
de-stained twice	Lane 22				0.16	
ethidium bromide	Lane 23	9431.93	1013.09	0.13		
de-stained once	Lane 23	12916.78	1727.37	0.16		
de-stained twice	Lane 23	12429.71	1605.78	0.15	0.09	0.15
ethidium bromide	Lane 24	12556.32	1143.68	0.11		
de-stained once	Lane 24	16370.4	1078.5	0.08		
de-stained twice	Lane 24	15748.39	852.49	0.07	0.04	0.09
ethidium bromide	Lane 25	7052.41	87.6	0.02		
de-stained once	Lane 25	10590.74	175.1	0.02		
de-stained twice	Lane 25	10186.02	177.03	0.02	0.01	0.02
ethidium bromide	Lane 26	9462.26	110.77	0.02		
de-stained once	Lane 26	13126.04	4.04	0.00		
de-stained twice	Lane 26	12619.76	771.9	0.08	0	0.03

Table_Apx C-40: Results for standard curve PCR 3 - ratiometric analysis

Standard curve

Standard	Band A	Band B	ratio squared	y value
1	31286.35	972104.2	1	0.977496
0.9	241781.6	941649.5	0.81	0.844833
0.8	349303.5	636079.6	0.64	0.717971
0.7	452541.4	460563.7	0.49	0.58725
0.6	685199.1	440694	0.36	0.473445
0.5	903416.6	366885.6	0.25	0.362138
0.4	1148361	323404.2	0.16	0.282489
0.3	1025607	165403.5	0.09	0.18398
0.2	1327537	125233.1	0.04	0.116514
0.1	1198757	42106.31	0.01	0.046806
0	1171727	1507.43	0	0.001795
1	21863.61	1094457	1	0.985912
0.9	174596.9	774910.1	0.81	0.861201
0.8	252062.7	490392.2	0.64	0.73117
0.7	497739.9	506636.9	0.49	0.587285
0.6	756588.4	496528.1	0.36	0.478478
0.5	1022896	415959	0.25	0.362445
0.4	1343940	375204.8	0.16	0.280729
0.3	1199560	192560.8	0.09	0.183283
0.2	1582240	154757	0.04	0.120288
0.1	1464074	51863.16	0.01	0.047186
0	1417755	3627.44	0	0.003564
1	58329.24	2225888	1	0.9816
0.9	349850.9	1764678	0.81	0.875801
0.8	547607.8	912380.4	0.64	0.69963
0.7	1726741	1795804	0.49	0.592487
0.6	2240431	1740907	0.36	0.520682
0.5	2708639	1351079	0.25	0.410837
0.4	3131069	1294833	0.16	0.366339
0.3	2949761	609680	0.09	0.224174
0.2	3275824	502402.4	0.04	0.176552
0.1	3052578	183880.9	0.01	0.077671
0	2575844	5790.64	0	0.003133

Table_Apx C-41: Results for standard curve - ratiometric analysis of control leukocyte samples

Results of the three PCRs for leukocytes

PCR template	transcription ratio squared= x-value	y-value	transcription ratioratio	mean transcription ratio squared	mean measured band intensity ratio	mean transcription ratio
gDNA 26	0.31	0.39	0.56			
gDNA 26	0.32	0.40	0.57			
gDNA 26	0.34	0.42	0.58			
gDNA 33	0.31	0.39	0.55			
gDNA 33	0.30	0.38	0.54			
gDNA 33	0.28	0.37	0.53	0.31	0.39	0.56
cDNA-Ex1	0.58	0.65	0.76			
cDNA-Ex1	0.30	0.38	0.55			
cDNA-Ex1	0.93	0.97	0.96	0.60	0.67	0.76
cDNA-Ex2	-0.11	0.00	0.00			
cDNA-Ex2	-0.10	0.01	0.00			
cDNA-Ex2	-0.09	0.01	0.00	-0.10	0.01	0.00
cDNA-Ex3						
cDNA-Ex3	0.93	0.98	0.97			
cDNA-Ex3	0.37	0.45	0.61	0.65	0.71	0.79
cDNA-Ex4	0.42	0.50	0.65			
cDNA-Ex4	0.47	0.55	0.69			
cDNA-Ex4	0.01	0.11	0.11	0.30	0.38	0.48
cDNA-Ex5						
cDNA-Ex5						
cDNA-Ex5	0.92	0.97	0.96			
cDNA-Ex6	0.95	0.99	0.97			
cDNA-Ex6	0.91	0.95	0.95			
cDNA-Ex6				0.93	0.97	0.96
cDNA-Ex7	0.23	0.32	0.48			
cDNA-Ex7	0.83	0.88	0.91			
cDNA-Ex7	0.28	0.37	0.53	0.45	0.52	0.64
cDNA-Ex8						
cDNA-Ex8	0.52	0.59	0.72			
cDNA-Ex8						
cDNA-In1	0.30	0.38	0.54			
cDNA-In1	-0.10	0.01	0.00			
cDNA-In1	0.55	0.62	0.74	0.25	0.34	0.43
cDNA-In2	0.48	0.55	0.69			
cDNA-In2	0.44	0.51	0.66			
cDNA-In2	-0.10	0.01	0.00	0.27	0.36	0.45
cDNA-In3	0.03	0.13	0.18			
cDNA-In3	0.93	0.97	0.96			

cDNA-In3	0.38	0.45	0.61	0.45	0.52	0.59
cDNA-In4	0.91	0.96	0.95			
cDNA-In4	0.49	0.56	0.70			
cDNA-In4	0.93	0.98	0.97	0.78	0.83	0.87
cDNA-In5	0.58	0.65	0.76			
cDNA-In5	0.93	0.98	0.96			
cDNA-In5	0.94	0.98	0.97	0.82	0.87	0.90
cDNA-In6	0.93	0.98	0.96			
cDNA-In6						
cDNA-In6						
cDNA-In7	0.94	0.98	0.97			
cDNA-In7	0.92	0.97	0.96			
cDNA-In7	0.93	0.97	0.96	0.93	0.97	0.96
cDNA-In8	0.56	0.63	0.75			
cDNA-In8						
cDNA-In8						

Table_Apx C-42: Results for leukocyte samples - ratiometric analysis of control leukocyte samples

Standard curve

allelic ratio squared	Band A	Band B	A*	measured band intensity ratio
1	9647.347	251943.9	6900.853	0.97334
1	22294.59	706510	15947.57	0.977926
1	17473.78	578036.9	12499.18	0.978834
0.81	45118.73	138313.3	32273.93	0.810807
0.81	174902.2	533710.4	125109.5	0.810101
0.81	175967.4	516757.2	125871.4	0.80413
0.64	82504.67	117342.4	59016.5	0.665361
0.64	332478.8	482542.7	237825.7	0.669855
0.64	336707.3	509606.5	240850.5	0.679062
0.49	274835.2	213880.9	196592.7	0.521059
0.49	829827.2	710547.8	593584.6	0.544843
0.49	294014.9	246399	210312.1	0.539507
0.36	296112	145881.5	211812.2	0.407839
0.36	1224445	629322.9	875859.3	0.418104
0.36	371566.1	187474.7	265785.3	0.413614
0.25	362012.7	115156.2	258951.7	0.307815
0.25	1574394	499394.3	1126181	0.307211
0.25	467782.9	148768.7	334610.3	0.307768
0.16	256206.2	51493.76	183267.1	0.219346
0.16	1925163	453642.5	1377090	0.247793
0.09	298882.1	38470.03	213793.6	0.152499
0.09	1892228	194268.2	1353532	0.125512
0.09	498974.8	48781.54	356922.1	0.120239
0.04	282939.5	15683.18	202389.7	0.071917
0.04	2100353	137710.1	1502406	0.083964
0.04	229588.7	13870.58	164227.3	0.077882
0.01	335201.4	7171.007	239773.3	0.029039
0.01	2219223	48138.71	1587435	0.029432
0.01	231679.3	4257.387	165722.7	0.025046
0	217244.5	116.3367	155397.3	0.000748
0	1683863	181.4467	1204486	0.000151
0	123872.6	290.32	88607.43	0.003266

Table_Apx C-43: Results for standard curve – ratiometric analysis of vein samples

Allelic Transcription Assay for vein samples

TIG	Band A	Band B	band A*	allelic ratio squared	y axis	allelic ratio	variance
2	338952.3	83784.19	242456.3	0.222379	0.256817	0.471571	
2	704243.3	443701.6	503753	0.442545	0.468309	0.665241	
2	146714.9	112579.9	104946.8	0.493801	0.517545	0.70271	0.015
3	6841.99	272602.6	4894.151	0.977684	0.982363	0.988779	
3	5085.337	120887.1	3637.597	0.965634	0.970788	0.982667	0
8	16708.31	218958.1	11951.64	0.942162	0.948241	0.97065	
8	772535.3	307.7167	552603	-0.04439	0.000557	0	0.471
9	266310.1	108309.3	190494.5	0.332372	0.362476	0.576517	
9	585784.5	551004	419018.1	0.546359	0.568032	0.739161	
9	186457.9	18715.3	133375.4	0.083129	0.123054	0.288321	0.052
11	303474	85256.54	217078.3	0.248588	0.281994	0.498586	
11	1106684	151907.8	791623.1	0.122631	0.160999	0.350187	
11	277573.3	99753.11	198551.2	0.303144	0.3344	0.550585	0.011
13	303480.5	109837.2	217082.9	0.304784	0.335976	0.552073	
13	513266.9	306741.3	367145.4	0.42888	0.455182	0.654889	
13	240589.6	32103.34	172096.4	0.118692	0.157215	0.344517	0.025
17	335440.3	54980.92	239944.1	0.149098	0.186423	0.386132	
17	618589.8	634856.6	442484.1	0.568479	0.589281	0.753976	
17	171379	17318.72	122589.3	0.083892	0.123786	0.289641	0.06
20	336808.9	38426.91	240923.1	0.098228	0.137558	0.313414	
20	929701.5	324621.4	665025.7	0.296499	0.328017	0.544518	
20	49404.27	165335.3	35339.42	0.812718	0.823897	0.901509	0.088
22	390613.3	1968.853	279410	-0.03769	0.006997	0	
22	82325.21	1067.067	58888.13	-0.02644	0.017798	0	
26	97015.83	194825	69396.49	0.722626	0.737355	0.850074	
26	572093.5	742628.5	409224.8	0.626197	0.644725	0.791326	
26	201825.7	171262.2	144368.2	0.519887	0.542604	0.721032	0.004
28	86783.15	306912.1	62076.94	0.820909	0.831765	0.90604	
28	1268338	542298.3	907256.3	0.344487	0.374114	0.58693	
28	798834.1	262208.7	571414.9	0.28247	0.314541	0.531479	0.041
37	821302.3	170296	587486.5	0.188975	0.224729	0.434713	
37	1668869	364662.5	1193760	0.19862	0.233995	0.445668	
37	169756	267125.9	121428.3	0.670713	0.687487	0.818971	0.048
41	621996.8	319907	444921.2	0.390457	0.418273	0.624866	
41	877168.2	900700.9	627448.1	0.56861	0.589406	0.754062	
41	653960.8	74426.33	467785.3	0.097922	0.137264	0.312926	0.051
43	706003.4	1150.73	505012	-0.04261	0.002273	0	
43	1000978	742044.4	716010.4	0.48483	0.508928	0.696297	
43	135863.3	109.1333	97184.54	-0.0438	0.001122	0	0.162
44	743409.4	76105.2	531768.9	0.085362	0.125199	0.292168	
44	1777383	786025	1271381	0.352745	0.382047	0.593923	
44	419956.8	137318.6	300399.7	0.28161	0.313715	0.530669	0.025
46	553062.9	238169.4	395612	0.346233	0.375791	0.588415	
46	1608463	690014.5	1150552	0.345297	0.374893	0.58762	
46	201428.5	381244.1	144084	0.71052	0.725726	0.842924	0.022

Table_Apx C-44: Results for vein samples – ratiometric analysis of vein samples

TIG	Band A	Band B	Band A*	x-axis = allelic ratio squared	y-axis	allelic ratio	Variance
3	642089.53	177162.92	459293.71	0.15	0.28	0.38	
3	549464.55	294184.50	393038.04	0.32	0.43	0.56	0.016
8	12221.21	319054.61	8741.97	0.93	0.97	0.96	
8	8769.50	84856.78	6272.92	0.88	0.93	0.94	
8	16972.46	346759.73	12140.58	0.92	0.97	0.96	0
9	347687.61	295465.58	248704.77	0.45	0.54	0.67	
9	450500.91	114000.45	322248.26	0.13	0.26	0.36	
9	549440.99	193055.61	393021.18	0.21	0.33	0.45	0.025
13	14584.78	366950.83	10432.65	0.93	0.97	0.96	
13	7494.71	79617.75	5361.05	0.89	0.94	0.94	0
22	190984.30	427494.66	136613.17	0.69	0.76	0.83	
22	292690.89	399531.28	209365.01	0.57	0.66	0.76	
22	28757.85	510935.15	20570.80	0.92	0.96	0.96	0.01
26	18850.31	358287.13	13483.83	0.92	0.96	0.96	
26	7033.08	50378.19	5030.84	0.86	0.91	0.93	
26	20537.76	379713.93	14690.88	0.92	0.96	0.96	0
28	554685.47	367257.57	396772.62	0.38	0.48	0.61	
28	400189.42	386957.50	286259.90	0.48	0.57	0.69	
28	656161.23	444170.12	469359.35	0.38	0.49	0.62	0.002
44	530601.12	222952.68	379544.82	0.25	0.37	0.50	
44	321617.40	239106.32	230056.47	0.41	0.51	0.64	
44	205821.55	92631.61	147226.42	0.27	0.39	0.52	0.006

Table_Apx C-45: Results for allelic transcription assay for adipose tissue.

Appendix D: Supplementary Material

Patient Follow up Questionnaire

TIG:_____
Date:_____

Study Group □ Control Group□

Number of weeks post op: _____
Date of operation:_____

Recruitment

How do you feel about the way you were recruited to the study?

Very satisfied satisfied unsatisfied very unsatisfied

Give reasons why not satisfied:_____
_____

Would you take part in clinical studies in the future: □Yes □No

Surgery

Did you have any complications from the operation which required you to seek medical advice?

□No □Yes-Give details: _____

How long after your surgery were you able to resume your normal daily activity?
□<1 week □ 1-2 weeks □ 2-3 weeks □ 3-4 weeks □ other:

On the first day after your operation, how sever was your pain?

0 1 2 3 4 5 6 7 8 9 10

On the second day after your operation, how sever was your pain?

0 1 2 3 4 5 6 7 8 9 10

Did you feel that you suffered any ill side effects due to taking part in this study?

□No □Yes, Give Reasons why: _____

Figure_Apx D-1: Patient Questionnaire

Correspondence

Ref: CPW/sta

01 March 2004

Professor I Day
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Duthie Building
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Dear Professor Day

REC Ref: 040/04/w – Tissue specific genetic regulation of interleukin-6 synthesis

The Southampton & South West Hampshire LREC reviewed your application on 25th February 2004. The documents reviewed were as follows:

Application Form version 2.2 – signed on 3 February 2004
Patient Information Sheet – version 2 dated 4 February 2004
Consent Form – version 2 dated 4 February 2004
GP Letter – version 1 dated 4 February 2004
Copy of Invoice for Reimbursement of Dr D Cumming Salary dated 4 November 2003
Letter from British Heart Foundation dated 3 August 2001
R&D Project Peer Review Report dated 16 December 2003
R&D Project Detail Form
Protocol

The members of the Committee present gave approval for your research on ethical grounds providing you comply with the conditions set out below:

The committee felt this to be a well-written study.

- **There is a spelling error in paragraph 9 of the consent form.**
- **B46 of the application form should state the number of years i.e. 15 years, which is in line with trust policy.**
- **The Patient Information Sheet should include that a small biopsy of Skeletal Muscle and Fat tissue will be taken.**

A copy of the revised documents should be sent to the Ethics Committee for its records.

Conditions of approval:

- *(Where approval is given before receipt of CTX)* Please let the LREC have a copy of the CTX when it is available. If changes to the protocol are required by the MHRA (Medicines and Healthcare Products Regulatory Agency), the LREC approval will become void until those changes have been made and the revised protocol will need to be approved.

- You do not undertake this research in any NHS organisation until the relevant NHS management approval has been received.

- You do not deviate from, or make changes to, the protocol without the prior written approval of the LREC, except where this is necessary to eliminate immediate hazards to research participants or when the change involves only logistical or administrative aspects of the research. In such cases, the LREC should be informed within seven days of the implementation of the change. Likewise, you should also seek the relevant NHS management approval for the amendment, or inform the NHS organisation of any logistical or administrative changes.

- You complete and return the standard progress report form to the LREC one year from the date of this letter and thereafter on an annual basis. This form should also be used to notify the Committee when your research is completed and should be sent to the LREC within three months of completion. For a copy of the progress report please see www.corec.org.uk.

- If you decide to terminate this research prematurely, a progress report form should be sent to the LREC within 15 days, indicating the reason for the early termination. For a copy of the progress report please see www.corec.org.uk.

- You must advise the LREC of all Suspected Serious Adverse Reactions (SSARs) and all Suspected Unexpected Serious Adverse Reactions (SUSARs).

- You advise the LREC of any unusual or unexpected results that raise questions about the safety of the research.

- The project must be started within three years of the date of this letter.

'Lead' LREC – other local submissions

Where this LREC is taking the role of 'Lead' LREC, it is your responsibility to ensure that any other local researchers within the Hampshire & Isle of Wight Strategic Health Authority seek the approval of the relevant LREC before starting their research. To do this you should submit **one copy** of the following documents to the relevant LRECs:

- This approval letter
- Part C of the REC Application form (with pertinent local details)
- LREC-approved version of the patient information sheet and consent form, in the appropriate local format (ie on applicable headed paper and showing pertinent local contact details)
- Principal (local) investigator's CV.

No other documents are required by the LREC to consider locality issues.

NHS LRECs are compliant with the International Conference on Harmonisation/Good Clinical Practice (ICH GCP) Guidelines for the conduct of trials involving participation of human subjects.

Your application has been given a unique reference number, please use it on all correspondence with the LREC.

Yours sincerely

Mrs Clair Wright
LREC Manager
Southampton & South West Hampshire

Enc: List of members present / members who submitted written comments

Our Ref: CPW/hph

09 March 2005

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Professor C P Shearman
Consultant Vascular Surgeon
University of Southampton School of Medicine
Vascular Surgery
Level F, Mailpoint 816, Centre Block
Southampton General Hospital

Dear Professor Shearman,

Study title: **Tissue specific genetic regulation of IL-6 synthesis**
REC reference: **040/04/w**
Protocol number: **n/a**
EudraCT number: **n/a**

Amendment number: **2**
Amendment date: **9th February 2005**

The above amendment was reviewed at the meeting of the Sub-Committee of the Research Ethics Committee held on 23rd February 2005.

Ethical opinion

The members of the Committee present gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Notice of Amendment Form, amendment 2 signed 9 February 2005
Information Sheet, version 3 dated 4 January 2005
Consent Form, version 3 dated 4 January 2005

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Management approval

All investigators and research collaborators in the NHS should notify the R&D Department for the relevant NHS care organisation of this amendment and check whether it affects local management approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

REC reference number:	**040/04/w**	**Please quote this number on all correspondence**

Yours sincerely,

Mrs Clair Wright
REC Manager

E-mail: clair.wright@nhs.net

Enclosures *List of names and professions of members who were present at the meeting and those who submitted written comments*

Our Ref: CPW/hph

09 March 2005

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Professor C P Shearman
Consultant Vascular Surgeon
University of Southampton School of Medicine
Vascular Surgery
Level F, Mailpoint 816, Centre Block
Southampton General Hospital

Dear Professor Shearman,

Study title: **Tissue specific genetic regulation of IL-6 synthesis**
REC reference: **040/04/w**
Protocol number: **n/a**
EudraCT number: **n/a**

Amendment number: **3**
Amendment date: **9th February 2005**

The above amendment was reviewed at the meeting of the Sub-Committee of the Research Ethics Committee held on 23rd February 2005.

Ethical opinion

The members of the Committee present gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Letter dated 5 January 2005
Notice of Amendment Form, amendment 3, signed 9 February 2005
Amended Page 18 Question B21 of the Standard NHS REC Application Form

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Management approval

All investigators and research collaborators in the NHS should notify the R&D Department for the relevant NHS care organisation of this amendment and check whether it affects local management approval of the research.

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Yours sincerely,

Mrs Clair Wright
REC Manager

E-mail: clair.wright@nhs.net

Enclosures *List of names and professions of members who were present at the meeting and those who submitted written comments*

Southampton NHS
University Hospitals NHS Trust

Patient Information Sheet

Tissue Specific Genetic Regulation of Interleukin-6 Synthesis[1]

Introduction:
You are being invited to take part in a research study. Before you decide it is important that you understand why the research is being done and what it will involve. Please take time to read this information and discuss it with others if you wish.

Background Information:
Various diseases including diseases of the heart are made worse in people who produce too strong an inflammatory response. A chemical known as IL-6 is produced at different levels in different tissues in different people, and may be a key culprit, contributing to the chance of dying from e.g. a heart attack. We want to find out more about IL-6 by studying healthy people. We will undertake studies of IL-6 and related chemicals using small samples taken as biopsies from routine operations (these samples would normally be removed and discarded at the time of operation). In the laboratory we will study the time course of production of our possible culprit: IL-6 - in response to specific noxious stimuli. With such a strategy, using samples from healthy individuals producing the various types of IL-6, we will have a much clearer idea of the role of IL-6 and how best to design drugs that could combat its harmful effects and thus help prevent people dying.

Why have I been chosen?
You are already scheduled to undergo an operation on the varicose veins that are currently causing you problems. Other than the trouble with your varicose veins you are deemed medically fit and stable. Therefore you would be an ideal candidate for this research on normal individuals free from significant disease.

What the study involves:
Patients under the care of Consultant Vascular Surgeons, from Southampton General Hospital and the Royal South Hants Hospital will be considerer for the inclusion in this study.
You will continue to receive all the standard care normal for patients with varicose vein disease and your surgery will be performed by your vascular surgeon in exactly the same manner as in routine with all other patients.
If you agree to take part in the study and have read and signed the consent form, then at the time of your operation confirmation of your medical history and any drugs that you are currently taken will be obtained. A blood test will also be

[1] Patient Information Sheet – Interleukin-6 Study Dated 04.01.2005 Version 3 L REC 040/04/W

required for us to ascertain your profile for these inflammatory markers. During your operation to remove the varicose vein a portion of the excised tissue will be made available for cell culture and for analysis at the molecular level. A biopsy of skeletal muscle and fat will also be taken for similar analysis. Specific cell types isolated form the blood sample will also be grown short term in culture. With your permission blood and tissue samples will be frozen and stored anonymously for future studies relating to cardiovascular and inflammatory disease.

What are the risks / benefits in taking part in this study?

Your operation will be carried out as normal. The material made available for study is material that would normally be excised and disposed of. An additional sample of blood will be required but that should not cause any additional significant discomfort. Skeletal muscle and fat samples will only be taken if appropriate.

The benefits of this work will not be realized immediately. You individually will not receive any benefit as a result of your participation in the study. The impact of the study will only be realized when all the data from our normal individual study population is analysed as a whole.

Is there anything else I should do?

If you agree to be part of this study there are no specific requirements for you to do anything other than to allow the use of the biopsy material and additional blood sample.

Will my taking part in this study be kept confidential?

If you consent to take part in the study your medical records may be inspected by a member of the research team for the purpose of analysing the results. They may also be looked at by the regulatory authorities to ensure that the study is being carried out correctly. All information that is collected about you during the course of the research will be kept strictly confidential. Any information and samples that leave the hospital will have your name and contact details removed so that you cannot be recognized from it.

With your permission we will inform your GP on your behalf that you are taking part in this study. As you know you GP will also keep this information confidential.

What happens if something goes wrong?

Compensation for any injury caused by taking part in this study will be in accordance with the guidelines of Southampton General Hospital Indemnity Policy.

Who has reviewed this study?
This study has been reviewed and approved by Southampton and South West Hampshire Local Research and Ethics Committed submission number 040/04/W. This is a group of people not involved in this study, which reviews the science and ethics of the study before it is allowed to start.

If there is anything that is not clear or if you would like more information please contact:

Consultant Vascular Surgeons
E level West Wing
Southampton General Hospital
Tel: 023 8079 8801

Professor I Day
Division of Human Genetics
Duthie Building
Southampton General Hospital
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Jenny Williams
Sabine Sonnenberg
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F level Centre Block
Southampton General Hospital
Tel.: 023 8079 8558

Southampton NHS
University Hospitals NHS Trust

Patient Identification for Study

CONSENT FORM[2]

Title of Project: **Tissue Specific Genetic Regulation of Interleukin-6 Synthesis**

Name of Researchers: **Professor C Shearman, Professor I Day Dr H Montgomery, Dr D Cumming**

PLEASE **INITIAL** BOXES IF YOU AGREE WITH EACH SECTION.

1. ☐ I confirm that I have read the information sheet dated 04/01/05 (version 3) for the above study, have had the opportunity to ask questions, understand why the research is being done and any possible risks have been explained to me.

2. ☐ I understand that my participation is voluntary and that I am free to withdraw at any time by contacting Professor Shearman or a member of the research team, without giving any reason and without my medical care or legal rights being affected. If I withdraw I understand that any unused donated tissue will be disposed of, in the case of unlinked anonymised samples.

3. ☐ I understand that sections of my medical notes may be looked at by responsible individuals of the research team of from regulatory authorities where it is relevant to my taking part in this study. I give permission for these individuals to have access to my records.

4. ☐ I understand that my own GP will be informed if any of the results of the medical tests done as part of the research are important for my health.

5. ☐ I agree to take part in the above study for collection of blood and tissue biopsies

[2] Consent Form Interleukin-6 Study Dated 04.01.05 Version 3 L REC 040/04/W

Unlinked Anonymised Samples:

7. ☐ I give permission for the samples to be used for investigations of medical conditions relating to inflammatory and cardiovascular disease.

8. ☐ I give permission for the samples to be stored for use in other unrelated research studies the precise nature of which will depend upon future scientific advances but excluding germ line research.

9. ☐ I understand that future research using samples I give may include genetic research aimed at understanding the genetic influences on the susceptibility and progression of disease, particularly inflammatory and cardiovascular disease.

10. ☐ I understand that the samples my be used for Commercial development, without financial or other benefit to myself for the investigation of medical conditions, potentially leading to new preventative measures against such conditions in keeping with the gift nature of my sample

_____	_____	_____
NAME OF PATIENT	**DATE**	**SIGNATURE**
_____	_____	_____
NAME OF PERSON TAKING CONSENT IF DIFFERENT FROM RESEARCHER	**DATE**	**SIGNATURE**
_____	_____	_____
NAME OF RESEARCHER	**DATE**	**SIGNATURE**

Appendix E: Technical Notes

a: Standard Curve

When making a standard curve it is important to avoid overspill from one well to the next as this would alter the intensity of the measured fluorescence. Several point will help ovoid this error.

1. Use of high concentration loading dye
2. Allowing the surface of the gel to dry slightly before filling the well
3. Ensuring the well is empty by aspiration with a pipette before inserting the sample

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