PHENOTYPE AND GENOTYPE RELATIONSHIP IN NITRIC OXIDE AND PTERIN PATHWAYS IN MAN

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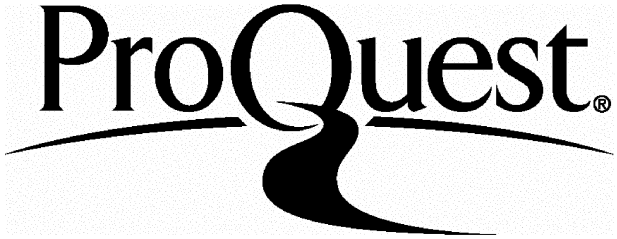
UNIVERSITY COLLEGE LONDON

JANUARY 2001

A thesis submitted in accordance with the regulations of the University of London for the degree of Doctor of Philosophy



To Feizal,

Ma* and *Pa

ABSTRACT

In the vascular endothelium, nitric oxide (NO) is synthesised from L-arginine by endothelial nitric oxide synthase (eNOS). Tetrahydrobiopterin (BH_4) is an essential cofactor of eNOS and NO production is enhanced by increased concentration of BH_4. The latter is synthesised from guanosine triphosphate (GTP) by the rate-limiting enzyme of the pterin pathway, GTP cyclohydrolase I (GTPCH). Neopterin is a side reaction product of the pterin pathway.

In the vasculature, NO exerts a tonic vasodilator influence, regulates regional blood flow and systemic blood pressure (BP), and also confers thromboresistant and atheroprotective properties to the endothelium. A reduction in its synthesis or availability might underlie the impaired endothelium-dependent vasodilatation, which is observed in blood vessels from individuals with cardiovascular disease (CVD) risk factors, including active and passive smokers as well as patients with hypertension and hypercholesterolaemia. Furthermore, loss of NO-mediated effects may predispose to the development of atherosclerosis. Conversely an increase in inflammatory NO, paralleled by a rise in BH_4 avaibility may cause endothelial damage and dysfunction.

We hypothesised that sequence variation in the eNOS gene (*NOS3*) and/or GTPCH gene (*GCH1*) would influence the generation of NO *in vivo* and in so doing would determine both endothelial vasodilator function and the risk of ischaemic heart disease (IHD). Because NO production might be regulated by BH_4 availability, which could be genetically determined, we searched for polymorphisms in *GCH1* and investigated associations with activity of the pterin pathway, NO production and IHD risk. The

relationship between NO and neopterin production, classical CVD risk factors, IHD events and both *NOS3* and *GCH1* polymorphisms were studied in a cohort of 3179 healthy men aged 50-61 years from the second **N**orthwick **P**ark **H**eart **S**tudy (NPHSII).

High throughput assay methods were established for measuring plasma NO_x (i.e. NO_3^- and NO_2^-) and neopterin, as an index of eNOS and GTPCH activities respectively. Three novel polymorphisms, i.e. -577 (G/A), -741 (T/C) and -796 (G/A), were identified in the promoter of *GCH1* by **S**ingle **S**trand **C**onformation **P**olymorphism (SSCP). Genotyping of *GCH1* variants at positions -577 and -796 as well as four previously described *NOS3* polymorphisms, i.e. -786 (T/C), -922 (A/G), +894 (G/T) and a 27-bp tandem repeats in intron 4 (eNOS4b/4a), were performed by **P**olymerase **C**hain **R**eaction-**R**estriction **F**ragment **L**ength **P**olymorphism (PCR-RFLP) based methods combined with **M**icroplate **A**rray **D**iagonal **G**el **E**lectrophoresis (MADGE) technique.

New data on allelic association of *NOS3* polymorphisms was obtained. NO_x levels were found to be lower in smokers compared to non-smokers but were not influenced by *NOS3* genotype. Both -577 (G/A) and -796 (G/A) variants influenced plasma neopterin levels significantly. Neopterin was positively associated with fibrinogen, an acute-phase protein and an established cardiovascular risk factor. This finding supports the view that an inflammatory reaction might be the basis of the pathology of CVD. Over 9 years of follow-up, there was no influence of *NOS3* and *GCH1* polymorphisms on IHD risk. Genetic variations in *GCH1*may influence the activity of the pterin pathway and further studies are required to confirm this finding and to determine their influence on vascular function in health and disease.

ACKNOWLEDGEMENTS

Ph.D. studies are reputed for their long spells of frustration with intermittent bouts of happiness. As my studies are over and my suitcases are already packed, I feel very nostalgic. I was very privileged to be supervised by three fantastic individuals, namely Professor Steve Humphries, Professor Patrick Vallance and Dr Aroon Hingorani. I would like to express my deepest gratitude to Steve and Patrick for giving me the opportunity to carry out my Ph.D. within their respective departments. Steve had always 'one minute' to listen to spare for my incessant queries and Patrick was always encouraging and supportive. I am deeply indebted to Aroon for his constant enthusiasm, patience, friendship, advice and guidance all throughout the project.

I thank Dr George Miller for access to the precious NPHSII samples, Jackie Cooper and Sarah Bujac for statistical analysis, Jackie Mitchell for her help and Dr Dawn Waterworth for her advice. I acknowledge the help of Lisa Jones, Ros Whitall and Lee. During the three years, I have made many friends and I am grateful to all members, past and present, of Cardiovascular Genetics and Clinical Pharmacology groups as well as the Cruciform Project, for their help and generosity. *Merci beaucoup et au revoir!!*

Many thanks to my family and friends in Mauritius for all their support and affection. I would like to thank the *British Council*, the *Government of Mauritius*, especially the staff at the *Ministry of Education* and the *Association of Commonwealth Universities* for giving me this award. I am grateful to my sister Ridwana for her encouragement and help. I dedicate this thesis to Feizal who made this Ph.D. possible through his unceasing love and friendship.

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ABBREVIATIONS

A	Adenine
A_{540}	Absorbance at 540 nm
ACh	Acetylcholine
AP-1	Activator protein 1
APS	Ammonium persulfate
Arg	Arginine
Asp	Aspartic acid
ATP	Adenosine triphosphate
bp	Base pair
BH_4	5,6,7,8-Tetrahydrobiopterin
BMI	Body Mass Index
BP	Blood pressure
dBP	Diastolic blood pressure
sBP	Systolic blood pressure
BPB	Bromophenol blue
χ^2 test	Chi-squared test
C	Cytosine
Ca^{2+}	Calcium ions
CAD	Coronary artery disease
CaM	Calmodulin
CAV	Caveolin
C/EBP	CCAAT-box enhancer binding protein
COOH	Carboxy
CVD	Cardiovascular disease

Cys	Cysteine
ddH_2O	Double distilled water
ds	Double stranded
DBP	Diastolic blood pressure
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
cDNA	Complementary deoxyribonucleic acid
DRD	Dopamine responsive dystonia
ED	Endothelial dysfunction
EDD	Endothelium-dependent vasodilatation
EDTA	Ethylene-diamine tetra-acetic acid
EDRF	Endothelium-derived relaxing factor
ELISA	Enzyme Linked ImmunoSorbent Assay
EH	Essential hypertension
ERE	Oestrogen response element
EtBr	Ethidium bromide
FAD	Flavin adenine dinucleotide
FMD	Flow-mediated dilatation
FMN	Flavin mononucleotide
G	Guanine
GC	Guanylate cyclase
sGC	Soluble guanylate cyclase
G-6-PDH	Glucose-6-phosphate dehydrogenase
Glu	Glutamic acid
GCH1	Gene coding for GTPCH protein
GFRP	GTPCH Feedback Regulatory Protein
cGMP	Cyclic guanosine-3', 5'-monophosphate

GTN	Glyceryl trinitrate
GTP	Guanosine triphosphate
GTPCH	GTP cyclohydrolase I
H_2O_2	Hydrogen peroxide
Hb	Haemoglobin
HNF-1	Hepatic nuclear factor 1
HPD	Hereditary progressive dystonia
HPLC	High Pressure Liquid Chromatography
In4	Intron 4
IHD	Ischaemic heart disease
IL-6	Interleukin alpha
IFN-γ	Interferon gamma
kb	Kilobases
K-W test	Kruskal-Wallis test
L-NAME	N^G monomethyl-L-arginine
LPS	Lipopolysaccharide
LVH	Left ventricular hypertrophy
μM	Micromolar
mRNA	Messenger ribonucleic acid
M_w	Molecular weight
MADGE	Microtiter Array Diagonal Gel Electrophoresis
MI	Myocardial infarction
N	Frequency
NADPH	Reduced adenine dinucleotide phosphate
NED	N-(1-naphthyl) ethylene diamine
Neop/Creat	Neopterin:creatinine ratio
NF-1	Nuclear factor 1

NF-κB	Nuclear factor kappa B
NH_2	Amino
7,8-NH_2	7,8-Dihydroneopterin
7,8-NH_2TP	7,8-Dihydroneopterin triphosphate
nM	Nanomolar
nt	Nucleotide
NO	Nitric oxide
NO_2^-	Nitrite
NO_2	Nitrogen dioxide
N_2O_3	Dinitrogen trioxide
NO_3^-	Nitrate
NOS	Nitric oxide synthase
ecNOS	Endothelial constitutive nitric oxide synthase
iNOS	Inducible nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
NOS1	Type 1 nitric oxide synthase (neuronal)
NOS2	Type 2 nitric oxide synthase (inducible)
NOS3	Type 3 nitric oxide synthase (endothelial)
NOS1	Gene coding for nNOS
NOS2	Gene coding for iNOS
NOS3	Gene coding for eNOS
NPHSII	Second Northwick Park Heart Study
NR	Nitrate reductase
O_2	Oxygen
O_2^-	Superoxide
O_2Hb	Oxyhaemoglobin
$ONOO^-$	Peroxynitrite

PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PTPS	6-Pyruvoyl tetrahydropterin synthesis
QBH_2	Quinonoid tetrahydrobiopterin (reduced form)
RNA	Ribonucleic acid
RPLP	Restriction Fragment Length Polymorphism
RE	Restriction endonuclease
SA	Sulphanilic acid
SD	Standard deviation
SEM	Standard error of the mean
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
SR	Sepiapterin reductase
SRF	Serum response factor
ss	Single stranded
SSCP	Single Strand Conformation Polymorphism
SSRE	Shear stress responsive element
T	Thymine
T_a	Annealing temperature
T_m	Melting temperature
$t_{1/2}$	Half life
TAE	Tris-acetate-EDTA buffer
TBE	Tris-borate-EDTA buffer
TEMED	NNN', N tetramethylethylene-diamine
TF	Transcription factor
TNF-α	Tumour necrosis factor alpha
tsp	Transcription starting point

U	Unit
USF	Upstream stimulatory factor
UTR	Untranslated region
uv	Ultraviolet
V	Volt
VNTR	Variable number of tandem repeats
VSMC	Vascular smooth muscle cell
WBC	White blood cell
w/v	weight by volume
XC	Xylene cyanol
YY-1	Yin yang 1

PUBLICATIONS

Publications and presentations arising from work conducted as part of this thesis:

Jeerooburkhan, N., Jones, L. C., Bujac, S., Cooper, J. A., Miller, G. J., Vallance, P., Humphries, S. E., Hingorani, A. D. Genetic and environmental determinants of plasma nitrogen oxides and risk of ischaemic heart disease. (Hypertension, 2001).

Leeson, C. P. M., Hingorani, A. D, Mullen, M. J., Jeerooburkhan, N., Kattenhorn, M., Cole, T. J., Lucas, A., Muller, D. P. R., Humphries, S., Deanfield, J. E. The E298D endothelial nitric oxide synthase gene polymorphism interacts with environmental and dietary factors to influence endothelial function. (Circulation Research, 2002).

Jeerooburkhan, N., Jones, L. C., Bujac, S., Cooper, J. A., Miller, G. J., Vallance, P., Humphries, S. E., Hingorani, A. D. Genetic contribution of GTP cyclohydrolase I gene to plasma neopterin levels. (Manuscript in preparation).

- Genetic and Environmental Determinants of Nitric Oxide in Man.

- Oral presentation to the *British Society for Cardiovascular Research* - Glasgow / March 1999.

- Phenotype and Genotype Relationship of NO and Pterin Pathways in Man.

- Poster presentation at the *Nitric Oxide Conference* - Paris / October 1998; and

- Poster presentation at the *Postgraduate Poster Competition* - UCL / Summer 1999.

CHAPTER 1

NITRIC OXIDE:

BIOCHEMISTRY AND ENDOTHELIAL CELL DYSFUNCTION

1.1 Historical perspective

Within two decades of its discovery, nitric oxide (NO) has created a revolution in medical research - clinical, biochemical and pharmacological. NO, nicknamed "mediator", "murderer" and "medicine" because of its signalling, toxic and therapeutic properties, has been the subject of more than 20 000 scientific papers in the worldwide literature (Änggård, 1994). The discovery of endogenous NO formation in a variety of tissues, where it has been demonstrated to play many key roles has led to a proliferation of literature addressing its biological roles (Snyder and Bredt, 1992; Nathan, 1992).

In 1980, Furchgott and Zawadzki reported that the relaxation of vascular smooth muscle cell (VSMC), mediated by acetylcholine (ACh), only occurred in the presence of an intact endothelial lining (Furchgott and Zawadski, 1980). When the endothelial lining was damaged or removed, the relaxation to ACh was blunted whereas the dilator response to glyceryl trinitrate (GTN) and contractile response to noradrenaline were both preserved. They hypothesised that the endothelial cells release an unstable humoral substance ($t_{1/2}$ = few seconds), which they called aptly endothelium-derived relaxing factor (EDRF). Several candidate mediators were thought to be EDRF, but by

1986, both Furchgott (Furchgott, 1988) and Ignarro (Ignarro *et al.*, 1987) showed that NO and EDRF were quenched by haemoglobin (Hb) and superoxide (O_2^-) and both relax VSMC. They concluded that the vascular endothelium releases NO and consequently proposed that EDRF is NO or a labile compound releasing NO (Furchgott, 1990; Ignarro *et al.*, 1988).

During that same period, several scientists demonstrated the following: vascular endothelial cells synthesise NO (Palmer *et al.*, 1987), NO maintains the resistance vasculature in an active state of vasodilation (Vallance *et al.*, 1989), NO curtails platelet aggregation (Radomski *et al.*, 1987) and L-arginine is the physiological precursor of NO (Palmer *et al.*, 1988). NO was shown to diffuse freely across cell membranes to its sites of action (Lohse *et al.*, 1998), where it is involved in pharmacological and biochemical processes such as vascular regulation, thrombosis, neurotransmission, inflammation and immunity (Beckman and Koppenol, 1996).

1.2 L-arginine nitric oxide (NO) pathway

The synthesis of NO in the human vasculature uses L-arginine (L-arg) and molecular oxygen, with the formation of equimolar amounts of NO and L-citrulline (***Figure 1.1***). This enzymatic reaction is catalysed by nitric oxide synthase [NOS; EC 1.14.13.39] (Moncada and Higgs, 1993; Knowles and Moncada, 1994). Production of free radical, gaseous, NO occurs via a five-electron stereospecific oxidation of a guanidino nitrogen of L-arg (Palmer and Moncada, 1989; Marletta, 1993). This reaction, which is oxygen-dependent, occurs via two separate steps (Knowles and Moncada, 1994). The initial reaction involves the hydroxylation of the guanidino nitrogen (Body *et al.*,

1995) and results in an unstable intermediate N-hydroxy-L-arginine (N^{ω}-OH-L-arg) followed by the formation of L-citrulline and NO (Tayeh and Marletta, 1989). NOS is stereospecific for L-arg isomer since D-arg is not a substrate (Hobbs *et al.*, 1994).

Figure 1.1: Biosynthetic pathway for NO production in humans.

Proposed mechanism for the sequential oxidation of L-arginine by NOS isoforms. L-arginine is hydroxylated to N-hydroxy-L-arginine and then the latter is cleaved to L-citrulline.

$$\text{L-arg} + \text{NADPH} + O_2 \longrightarrow N^{\omega}\text{-OH-L-arg} + \text{NADP}^+ + H_2O$$

$$N^{\omega}\text{-OH-L-arg} + {}^1/_2\text{NADPH} + O_2 \longrightarrow \text{L-citrulline} + {}^1/_2\text{NADP}^+ + H_2O + \text{NO}$$

1.3 Nitric oxide synthase (NOS) isoforms

Mammalian NO synthesis is mediated by at least three NOS isoforms (Nathan, 1992; Kiechle and Malinski, 1993). For the nomenclature of these isoforms, three terminologies are currently used. The first nomenclature describes the cells or tissues from which the proteins were originally identified: neuronal NOS (nNOS) from the neurons, endothelial NOS (eNOS) from the vascular endothelium and inducible NOS from immune-activated macrophages (iNOS) (Body *et al.*, 1995). A second terminology designates NOS as either dependent on calcium (Ca^{2+}) and calmodulin (CaM) (eNOS and nNOS) for activation or independent of Ca^{2+} and CaM (iNOS) (Klee *et al.*, 1986). The final terminology, developed by the *Human Genome Nomenclature Committee*, assigns roman numerals to the genes encoding the respective enzymes according to the order of their isolation and characterisation. Each

isoform is encoded by a distinct gene: nNOS by *NOS1* (29 exons located on chromosome 12), eNOS by *NOS3* (26 exons located on chromosome 7) and iNOS by *NOS2* (26 exons located on chromosome 17) (Marsden *et al.*, 1993; Förstermann *et al.*, 1994; Hattori *et al.*, 1994; Miyahara *et al.*, 1994; Nadaud *et al.*, 1994; Vallance and Moncada, 1994).

Basal levels of NO are constantly synthesised by eNOS and nNOS to regulate vascular tone and synaptic plasticity respectively (Lowenstein *et al.*, 1994). These two constitutive enzymes are activated in response to transient elevations in intracellular Ca^{2+} and CaM within the cytosol. In contrast, iNOS is solely regulated at the transcriptional level and its messenger ribonucleic acid (mRNA) is induced by bacterial products and inflammatory mediators (Vallance and Moncada, 1993). Ca^{2+}-independent iNOS contains a tightly bound CaM molecule, allowing the enzyme once transcribed to be fully active at basal levels of Ca^{2+} (Knowles and Moncada, 1994). Macrophages, in response to cytokines, express nanomolar (nm) concentrations of NO induced by iNOS for several hours (Wong and Billiar, 1995). The nNOS and iNOS proteins are located in the cytosolic soluble cell region as opposed to eNOS, which is membrane-bound (Lamas *et al.*, 1992; Busconi and Michel, 1993). Endothelial NOS resides on the Golgi complex and in small invagination of the plasma membrane, called caveolae, which are characterised by the presence of a structural protein called caveolin (Shaul *et al.*, 1996; Michel and Feron, 1997).

All three human isoforms share about 50-60 % homology but they are all homodimeric, exhibit identical cofactor requirements, catalyse enzymatic reactions by identical mechanisms and exhibit a common genomic organisation (Marletta, 1993)

(***Table 1.1***). A conservation of exon/intron (50-60%) structure across human *NOS1*, *NOS2* and *NOS3* suggest that these genes are derived from a single ancestral gene (Hall *et al.*, 1994). For enzymatic activity, the homodimers associate into dimeric structures of about 260 kDa (Baek *et al.*, 1993; Wang and Marsden, 1995).

A canonical feature of the translated NOS proteins is a distinct organisational structure: a reductase domain located at the carboxy-terminal (COOH) and an oxidative domain at the amino-terminal (NH_2) (Wang and Marsden, 1995) (***Figure 1.2***). The two domains are linked by a 'bridge' of about 25-35 residues, which is a Ca^{2+}/CaM binding site (Moncada and Higgs, 1995). The COOH-terminal is homologous in structure and function to Cytochrome P450-like haemoproteins and contains the cofactors flavin mononucleotide (FMN), flavine adenine dinucleotide (FAD) and reduced nicotine adenine dinucleotide phosphate (NADPH) binding sites (Bredt *et al.*, 1991; Furchgott and Vanhoutte, 1989). The N-terminal contains consensus sequences for the binding of L-arg (substrate), 6*R*-L-erythro-5,6,7,8-tetrahydrobiopterin (BH_4; cofactor) and haeme (catalytic) (Wang and Marsden, 1995). The reductase domain of all three NOS comprises about 600-700 amino acids, encoded by the following exons: 15-29 (*NOS1*), 13-26 (*NOS3*) and 14-26 (*NOS2*) (Knowles and Moncada, 1994; Hobbs *et al.*, 1999).

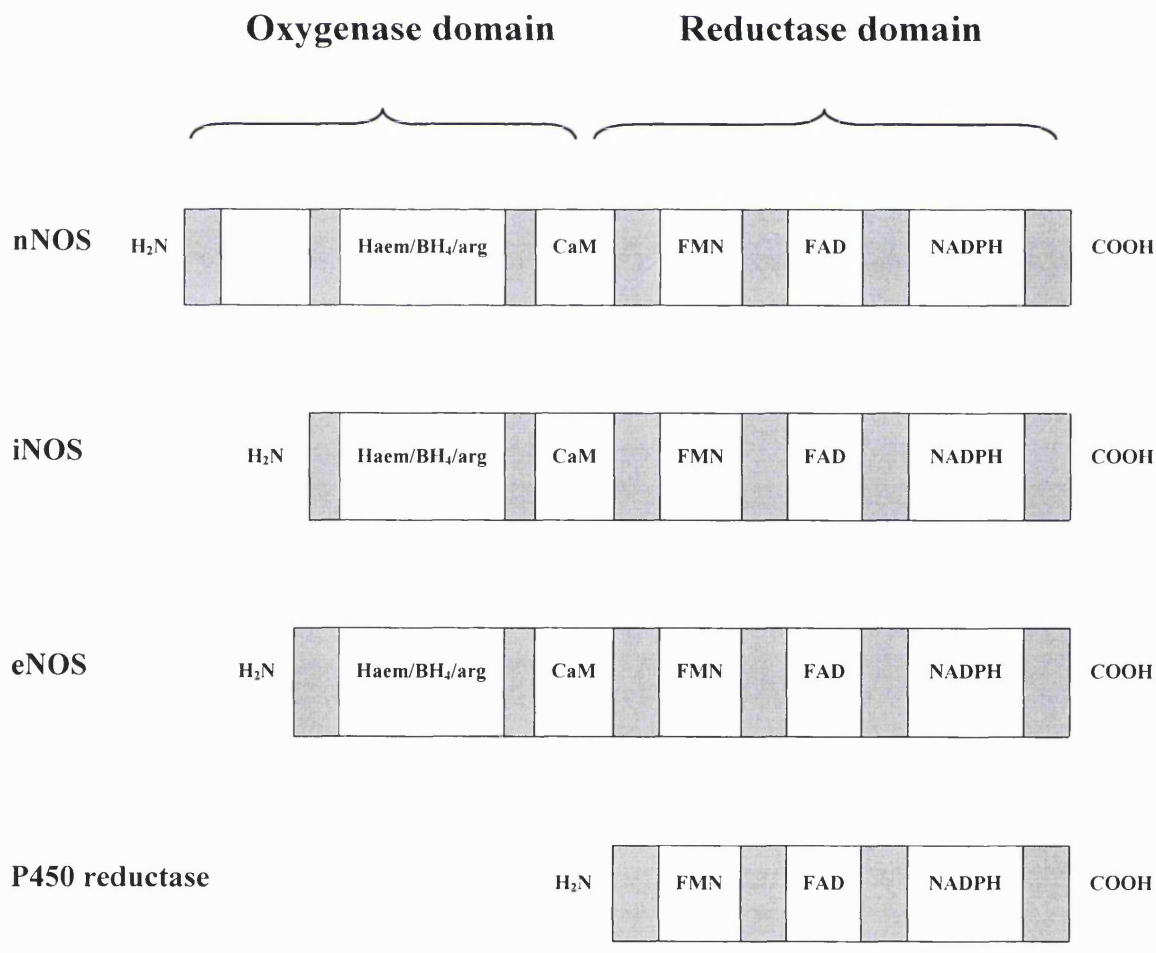
Table 1.1: Properties and characteristics of NOS isoforms and their genes.

Properties	Isoform		
Type	**I**	**II**	**III**
Enzyme	nNOS	iNOS	eNOS
Cell prototype	Neuron	Macrophage	Endothelial cell
Cellular localisation	Cytosolic / soluble	Cytosolic / soluble	Membrane
M_w (kda)	160	130	135
Amino acids	1433	1144	1203
Regulation	Ca^{2+}/CaM	*Ca^{2+} independent	Ca^{2+}/CaM
Quantity NO released	Picomole	Nanomole	Picomole
	Small pulses	Continuous	Small pulses
Main function	Cell signalling	Cytotoxicity	Cell signalling
Cofactors	NADPH, FAD, FMN, BH_4, haeme		
Encoded by gene	*NOS1*	*NOS2*	*NOS3*
Chromosome location	12q24.2	17q11.2-q12	7q35-q36
Gene size (kb)	>200	~37	~21
Number of exons	29	26	26

*: CaM is tightly bound to this isoform

Figure 1.2: Schematic representation of the human NOS isoforms illustrating functional homology and relationship to Cytochrome P450 reductase.

The three isoforms exhibit about 60% of sequence homology at their N-terminal portion, which contains the putative binding sites for L-arginine, tetrahydrobiopterin and haeme. Consensus sequences for the binding of NADPH and flavins (FAD and FMN) are located at the C-terminal region. The latter is conveniently called the reductase domain as it closely resembles and performs basically the same functions as cytochrome P-450 reductase.



It is believed that the CaM binding site acts as a switch to funnel electron flow from the reductase to the oxygenase domains for the activation of molecular oxygen (Abu Soud and Stuehr, 1993) (***Figure 1.3***). The NOS flavins are thought to serve as an electron storage pool, accepting two electrons at a time from NADPH and delivering them one at a time to haeme during the catalytic oxidation of L-arg (***Figure 1.4***) (White and Marletta, 1992). There is evidence that BH_4 participates in a redox-manner (Hevel and Marletta, 1992) and has a conformational role for the binding of NOS isomers into the active dimeric form (Mayer and Werner, 1995; Baek *et al.*, 1993). Chen *et al.* found that basal purified NOS activity correlates linearly with its endogenous pterin content and a variable concentration of BH_4 remains always tightly bound to purified NOS, with cysteine 99 being critical for the binding (Chen *et al.*, 1995). BH_4 maintains haeme NOS in the ferrous state through electron transfer. This redox role protects the enzyme from feedback inhibition from NO, which preferentially binds to haeme iron in the ferric state (Griscavage *et al.*, 1994).

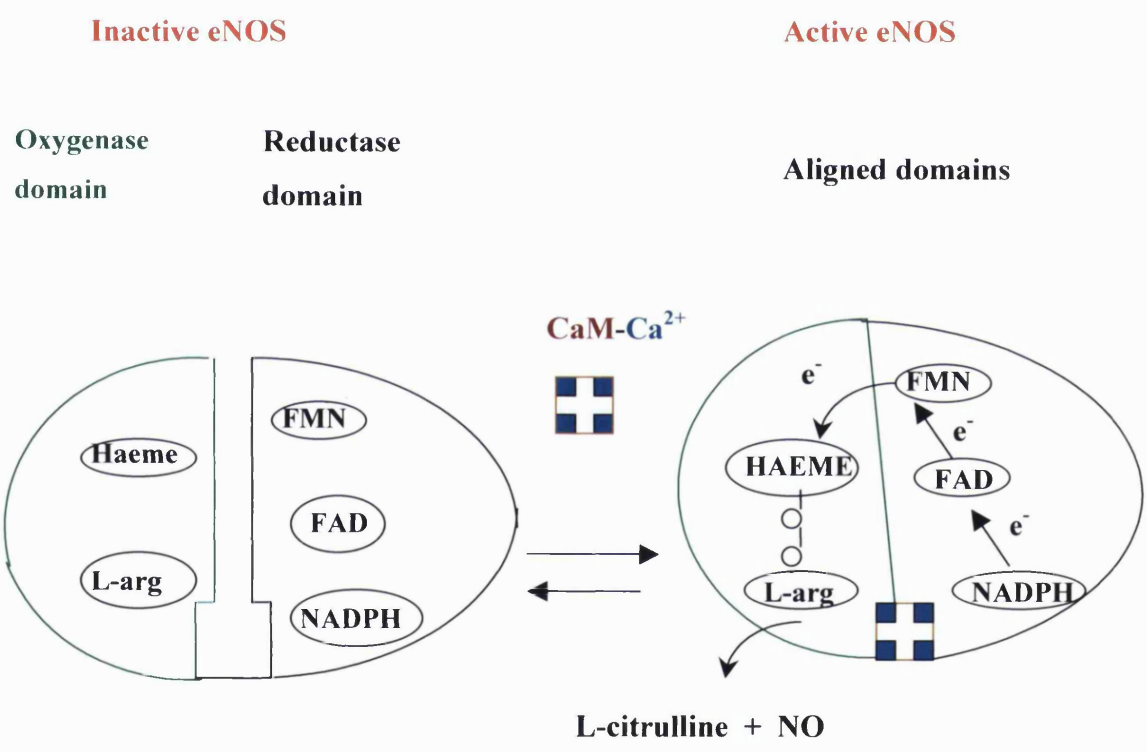
Figure 1.4: Shuttle of electrons by NOS flavins.

Flavin pool accepts two electrons at a time from NADPH and delivers one at a time to haeme.

$$NADPH \rightarrow 2e^- \rightarrow [\ FAD \rightarrow FMN\ ] \rightarrow 1e^- \rightarrow HAEME$$

Figure 1.3: Representation of electrons flow accompanying catalytic NO synthesis.

When calmodulin, associated with four calcium atoms, binds to the hinge region of the NOS protein, the oxygenase domain (*left*) is aligned with the reductase domain (*right*) thereby enabling the flavins to shuttle the electrons from NADPH to haeme, in the presence of saturating amounts of tetrahydrobiopterin and L-arginine. (*Adapted from Abu Soud and Stuehr, 1993*)



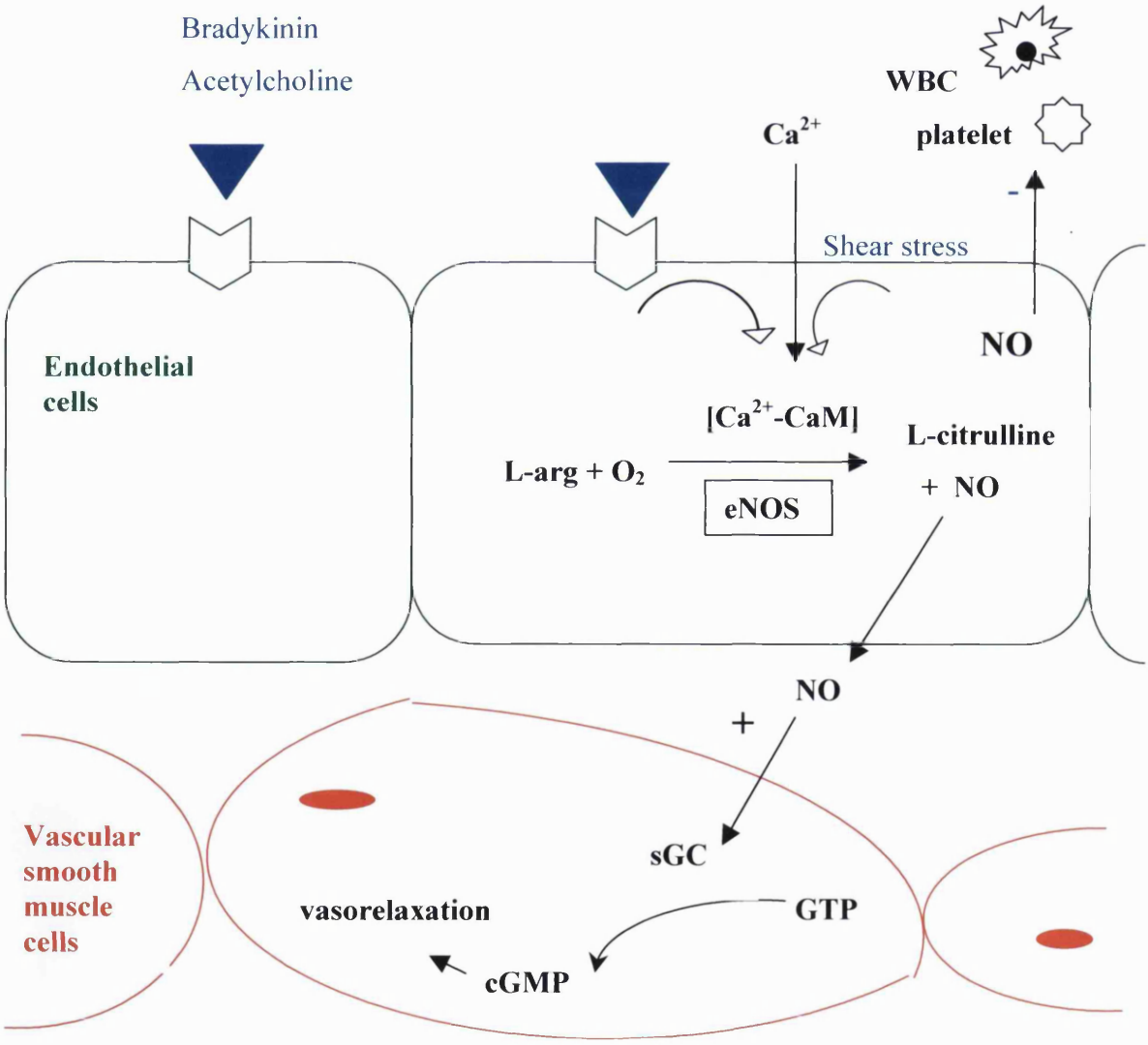
1.4 Haemodynamic and cytotoxic effects of NO

Several physiological functions of NO have been identified within the vessel wall (Änggård, 1994; Lowestein *et al.*, 1994; Fleming and Busse, 1999). It increases blood flow through vasodilatation and disaggregates aggregating platelets thereby suppressing microvascular thrombosis (Radomski *et al.*, 1990). NO also modulates vascular growth, prevents the replication and migration of VSMCs (Sarkar *et al.*, 1996), inhibits the adherence of lymphocytes, monocytes and neutrophils to the endothelium (Radomski *et al.*, 1987). Furthermore, NO is responsible for the vasorelaxant and anti-platelet action of nitrovasodilator agents such as GTN (Feelisch and Noack, 1987; Harrison and Bates, 1993).

NO initiates its physiological responses by stimulating soluble guanylate cyclase (sGC) [EC 4.6.1.2] (Murad *et al.*, 1990; Hobbs, 1997), a heterodimeric protein containing distinct catalytic- and haeme-binding domains (Bhagat and Vallance, 1996). On release from the endothelial cells, NO diffuses to the VSMCs where it activates sGC by binding to its haeme moiety to form a nitrosyl-haeme complex (Ignarro, 1990; Hobbs, 1997). The conformational change induced in the heterodimer exposes the catalytic domain of sGC to its substrate, guanosine-5-triphosphate (GTP), and catalyses its cyclisation (Murad, 1986). This results in the concomitant formation of cyclic guanosine-3',5'-monophosphate (cGMP), which acts as an intracellular second messenger affecting the function of protein kinases and phosphorylates intracellular proteins including myosin light chain kinases (Rapoport and Murad, 1983). These processes lead to VSMC relaxation, which mediates vasodilation (Waldman and Murad, 1987) (***Figure 1.5***).

Figure 1.5: Vascular relaxation mediated by endothelial NO.

Increases in blood flow (shear stress) or an agonist such as acethylcholine binds to its receptor on the endothelial cell surface, causing a transient increase in intracellular calcium. Calcium binds to calmodulin and the Ca^{2+}-CaM complex upregulates eNOS, which synthesises NO. NO released abluminally, diffuses into adjacent smooth muscle cells, where it binds to the haeme group of soluble guanylate cyclase. This leads to enhanced production of cyclic GMP, which induces vasorelaxation through a cascade of protein kinases. NO, released into the lumen of the endothelium, inhibits leucocyte and platelet activation as well as adhesion to the endothelial wall.

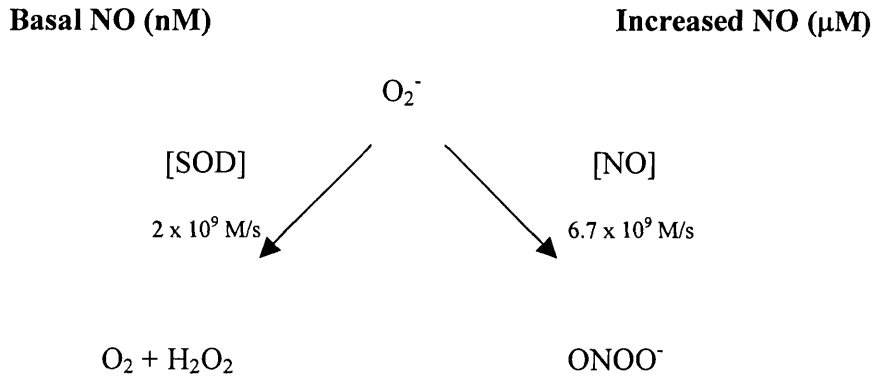


During inflammation or infection, activated macrophages combat intra- and extra-cellular pathogens by producing large amounts of NO (Hibbs *et al.*, 1988). NO mediates anti-microbial and tumoricidal effects by impairing the synthesis of DNA and protein as well as certain enzymes such as cytochrome *c* oxidase involved in mitochondrial function (Kwon *et al.*, 1991). As a result, cellular ATP stores are depleted leading to cytotoxicity (Nathan and Hibbs, 1991). Unfortunately during inflammation, the cytotoxic or cytostatic actions of NO are also associated with tissue damage, as it does not discriminate between the invading pathogens, the cells producing it and those located within the vicinity (Moncada, 1994).

Beckman and co-workers suggested that the toxicity of NO may be due to its ability to interact with O_2^- anion to yield the potent, cytotoxic oxidant peroxynitrite ($ONOO^-$) (Beckman *et al.*, 1990; 1993). Under normal physiological conditions, superoxide dismutase (SOD) prevents the formation of significant amounts of $ONOO^-$ (Beckman *et al.*, 1994). However, the production of both NO and $ONOO^-$ species are considerably increased during inflammation and sepsis (Moncada *et al.*, 1991; Haddad *et al.*, 1994) (***Figure 1.6***). $ONOO^-$ has been shown to alter protein function thereby disrupting cellular activity, by inducing toxicity through nitration of tyrosine and cysteine residues (Beckman and Koppenol, 1996; Bhagat and Vallance, 1996).

Figure 1.6: Formation of peroxynitrite by NO.

At basal levels of NO, superoxide is effectively removed by superoxide dismutase, present at micromolar (μM) concentrations. At increased levels, NO competes with superoxide dismutase for superoxide due to its faster rate constant, resulting in peroxynitrite formation.



1.5 NO, endothelial dysfunction, inflammation and atherosclerosis

Endothelial NO is considered to be an antiatherogenic and antithrombotic factor (Moncada *et al.*, 1991; Anggard, 1994; Lowenstein *et al.*, 1994; Vallance and Moncada, 1994) as well as a blood pressure (BP) regulator (Haynes *et al.*, 1993; Baylis and Vallance, 1996). NO regulates vascular tone and maintains the integrity of the endothelium through vasodilation, by opposing platelet aggregation and inhibiting VSMCs migration and growth (Radomski *et al.*, 1987; Garg and Hassid, 1989; Vallance *et al.*, 1989; Bath *et al.*, 1991). Individuals with atherosclerotic risk factors, such as hypercholesterolemia, cigarette smoking and hypertension have an impairment in their endothelium-dependent vasodilation (EDD) responses (Creager *et al.*, 1990; Calver *et al.*, 1992; Celermajer *et al.*, 1994; Heitzer *et al.*, 1996; Benjamin and Vane, 1996) and a reduction in vascular NO bioactivity has been implicated (Quyyimi *et al.*,

1995a; Glasser *et al.*, 1996; Mullen *et al.*, 1997). It is believed that hypertensives have a defect in their endothelial L-arginine-NO pathway (Calver *et al.*, 1994; Baylis and Vallance, 1996) while in a rabbit atherosclerotic model, systemic inhibition of NO production with the NOS inhibitor N^G monomethyl-L-arginine (L-NAME), initiates the formation of intimal atherosclerotic lesions (Cayatte *et al.*, 1994; Naruse *et al.*, 1994).

However, it is unclear whether a diminution in vascular NO synthesis, an increase in NO destruction or both that underlie the process of "endothelial dysfunction" (ED) (Bhagat and Vallance, 1996; Harrison, 1997). Impaired EDD is considered to be an index of ED, which may be a marker for an impairment of the normal atheroprotective and antiproliferative actions of the endothelium (Cosentino and Lüscher, 1997; Harrison, 1997). Several hypotheses have been proposed to account for ED, accompanied by an impairment in NO-mediated dilator responses. Possible explanations are: synthesis of an endogenous eNOS inhibitor (Vallance *et al.*, 1992), decreased substrate availability (Cooke and Dzau, 1997; Stroes *et al.*, 1997), imbalance between production of endothelium-derived relaxing and constricting factors (Moolenaar, 1994), over-production of oxygen-derived free radicals (Ignarro, 1990; Hamilton *et al.*, 1997), decreased expression of eNOS (Oemar *et al.*, 1998), inhibition of NOS activity (Fleming and Busse, 1999) and a deficiency in BH_4 availability and/or synthesis (Tahey and Marletta, 1989; Gross and Levi, 1992; Werner-Felmayer *et al.*, 1993).

ED due to abnormal endothelial functions (i.e. unopposed platelet aggregation, vasoconstriction and leucocyte infiltration) may be an early event in atherogenesis

(Bhagat and Vallance, 1995; Lloyd-Jones and Bloch, 1996; Glasser *et al.*, 1996). It is present before the development of atherosclerotic plaques in patients with a family history of atherosclerosis (Celermajer *et al.*, 1994) and certainly well before the clinical detection of disease (Cooke and Tsao, 1994). It was shown that although the endothelium was physically intact, the amount of bioactive NO released from the endothelial cells was decreased (Quyyumi *et al.*, 1995b). Indeed one of the earliest abnormalities to occur in hypercholesterolemic animals and humans is a reduction in the activity of vascular NO (Naruse *et al.*, 1994), as manifested by impaired EDD and this occurs well before any structural changes in the vessel wall were apparent (Celermajer *et al.*, 1992). This impairment in vasodilation is reversible by local or systemic administration of L-arginine, substrate for NO synthesis, and is paralleled by a striking sustained enhancement in vascular NO activity (Creager *et al.*, 1992; Cayatte *et al.*, 1994; Cooke *et al.*, 1992).

Atherosclerosis is a progressive and diffuse process, believed to start in early childhood and becoming clinically manifest later in life (Wick *et al.*, 1995; Zeiher *et al.*, 1991). It is essentially a multifactorial response caused by metabolic, physical or environmental injuries to the endothelium, operating through exposures such as hypercholesterolemia, hypertension or cigarette smoking (Harrison, 1997; Glasser *et al.*, 1996). Endothelial injury predisposes to leucocyte adhesion, proliferation and media-to-intima migration of VSMCs and thrombosis in the arterial wall (Vanhoutte and Shimokawa, 1989). It is also associated with an impaired responsiveness in blood flow to endothelium-dependent vasodilators (i.e. ACh and bradykinin) and dysregulated endothelial functions, including NO release and actions (Schmidt et Walter, 1994; Fleming and Busse, 1999).

A complex network of risk factors, intricately linked, account for the pathogenesis of atherosclerosis (Ross, 1986; Boerwinkle *et al.*, 1996; Harrison, 1997). While reduced NO plays a key role in its development (Cosentino and Lüscher, 1997), additional processes are also likely to be important (Alexander, 1994). Inflammation, at distant sites or generalised low-grade inflammation perhaps within the vessel wall itself is associated also with atherosclerosis, and it is undoubtedly one of the cornerstones of atherogenesis as many features implicated in atherosclerosis are also common to inflammation (Ross, 1999; Libby and Hansson, 1991). Cytokines, such as interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) are involved in the initiation and progression of atherosclerosis (Ruszczak and Schwarz, 1996), while macrophages, monocytes and lymphocytes have been identified in the human atherosclerotic plaque (Wick *et al.*, 1995). Atherosclerosis appears to be a silent chronic inflammatory process that can develop to an acute clinical event by induction of a plaque rupture by enzymes released from activated macrophages (Vallance *et al.*, 1997; Berliner *et al.*, 1995).

1.6 Genetic susceptibility to IHD

Atherosclerosis, leading to ischaemic heart disease (IHD), is a multifactorial disorder, resulting from an interplay between environmental and genetic factors (Humphries *et al.*, 1999) as attested by results of the twin studies (Marenberg *et al.*, 1994), adoptees (Sorensen *et al.*, 1988) and familial hypercholesterolemia (Sorensen *et al.*, 1994). Several genes have been investigated as candidate atherosclerotic susceptibility genes. DNA sequence polymorphisms within the low-density lipoprotein (LDL)-receptor

gene (Brown and Goldstein, 1986), IL-6 (Ruszczak and Schwarz, 1996) and fibrinogen (Humphries *et al.*, 1995) genes, among others, have been shown to influence the levels or activity of their respective encoded proteins and to be associated with atherosclerosis.

Several *in vivo* and *in vitro* studies have shown that endothelial NO production may be regulated by *NOS3* expression at the transcriptional level (Busconi and Michel, 1994; Wang and Marsden, 1995). Therefore *NOS3* is a potential candidate atherosclerosis-susceptibility gene and polymorphisms in *NOS3* could result in differences in the expression or activity of the encoded eNOS protein (Hingorani, 2000). These functionally important variants may affect the synthesis of endothelial NO (Oemar *et al.*, 1998; Soma *et al.*, 1999) and influence an individual's susceptibility to ED and in turn IHD (Soubrier, 1999; Lüscher and Noll, 1999).

NO may have a dual role in the development of atherosclerosis. Since inflammation is classically associated with elevated NO, it is possible that both a decrease in physiological endothelial NO production as well as an increase in inflammatory NO generation may lead to the development of endothelial damage and dysfunction (Luscher *et al.*, 1995; Quyyimi *et al.*, 1995a; Nathan and Xie, 1994; Schmidt and Walter, 1994). Common genetic variation identified in *NOS3* have been associated with atherosclerosis and coronary artery disease (CAD) (Wang *et al.*, 1997; Hingorani *et al.*, 1999a; Shimasaki *et al.*, 1998; Ichihara *et al.*, 1998; Nakayama *et al.*, 1999). However, few studies have addressed directly the effects of these variants, if any, on endothelial NO production (Wang *et al.*, 1997; Tsukada *et al.*, 1998). Four previously identified polymorphisms in the promoter and coding region of *NOS3* have been

studied as they have the potential to influence eNOS expression or function. They are: -922 (A/G), -786 (T/C), +894 (G/T) and a 27-bp variable number of tandem repeats (VNTR) in intron 4 (In4). The -922 (A/G) bi-allelic polymorphism is of interest as it is found in a region known to influence basal transcription (Zhang *et al.*, 1995) and the variant sequence creates a consensus motif for the transcription factor (TF) CF-1 (Riggs *et al.*, 1991). The other promoter variant, -786 (T/C) was associated with a reduction in *NOS3* gene transcription and diminished NO production in Japanese subjects (Nakayama *et al.*, 1999). In4 VNTR was associated with CAD in Australian smokers (Wang *et al.*, 1996) and influenced plasma NO_x levels (Wang *et al.*, 1997).

The polymorphism identified in exon 7 at nucleotide (nt) 894 (+894 G→T) causes an amino acid substitution of glutamic acid by aspartic acid (Glu→Asp) at codon 298. Amino acid 298 is located within a loop on the external surface of eNOS crystal structure and Asp298 was found to be more susceptible to proteolytic cleavage than Glu298 eNOS (Tesauro *et al.*, 2000). By enzymatic studies, Hingorani *et al.* confirmed that the Asp298 does not influence enzyme catalysis and substrate or inhibitor binding (Hingorani, *manuscript in publication*). The Asp298 allele, a risk factor for CAD in Caucasians (Hingorani *et al.*, 1999a) as well as Japanese subjects (Shimasaki *et al.*, 1997) was found to be associated with reduced flow-mediated dilatation (FMD) in male smokers (Leeson *et al.*, *manuscript in publication*). Therefore, carriers of Asp298 could have reduced amount of functional eNOS, with a resultant reduction in capacity for NO synthesis.

One of the major aims of the current thesis was to understand whether eNOS polymorphisms lead to individual differences in NO synthesis. An additional aim was

to see whether atherosclerotic risk factors are also associated with differences in steady state NO. For the investigation of this type of association, a reliable *in vivo* index of NO synthesis that could be assayed in large sample sets was required and a method which utilises the measurement of steady state levels of the oxidative break down products was used.

1.7 Plasma NO_x: Index of NO synthesis

Although several studies have suggested that NO bioactivity is reduced in atherosclerosis (Quyyimi *et al.*, 1995a), it is difficult to measure NO directly because of its short half-life ($t_{1/2}$ = 10-60 seconds), it exists in minute amounts and is unstable in body fluids (Knowles and Moncada, 1992; Archer, 1993). On release, NO spontaneously auto-oxidises to a variety of nitrogen oxides (Ignarro, 1990; Wishnok *et al.*, 1996) (***Figure 1.7***). NO binds with O_2^- or oxyhaemoglobin (O_2Hb) and is rapidly oxidised to inorganic nitrate (NO_3^-) and nitrite (NO_2^-) in the bloodstream and this is the major route of NO decomposition *in vivo* (Green *et al.*, 1982; Rhodes *et al*, 1995). NO_2^- reacts with further O_2Hb stoichiometrically to form NO_3^- and methaemoglobin in the vascular system (Ignarro *et al.*, 1993). These stable metabolites have been frequently used as a reliable plasma measurement of NO production (Wong *et al.*, 1995; Schoedon *et al.*, 1995a; Moshage *et al.*, 1995).

Figure 1.7: Proposed cascade pathway triggered when NO reacts with oxygen.

Cascade of reactions triggered in physiological solutions when NO reacts with molecular oxygen to form nitrogen dioxide (NO_2) and dinitrogen trioxide (N_2O_3) and terminates with nucleophilic substances, such as nitrite (NO_2^-) and nitrate (NO_3^-), which is the predominant species *in vivo*. (*Adapted from Wishnok et al., 1996*).

$2NO + O_2 \rightarrow 2NO_2$

$2NO + NO_2 \rightarrow N_2O_3$

$N_2O_3 + H_2O \rightarrow NO_2^-$

$N_2O_3 + \text{amine} \rightarrow N\text{-nitroso compound} + NO_2^-$

$NO + O_2^- \rightarrow ONO_2^- \rightarrow NO_3^-$

$NO + O_2Hb \rightarrow NO_3^-$

$NO_2^- + O_2Hb \rightarrow NO_3^-$

$N_2O_3 + H_2O \rightarrow NO_3^-$

Therefore, steady-state plasma levels of nitrate and nitrite (i.e. NO_x) were assayed by the Griess assay (Green *et al.*, 1982). This assay can be adapted and optimised to measure inorganic NO_3^- and NO_2^- from plasma samples of an epidemiological study using the 96-well microtitre plate system (Snell *et al.*, 1996). It is simple and sensitive (micromolar range), with samples not requiring extensive preparation prior analysis, thereby considerably eliminating the risks of contamination (Kiechle and Malinski, 1993; Leone *et al.*, 1994; Moshage *et al.*, 1995). Nitrate was reduced to nitrite by enzymatic nitrate reductase (NR) in the presence of NADPH, which was regenerated by glucose-6-phosphate dehydrogenase (G6PDH) (Green *et al.*, 1982; Gilliam *et al.*,

1993; Verdon *et al.*, 1995). The NO_2^- species was mixed with acidified sulphanilic acid (SA) *in vitro* to form a diazonium salt, followed by derivatization with N-(1-naphthyl) ethylenediamine (NED) to form a purple chromophore detectable at 540 nm by spectrophotometry (***Figure 3.2, Chapter 3***) (Green *et al.*, 1982; Jaekle *et al.*, 1994).

1.8 GTP Cyclohydrolase I: Gene (*GCH1*) and enzyme (GTPCH)

A deficiency of BH_4, an essential cofactor of NO biosynthesis, has also been implicated in ED (a feature of atherosclerosis) and this hypothesis is supported by several lines of evidence (***See Section 5.2.1, Chapter 5***) (Werner-Felmayer *et al.*, 1993; Cosentino and Katusic, 1995; Busse and Fleming, 1996).

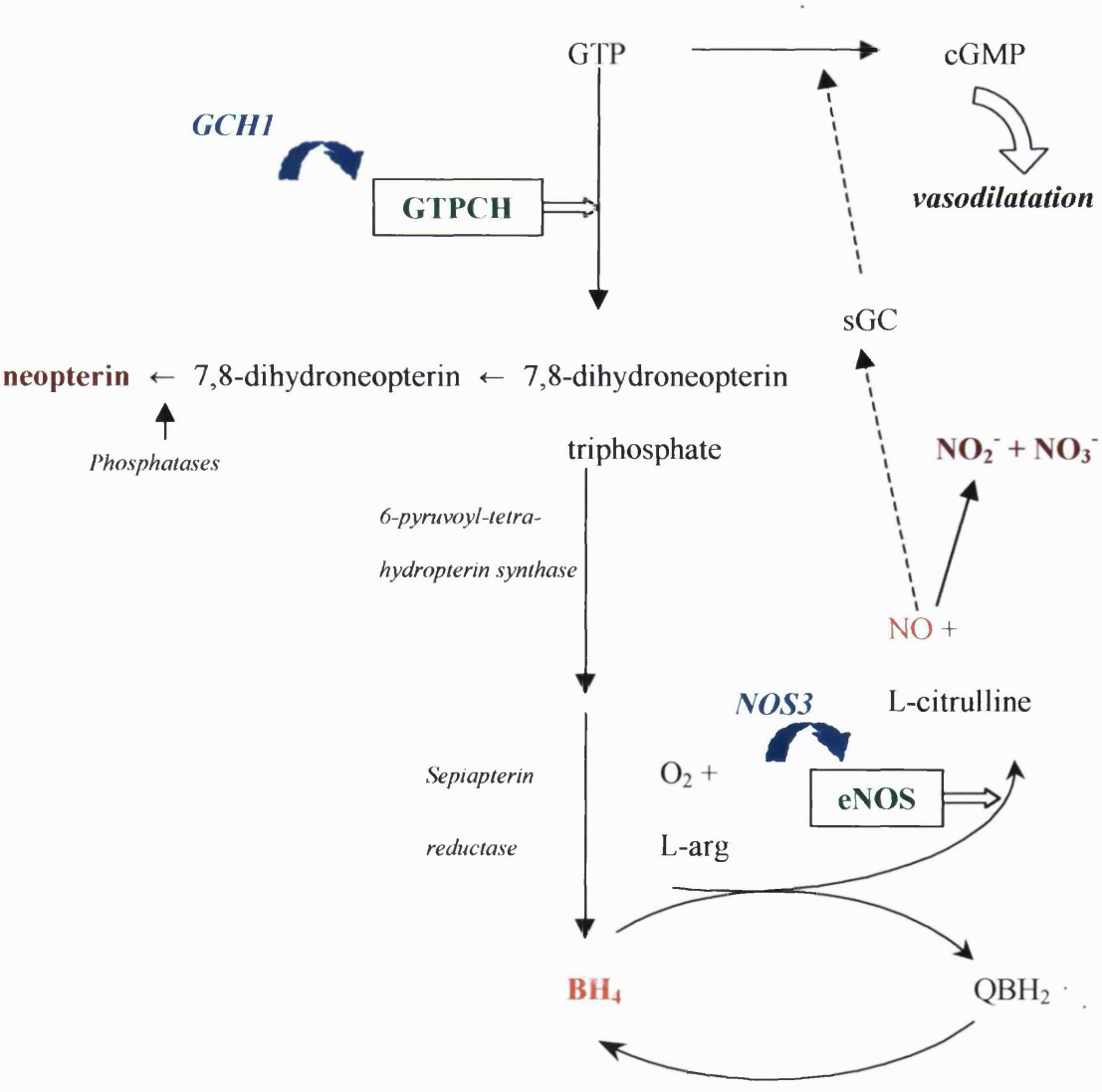
BH_4 is synthesised by GTP cyclohydrolase I [GTPCH; E.C. 3.5.4.16; M_w = 125 000], which is the first and rate-limiting enzyme of the pterin pathway (***Figure 5.1, Chapter 5***) (Tahey and Marletta, 1989; Kwon *et al.*, 1989, Gütlich *et al.*, 1994). GTPCH cleaves GTP to 7,8-dihydroneopterin triphosphate (7,8-NH_2-TP) which is converted to BH_4 by two consecutive enzymes: 6-pyruvoyl tetrahydropterin synthase (PTPS) and sepiapterin reductase (SR) (Nichol *et al.*, 1985; Kaufman, 1978; Blau and Niederweiser, 1985; Curtius *et al.*, 1986). Alternatively 7,8-NH_2-TP is cleaved to 7,8-dihydroneopterin (7,8-NH_2) by phosphorylases, which is further degraded to D-*erythro*-neopterin (Huber *et al.*, 1984; Werner *et al.*, 1990). In most human cells, the majority of 7,8-NH_2-TP formed is metabolised to BH_4. As PTPS and SR are constitutive enzymes, that is their activities are unaffected by cytokines, they are present in low amounts in monocytes and macrophages (Werner *et al.*, 1990; Werner *et al.*, 1993). These cells accumulate 7,8-NH_2-TP, which after hydrolysis by

ubiquitous phosphatases and partial oxidation is excreted as 7,8-NH_2 or neopterin (Werner *et al.*, 1989; Prior *et al.*, 1986). To regulate its own synthesis, BH_4 acts as a feedback inhibitor by forming a ternary 'inactive' complex with GTPCH and a *G*TPCH *F*eedback *R*egulatory *P*rotein (GFRP) in the liver and the kidney as well as in other tissues, such as lung, heart and spleen (Xie *et al.*, 1998). At low GTP concentrations, phenylalanine converts this inactive complex to an active complex (Harada *et al.*, 1993).

GTPCH is encoded by the gene *GCH1* (Blau and Niederweiser, 1985; Duch and Smith, 1991). Polymorphisms within *GCH1* may have the potential to influence BH_4 production, indirectly limiting endothelial NO production, by modulating GTPCH gene expression (***Figure 1.8***). However, surprisingly to date, there have been no studies on common sequence variation within the *GCH1* gene promoter and its coding sequences. Also, there is no information on the effects of any polymorphism on BH_4 production or with the development of IHD.

Figure 1.8: Hypothetical scheme demonstrating putative interconnections between L-arginine-NO pathway and pterins biosynthesis in eukaryotic cells.

GTP plays a dual in the biology of NO as it is a precursor to both BH_4 (cofactor of eNOS) and to cGMP (mediator of vasodilatation). NO activates sGC which catalyses the conversion of GTP to cGMP. BH_4 oxidation to its quinoid isoform ($Q\text{-}BH_2$) is believed to provide two of the five electrons necessary for the oxidation of L-arginine to L-citrulline and NO. Neopterin is formed as a by-product of the pterin pathway while NO_x (i.e. NO_3^- and NO_2^- anions) are formed as oxidation products of NO.



Ichinose *et al.* cloned human *GCH1* in 1995, which spans ~30 kb and is composed of six exons (Ichinose *et al.*, 1995a) (***Figure 5.2, Chapter 5***). The gene was mapped to region q21-22 of chromosome 14 (Thöny *et al.*, 1995). Each exon/intron boundary conforms to the GT/AG rule (Breatnach and Chambon, 1981). Canonical features of an eucaryotic promoter such as a CCAAT- and a TATA-box are located 64- and 21-bp upstream of the *tsp* and several consensus sequences for the binding of Sp1 are present (Witter *et al.*, 1996). Upstream of the *tsp*, there is a 452-bp GC-rich region acting as the promoter for the transcription of various housekeeping genes (Witter *et al.*, 1996). Analyses of the promoter shows the presence of potential binding sites for the following TFs: CCAAT-box/enhancer binding protein (C/EBP), hepatic nuclear factor-1 (HNF1), upstream stimulatory factor (USF), AP-1, serum response factor (SRF), and nuclear factor NF-κB (***Figure 5.3, Chapter 5***). Cytokines elicit increases in NO and BH_4 levels via NF-κB and AP-1 activation in murine cells (Togari *et al.*, 1998).

In humans, GTPCH molecules are encoded by at least three mRNAs, distinct in their 3' ends (Togari *et al.*, 1992). The different isoforms of *GCH1* mRNAs result from alternative use of the splice acceptor site at exon 6. Splicing between the 5'- and 3'-splices site of intron 5 produced type 1 mRNA, splicing between the 5'-splice site of intron 5 and the middle of exon 6 generates type 2 mRNA, whereas intron 5 is not spliced out in the transcript corresponding to type 3 mRNA (Ichinose *et al.*, 1995a). The latter derives most probably from an immature mRNA. However, only type 1 mRNA encodes an active GTPCH while the other two are defective in GTPCH activity (Gütlich *et al.*, 1994a; Ichinose *et al.*, 1995a). The total length of the *GCH1* mRNA was shown to be 3.6 kb with the 5'-untranslated region (5'-UTR) plus the open reading frame (ORF) accounting for 923 bp and the remaining 2.7 kb for the 3'-UTR (Gütlich

et al., 1992). At present, there is no information concerning the functional relevance of 5' UTR of *GCH1*. However, studies on 5'-UTR in other genes suggest that this region is important in the regulation of gene expression, and that sequence changes can lead to a decreased enzyme activity of the gene product (Bandmann *et al.*, 1998).

A mouse mutant deficient in GTPCH activity, generated by chemical mutagenesis, showed that the *hph-1* mutation affects the steady state level of *GCH1* mRNA instead of altering the sequences within the reading frame of the gene (McDonald *et al.*, 1988; Hyland et al., 1996). This occurs due to either a mutation occurring in the regulatory region of *GCH1* affecting either the binding of a TF to the promoter, or due to the release of an inhibitory factor, or a mutation in the gene of a regulatory factor thus causing a decreased hepatic GTPCH activity (Gutlich *et al.*, 1994b).

About 52 mutations have been found along human *GCH1*, mostly in exons 1 and 6, with considerable heterogeneity of phenotypic expression (Bandmann *et al.*, 1996; Brique *et al.*, 1999). A possible explanation for the varied clinical manifestations is due to alternative splicing of *GCH1* mRNA resulting in different levels of mutant/normal mRNA ratios (Hirano *et al.*, 1996; 1999). Deficiency in GTPCH activity in humans results in a lack of BH_4 thereby impairing both the biosynthesis of catecholamines and serotonin leading to hyperphenylalaninemia, dopamine responsive dystonia (DRD) and hereditary progressive dystonia (HPD) (Bandmann *et al.*, 1996; Brique *et al.*, 1999). DRD is a hereditary lower limb dystonia disorder (Nygaard *et al.*, 1993) while HPD is characterised by childhood-onset dystonia and marked diurnal fluctuation (Ichinose *et al.*, 1994; 1995b). However the effect of BH_4 deficiency on vascular tone, BP and ED in these patients has not been documented.

1.9 Tetrahydrobiopterin (BH_4): Essential cofactor of eNOS

BH_4 regulates endothelial NO formation and is an essential cofactor of eNOS (***Figure 1.8***). Endothelial deficiency of BH_4 (either from impaired synthesis or enhanced oxidative degradation) has been implicated in ED, that predisposes to atherosclerosis, whilst its avaibility is enhanced in sepsis and inflammation (Maier *et al.*, 2000; Tiefenbacher *et al.*, 2000; Werner *et al.*, 1998). Current experimental evidence suggests that BH_4 may play numerous roles in the integrity of NOS enzyme and activity and several hypotheses have been proposed:

- BH_4 acts as an electron donor in the monoxygenation of L-arginine and in the hydroxylation of aromatic amino acids (Tahey and Marletta, 1989; Kwon *et al.*, 1989; Mayer *et al.*, 1992). To date, no amino acid sequence homology between NOS and BH_4-dependent aromatic amino acid hydroxylase has been reported.
- BH_4 serves as an allosteric effector or a stabilising agent for eNOS subunit dimerization (Liu and Gross, 1996; Sahin-Toth *et al.*, 1997). Tightly bound BH_4 keeps macrophage NOS in its active dimeric form and the dissociated inactive monomers only reassociate when haeme, BH_4 and L-arginine are present simultaneously (Baek *et al.*, 1993).
- BH_4 autoxidises to superoxide which reacts with NO to form peroxynitrite, thus protecting NOS inactivation by NO (Mayer *et al.*, 1995).

However, the functional roles of BH_4 in NOS catalysis remain elusive and future studies are needed to clarify these crucial roles.

There is considerable evidence showing that an increase in NO production is accompanied by an increase in BH_4 synthesis (Gross and Levi, 1992; Hattori and Gross, 1993). During inflammation and sepsis, inflammatory cytokines i.e. interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) with co-stimulation of lipopolysaccharide (LPS) and interleukin 1 (IL-1), induce GTPCH activity through *GCH1* mRNA induction (Werner-Fermayer *et al.*, 1989; Gross and Levi, 1992). This is accompanied by an increase in BH_4 levels in human macrophages and an induction in L-arginine NO pathway within endothelial cells and macrophages. (Werner-Felmayer *et al.*, 1993; Hattori *et al.*, 1995). The NO formed stimulates sGC, which mediates vasorelaxation via cGMP production (Van Amsterdam and Werner, 1992) and acts as a powerful cytotoxic agent (Wachter *et al.*, 1992).

As the amount of BH_4 formed is controlled by GTPCH, the key enzyme of pteridine biosynthesis, it can therefore be used as a marker of GTPCH activity (Nichol *et al.*, 1987; Werner *et al.*, 1993). However, the pterin pathway may be genetically determined. Sequence variation(s) within *GCH1* could influence basal transcription rate of its mRNA and in turn limit the production and/or the activity of its protein, i.e. GTPCH. *GCH1* may therefore indirectly limit BH_4 production thereby influencing the amount of bioactive NO present in the vasculature and lead to the development of ED, a feature of atherosclerosis and IHD (Busse and Fleming, 1996; Cosentino and Katusic, 1995). Methods currently available for measuring BH_4 are not applicable for epidemiological studies. Therefore an alternative method is to measure plasma neopterin, which is formed as a by-product of the pterin pathway (Wachter *et al.*, 1992).

1.10 Plasma neopterin: Index of GTPCH activity

Exactly as plasma NO_x has been assayed as a marker of eNOS, so plasma neopterin has been used as an index of GTPCH activity (Schoedon *et al.*, 1987; Wachter *et al.*, 1992). Actually, the same cytokines that induce NOS also induce pterin synthesis, therefore levels of nitrite and nitrate (markers of NO production) might be expected to parallel the elevations in neopterin levels (Moncada *et al.*, 1991; Werner-Felmayer *et al.*, 1989; Milstien *et al.*, 1994). Conversely if endothelial dysfunction is due to a decrease in NO biosynthesis, that is secondary to a reduction in BH_4 avaibility, reductions in plasma NO_x might be expected to parallel reductions in BH_4.

Previous *in vitro* and *in vivo* studies show that neopterin biosynthesis correlates closely with the *in vivo* activation of the cellular immune system (Margreiter *et al.*, 1983; Schoedon *et al.*, 1987). Under specific inflammatory conditions (challenge by cytokines or endotoxin) within the human vasculature, GTPCH's activity is induced several-fold followed by an increase in neopterin release, which is considered to be an indicator of the stimulation of the *de novo* pathway for BH_4 biosynthesis (Werner-Felmayer *et al.*, 1993; Hattori *et al.*, 1995). This induction of pterin synthesis has been shown to accompany NOS induction and to be necessary for NO production (Hattori and Gross, 1993; Werner-Felmayer *et al.*, 1993; Schoedon *et al.*, 1993). This is followed by leakage of neopterin from monocytes and macrophages, two cells which play a central role in the inflammatory response (Huber *et al.*, 1984; Fuchs *et al.*, 1988a; Williams *et al.*, 1993).

Neopterin, which has no known function, is released in response to local stimuli and enters the bloodstream due to its small size (M_w = 253) and stability (Aulitzky *et al.*, 1988; Fuith *et al.*, 1991). On excretion by the kidney, it is not modified and does not bind to any receptors. Being diffusible and naturally fluorescent, it is easily measured from plasma by Enzyme Linked ImmunoSorbent Assay (ELISA) (Werner *et al.*, 1989; Rokos and Rokos, 1983; Fahey *et al.*, 1990).

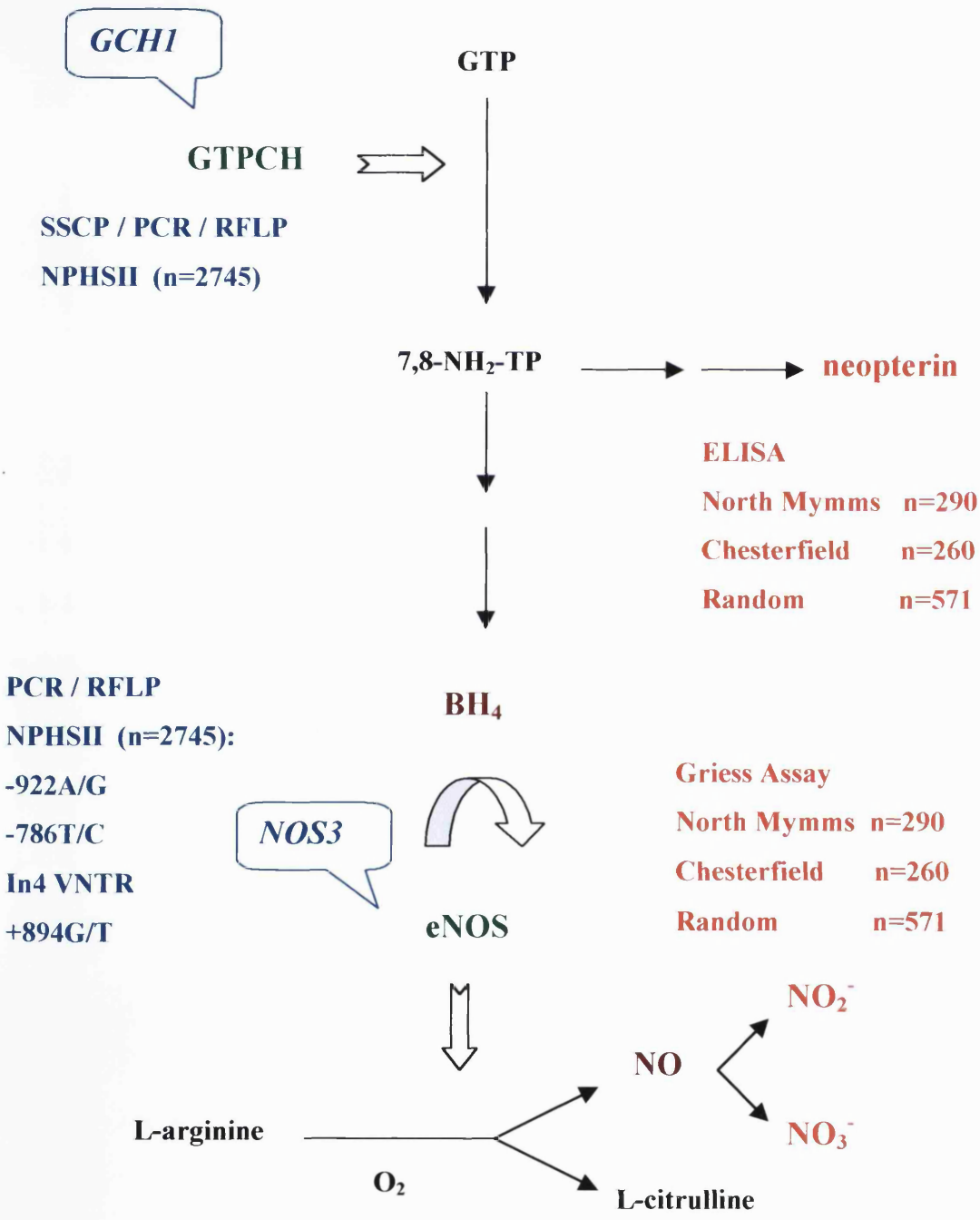
Although neopterin induction is not specific for any disease, its levels steadily during inflammatory disease progression and decreases towards normal with recovery (Werner *et al.*, 1993; Müller *et al.*, 1991). During the acute phase of infections, neopterin level peaks (up to 100 times higher) and even precedes the detection of specific antibodies in plasma (Fuchs *et al.*, 1988b; Schumacher *et al.*, 1997). Because of its predictive significance, neopterin is used as an objective non-invasive index of disease activity and is useful for monitoring follow-up treatment in response to therapy (Pacher *et al.*, 1989, Prior *et al.*, 1986; Fuchs *et al.*, 1988c; Candito *et al.*, 1993). As neopterin is an early and sensitive marker of the activation status of monocytes and macrophages (Schumacher *et al.*, 1992; Kuller *et al.*, 2000), it may serve as a valuable tool in the diagnosis and prognosis of atherosclerosis and/or IHD, especially as these pathologies are believed to be mediated by inflammatory processes (Tatzber *et al.*, 1991; Weiss *et al.*, 1994).

1.11 Aims of my thesis

At the time of thesis writing, there was a lot of evidence implicating a defect in the L-arginine-NO pathway in the development of ED, a feature of atherosclerosis and/or IHD. Similarly, BH_4 deficiency is believed to predate to ED. BH_4 plays a crucial role in NO formation and its synthesis is actively regulated by GTPCH. However the potential role of the pterin pathway in ED and IHD is unidentified.

The overall aim was to investigate genetic variations in two candidate genes, that is *NOS3* and *GCH1* and to examine the possibility that these sequence changes could lead to the production of impaired eNOS and GTPCH in the second Northwick Park Heart Study (NPHSII) study (***Figure 1.9***). The resulting altered bioactivities of these enzymes, if any, were then detected at cellular levels by assaying their corresponding phenotypic markers, i.e. NO_x and BH_4 respectively. However, neopterin (a by-product of the pterin pathway) was assayed as a plasma index of GTPCH activity instead of BH_4 (Wachter *et al.*, 1992). The possibility that variations in *NOS3* and *GCH1* expression may predispose to IHD was also studied.

Figure 1.9: Schematic diagram illustrating NO and BH_4 synthesis with the appropriate genes (*blue*) and plasma markers (*red*) investigated as well as the corresponding methodology and study group(s) used.



The principal aims of this thesis are as follows:

Aim 1: To optimise and validate the Griess assay so as to measure circulating plasma nitrite and nitrate (i.e. NO_x) as an index of endogenous NO production and indirectly as a marker of quantitating eNOS activity (***See Chapter 3***).

Aim 2: To set up Polymerase Chain Reaction in combination with Restriction Fragment Length Polymorphism (PCR/RFLP) based protocols for genotyping four previously identified poylmorphisms within *NOS3* gene (***See Chapter 4***).

Aim 3: To search for potentially important polymorphisms within the promoter of *GCH1* gene using Single Strand Conformation Polymorphism (SSCP) technique and to set up high-throughput genotyping protocols (***See Chapter 5***).

Aim 4: To measure plasma neopterin levels as an index of GTPCH activity by ELISA (***See Chapter 6***).

Aim 5: To look for association between the polymorphisms in *NOS3* and *GCH1* and plasma levels of NO_x and neopterin respectively (***See Chapters 4 and 5***).

Aim 6: To examine possible association between circulating plasma NO_x and neopterin with conventional IHD risk factors such as BP, cholesterol and smoking (***See Chapters 3 and 6***).

Aim 7: To study the effect of *NOS3* and *GCH1* genotypes and the risk of IHD events (***See Chapters 4 and 5***).

I carried out all the practical work mentioned in this thesis, except for the following: Miss Manjeet Bolla carried out the Intron 4 genotyping for 20 NPHSII plates; Ms Roslyn Whithall taught me the SSCP technique and Miss Lisa Jones helped with the NO_x and neopterin assays in the Chesterfield and Random sets from the NPHSII.

CHAPTER 2

SUBJECTS, MATERIALS AND METHODS

2.1 Study subjects: Second Northwick Park Heart Study (NPHSII)

2.1.1 Background to NPHSII

The second Northwick Park Heart Study (NPHSII) is a prospective cohort study and its main objective is to test the hypothesis that prior to a coronary event, there is a hypercoagulable state within the body (Meade *et al.*, 1986; Meade, 1994). Participants were from the following practices (***Table 2.1***): The Surgery, Aston Clinic, Upper Gordon Road, Camberley; The Health Centre, Carnoustie; Whittington Moor Surgery, Chesterfield; Potterells Medical Centre, North Mymms; The Health Centre, Harefield, Rosemary Medical Centre, Parkstone; The Health Centre, St Andrews and The Market Place Surgery, Halesworth. The regional distribution of these areas is shown on ***Figure 2.1***.

Figure 2.1: Regional distribution of the nine clinics of NPHSII.

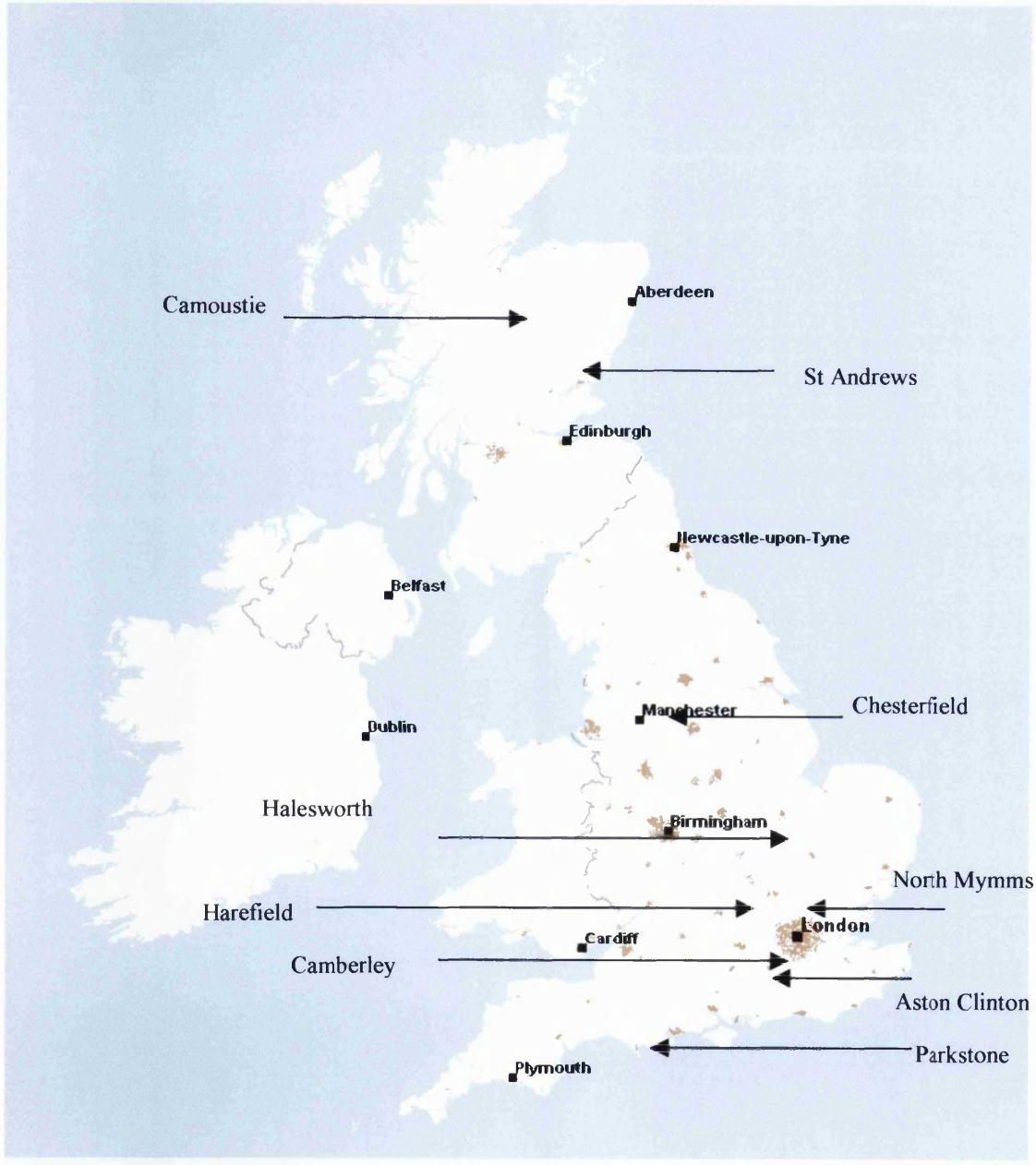


Table 2.1: Number of participants and geographical location of each clinic.

Sample area	Clinic number	Frequency	Geographical location
Camberley	1	416	South West of London
Carnoustie	2	408	North Scotland/Angus
St Andrews	3	386	East Scotland
Aston Clinic	4	111	Surrey
Harefield	5	291	North London
Chesterfield	6	261	Midlands/North
Halesworth	7	435	East Anglia
Parkstone	8	413	North West England
North Mymms	9	291	North London

Initially, 4600 healthy men aged 50 to 61 years, registered with nine general medical practices in England and Scotland, were screened for eligibility and 459 were excluded because of a history of acute myocardial infarction (MI) or unstable angina, regular medication with aspirin or anticoagulants, presence of cerebrovascular disease or any life-threatening illness, malignancy with the exception of skin cancer or other melanoma, any conditions, such as hepatitis B or other infectious disease, which may expose the medical staff to risks of infection, mental disorders and other rare characteristics precluding regular attendance for medical examination or informed consent.

Subsequently, 3179 (77%) of the 4141 respondents were considered eligible for study and recruited for prospective surveillance. About 228 (7%) of them were eventually excluded from the statistical analysis because of the following reasons: refusal of venepuncture, evidence of a previous MI on the electrocardiogram, missing results due to loss in blood samples during storage, transport or laboratory processing and unclear labelling of samples. In total, 2951 men, free of symptoms of coronary artery diseases (CAD), were invited for annual follow-up examination and 2745 were Caucasians.

2.1.2 Clinic attendance

Participants were reviewed annually in a non-fasting state, either in the morning or afternoon. Respondents examined in the morning were requested to have only one cup of tea or coffee and to avoid a cooked breakfast prior examination, whereas those seen later were asked to have a light snack during lunch with only a cup of tea or coffee. All were instructed to avoid full-cream milk on the day of their visit and to refrain from smoking or vigorous exercise from the previous midnight. Participants who admitted to non-compliance were given a further appointment.

2.1.3 History

The subjects' medical history was assessed by completion of a questionnaire, which included chest pain, alcohol and smoking habit. From the demographic information, subjects were classified in three groups according to smoking habit: never smokers, ex-smokers (stopped for a year or more prior interview) and current smokers (***Table 2.2***).

Table 2.2: Baseline characteristics of NPHSII cohort at the start of the study.

Baseline Characteristics	Never Smoked N=937		Ex-Smoker N=1215		Current Smoker N=859		ANOVA *P* value
	Mean	SD	Mean	SD	Mean	SD	
Age (years)	56.0	3.4	56.2	3.5	56.0	3.4	0.17
BMI (kg/m^2)	26.2	3.3	27.0	3.5	25.9	3.8	**0.0001**
sBP (mmHg)	136.8	18.7	139.2	18.9	138.0	19.9	**0.01**
dBP (mmHg)	84.3	11.2	85.4	11.3	83.4	11.6	**0.0004**
Cholesterol (mM)	5.71	0.98	5.78	1.02	5.69	1.05	**0.11**
Triglyceride (mM)	1.66	0.85	1.87	1.00	1.85	0.96	**0.0001**
FVIIc (% standard)	105.6	27.7	111.1	29.5	110.2	29.1	**0.0001**
Fibrinogen (mg/dl)	2.60	0.47	2.66	0.48	2.92	0.55	**0.0001**
ApoA1 (mg/dl)	1.62	0.35	1.60	0.34	1.61	0.32	0.56
ApoB (mg/dl)	0.87	0.27	0.88	0.27	0.87	0.25	0.71

2.1.4 Measurements

After being seated for 5 minutes (min), systolic (sBP) and diastolic (dBP) blood pressure were measured twice with a random zero mercury sphygmomanometer and the results were averaged for statistical analysis. Weight (kg) and height (m) were measured on a balance scale and a stadiometer respectively and were recorded to the nearest 0.1 cm and 0.1 kg, after participants had removed their outdoor clothing and their shoes. Body mass index (BMI) was calculated as weight/height^2 (kg/m^2).

2.1.5 Blood sampling

Venepuncture was performed by the Vacutainer technique (Becton Dickinson, Cowley, Oxford). Blood was drawn through a 21-gauge needle and collected as follows:

- 4.5 ml into a silicone tube containing trisodium citrate as anticoagulant;
- 4.5 ml and 9 ml volumes into silicone tubes containing an anticoagulant mixture of trasylol, ethylene-diamine tetra-acetic acid (EDTA) and a thrombin inhibitor (Byk-Sangtec, Dietzenbach, Germany); and
- 5 ml into a glass tube with a clot activator for the separation of serum.

The tubes were immediately centrifuged at 1000x G for 10 min at room temperature (R_t). Plasma or serum was then transferred by plastic Pasteur pipette in 1.0 ml aliquots into plastic screw cap vials (Nunc) for immediate storage at -45°C, prior transfer for storage at -80°C to the Medical Research Council (MRC) laboratory. If the quality of venepuncture was unsatisfactory it was repeated at an another date if possible (Miller *et al.*, 1995).

2.1.6 Biochemical assays

Serum cholesterol (mM) and triglyceride (mM) were determined by enzymatic procedures with reagents from Sigma (Poole, UK) and Wako Chemicals (Alpha Laboratories, UK). Factor VII coagulant activity (VIIc, % standard) was measured by one-stage bioassay (Diagen, UK) on the citrated plasma in duplicates and the results averaged. Fibrinogen concentration (mg/dl) was determined by a thrombin-clotting method. Clinical progress was monitored by regular review of medical records and annual measures of BP, cholesterol and clotting factors. All IHD events are being recorded and any deaths occurring are automatically notified by the UK National Health Service Central Register. To date, most practices have completed about 8.1 years of follow up on 2965 men, with 159 men having developed an IHD event (***Table 2.3***). This study will continue monitoring until year 2000.

Table 2.3: Baseline demographic characteristics of study participants.

Characteristics	No IHD event	IHD event	*P*
n	2806	159	
Age (years)	56.0 (3.5)	56.7 (3.7)	0.02
BMI (kg/m^2)*	26.6 (3.4)	27.2 (3.5)	0.02
Current Smokers	28.7%	44.0%	<0.0001‡
Systolic BP (mmHg)*	139.5 (18.4)	145.6 (20.1)	<0.0001
Cholesterol (mmol/L)*	5.84 (1.01)	6.24 (1.07)	0.0001#
Triglyceride (mmol/L)*†	1.94 (1.02)	2.20 (1.16)	0.0002
Fibrinogen (g/L)* †	2.69 (0.51)	2.87 (0.51)	<0.0001

* adjusted for age and practice

† geometric means (approximate SD)

‡ By χ^2 analysis

Welch's test

2.2 General materials

2.2.1 Reagents

All reagents were of molecular biology grade and general purpose reagents were obtained from BDH Chemicals Ltd. (Merck Ltd., Poole, Dorset), Gibco BRL (Life Technologies Inc., Paisley, Scotland) and Sigma Chemical Co. (St Louis, MO, USA). Dimethyl sulfoxide (DMSO) was from BDH (Lutterworth, Leicestershire, UK), *Thermus Aquaticus* (*Taq*) thermostable DNA polymerase from Gibco-BRL (5kU/L, Cat. No. 18038-026; Renfrew, UK) and paraffin oil from BDH (Poole, UK).

2.2.2 Enzymes

Restriction endonucleases (RE) were obtained from Promega Limited (Madison, WI, USA) and New England Biolabs (NEB; Hitchin, UK).

2.2.3 DNA ladders

100 base pairs (bp) and 1 kilobase (kb) DNA ladders were from Gibco BRL (Life Technologies Inc., Scotland).

2.2.4 Equipment

0.5-10 µl 8-channel multipipette (Finnpipette, Life Sciences, Basington, UK), 100 µl repeater pipette (Biohit, Alpha Labs., Eastleigh, UK), deep 96-well plates (Beckman, High Wycombe, UK), 96-well Omniplates (Hybaid Ltd., Teddington, Middlesex, UK), 96-well microtitre plates and lids (Falcon 3911 and Falcon 3913, Becton Dickinson, Oxford, UK), centrifuge (Sorvall, T60000B: duPont, Newtown, CT), Omnigene PCR machine, (Hybaid) Incubator (Sanyo-Galleenkamp, Leicester, UK), horizontal gel electrophoresis (Model MPH, Cat No. 52000), MADGE gel formers (GenetiX, WinbourneMinster, UK), Hoefer Mighty Small™ electrophoresis apparatus (Hoefer Scientific Instruments, San Francisco, California), ultraviolet (*uv*) transilluminator (UVP Inc), charge-coupled device camera, UVP Imagestore 5000 frame grabber for digital images and video copy processor (UV Products, Cambridge, UK).

2.2.5 Solutions and buffers

All the solutions were made up in double distilled or de-ionised water and water employed in molecular biology analysis was autoclaved. The solutions and buffers were prepared as follows:

10x Tris-acetate-EDTA (TAE) buffer

0.4 M Tris; 0.01 M EDTA; pH 8.0 with glacial acetic acid.

10x Tris-borate-EDTA (TBE) buffer

89 mM Tris-borate; 2 mM EDTA; pH 8.3.

1 M $MgCL_2$

20.33 g $MgCL_2$ added to 100 ml of deionised water and autoclaved.

1 M Tris-HCL (pH 8.2)

12.11 g Tris added to 100 ml of deionised water, pH 8.2 with concentrated HCL and autoclaved. The procedure was repeated for Tris pH 7.5.

Reagent A (stored at 4°C)

109.54 g sucrose dissolved in 10 ml of 1M Tris pH 7.5, 5 ml of 1 M $MgCL_2$ and 10 ml of Triton-X-100. This mixture was made up to 1000 ml with deionised water.

0.5 M Na_2EDTA (pH 8.0)

37.22g EDTA dissolved in 200 ml of deionised water using a heated stirrer and adjusted to pH 8.0 with concentrated NaOH.

10% sodium dodecylsulphate (SDS)

10 g SDS made up to 100 ml with deionised water.

Reagent B

2.34 g NaCL combined to 1 ml Tris-HCL (1M), 0.4 ml Na_2-EDTA (0.5 M) and 90 ml deionised water. The mixture was autoclaved and 10 ml of 10% SDS added.

5 M Sodium perchlorate

70.24 g sodium perchlorate added to 100 ml of deionised water.

TE buffer (pH 7.6)

1.21 g Tris and 0.37 g EDTA are made up to 1000 ml with deionised water and adjusted to pH 7.6 with concentrated HCL and autoclaved.

'Sticky silane'

0.5% Gamma-methacryloxypropyltrimethoxysilane / 0.5% glacial acetic acid / ethanol (v/v). All the reagents were from Sigma Chemical Co, Poole, Dorset, UK.

25% Ammonium persulfate (APS)

2.5 g APS solid (Sigma Chemical Co., St Louis USA) was dissolved in 10 ml of ddH_2O.

Polmix (10x)

500 mM KCL; 100 mM Tris (pH 8.3); 0.01% Gelatin; 2 mM each dNTP (i.e. dATP, dCTP, dGTP and dTTP); ddH_2O to volume.

1/10 dCTP Polmix (10x)

500 mM KCL; 100 mM Tris (pH 8.3); 0.01% Gelatin; 2 mM each dNTP (i.e. dATP, dGTP and dTTP); 0.2 mM dCTP; ddH_2O to volume.

Xylene cyanol (XCFF) loading buffer

0.25% XCFF added to 15% Ficoll-400.

Bromophenol blue (BPB) and xylene cyanol (XCFF) loading buffer

0.25 g of 0.25% BPB; 0.25 g of 0.25% XCFF; 10 ml of 1.0% SDS; 20 ml of 0.1M Na_2EDTA (pH 8.0); 20 g 20% of Ficoll-400.

Formamide loading buffer (MADGE dye) (stored at 4°C)

49 ml of 95% formamide; 1 ml of 10 mM EDTA (pH 8.0); 12.5 mg of 0.025% BPB; 12.5 mg of 0.025% XCFF.

SSCP loading buffer (stored at 4°C)

7.0 ml of MADGE dye; 2.5 ml of 0.1% (w/v) SDS; 2.5 ml of 10 mM EDTA.

2.3 General methods

2.3.1 Primer design

For selective amplification of a specific DNA sequences, amplimers were constructed to that they bind to the complementary DNA sequences flanking the desired target region. Primers were designed according to the following principles:

- Length was 18-24 bases;
- GC base composition was 50-60%;
- Amplified product was chosen to be between 200-500 bp;
- Estimated melting temperature of forward and reverse primers were roughly equal;
- 3'-Ends of primer pairs were not complementary to reduce primer/dimer formation;
- 3'-Ends of primers must not have runs of three Cs or Gs to prevent mispriming; and
- Self-complementarity of primers leading to secondary structures (i.e. hairpins and loops) was avoided.

All oligonucleotides were obtained from Gibco BRL (Life technologies, Paisley, Scotland). For each PCR reaction, the primers were diluted in double distilled water (ddH_2O) and 10-15 picomole (pmole) were used per reaction mixture.

2.3.2 Choosing the optimum T_m and T_a

For PCR reactions, theoretical optimum melting (T_m) and annealing temperatures (T_a) were calculated for each set of primers according to the following formula:

and

$$T_m = 2 \Sigma(A + T) + 4 \Sigma(G + C)$$

$$T_a = T_m - 5°C$$

In practice, PCR amplification was optimised empirically after a 'temperature titration'.

2.3.3 DNA extraction

DNA from the NPHSII samples was previously extracted from whole blood by the staff of the *Department of Cardiovascular Genetics* at *University College London.* The 2745 DNA samples were stored in 32 96-well arrays at 4°C. I personally extracted DNA from white blood cells (WBCs) (n=262) and whole blood (n=37) for two genetic studies. Genomic DNA was prepared from WBCs or whole blood, collected into 10 ml EDTA tubes, by the 'salting-out' method (Miller *et al.*, 1988) with a few modifications.

1 For cellular lysis, whole blood was emptied into a 30 ml propylene tube and topped up with 10 ml of Reagent A (4°C). The tube was inverted several times to ensure thorough mixing and centrifuged (10,000 rpm / 10 min / 4°C. The supernatant was carefully discarded in a waste tube and the pellet re-suspended in 10 ml cold lysis buffer. Step 1 was repeated to obtain a pale pellet.

2 Two ml of Reagent B were added to the nuclear pellet and the tube vortexed briefly for complete nuclear lysis. For deproteinisation, 1 ml of 5 M sodium perchlorate was added and the contents were thoroughly mixed.

3 Extraction of contaminated protein was carried out by adding 2 ml of chloroform (-20°C) with a glass pipette and the tube inverted several times, followed by centrifugation at (3,000 rpm / 5 min) at room temperature (R_t). The upper aqueous phase was carefully transferred into a propylene tube whilst the lower organic phase containing the precipitated protein was discarded.

4 Ten ml of ice-cold 100% ethanol (-20°C) were slowly added to the supernatant tube, and after thorough mixing, the DNA was seen precipitating as fine, stringy, white woolly fibres at the interface.

5 The DNA was removed using a sterile Pasteur pipette and rinsed in 70% ethanol for a few seconds to remove salts and small organic molecules. The washed DNA was re-suspended in 1 ml of sterile TE buffer in sterile 2 ml screw cap Apex tubes (Alpha Laboratories Ltd, Eastleigh, UK) and incubated overnight at 37°C.

6 DNA samples were diluted 1 in 10 in sterile water and transferred to 96-well Beckman arrays for storage at 4°C. Each of these Master Arrays, contains two empty wells for negative and positive controls for subsequent PCR (***Figure 2.2***).

Figure 2.2: A 96-well Master Array containing NPHSII samples.

well	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	-	-	-	-	-	-	-	-	X	X
B	-	-	-	-	-	-	-	-	-	-	-	-
C	-	-	-	-	-	-	-	-	-	-	-	-
D	-	-	-	-	-	-	-	-	-	-	-	-
E	-	-	-	-	-	-	-	-	-	-	-	-
F	-	-	-	-	-	-	-	-	-	-	-	-
G	-	-	-	-	-	-	-	-	-	-	-	-
H	-	-	-	-	-	-	-	-	-	-	-	-

'-' : NPHSII samples **'X' : Empty wells**

2.3.4 Measuring DNA concentration by spectrophotometry

For a 1/10 dilution, 40 µl of stock genomic DNA were diluted with 360 µl Sigma water (Molecular Sigma® Biology, St Louis, USA). An aliquot was transferred to a quartz cuvette and placed in a spectrophotometer (Du®-64, Beckman PAC™ Module, Softpac Module Nucleic acid). For each sample, light absorbance was recorded at 260 nm (A_{260}) for DNA and 280 nm (A_{280}) for protein. TE buffer was used as a blank. The ratio A_{260}/A_{280} was calculated by a software (Nucleic acid Softpac™ Module, Beckman Instruments Inc., Fulterton CA 92634) and the results printed (Epson Fx-850). The quality of the prepared DNA was evaluated by the ratio A_{260}/A_{280} with

highly pure DNA having an A_{260}/A_{280} between 1.6-2.0 (ideally 1.8) whereas protein impurities cause A_{260}/A_{280} ratio to rise towards 2.

The DNA concentration in the stock solution was calculated according to the following formula with the conversion factor equal to 50 for genomic DNA:

DNA concentration (μg/ml) = A_{260} x dilution factor x conversion factor

The mean concentration of DNA in Master Arrays, stored at -20°C was 300 μg/ml. The NPHSII samples were 1/20 diluted (i.e. 15 μg/ml) and stored in replica arrays at 4°C, until used. For PCR, 2 μl of DNA was used per well, i.e. 0.03 μg per reaction. Prior to use, the Beckman array containing the DNA samples was removed from the fridge and centrifuged (1 min / 3000 rpm) so as to collect any condensation from the lid and the sides of the well. With a multichannel pipette, 2 μl DNA were transferred to a 96-well microtiter plate (Omniplate), which was centrifuged (30 sec / 1000 rpm) to ensure that the suspended DNA collected at the base of the wells and dried (10 min / 75°C) on the DNA Tetrad PCR machine (MJ Research). The pre-PCR dried DNA template is an exact replica of the 96-well Master Array with the same samples configuration.

2.4 Molecular biology techniques

2.4.1 DNA amplification by Polymerase Chain Reaction (PCR)

For the genotyping of *NOS3* polymorphisms, Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) based protocols were developed (Saiki *et al.* 1988). The conditions were optimised to amplify specific region of interest on the genomic DNA so as to obtain an effective concentration and specificity for eventual analysis of sequence changes. Oligonucleotide primers were designed using the published sequence of the human *NOS3* gene (*EMBL L10693-L10709*) from Genbank.

For *NOS3*, PCR was performed in a volume of 30 μl containing 50 ng of dry DNA (to minimize variability) as template, about 10-15 pmole of each primer, 2 U of *Taq* polymerase (Gibco BRL) in a buffer containing 50 mM KCL, 10 mM Tris-HCL (pH 8.3), 0.1% gelatin and 0.2 mM of each dNTP (Pharmacia, Uppsala, Sweden) and varying concentration of $MgCL_2$ individually optimised for each primer pair. The PCR mix was overlaid with 20 μl paraffin oil to prevent evaporation.

PCR was performed in automated thermal cyclers (MJ Research, PTC-225 Peltier Thermal Cycler). After an initial denaturation step of 95°C for 5 min, 35 cycles of three consecutive steps each lasting for 45 sec were performed: denaturation at 95°C, annealing step with an T_a optimised for each primer set and a final extension (DNA synthesis) at 72°C. Primer sequences and PCR conditions optimised for amplification

are shown in ***Table 2.4***. The primer sequences and conditions used for the genotyping of Intron 4 (In4) VNTR was adapted from Wang *et al.*, 1997.

Table 2.4: Primer pairs and PCR conditions for *NOS3* polymorphisms.

Variant	Primer Pairs Sense / Antisense	Mg^{2+} mM	T_a °C	PCR size (bp)
-922 (A/G)	5'-CAGCTAGTGGCCTTTCTCC-3' 5'-AGAGCTTGATGCCCTGGTG-3'	2.0	60	138
-786 (T/C)	5'-AGTTTCCCTAGTCCCCCATGC-3' 5'-CCACACCCCCATGACTCAAGT-3'	1.5	61	194
In4 VNTR	5'-AGGCCCTATGGTAGTGCCTTT-3' 5'-TCTCTTAGTGCTGTGGTCAC-3'	1.2	56	393, 420
+894 (G/T)	5'-AGGAAACGGTCGCTTCGACGTGCTG-3' 5'-CCCCTCCATCCCACCCAGTCAATCC-3'	2.0	63	151

2.4.2 Detection of Restriction Fragment Length Polymorphism (RFLP)

For restriction enzyme analysis, 10 µl of amplified-PCR DNA was digested with 2 U of the appropriate allele specific RE. The conditions used were according to the manufacturer's instructions. The enzymes from Promega and NEB were chosen to

cleave the rare allele for any given polymorphism into two distinct fragments. Conditions for RFLP analysis are shown in ***Table 2.5***.

Table 2.5: Enzyme digestion and MADGE conditions for *NOS3* polymorphisms.

Variant	Restriction enzyme	Digestion conditions	Recognition sequence	Gel %	RFLP (bp)
-922 (A/G)	*Bsl* I	55°C / 3 hrs	5'..CCNNNNN↓NNGG..3' 3'..GGNN↑NNNNNCC..5'	7.5	28,110
-786 (T/C)	*NgoM* I	37°C / 16 hrs	5'...G↓CCGGC...3' 3'...CGGCC↑G...5'	10	50, 144
In4a VNTR	-	-		10	393
In4b VNTR					420
+894 (G/T)	*Dpn* II	37°C / 16 hrs	5'...↓GATC...3' 3'...CTAG↑...5'	10	51,100

The digested DNA were loaded on a MADGE gel for electrophoresis in 1x TBE buffer for about 45 min and subsequently visualised by *uv* (260 nm). A 'blind' observer confirmed all genotypes. Discrepancies were resolved by repeat PCR and enzyme digestion. In 15% of the NPHSII cohort, DNA was unsuitable for genotyping.

2.4.3 Gel electrophoresis

2.4.3.1 Agarose gel electrophoresis

PCR products were size separated by agarose gel electrophoresis. For DNA fragments between 100-2000 bp, a 2% (w/v) agarose concentration was used. A agarose gel was prepared by melting 2 g of agarose powder in 100 ml 1x TAE buffer (i.e. 10 ml of 10x TAE and 90 ml ddH_2O). The agarose was cooled to about 55-60°C and 2 µl (0.5 µg/ml) of ethidium bromide (EtBr), a fluorescent dye that intercalates into DNA, was added.

A perspex gel tray (10 cm x 10 cm) was sealed with adhesive tape at the ends to prevent leakage and 2 combs with 15 lane well-formers were inserted onto the tray. The molten gel solution was poured into the tray to a thickness of about 3-5 mm while ensuring that no air bubbles were trapped in the gel. The gel was allowed to set for 30 min at R_t and then it was immersed in a horizontal electrophoresis tank filled with 1 litre (L) of 1x TAE, after removing the adhesive tape. The combs were carefully removed.

PCR product (10 µl) were mixed with 2 µl of XCFF or BPB loading buffer and 10 µl of this mixture were loaded sequentially in each well of the gel. The loading buffers enabled visualisation of the tracking of electrophoretic migration and raised the sample density so that it settled in the bottom of the well. XCFF ran slower compared to BPB, which was used when running smaller fragments.

About 3 µl of a suitable DNA ladder (100 bp or 1 Kb) were loaded in the first well. This was used as a reference size marker and ran simultaneously with the PCR products on the gel. Electrophoresis was carried out for 30-45 min at constant voltage (100-120 mV). The gels were visualised by *uv* and photographed. If PCR amplification was successful, a band of the appropriate size was observed. For the genotyping of In4 VNTR, a 3% agarose gel was used. It was prepared by suspending 9 g agarose in 300 ml of 1x TAE and a 20 cm x 24 cm gel mould was used with each comb containing 30 wells.

2.4.3.2 Polyacrylamide gel electrophoresis

For detecting small size differences such as in microsatellite repeats of In4 VNTR, PCR samples were ran on a 10% polyacrylamide gel of a Hoefer Mighty Small™ apparatus. The gel was made of 17.2 ml ddH_2O, 3 ml of 10x TBE and 10 ml acrylamide (30% w/v acrylamide; 1.579% w/v bis-acrylamide; Ratio 19:1, Severn Biotech Ltd). Polymerisation was initiated by 100 µl APS and the reaction catalysed by 100 µl N,N,N', N tetramethylethylene-diamine (TEMED) (BDH Chemicals Ltd, Poole, Dorset).

The gel mixture was poured slowly down the edge of the space in between the vertical clamped plates, a comb was gently inserted and more gel added on top to prevent any air bubbles leaking through the gel. It was allowed to set for 30 min and the plates were tightly clamped. The comb was removed and the wells flushed with 1x TBE. The vertical electrophoresis gel tank was filled with 1x TBE buffer whilst ensuring

that no air bubbles were trapped at the bottom of the gel as these would impair conduction.

The gel was placed on the tank and 5 µl of appropriate marker as well as 10 µl of PCR product, added to 2 µl of loading buffer (either XCFF or BPB alone, or a mixture of XCFF + BPB), were loaded. The 'Mighty Small™' gel was pre-run for 15 min to remove any debris from the wells and then electrophoresed at 150mV. After an hour, the gel plates were prised apart cautiously, the acrylamide gel peeled from the glass plate, stained for 15 min in 1x TBE and EtBr, visualised under *uv* light and photographed.

2.4.3.3 Microtiter Array Diagonal Gel Electrophoresis

Microtiter Array Diagonal Gel Electrophoresis (MADGE) enabled the loading of 96-well microtitre plate (8 columns x 12 arrays) on a horizontal polyacrylamide gel, thus preserving the exact configuration of the 96-well Master Array plate. As PCR and digestion were done in the same plate, there was a high resolution for the genotyping recognition pattern. The method allowed polyacrylamide gel, which offered the highest resolution for small PCR products and digests of PCR products (50-500 bp), to be prepared and manipulated in a horizontal position. MADGE gel formers with teeth in a 96-well standard format were made from Perspex blocks (Mazak, Worcester, UK) and with the teeth cut at an angle of 71.6° to the microtitre plate row axis but perpendicular to the long edge of the block. Each tooth measured 2 x 2 x 2 mm.

Glass plates (dimensions: 150 x 100 x 2 mm) were washed with detergent (Fairy Liquid Excel, Proctor and Gamble Ltd., UK), rinsed with tap water followed by 70% ethanol, double distilled water and dried. One side of the glass plate was covered with 'sticky silane' and dried. Acrylamide mix was prepared according to the % of polyacrylamide matrix gel required with acrylamide bis-acrylamide (30% w/v acrylamide, 1.579% w/v bis-acrylamide, Ratio 19:1, Severn Biotech Ltd). Polymerisation was initiated by 200 µl APS and the reaction accelerated by 50 µl TEMED (***Table 2.6***).

Table 2.6: Preparations of different % MADGE gels.

% MADGE gel	**5%**	**7.5%**	**10%**
Acrylamide (µl)	9.0	12.5	16.0
TBE 1x (µl)	5.0	5.0	5.0
ddH_2O (µl)	36.0	32.5	29.0

Volume of acrylamide solution required was calculated according to the following formula, with % Acrylamide = 30%:

$$\text{Volume of acrylamide required} = \frac{\%\ \text{MADGE gel required}}{\%\ \text{Acrylamide}} \times \text{Final volume}$$

The gel mixture was poured onto the MADGE moulds, with the teeth jutting upwards. The silanized side of the glass plate was rested carefully on the comb so as to ensure

that no air bubbles were trapped in between the plates. A 200-250g weight was put on the plate to maintain an equal pressure between the gel and the MADGE comb and the gel was allowed to set for about half an hour. The glass plate was prized off the gel former using a spatula and the polymerised gel remained anchored to the silanized glass plate.

Pre-staining was carried out by soaking the gel for 30 min, in the dark, in a buffer mix containing 200 ml of 1x TBE and 15 µl of EtBr (0.5 µg/ml). The gel was immersed horizontally in the electrophoresis tank containing about 3 L 1x TBE. Eight µl sample added to 2 µl MADGE dye were loaded onto the gel with a multichannel pipette. Electrophoresis was carried out in tracks following a 71.6° diagonal between wells at 150V. For observation, the gel was placed side down on the UV transilluminator and a digital image of the gel acquired with a camera. The patterns on the photograph were easily recognisable (***Section 4.4.1***, ***Chapter 4***).

2.4.3.4 Polyacrylamide gel for variant detection

For the preparation of a double 7.5% polyacrylamide non-denaturing gels, of dimensions 40 cm x 30 cm x 0.4 mm, the mix was made up as follows: 15 ml of 10x TBE (pH 8.3)' 22.5 ml of 49% acrylamide: Bis acrylamide, 3 ml of 0.5M EDTA, 7.5 ml of glycerol, 103 ml of water, 130 µl of 25% APS and 130 µl of TEMED.

2.4.3.5 Acrylamide-urea gel for sequencing

The following reagents were mixed in a beaker: 18 g of urea, 5.2 ml of 40% acrylamide stock, 27.5 ml of ddH_2O and 0.5 g of mixed-bed ion exchange resin. Five ml of filtered 10x TBE were poured into the gel solution, which was degassed for 5 min followed by addition of 250 µl of 10% APS and 35 µl TEMED. For polymerisation, the gel was poured between two clean glass plates and mounted in a gel cassette.

2.4.4 Single Strand Conformation Polymorphism (SSCP)

2.4.4.1 Minimum use of radioisotope α-^{32}P

α-^{32}P deoxycytosine triphosphate (α-^{32}P dCTP) was used to label the PCR products. To minimise the exposure to radioactivity, a PCR polymerase mix containing one tenth (1/10) the usual concentration of dCTP was used (0.02 mM dCTP, 0.2 mM dATP, 0.2 mM dGTP and 0.2 mM dTTP) together with a ten-fold reduction of neat α-^{32}P dCTP ($t_{1/2}$ =14 days). Therefore, 2 µl of hot α-^{32}P dCTP at 250 µCi/µL at 9.25 MBq (Amersham, UK) was added to 1.5 ml of PCR mix.

2.4.4.2 Sample preparation for SSCP analysis

For the detection of genetic variability within GTP cyclohydrolase 1 gene (*GCH1*) by SSCP, the promoter region was amplified by using five overlapping primer pairs. PCR was performed in five microtitre plates as described in Section 2.4.1 with few modifications. The protocol adopted for each amplification was as follows:

1. Two µl of 44 DNA samples from Master Array 1 (NPHSII) was dispensed in a 96-well plate (Hybaid Omnigene) and dried (75°C / 10 min).
2. 1500 µl PCR cocktail was prepared for 50 samples, that is enough for half a microtitre plate, i.e. 44 samples, 4 blanks (negative controls) and 2 extras. See ***Table 5.2*** (***Section 5.3.3, Chapter 5***) for PCR cocktail preparation.
3. 2 µl of α-^{32}P dCTP was added to 1.5 ml (30 µl x 50 samples) of cocktail mix and the radioactive mix was distributed into the blank and dried DNA templates wells. The cocktails were overlaid with 20 µl paraffin oil.
4. The amplification cycles were 95°C for 5 min followed by 35 cycles of three consecutive steps each lasting 45 sec: denaturation at 95°C, annealing step with a T_a optimised for each primer set (***Table 5.1, Section 5.3.2, Chapter 5***) and extension at 72°C. The same program was used for both radioactive and non-radioactive PCRs.

2.4.4.3 SSCP double gel set-up

For convenience and high sample throughput, samples were run on two gels simultaneously on a vertical electrophoresis apparatus. The gels, prepared as described in Section 2.4.3.4, were poured between three glass plates. When clamped, the arrangement of the plates was a large plate, which was the 'lowest' and outermost one and two smaller plates, which were 'middle' and 'uppermost'. The glass plates were scrubbed with mercury chloride and cleaned with detergent (Fairy Liquid Excel, Proctor & Gamble Ltd) followed by 100% ethanol and distilled water. Repelcote (BDH, Poole, UK) was applied thinly to the four glass faces which make contact with the two gels.

A pair of spacers (0.4 x 10 x 400 mm) were placed on top of the silanized side of the large 'lowest' plate (33 x 42 cm). The small plate (33 x 39 cm) which had both sides repelcoted was put on top of the spacers and a second set of spacers were arranged on top of this 'middle' plate. The silanized side of the 'uppermost' small plate (33 cm x 39 cm) was placed on top of this arrangement. The sides of the three plates were aligned and clamped with bulldog clips. The bottom gel was injected on the large plate by using a syringe, whilst tapping gently at the gel front so as to avoid any bubbles. The flat side of a shark comb was inserted and the gel allowed to set for 10-15 min. The second gel was then poured in a similar manner and another comb inserted. To ensure that both gels were properly set and with no leakage, the gels were allowed to set for 2-3 hours.

2.4.4.4 SSCP gel electrophoresis

The amplified PCR samples were subject to SSCP on 7.5% polyacrylamide non-denaturing gels. The procedures were the same for all the five gels. Three µl of radioactive PCR mix of all samples were transferred into a 96-well plate containing 6 µl of SSCP loading dye in separate wells. The mixture was mixed gently with the pipette. The samples were denatured (95°C / 5 min) and chilled immediately on wet ice. The top buffer tank of the electrophoresis apparatus was filled with 1x TBE and after ensuring that the top tank had no leakage, the bottom tank was filled with 1x TBE. The two combs were removed, rinsed and the teeth of both combs were inserted gently in both gels and the samples loaded in the wells with the multi pipette.

Electrophoresis conditions were 300 V at 21-25°C for about 21 hours and after that period the electrophoresis apparatus was stopped and the top buffer tank emptied. The plates were removed and prized apart. The gels, which remained stuck to one of the plates, were transferred to Whatman 3 mm chromatographic paper and dried (80°C / 30 min). They were exposed to a Hyperfilm MP (Amersham, UK) for 2 days at -70°C with an intensifying screen in a film cassette. The film was developed by dipping it respectively in silver nitrate solution (which deposits on the DNA bands), in diluted acetic acid (to stop the reaction) and finally in a solution of formaldehyde and sodium carbonate (which reduces the silver). The film was washed with tap water and dried.

2.4.4.5 Reading an SSCP profile

The X-ray films were analysed for SSCP variants, which have a different pattern of migration on the polyacrylamide gel due to conformational changes. A few PCR amplified samples, exhibiting the three types of mobility shifts on the SSCP polyacrylamide gel, were purified and sequenced (***Section 5.3.6, Chapter 5***).

2.4.4.6 Template preparation for DNA sequencing

Prior to sequencing, the QIAquick™ Gel Extraction Kit (QIAGEN) was used to extract PCR fragments for direct use as templates during sequencing. The main steps were solubilisation of the gel slice, binding of the double-stranded DNA to a silica membrane, washing of all contaminants (i.e. salts, excess primers, agarose, dyes, ethidium bromide, oils, unincorporated nucleotides...) and elution of the pure bound DNA with ddH_2O.

2.4.4.7 Cycle sequencing of purified DNA fragments

Both the forward and reverse strands of the purified DNA fragments were used as templates for further amplification by cycle sequencing using the primers from Section 2.4.4.2. A commercial kit, the ABI PRISM™ dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems), was used and for each sequencing reaction, the cocktail was prepared according to the manufacturer's instructions.

Briefly, about 60 ng (~ 1 μl) of PCR template (amplified and purified as described in Sections 2.4.4.2 and 2.4.4.6), 3.2 pmole (3.2 μl) of forward or reverse primer, 8 μl of pre-mix (buffer, enzyme, dNTPs and dye-labeled ddNTPs) and 7.8 μl ddH_2O were added to labelled sequencing tubes. The reactions were overlaid with 20 μl mineral oil. This amplification process employs only a single primer during the repeated cycles of thermal denaturation, primer annealing and polymerisation within each sequencing tube.

The plate was placed in the thermocycler (GeneAmp PCR 9700, PE Applied Biosystems) with the following cycle parameters:

Step 1: 96°C for 10 sec (i.e. denaturation step)

Step 2: 50°C for 5 sec (i.e. annealing step)

Step 3: 60°C for 4 min (i.e. extension step)

Step 4: Repeat Steps 1, 2 and 3 sequentially for 25 cycles

2.4.4.8 Automated DNA sequencing

DNA sequencing method used is a variation of the chain-termination method developed by Sanger and colleagues (Sanger *et al.*, 1977). The DNA fragment to be sequenced acts as a template for the enzymatic synthesis of new DNA by primers. A mixture of both deoxy- (dNTP) and labelled dideoxynucleotide (ddNTP) was used in the reaction mixture. Incorporation of the fluorescent dye labels on the growing chain blocked further elongation and resulted in a population of truncated DNA fragments of

varying length, which were detected on a denaturing high-resolution polyacrylamide gel.

In automated sequencing, different dye labels were used to tag the four ddNTP and a laser was used to detect the DNA fragments labeled with fluorescent dyes to give an electronic gel image. The signal strength of each band was measured and extracted to give a clear electropherogram to which an automated 'base calling' was applied.

2.4.4.9 Loading and electrophoresis of ABI gel

The sequencing products were added to 100% ethanol to remove all labeled ddNTPs, washed with 70% ethanol, resuspended in 4 µl of loading buffer, denatured (70°C / 5 min), quenched on ice and loaded onto a 4.1% acrylamide-urea gel. The sequencing gels were electrophoresed for 12 hours in the sequencer (ABI prism™ 377 DNA Sequencer). The DNA sequence was analysed using the Sequence Navigator (ABIPrism™, GeneScan®), Sequence Editor and Factura™ software packages for the Apple Macintosh and clear electropherograms were obtained (***Section 5.4.1, Chapter 5***).

2.5 Measuring nitrite and nitrate (NO_x) by Griess assay

2.5.1 Materials and reagents

Prior to analysis, all laboratory wares i.e. pipette tips, eppendorfs and containers were rinsed thoroughly (at least three times) with ultrapure deionized water (milli-Q+, Millipore, Watford, UK) to remove any prevalent nitrite (NO_2^-) and nitrate (NO_3^-) anions. To minimise background values, milli-Q+ water was used to prepare all solutions and while nitrite and nitrate standards were made up in PBS buffer (GIBCO BRL).

The following reagents were purchased from Sigma Chemical Co. (St Louis, MO): sodium nitrate ($NaNO_3$) (Cat. S5506), sodium nitrite ($NaNO_2$) (Cat. S2252), phosphoric acid (PA), nitrate reductase (NR) (Cat. N7265), glucose-6-phosphate dehydrogenase (G6PDH) (Cat. G4134), nicotinamide adenine dinucleotide phosphate (NADPH) (Cat. N6505/7265), glucose-6-phosphate (G6P) (Cat. S7250), sulphanilamide (SA) (Cat. S9251) and N-(1-naphthyl)ethylenediamine dichloride (NED) (Cat. N5889). Assay plates were from Falcon (Cat. 3912).

Preparation of sodium nitrate ($NaNO_3$) and sodium nitrite ($NaNO_2$) standards

Stock of nitrate and nitrite calibration solutions (1 mM) were made up fortnightly in PBS and stored at 4°C. They were diluted to cover a working range of 0-50 μmol/L. The range used in the wells was 0-500 μM, including 0.5, 10, 50, 100, 200 and 250 μM.

1 mM $NaNO_2$ standard was prepared by dissolving 6.9 mg solid in 100 ml PBS

1 mM $NaNO_3$ standard was prepared by dissolving 8.499 mg solid in 100 ml PBS

Preparation of Griess reagent, made up of solutions A and B:

Both solutions were stable for 2 weeks if stored at 4°C and protected from light.

Reagent A: 1% Sulphanilamide (SA) solution

5% solution of PA was made by adding 0.5 ml PA to 10 ml milli-Q+.

Reagent A was then prepared by dissolving 100 mg SA into 10 ml 5% PA

Reagent B: 0.1% Naphthalethylene diamine dichloride (NED) solution

Reagent B was made up by dissolving 10 mg NED into 10 ml milli-Q+.

Preparation of NADPH Solution

A concentration of 1 μM was required in the well, therefore 10 μM stock solution was prepared. It was prepared by dissolving 1.67 mg of NADPH solid into 200 ml PBS. As it is stable for 12 hours, the solution was prepared fresh daily and was kept on ice.

Preparation of Nitrate Reductase (NR)

Each vial of NR contains set units, e.g. 10 U. Each vial was reconstituted by dissolving the lyophilised powder in 1 ml of PBS to give 10 U/ml.

3 U were required for each 96-well plate, i.e. 300 μl

Preparation of Glucose-6-phosphate dehydrogenase (G6PDH)

Each vial of G6PDH contains set units, e.g. 100 U. Each vial was reconstituted by dissolving the lyophilised powder in 1 ml of PBS to give 100 U/ml.

2 U were required for each 96-well plate, i.e. 20 µl

Preparation of Reaction Buffer

For each assay (i.e. 96-well plate), a reaction buffer was prepared fresh and stored on ice. For the buffer, 1.9 mg G6P was dissolved into 5 ml PBS followed by the addition of 300 µl (3 U) of NR and 20 µl (2 U) of G6PDH respectively.

2.5.2 Optimising assay for NO_x analysis

Nitrite (NO_2^-) is rapidly oxidised to nitrate (NO_3^-), >95 % in about an hour (Wong *et al.*, 1995). It is more meaningful to assay the sum of nitrite and nitrate (i.e. NO_x) content as NO_2^- determination alone will lead to a serious underestimation of the whole NO_x production (Archer, 1993). Therefore, no attempt was made to differentiate between these two anions and results were reported as total NO_x. The microassay developed for high throughput analysis of human plasma samples was adapted from the method used by Verdon *et al* for the measurement of NO_2^-/NO_3^- in rat plasma (Verdon *et al*, 1995) and was modified accordingly. Two stepwise procedures were performed:

1. An enzymatic reduction of NO_3^- to NO_2^- anion; and
2. A spectrophotometric analysis of the NO_2^- species with the Griess Reagent.

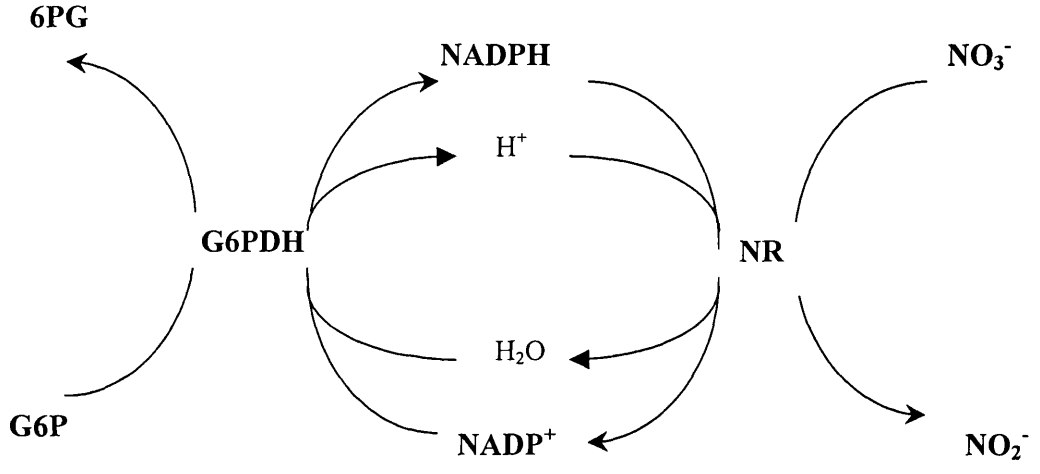
This approach provides a sensitive and reliable measurement of NO_x in the physiological range (0.5-50 μM), with minimal volume of plasma (40 μl) required. The assay was adaptable to a 96-well plate format, allowing the analysis of a large number of samples simultaneously. As both reduction and absorbance measurement were carried out in the same 96-well microassay plate, there was minimum handling of the sample thereby reducing considerably the risks of contamination, facilitating handling and preserving homogeneity

NADPH serves as an electron donor during the conversion of NO_3^- to NO_2^- by NR. However, when used at concentration required to drive the reduction to completion (200 μM), NADPH interferes with the subsequent reaction of the NO_2^- species with the Griess reagent causing a signal reduction. To counteract this problem, 1 μM NADPH was used but this concentration of cofactor is limiting and not all NO_3^- was converted to NO_2^-. Therefore the $NADP^+$ formed was recycled to NADPH by coupling with G6PDH, which catalysed the dehydrogenation of G6P to 6-phosphoglucolactone (6PG) (***Figure 2.3***).

Figure 2.3: Schematic representation of NO_3^- enzymatic conversion to NO_2^-.

$$NO_3^- + NADPH + H^+ \rightarrow NO_2^- + NADP^+ + H_2O$$

$$H_2O + NADP^+ + G6P \rightarrow 6\text{-}PG + NADPH + H^+$$



On the spectrophotometer, reading with the microplate reader software package Anthos, resulted in more background values. With the new SOFTmax Pro program (Molecular Devices, Menlo Park, CA, USA) and with all the reagents and standards made up in PBS buffer, the background noise was considerably reduced. The stepwise procedure and the principles of this assay are highlighted in ***Figure 3.2*** (***Section 3.2, Chapter 3***).

2.5.3 Griess assay protocol

NPHSII plasma samples were available for NO_x measurement by the Griess assay. The precautions to be observed at all times during the protocol were to ensure that no

air bubbles were trapped in the wells when adding the reagents and samples; and prior to use, all solutions were kept on ice. The optimal conditions for the amount of enzymes used and the times of incubation were determined for plasma. For maximum activity, the enzymes were reactivated by shaking the reconstituted vials gently at R_t for 5 min.

The protocol adopted was as follows:

1. The number of 96-well plates required for the assay was determined and each sample was tested in duplicate. NO_3^- standards were always run in duplicates, in the first two columns of each assay plate, in wells from A to H of lanes 1 and 2, i.e. (A-H) (1-2).
2. The $NaNO_2$ and $NaNO_3$ calibration standards were prepared by dilution in PBS as well as the reaction buffer and NADPH solution.
3. 10 µl of standards and 40 µl PBS were aliquot in wells (1-2) (A-H) according to ***Figure 2.4***. 40 µl of plasma samples and 10 µl PBS were added to the appropriate wells followed by 10 µl of NADPH solution and 40 µl of reaction buffer respectively.
4. The contents of the plate were mixed gently and incubated at 37° C for an hour.
5. Equal amounts (50 µl) of Reagents A and B were added serially to each well and the plate was incubated at R_t for 15 min, as longer reaction times may result in artefact values. A magenta colour developed immediately indicating the presence of NO_2^-.
6. The absorbance was read using the SoftmaxPro program on a microplate reader at 540 nm. Data were automatically transferred to the computer for post-run analysis.

7. The concentration of NO_3^- was read automatically from the $NaNO_3$ standard curve generated by the computer after removal of background values, i.e. 200 µl of PBS.

Figure 2.4: Arrangement of reagents and samples for Griess assay.

The final concentration of nitrate standards are shown in lanes/wells (1-2) (B-H) together with wells (1-2) (A) which contain 2OOµl PBS for background purposes.

Well	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
A	2OOµl PBS	2OOµl PBS	-	-	-	-	-	-	-	-	-	-
B	0.5 µM	0.5 µM	-	-	-	-	-	-	-	-	-	-
C	1 µM	1 µM	-	-	-	-	-	-	-	-	-	-
D	5 µM	5 µM	-	-	-	-	-	-	-	-	-	-
E	10 µM	10 µM	-	-	-	-	-	-	-	-	-	-
F	20 µM	20 µM	-	-	-	-	-	-	-	-	-	-
G	25 µM	25 µM	-	-	-	-	-	-	-	-	-	-
H	50 µM	50 µM	-	-	-	-	-	-	-	-	-	-

A 96-well plate requires (i.e. 96 samples):

- 0.96 ml (i.e. 96 x 10 µl) NADPH solution
- 3.84 ml (i.e. 96 x 40 µl) reaction buffer
- 9.6 ml (i.e. 96 x 100 µl) Griess Reagents

2.5.4 Calculating NO_x concentrations

Total NO_x (i.e. NO_2^- and NO_3^-) concentrations in unknown samples were calculated after generating a standard curve relating absorbancies to known NO_3^- concentrations (Range 0-50 μM) (***Section 3.4.2, Chapter 3***). The standards contain NO_2^- when measuring NO_2^- concentration only and NO_3^- when measuring the total of NO_2^- plus NO_3^- concentrations in the sample aliquot. $NaNO_2$ standards were run daily with $NaNO_3$ standards to check for the completeness of the conversion reaction and the efficacy of the reagents. When measuring NO_2^- concentration, the assays can be performed without adding any enzymes but only the Griess reagents. The difference between both aliquots gave the NO_3^- concentration in the sample, i.e. [$NaNO_2$ + $NaNO_3$] - [$NaNO_2$]

2.6 Neopterin measurement by ELISA

Quantitative determination of neopterin in plasma was carried out by a commercially available kit, the ELItest® Neopterin. The kit is a competitive enzyme immunoassay and uses the principle of an Enzyme-Linked ImmunoSorbant Assay (ELISA). The plasma samples were assayed directly without any preparation and direct measurement of the aromatic neopterin molecule was carried out, regardless of its reduced forms. See ***Figure 6.2, Section 6.3.2, Chapter 6*** for the principle of the ELISA.

The wells of the microtiter plate were pre-coated with polyclonal sheep anti-neopterin-antibodies. 150 μl enzyme conjugate (neopterin~alkaline phosphatase conjugate) were

added to 50 µl neopterin standards in the range 2-250 nmol/l (i.e. 2.0, 6.4, 16, 40, 100 and 250 nmol/l), 50 µl control sera or patient samples. Neopterin present in patients' samples compete with the neopterin~enzyme conjugate for the binding sites of the antibodies. An immune complex is formed consisting of both anti-neopterin-antibody/neopterin and anti-neopterin-antibody/neopterin~alkaline phosphatase conjugate. All unbound components were removed by the addition of 1750 µl of washing solution followed by 100 µl of 4-nitrophenylphosphate substrate solution, which catalysed the separation of the phosphate from the 4-nitrophenylphosphate and setting free the 4-nitrophenol.

The reaction was stopped with 100 µl sodium hydroxide. The optical density (OD) of the yellow colour of the 4-nitrophenole was measured by a photometer at 405 nm by the SoftmaxPro software, and it was dependent on the amount of enzyme bound in the cavities. This value was inversely proportional to the neopterin concentration of the patient samples, thus a low neopterin value correspond to a high OD value. A standard curve was created (OD *vs* concentrations of neopterin standards) and the concentration of the patient samples' neopterin could be read off directly. The control values were checked to ensure that they were within the range provided.

2.7 Data storage and handling

NPHSII subjects' phenotypic and genotyping data were stored on a spreadsheet (Microsoft Excel) at the MRC Epidemiology Unit, Wolfson Institute of Preventive Medicine (St. Bartholomew's Hospital, London). Statistical analyses were carried out by qualified statisticians, Miss Jacqueline Cooper and Miss Sarah Bujac. I had access

to only parts of the NPHSII database and personally carried out part of the analysis. The analyses were conducted using the following packages: Microsoft Excel 97, STATA (Intercooled Stata 5.0), SPSS 6.0 for Windows and Prism 2.01 (GraphPad Software).

2.8 Statistical analysis

2.8.1 Tests used for analysis

Only white Caucasian individuals of European ancestry were included in the analysis. Normally distributed data were expressed as mean and standard deviation (SD) or standard error of the mean (SEM). Concentrations of NO_x, neopterin, serum triglyceride and fibrinogen were log transformed, prior to analysis, to normalise their distributions. Geometric means and approximate SD are reported for these variables. Non-normally distributed data were expressed as median and range. Univariate analysis of continuous variables was carried out by one-way analysis of variance (ANOVA). Welch's test was used where there was evidence of unequal variances between groups.

As the distribution of plasma NO_x and neopterin were highly skewed, Kruskal-Wallis (KW) non-parametric tests were used to determine the relationship with genotype in men who developed an IHD event and those who remained free. Medians with interquartile range are presented. Geometric means and appropriate SD are reported for these variables. The KW test was used to assess the effect of genotype on NO_x and neopterin levels. Confounding influences of other factors were assessed using

multiple logistic regression models. The effect of possible confounding factors such as age and BMI were assessed using the Spearman Correlation coefficient.

The student's T-test (two group comparisons) or ANOVA (three group comparisons) were used to compare normally distributed variables. Non-normally distributed variables were compared by the Mann-Whitney test (two group comparisons) or the KW test (three group comparisons). Differences in clinical and biochemical characteristics according to smoking status were analysed using one-way ANOVA. Non- and ex-smokers were combined for analysis and compared with smokers.

For association studies, candidate gene approach was used It tests the hypothesis that a specific allele occurs more frequently in diseased subjects (those who suffered an IHD event) than in those without the disease (i.e. no IHD event). Frequency of a particular marker allele in the group of unrelated 'cases' were compared to a group of unrelated 'controls', who were closely matched for biochemical factors such as age and BMI so as to avoid differences between allele frequencies bearing no causal relationship at all with the disease.

An increase in allele frequency in the cases compare to the controls suggests the probability of a cause/effect relationship between the 'associated' alleles and the disease. Either the gene itself or a nearby gene has a functional genetic variant, which might contribute to the disease process. A significant association between a genetic marker and a disease implies that either the genetic marker either resides in the disease locus itself or within 500 kb of the disease DNA locus (Kurtz *et al.*, 1992).

The relative risk (RR) gives a convenient summary of the outcome of an epidemiological study and represents a quantitative value of an approximate estimate of the RR for the disease associated with the factor. For all the polymorphisms, risk was estimated in rare allele carriers and homozygotes (i.e. 'exposed' individuals) combined and were adjusted for age, BMI, baseline levels of plasma cholesterol, triglycerides, fibrinogen and sBP, using Cox's proportional hazard model. Survival analysis was carried out using Cox's model, thereby allowing for varying follow-up intervals and failure time was taken as time to the first IHD event. Significance was assessed using the Likelihood Ratio (LR) test and 95% confidence intervals (CI) for the parameters were calculated from the standard errors by assuming normality. Results are exponentiated and are presented as the RR representing the hazard ratios (HR) and their 95% CI. Binary variables were analysed using the chi-squared (χ^2) test for difference in proportions. χ^2 test, used for unpaired sample data, determines whether there is a hypothesised statistical difference between observed expected values of the data.

2.8.2 Hardy-Weinberg (H-W) equilibrium

Hardy-Weinberg (H-W) law states that the genotype frequencies in any generation can be predicted by knowing only the allele frequencies in the parental generation, assuming random mating of parents and Mendelian segregation ratios for each mating type in a large population. If p is the frequency of an allele (X) and q is the frequency of a second allele (Y) in the parental generation, then $q = 1\text{-}p$. The H-W law states that the genotype frequencies expected in the offspring are given by the binomial expression $(p + q)^2$. Thus:

- Relative frequency of common homozygous genotype, XX = p^2
- Relative frequency of heterozygous genotype, XY = $2pq$
- Relative frequency of rare homozygous genotype, YY = q^2
- By definition, $p^2 + 2pq + q^2 = 1$

A population is said to be in H-W equilibrium if the observed frequencies of genotypes XX, XY and YY are equal to the expected H-W frequencies of p^2, $2pq$ and q^2. Deviation from the equilibrium in a large random mating population occurs when there is mutation, change, selection or migration. The observed genotypes frequency for a 2-allele polymorphism were counted and all data were checked to be in H-W equilibrium. χ^2 analysis was used to compare categorical data between two groups and to assess whether genotype frequencies deviated from H-W equilibrium and χ^2 values and the number of degrees of freedom were calculated. A p value > 0.05 was taken to be not significant and to conform to H-W law.

2.8.3 Allelic association and linkage disequilibrium coefficients

Allelic association and linkage disequilibrium between alleles at two different polymorphisms in the same gene cluster was estimated as described elsewhere (Chakravarti *et al*, 1984). It was determined as follows, with a, b, c and d are the frequencies of the haplotypes A1B1, A1B2, A2B1 and A2B2 respectively:

Number of A1B1 chromosomes = a

Number of A1B2 chromosomes = b

Number of A2B1 chromosomes = *c*

Number of A2B2 chromosomes = *d*

The haplotype of an individual heterozygous for two alleles (1 and 2) at each of two loci A and B could be A1B1/A2B2 or A1B2/A2B1. A correlation coefficient delta (Δ) was estimated, where:

$$\Delta = \frac{ad-bc}{\sqrt{(a+b)(c+d)(a+c)(b+d)}}$$

A *p* value of <0.05 was taken as statistically significant. Δ can range between 0 and 1, with the higher the value of Δ the greater the degree of linkage between the 2 alleles. A negative Δ value means that the rare form of one allele is in complete linkage disequilibrium with the common form of the other allele.

The haplotypes of both heterozygous individuals, A1B2 and A2B1, were estimated by maximum likelihood analysis (Thompson *et al.*, 1988). The probability of the double heterozygous being distributed as A1B1/A2B2 is:

$$P = \frac{\text{number of A1B1/A2B2 haplotypes}}{\text{total number of haplotypes (n)}}$$

$$P = \frac{a/n \times d/n}{(a/n \times d/n) + (b/n \times c/n)}$$

CHAPTER 3

NO_x ASSAY AND ASSOCIATION WITH IHD RISK FACTORS

3.1 Objectives

- To develop an assay, appropriate for population scale studies, in order to measure plasma levels of nitrite and nitrate (that is NO_x) as an index of nitric oxide (NO) bioactivity.

- To test the hypothesis that prevailing levels of cholesterol and blood pressure (BP) as well as smoking status, all conventional ischaemic heart disease (IHD) risk factors known to decrease endothelial function, might influence circulating plasma NO_x.

3.2 Background

Since the 1980s, there has been an explosive growth in NO research and scientific interest in its myriad physiological, pharmacological, and pathological actions, has multiplied exponentially (Nathan, 1992; Lowenstein *et al.*, 1994; Bhagat and Vallance, 1996). Overproduction of NO has been implicated in the pathogenesis of sepsis (Wong *et al.*, 1995) while underproduction in the development of hypertension and atherosclerosis (Naruse *et al.*, 1994; Quyyumi *et al.*, 1995a; Forte *et al.*, 1997). For these reasons, interest in developing analytical methods for the quantitation of NO in

biological systems has soared considerably. Being a radical, NO is unstable in an aerobic environment, thus making its accurate evaluation in physiological solutions challenging (Kiechle and Malinski, 1993). *In vivo* NO is produced in minute amounts, has a very short half-life ($t_{1/2}$= 10-60 seconds) and is labile in the presence of oxygen (Furchgott, 1990; Moncada *et al.*, 1991; Knowles and Moncada, 1992).

Accordingly, there has been an active effort to develop reliable and sensitive methods to measure NO and its reaction products. Main analytical methods based on NO physicochemical and biochemical properties, include chemiluminescence (Gustafsson *et al.*, 1991), amperometry (Malinski and Taha, 1992), high performance liquid chromatography (Kaku *et al.*, 1994) electron paramagnetic resonance spectrophotometry (Wang *et al.*, 1991) and gas chromatography-mass spectrometry (Green *et al.*, 1982; Tesch *et al.*, 1976). However, these techniques are unsuitable for routine laboratory measurements due to several reasons: they require extensive sample preparation prior to analysis inevitably risking contamination, are labour intensive, time consuming, unsuitable for processing large number of samples, require sophisticated and expensive equipment (Leone *et al.*, 1994).

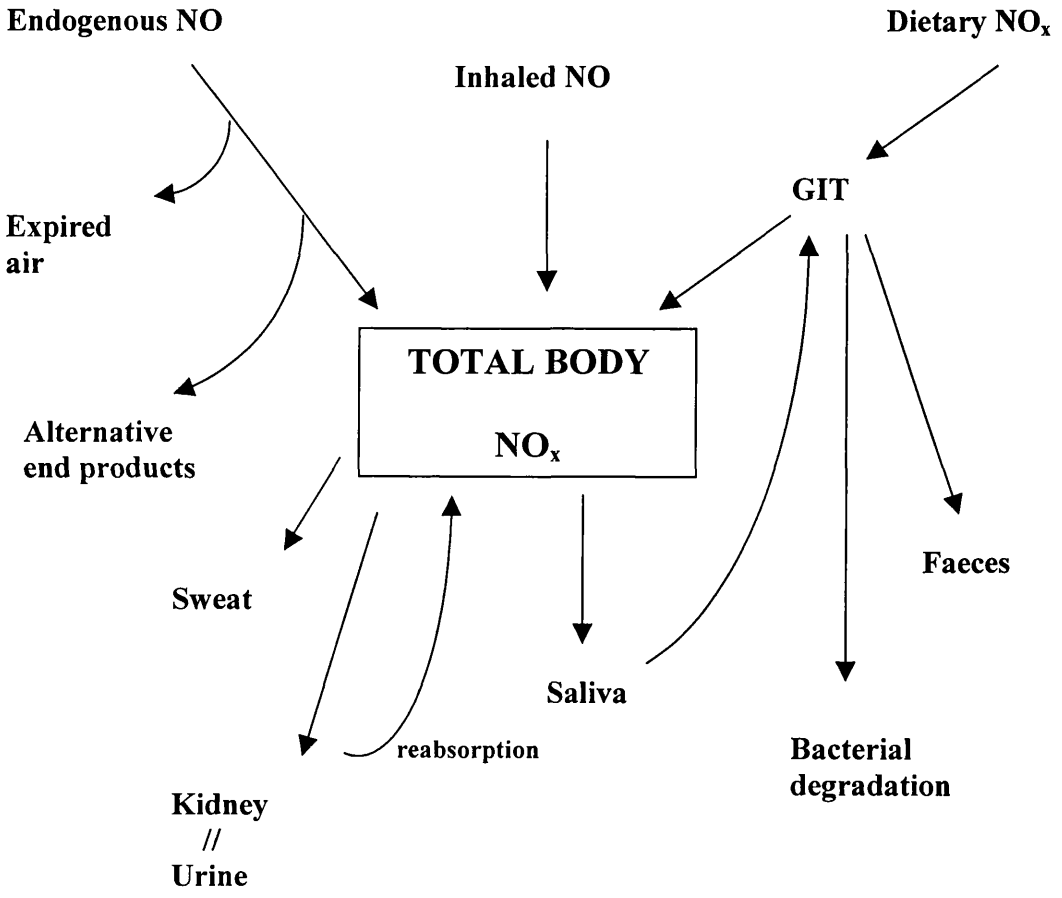
For these reasons, methods for NO measurement have largely focused on two products of NO metabolism: nitrite (NO_2^-) and nitrate (NO_3^-). These inorganic anions are commonly used as markers of NO generation as they are the final products of the chemical oxidation pathways of NO (***Figure 1.7, Chapter 1***) (Stuehr and Marletta, 1985; Wong *et al.*, 1995). On release NO participates in a variety of chemical reactions including reaction with haemoglobin leading to the formation of methaemoglobin and nitrite (Body *et al.*, 1995). The latter being highly unstable,

undergoes rapid oxidation to nitrate. Under appropriate *in vivo* and *in vitro* conditions, the accumulation of NO_2^- and NO_3^-, is a useful indirect way to quantitate nitric oxide synthase (NOS) activity non-invasively and as an index of endogenous NO production (***Figure 3.1***) (Granger *et al.*, 1996). Therefore, to measure plasma NO_x as an index of NO bioactivity, an assay convenient for high-throughput analysis of a large number of plasma samples was sought. Furthermore, to explore the influence of environmental factors known to reduce endothelial function on plasma NO_x levels, its association with well-established IHD risk factors were studied.

The Griess reaction, a simple and sensitive method which can simultaneously quantify the nanomolar (nM) concentration of NO_2^- to the micromolar (μM) concentration of NO_3^- in blood, was found to be most appropriate (Archer 1993; Verdon *et al.*, 1995). It involves diazotization reaction, a very reliable method to determine NO_2^- concentration (Ignarro *et al.*, 1987). However, quantitation of NO_2^- alone in biological fluids is an underestimation of NO metabolism (Archer, 1993) and several studies have shown that there is no correlation between plasma NO_2^- and NO_3^- concentrations (varying from 3.9% to 88%) (Moshage *et al.*, 1995). Vascular NO_2^- is rapidly oxidised to NO_3^- by haemoglobin (Hb), which is stable in frozen biological samples at -20°C (Moshage *et al.*, 1995). Considering the time interval between blood withdrawal from patients and plasma preparation in the nine medical centres of the second Northwick Park Heart Study (NPHSII), most probably NO_2^- determination alone will be meaningless and the measurement of total NO_2^- and NO_3^- (i.e. NO_x) in stored plasma will be a better indicator of NO formation. Therefore, plasma NO_3^- was reduced enzymatically to NO_2^- and then quantitated by the Griess reaction.

Figure 3.1: Drawing illustrating origin and excretion of endogenous NO_x.

(From Baylis and Vallance, 1998).



3.3 Materials and methods

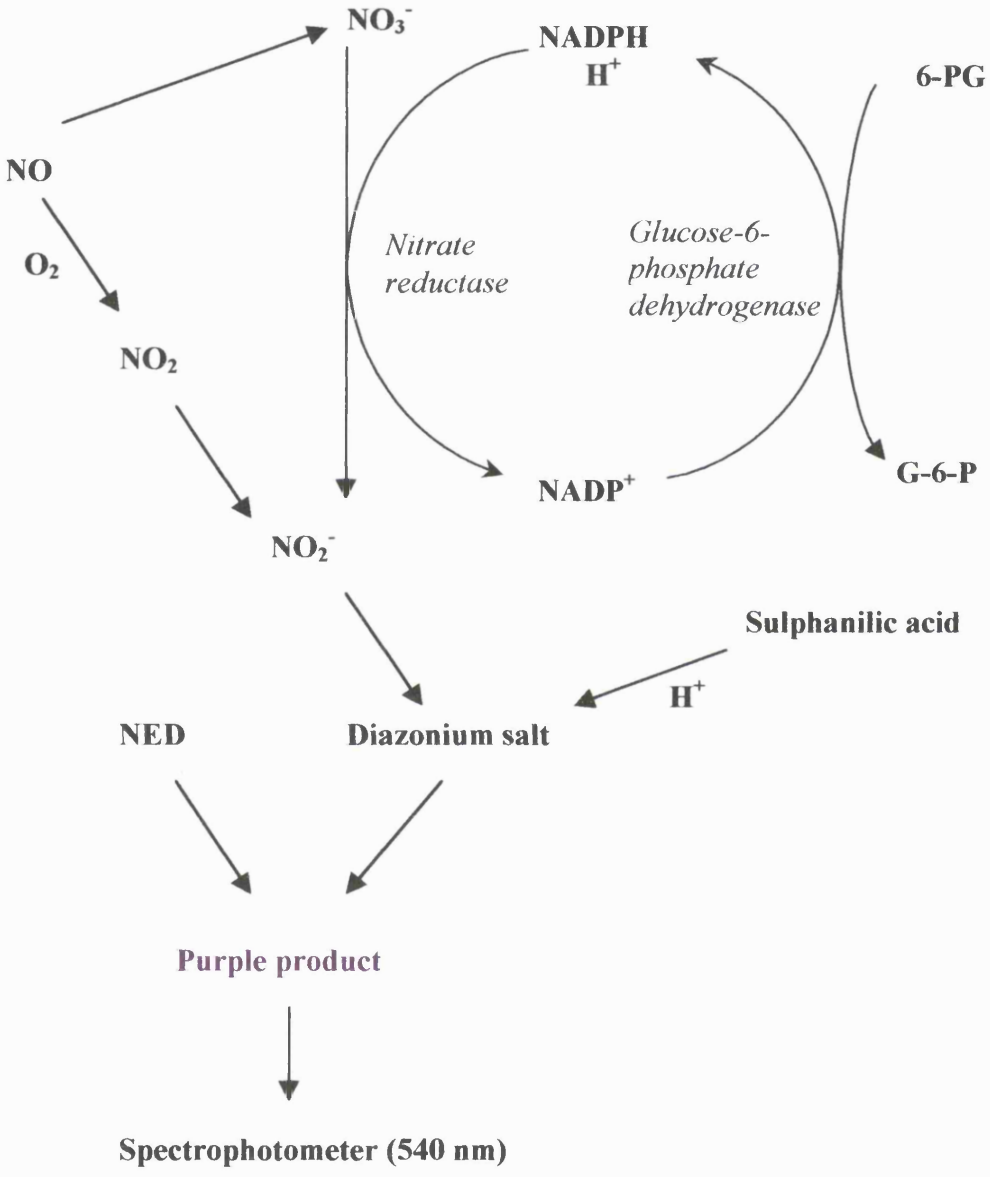
3.3.1 Subjects

Plasma NO_x levels were measured on frozen samples from the NPHSII study, which were obtained on entry in 1993. The samples were from two general practices: North Mymms (n = 290) and Chesterfield (n = 260) as well as a further 571 randomly computer-selected from all nine practices of the study, denoted as 'Random' sample.

3.3.2 Measuring plasma NO_x by Griess assay

The Griess reagent is a mixture of sulphanilic acid (SA) and N-(1-naphthyl) ethylenediamine (NED). Acidified SA reacts with the NO_2^- species *in vitro* to form a diazonium salt, which couples with the heterocyclic amine (NED) to form a purple chromophore with a specific absorption spectrum, measurable by UV-visible spectroscopy. The azo-dye peaks at 540 nm and the absorbance of this band is proportional to the NO_2^- concentration. However, NO_3^- does not undergo diazotization reaction with SA. Nitrate was quantitatively reduced to NO_2^- by nitrate reductase (NR) from *Aspergillus sp* (EC 1.6.6.2.) in the presence of glucose-6-phosphate dehydrogenase (G6PDH), followed by derivatization with Griess reagent and spectrophotometric detection (***Figure 3.2***). As both reduction and absorbance measurement were carried out in the same 96-well microassay plate, there was minimum handling of the samples thereby reducing considerably the risks of contamination. The protocol was described previously in ***Section 2.5, Chapter 2***.

Figure 3.2: Schematic representation of plasma NO_x assay by Griess assay.



3.3.3 Statistical analysis of data

The influence of conventional IHD risk factors on NO_x levels was examined by multivariate linear regression: age, weight, body mass index (BMI), fibrinogen, triglycerides, Factor VIIc, diastolic BP (dBP; >90 mmHg), systolic BP (sBP; >120 mmHg), cholesterol (> 220 mg/dl) and smoking status. To compensate for any confounding effect due to renal impairment, which could lead to raised plasma NO_3^- and NO_2^- levels, NO_x values were indexed to creatinine concentration (i.e. NO_x / Creatinine). Therefore, the results for the association studies were also presented as NO_x / Creat.

3.4 Results: Method validation and optimisation

3.4.1 Validating the NO_x assay

The conventional Griess assay, combined with the nitrate reductase-glucose deshydrogenase (NR-GD) enzymatic system, was optimised. To validate its performance, the bioassay was compared against a commercially available NO assay kit (Caymans Chemical, Cat. 780001), which had been thoroughly tested by the manufacturer.

3.4.2 Optimising the NO_x assay

3.4.2.1 Recovery

For recovery evaluation, plasma samples were either non-filtered (non-UF) or ultra-filtered (UF) through either a 5- or 10-kda filter (Ultrafree microcentrifuge filter unit, Biomax-5 or Biomax-10, Millipore). Basal replicate plasma samples (n = 3) were spiked with 10 µl of a stock of 0.05 mM NO_3^- standards (i.e. Final concentration x dilution = 5 x 5 = 50 µM). Recovery of the assay was then determined by the following formula:

$$\textbf{Recovery (\%)} = \frac{\textbf{Spiked sample concentration (µM) x 100}}{\textbf{Expected spiked concentration (µM)}}$$

The results are shown in ***Table 3.1***. There was about 20-40% (Minimum: 20.23; Maximum: 37.22) decrease in NO_2^- and NO_3^- concentration with non-UF compared to UF plasma. Interference by plasma proteins caused either high or low artefactual values to be measured. UF and non-UF plasma gave a recovery of 85-100% and 75-90% respectively. The recovery of NO_3^- as NO_2^- in calibration solutions after reduction by enzymatic NR was 99% (n > 10) over the concentration range tested, i.e. 0-50 µM.

Table 3.1: Recovery of spiked and unspiked plasma samples.

Sample	$[NO_2^- + NO_3^-]$ (μM)		% Recovery
	Obtained	Expected	
UF	55.365		
UF + spiked	98.5	105.365	93.5
Non-UF	45.16		
Non-UF + spiked	78.645	95.16	82.7

3.4.2.2 Precision

The precision of the method was determined by the inter-assay and intra-assay coefficient of variation (*CV*). The intra-day precision was determined by assaying UF samples, on the same day, but in three different assays whereas the inter-day *CV* was evaluated by analysing plasma samples on four separate days. The results as well as the confidence interval (CI) are presented in ***Table 3.2***. To determine the *CV* of the assay, the following formula was used, with SD representing the standard deviation:

CV **= (SD / mean concentration) x 100%**

Table 3.2: Intra-and inter-assay precision of ultra-filtered plasma samples.

Intra-assay precision		Inter-assay precision	
Variables	**Results**	**Variables**	**Results**
Mean (μM)	33.99	**Mean (μM)**	46.13
SD	1.17	**SD**	1.85
Std. Error	0.68	**Std. Error**	1.07
***CV* (%)**	3.4	***CV* (%)**	4.01
Lower 95% CI	31.09	**Lower 95% CI**	41.53
Upper 95% CI	36.90	**Upper 95% CI**	50.73

When the assays were performed on four separate days, the standard error varied negligibly, so I was confident that the sample mean was close to the population mean. On the basis of the results obtained, judgement was made to carry out NO_3^- standards analysis in triplicates so as to take into account the occasional anomalous result. As plasma samples gave almost the same results, they were assayed in duplicates.

3.4.2.3 Linearity

Figure 3.3 is a typical calibration curves from 0-50 μM of NO_2^- and NO_3^- standards. The signal was linear from $5x10^{-8}$ to $5x10^{-5}$ mol/L. Both calibration curves displayed a linear response within the range analysed, with $r^2 > 0.998$ ($n > 10$).

UF and non-UF plasma (40 μl) were then added to a range of NO_3^- standards so as to test the linearity of NADPH oxidation. A significant loss of linearity was observed with higher volumes of pure plasma used, i.e. > 50 μl (***data not shown***). Each data point on ***Figure 3.4*** represents the mean of three replicate determinations.

The relationship between NO_3^- standards spiked with plasma followed a linear relationship over the range 0-50 μm with r^2 >0.98 (n > 3). This result suggested that there were no metabolites within the plasma interfering with both the NO_3^- reduction and the azo chromophore formation. At lower concentrations, the graph of non-UF plasma was not linear possibly due to absorbance quenching by interfering substances within the plasma.

The reference wells contained 200 μl of phosphate buffered saline (PBS) for obtention of blank values and this enabled a background for data calculation. The blank was subtracted from each value obtained in the presence of the enzyme.

Figure 3.3: Nitrite and nitrate (NO_x) standard curve.

Standard curve showing the relationship between NO_x concentration (nM) and light absorbance at 540 nm in our adaptation of the Griess reaction.

For calculation of results, a standard curve is created (A_{540} versus concentrations of the nitrate standards), allowing the determination of the NO_x concentrations within the NPHSII plasma samples. The assay is linear in the physiological range.

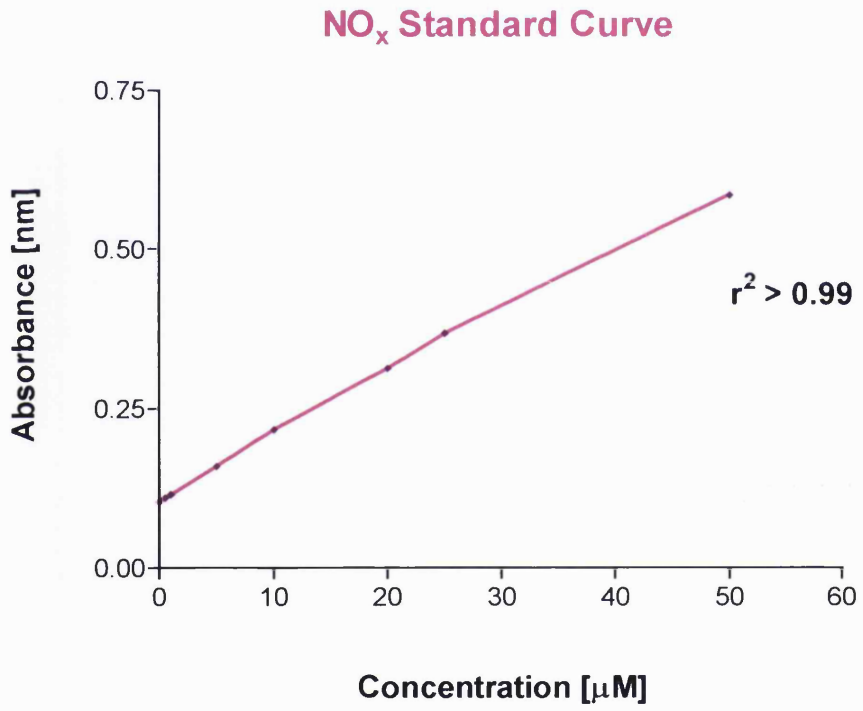
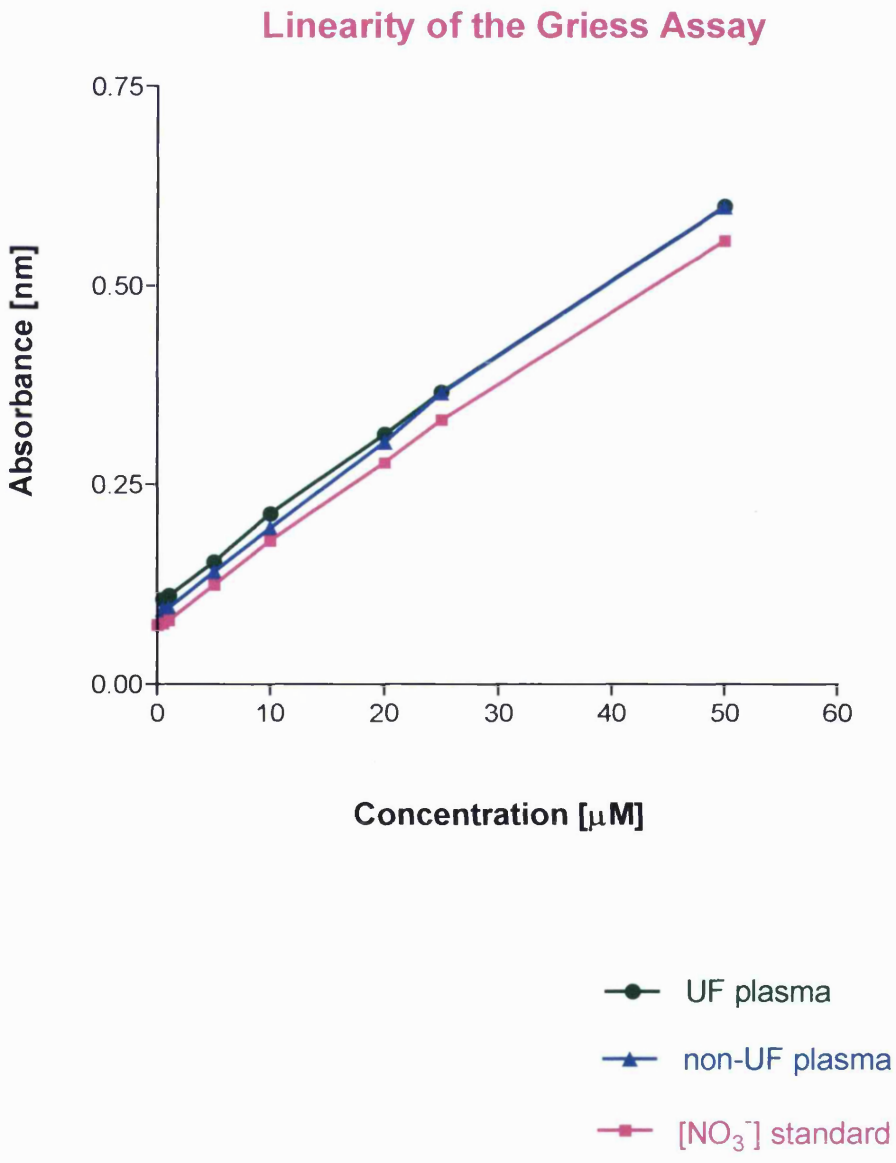


Figure 3.4: Standard curve of nitrate standards spiked with plasma.

Nitrate standards followed a linear relationship, with $r^2 > 0.98$.

When the standards were spiked with 40 µl of ultra-filtered (UF; $r^2 > 0.99$) and non-filtered (non-UF; $r^2 > 0.99$) plasma, the linearity was conserved except at lower concentrations for the latter.



3.4.2.4 Detectable range

A linear relationship between concentration of NO_2^- standards and absorbance was found at a concentration as low as 0.5 μM, although the true detection threshold appeared to be lower than 0.5 μM. A conservative estimate based on the results showed the lower detection limit of the assay to be approximately 1 μM for NO_2^- and NO_3^-.

During assay optimisation, it was found that the range of NO_2^- and NO_3^- concentrations of three healthy adults were about 30-58 μmol/L in UF and 25-38 μmol/L in non-UF plasma samples respectively. Therefore, it was decided to filter all plasma samples prior analysis. ***Table 3.3*** lists the mean values (μmol/L) for NO_2^- and NO_3^- in serum from healthy adults reported in the literature with the type of method used. The reference values for these anions in the literature varied and may be due to methodological as well as geographical factors (Cortas *et al.*, 1990).

Table 3.3: NO_x values determined by different methods in past literature.

Mean (SD)	No. specimens	Method used	Reference
15-60	?	Cu-coated Cd column	Green *et al.*, 1982
37.7 (15.4)	40	Cd column	Hegesh and Shiloah 1982
38.9 ± 19.1	12	Griess rxn / autoanalyzer	Endo *et al.*, 1996
36 (17)	40	Ion chromatography	de Jong and Burggraaf, 1983
34 (1)	9	HPLC	Lee *et al.*, 1986
51.2 (26.4)	38	Cu-coated Cd, kinetic	Cortas and Wakid, 1990
34-46	5	Capillary electrophoresis	Leone *et al.*, 1994
36.4	8	Chemiluminescence	Gilliam *et al.*, 1993
23.3	8	*Asp. spp.* NR assay	Gilliam *et al.*, 1993
23.9	26	*Asp. spp.* NR assay	Moshage *et al.*, 1995
30-58 / UF	6	*Asp. spp.* NR assay	This thesis
25-38/non-UF	6	*Asp. spp.* NR asssay	This thesis

Note:

- It is interesting to note that two methods assaying the same serum samples gave different results (Gilliam *et al.*, 1993).
- All the NR assays involved the use of the purified enzyme from *Aspergillus* species.
- Moshage and colleagues used plasma samples deproteinized by chemicals (zinc sulfate solution) and 50 μM final NADPH (Moshage *et al.*, 1995). To prevent interference with the assay, excess NADPH was further oxidized by lactate dehydrogenase/pyruvate.

- Gilliam and workers used non-deproteinized serum with 100 μM final NADPH concentration (Gilliam *et al.*, 1993).

3.4.2.5 Correlation

No correlation was observed between NO_2^- and NO_3^- in plasma. In fact, NO_2^- concentration varied from 1.67% to 20.19% as a percentage of the total amount of $[NO_2 + NO_3]$. Previous reports showed that there was no correlation between the two concentrations (Wennmalm *et al.*, 1993). As NO_2^- is rapidly oxidized to NO_3^- by Hb in red blood cells and considering the time interval between plasma acquisition among the medical centres, the NO_2^- concentration will most surely not be detectable (Moshage *et al.*, 1995).

3.5 Results: Associations between NO_x and IHD risk factors

The NO_x range studied was 2.79-63.4 μM after practice adjustment. Only 287, 241 and 532 mean values were valid from the North Mymms (n = 290), Chesterfield (n = 260) and random samples (n = 571) respectively. The mean NO_x levels from North Mymms, Chesterfield and random sets were pooled and denoted as 'Total NPHSII' (n = 955).

3.5.1 Plasma NO_x and smoking status

NO_x levels were 8.5% and 17.0% lower in current smokers when compared to non-smokers in North Mymms ($P = 0.17$) and Chesterfield ($P = 0.01$) subsets respectively (***Table 3.4***). When NO_x levels in both smokers and non-smokers were compared for both clinics together (***Table 3.5***), there was still strong statistical evidence ($P = 0.0004$) that mean values were 13.9% lower in current smokers compared to non-smokers.

Table 3.4: Plasma NO_x levels in smokers and non-smokers.

(NO_x) levels	**North Mymms**	**Chesterfield**	**Random**	**Total Sample***
	Mean (SD)	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**
	N	**N**	**N**	**N**
Non-Smokers	11.7 (4.6)	10.0 (4.0)	11.7 (4.7)	11.8 (4.7)
	180	132	405	717
Smokers	10.7 (4.6)	8.3 (3.7)	10.7 (5.1)	10.6 (4.8)
	54	57	127	238
P value	**0.17**	**0.01**	**0.03**	**0.0005**

*: adjusted for practice

Table 3.5: Comparing NO_x levels in smokers and non-smokers for both clinics.

North Mymms & Chesterfield	Mean (SD) N
Non-Smokers	10.91 (1.5) 388
Smokers	9.39 (1.5) 134
***P* value**	**0.0004**

To investigate whether this difference was due to a laboratory and/or assay difference or a real regional effect, a random sampling of NPHSII was carried out. A list of 600 random samples from the nine sample areas of the NPHSII was computer generated and 571 samples were selected. Only 532 samples had reliable NO_x measurements. A Kruskal-Wallis test (equality of populations) showed that NO_x levels were significantly (P = 0.0001) different in all the nine sample areas due to heterogeneity among the clinical practices (***Table 3.6***). St Andrews had the lowest NO_x levels (mean = 8.1 μM) whereas Camberley had the highest value (mean = 13.1 μM).

Table 3.6: NO_x levels in all nine NPHSII practices.

Practice	n	Mean (SD)
Camberley	74	13.1 (5.3)
Carnoustie	73	8.5 (4.3)
St Andrews	75	8.1 (3.5)
Aston Clinic	19	12.4 (4.2)
Harefield	47	10.3 (4.0)
Chesterfield	241	9.3 (3.8)
Halesworth	76	11.4 (5.3)
Parkstone	63	13.0 (6.0)
North Mymms	287	11.5 (4.5)
P value		**0.0001**

Similarly, when the mean NO_x values of non-smokers and smokers were examined separately according to clinical practice, there was still heterogeneity between the values in the individual practices (***Table 3.7***). For non-smokers, Carnoustie had the lowest NO_x levels (mean = 8.1 μM) whereas Parkstone had the highest value (mean = 13.7 μM). St Andrews had the next lower NO_x levels (mean = 8.4 μM) whereas Camberley had the next highest value (mean = 13.1 μM). For smokers, St Andrews had the lowest NO_x levels (mean = 7.0 μM) whereas Camberley had the highest value (mean = 12.8 μM).

Table 3.7: NO_x levels in non-smokers and smokers according to clinical practice.

Practice	Non-smokers		Smokers	
	n	G. Mean (SD)	n	G. Mean (SD)
Camberley	61	13.1 (4.9)	13	12.8 (7.1)
Carnoustie	51	8.1 (4.1)	22	9.3 (4.7)
St Andrews	59	8.4 (3.3)	16	7.0 (3.7)
Aston Clinic	16	12.6 (4.2)	3	11.1 (4.7)
Harefield	34	10.9 (4.0)	13	8.7 (3.6)
Chesterfield	167	9.8 (3.9)	74	8.4 (3.5)
Halesworth	54	11.7 (4.9)	22	10.7 (6.2)
Parkstone	49	13.7 (6.2)	14	10.7 (5.1)
North Mymms	226	11.7 (4.6)	61	10.6 (4.3)
P value		**0.0001**		**0.003**

However, there was still strong evidence that mean NO_x levels were lower in current smokers compared to non-smokers (***Table 3.4***) in this random cohort after practice adjustment. Plasma NO_x was 8.5% lower among current smokers (P= 0.03) with a mean value of 10.7 μM in smokers (n = 127) and 11.7 μM in non-smokers (n = 405) after practice adjustment.

When plasma NO_x in the total NPHSII samples assayed was examined (n = 955), there was 10.2% reduction in NO_x levels (***Table 3.4***). This result was statistically significant

(P = 0.0005) after practice adjustment. Smokers (mean = 10.6 μM; n = 238) had lower NO_x levels compared to non-smokers (mean = 11.8 μM; n = 717).

3.5.2 Plasma NO_x and serum cholesterol

There was a weak but nevertheless significant inverse relationship between serum total cholesterol (units: mM) and plasma NO_x in both the North Mymms (r = -0.13, P = 0.04) and the Chesterfield data sets (r = -0.18, P = 0.02). However, there was no statistical significant correlation detected in the random set (r = 0.009, P = 0.83) after practice adjustment (***Table 3.8***). These correlation were unaffected after compensation with serum creatinine.

When the correlation was tested in the total NPHSII cohort, there was still an inverse relationship which did not achieve statistical significance (r = -0.06, P = 0.09). However, this correlation became significant after adjustment with serum creatinine.

Table 3.8: Correlation coefficients (r) for cholesterol and plasma NO_x.

Cholesterol and	North Mymms r (P)	Chesterfield r (P)	Random r (P)	Total NPHSII* r (P)
NO_x	-0.13 (0.04)	-0.18 (0.02)	0.009 (0.83)	-0.06 (0.09)
NO_x / Creat	-0.14 (0.04)	-0.15 (0.04)	-0.002 (0.97)	-0.07 (0.04)

*: adjusted for practice

3.5.3 Plasma NO_x and other IHD risk factors

Conventional ischaemic heart disease (IHD) risk factors, such as sBP, dBP, age, weight, BMI, triglycerides, fibrinogen and haemostatic factors such as factor VIIc, had no influence on NO_x levels. Even when the NO_x values were normalized by creatinine, the correlations did not achieve significance (***negative results not shown***).

Serum creatinine (units:μM) associated positively with plasma NO_x in all four data sets studied, with significant correlations achieved only in Chesterfield practice and pooled NPHSII data set (***Table 3.9***).

Table 3.9: Correlation of serum creatinine with plasma NO_x.

Creatinine and	North Mymms r (p)	Chesterfield r (p)	Random* r (p)	Total NPHSII* r (p)
NO_x	0.10 (0.08)	0.15 (0.02)	0.05 (0.29)	0.08 (0.01)

*: adjusted for practice

3.6 Discussion

3.6.1 Optimising the NR-GD Assay

Throughout a six-month period, a protocol commonly used for the measurement of urinary NO_2^- / NO_3^- in rats (Verdon *et al.*, 1995) was improved and adapted for human plasma NO_x. Various changes were made to the original method as assaying NO_2^- / NO_3^- in rat and humans are considerably different due to the presence of a variety of proteins in human plasma.

Before NO_x analysis, the samples were deproteinised to remove Hb (M_w = 64,000) resulting from cell lysis and other proteins that may interfere with diazotisation reaction causing a reduction in signal intensity. As chemical deproteinisation carried out with an alkaline zinc sulphate solution (Cortas and Wakid, 1990) was unsuccessful, physical deproteinisation was attempted. This was accomplished by ultrafiltration using a variety of microcentrifuge filters from different companies. However, the Biomax-5 or -10, with 5,000 or 10,000 nominal molecular weight limit (NMWL) respectively (0.0005 or 0.001 μm pore size) Ultrafree microcentrifuge filter (Millipore) proved to be most suitable and were adopted for future assays. Ultrafiltration of pure plasma (12,900 rpm, 60 minutes, 4°C) helped to eliminate background absorbance due to the presence of haeme.

Many investigators reported premixing equal volumes (1:1) of the two components of the Griess reagents prior to sample addition. However, I observed that premixing attenuated the reaction due to colour quenching while serial addition of the two

components resulted in the formation of about 50% more of diazonium salt. Most probably by adding the Griess components separately, there is maximum diazonium salt formation (dependent on pH and reagent concentration) followed by chromogenic azo compound.

Analysis were performed on NPHSII samples previously collected in citrated tubes as phosphate and heparin were reported to interfere with the reduction of NO_3^- to NO_2^- (Green *et al.*, 1982). Heparinized plasma may form a precipitate on the addition of the highly acidic Griess reagent, thus rendering these samples unsuitable for analysis.

There was little volume of plasma samples available for NO_2^- / NO_3^- analysis. Diluting the plasma samples on several occasions, either by 0.5, 0.2 and 0.1 gave inconsistent results and consequently it was decided to use pure plasma. The optimum volume used without losing any sensitivity was 40 µl. According to Gilliam and colleagues, human serum has an inherently a low NO_3^- content and its original volume should be refrained from dilution as its NO_3^-concentration will then fall below the lower threshold of the assay (Gilliam *et al.*, 1993).

The analytical system responded in a quantitative linear fashion to NO_3^- and NO_2^- aqueous standards within the range 0-50 µM. Similarly, plasma spiked with increasing concentrations of NO_3^- (0.5-50 µM) resulted in a linear increase in absorbance values. There was nonetheless a reduction in signal intensity above 50 µM. The same phenomenon was reported by Misko *et al.* when assaying NO_x by fluorometric detection (Misko *et al.*, 1993).

It was reported unacceptable to use a NO_3^- standard prepared in water when quantitating NO_3^- content in serum (Gilliam *et al.*, 1993). It would be ideal to prepare the standards in the same medium as the samples whenever possible, i.e. ultrafiltered plasma or serum, so as to correct internally for interference from protein or colour. However, Moshage *et al.* (1995) showed that the standards could be prepared in both PBS or Milli-Q+ water, with the NO_2^- recoveries yielding comparable and reliable results as when prepared in plasma. For convenience, the NO_2^- and NO_3^- standards were prepared in PBS.

To validate this NR-GD assay method, the results were compared to another procedure, i.e. a commercially available validated kit. Actually, the concentration detected by the Cayman's NO_2^- / NO_3^- kit was considerably less than quantitation by the NR/GD assay and this result was confirmed by two previous studes (Giovannoni *et al.*, 1997; Marzinzig *et al.*, 1997). This was due to differing cofactors concentration used in the assay kit. Nicotinamide adenine dinucleotide phosphate (NADPH) provided was at the concentration of 0.5 μM and no system (i.e. enzymatic, chemical...) was provided to recycle the oxidized $NADP^+$ formed during the NO_3^- reduction to NO_2^-. The concentration used was insufficient to quantitatively drive the NR reaction to completion as NADPH was limiting (Verdon *et al.*, 1995).

3.6.2 Confounding factors in plasma NO_x assay

A critical approach must be adopted during NO_x measurement as not all the plasma NO_x measured from the assay was released as free NO (Schmidt & Kelm, 1996). A proportion of the NO may be released as nitrogen intermediates, i.e. such as

peroxynitrite and the NO_x may be derived from alternative sources such as anaerobic bacterial flora within the intestine, or airway-associated bacteria, ingested with the food or fluids or inhaled as NO/NO_2 (***Figure 3.1***).

Mammals excrete more NO_3^- than can be accounted for by dietary intake and there is evidence of *de novo* endogenous nitrogen biosynthesis in humans with an excess of NO_2^-/NO_3^- excreted in humans on low NO_2^-/NO_3^- diets (Green *et al.*, 1981). The daily production of NO_3^- from NO is about 700 μM and accounts for the majority of NO_3^- excretion, with the remainder coming from dietary sources (Jaekle *et al.*, 1994). Similarly, infants with acute diarrhoea excrete up to ten times more NO_3^- than they ingested daily due to bacteria in their gastrointestinal tract (Hegesh and Shiloah, 1982).

The results may be distorted by the ingestion of NO_3^--rich nutrients, i.e. cauliflower, spinach and broccholi (Marzinzig *et al.*, 1997; Radomski *et al.*, 1977). British surveys estimated a mean daily intake from food of 95 mg NO_3^- and 1.4 mg NO_2^- (Knight *et al.*, 1987), equivalent to plasma NO_3^- concentrations of 30 μM (Green *et al.*, 1982) and undetectable concentrations of NO_2^- (Wagner *et al.*, 1983). Exogenous dietary sources, depending on the season, may add up to 70% of the NO_3^- level, with 30% formed endogenously from NO (Hartmann *et al.*, 1982; Green *et al.*, 1981).

Rhodes *et al.* (1995) showed that although diet is a major contributor to human plasma NO_3^-, its concentration fell by about 54% after a 12 hour fast in healthy volunteers and that approximately 90% of the circulating NO_2^- / NO_3^- were derived directly from the L-arginine-NO pathway with plasma NO_3^- having a $t_{1/2}$ = 3-5 hours (Zeballos *et al.*, 1995). A recent study showed that fasting for 15 hours gave a better insight into the

basal endogenous NO production (Leone *et al.*, 1994). In this study subjects were non-fasted but had been asked to consume no more than a light meal on the morning of recruitment. Therefore any confounding result caused by dietary factors would be expected to be randomly distributed in a large study such as NPHSII.

The ideal situation to quantify NO synthesis *in vivo* would be to maintain human volunteers subjects on a chemically defined NO_3^- / NO_2^- free diet or a diet with a controlled NO_3^- / NO_2^- content (< 180 μM/day for humans). Under these stringent conditions, the accumulation of NO_3^- / NO_2^- is proportional to the total activity of NO synthesis per unit time. An alternative method would be measuring the generation of exogenously administered ^{15}N-labelled arginine to ^{15}N-nitrate (Forte *et al.*, 1997). The advantage of this technique is that all the nitrate will be derived exclusively from the L-arginine:NO pathway but there is no indication of the proportion of biologically active NO formed and its cellular source.

Nevertheless, plasma NO_x represents the primary metabolite of NO (Takahashi *et al.*, 1992; Wennmalm *et al.*, 1993; Winlaw *et al.*, 1994) and the use of NO_3^- and NO_2^- as quantitative indices of NO production in plasma in humans is to be endorsed. The accuracy obtained with this assay is sufficient for our purposes and NO_2^- and NO_3^- can be used as surrogate markers.

3.6.3 Influence of IHD risk factors and the NO pathway

A major finding of the current study is that plasma NO_x levels were about 8-18% lower in current smokers than non-smokers in three subsets of the NPHSII cohort.

This result is particularly striking since cigarette smoke itself contains NO (Epperlein *et al.*, 1996) and, acutely, cigarette smoking would be expected to result in an elevation rather than a reduction in the plasma NO_x levels (Chambers *et al.*, 1998; Sarkar *et al.*, 1999). However, the present results suggest strongly that chronic cigarette consumption is associated with depressed plasma levels of nitrogen oxides *in vivo*, a finding supported by the observation that salivary nitrate is also reduced in smokers compared to non-smokers (Bodis and Haregewoin, 2000).

Reduced NO_x levels in smokers may provide an explanation for the well-documented association between cigarette smoking (active and passive) and impaired endothelium-dependent vasodilation *in vivo* (Celermajer *et al.*, 1993; 1996), in clinically healthy smokers, irrespective of the presence or absence of overt coronary atherosclerotic lesions (Zeiher *et al.*, 1995). The results are in line with numerous reports which have established smoking as a primary risk factor for CHD (Molstad, 1991). The exact mechanisms of smoking-related endothelial damage are not yet fully elucidated but there are several possible explanations: nicotine may cause structural damage to the endothelial lining (Zimmerman and McGeachie, 1987), long-term smoking generates oxygen free radicals and plasma peroxidation (Adachi and Wang, 1998), which affect NO mediated regulation of coronary artery tone in smokers, and cigarette smoke extract inactivates endothelium-derived NO by increased production of superoxide anions (Kugiyama *et al.*, 1996). Increased superoxide anions is a characteristic feature of atherosclerosis in experimental models.

Elevations in serum cholesterol are also associated with impaired endothelium-dependent vasodilation (Chowienczyk *et al.*, 1994; Creager *et al.*, 1992) and, in this

study, apart from the random group, an inverse correlation was found between serum total cholesterol and plasma NO_x in the North Mymms, Chesterfield and the total NPHSII data sets. These results were replicated after NO_x values were adjusted with serum creatinine. The inconsistent result obtained in the random set is weak, suggesting either that the effect of cholesterol on endothelial function is mediated independently of NO synthesis (possibly via other endothelial dilator systems or by increased generation of oxidative free radicals), or that a small effect of cholesterol on NO synthesis was masked by the influence of dietary nitrate in this particular practice. The latter explanation sounds more plausible.

Hypercholesterolemia, a major IHD risk factor, has been associated with damage to the vascular endothelium, a process believed to be involved in the onset of atherogenesis (Anderson *et al.*, 1987). Increased serum cholesterol is associated with endothelial dysfunction (ED) and reduced NO activity, before any structural changes in the arterial wall are even detectable by angiography and this damaging interaction may begin very early, even in the first decade of life (Sorensen *et al.*, 1994).

Several hypothesis have been proposed to account for the impaired endothelium-dependent vasodilation in hypercholesterolaemics: reduced affinity of L-arg for eNOS, intracellular substrate depletion bstrate due to defective transmembrane transport system, increased NO inactivation by NO superoxide and enhanced NO destruction, which will drive the synthetic pathway. It is believed that in asymptomatic hypercholesterolemic subjects, elevated cholesterol generate oxygen free radicals, which inactivate NO or there is limited NO production (Clarkson *et al.*, 1996).

No clear correlation was observed between BP and plasma NO_x in this study, a result at odds with a previous study in Japanese (Node *et al.*, 1997). However hypertension has been shown to depress endothelium-dependent vasodilation in some (Calver *et al.*, 1992) but not all studies (Cockcroft *et al.*, 1994). This suggests that the effect of BP on endothelial function is variable, and that when seen, may result from processes that are either dependent (Forte *et al.*, 1997) or independent of endothelial NO synthesis.

Regarding other CVD risk factors, such as age, BMI, fibrinogen, triglycerides and Factor VIIc, they did not correlate with NO_x levels. These factors may play a role in the pathogenesis of atherosclerosis and thrombosis through other mediators but may themselves not be directly related to NO metabolism/pathway. Plasma NO_x correlated positively with serum creatinine, an indicator of renal function (Wong *et al.*, 1995). However, Cortas *et al* observed no correlation between NO_3^- and creatinine concentrations (Cortas and Wakid, 1990). This issue needs further investigation.

A defect in endothelium-dependent vasodilation has been demonstrated reproducibly in arteries from individuals with essential hypertension (Calver *et al.*, 1992), hypercholesterolaemia (Chowienczyk *et al.*, 1994; Creager *et al.*, 1992), and in those exposed to cigarette smoke (Celermajer *et al.*, 1993; 1996), as well as in individuals with established atherosclerosis. This abnormality is considered a marker for a generalised impairment of the vasculoprotective actions of the endothelium and may be the result of a reduction in NO synthesis or bioavailability.

Heitzer *et al.* (1996) demonstrated that cigarette smoking and hypercholesterolemia acts synergistically to impair endothelial function by enhancing the formation of

oxidized low density lipoprotein (LDL) and increased circulating products of lipid peroxidation by free radicals, hence increasing the risk of CAD (Kugiyama *et al.*, 1996). Furthermore in the presence of atherosclerotic wall thickening, smokers exhibited further impairment in flow-dependent coronary arterial dilation indicative of an additive adverse effect on endothelium-dependent coronary vasodilator function (Zeiher *et al.*, 1995) as endothelial vasodilator dysfunction merely reflects the severity of the atherosclerotic disease process.

In summary I have found evidence in a large cohort of male subjects that cigarette smoking and cholesterol levels, but not prevailing BP, are associated with a reduction in NO generation *in vivo*. Such a finding endorses the view that the ED seen following exposure to smoking is due a reduction in endothelial NO generation.

In summary, the main conclusions from this chapter are:

- Plasma NO_x an index of endogenous NO production, was measured by an optimised and validated biochemical assay;
- Plasma NO_x levels were reduced in smokers compared to non-smokers;
- Plasma NO_x correlated inversely with serum total cholesterol and positively with creatinine; and
- Conventional IHD risk factors such as BP, age, BMI, triglycerides had no influence on NO_x levels.

CHAPTER 4

RELATIONSHIP BETWEEN *NOS3* VARIANTS AND NO PRODUCTION

4.1 Objectives

- To set up high throughput genotyping protocols for four previously identified polymorphisms within endothelial nitric oxide synthase gene (*NOS3*) gene.
- To test the hypothesis that polymorphisms within the *NOS3* gene might influence prevailing levels of cholesterol and blood pressure (BP) as well as smoking status and nitric oxide (NO) production in the second Northwick Park Heart Study (NPHSII).
- To investigate whether indices of NO production or *NOS3* genotype predicted the risk of ischaemic heart disease (IHD) in healthy middle-aged British men.

4.2 Background

Endothelium-derived NO is believed to possess anti-atherogenic properties, due to its anti-aggregatory and anti-proliferative effects (Radomski *et al.*, 1987; Moncada *et al.*, 1991; Sarkar *et al.*, 1996). Indirect evidence points out that alterations in the endothelial NO pathway might be involved in endothelial dysfunction (ED) due to a reduction in bioavailable NO in atherosclerosis (Celermajer *et al.*, 1994; Quyyimi *et al.*, 1995b; Glasser *et al.*, 1996). As *NOS3* encodes an endothelial constitutive NOS

(ecNOS) responsible for the enzymatic generation of NO from L-arginine in various cells and stimuli conditions, this role defines its place as a candidate gene in cardiovascular diseases (CVD) (Soma *et al.*, 1999; Soubrier, 1999).

Genetic variability within *NOS3* has also been implicated in affecting eNOS activity, which is believed to contribute significantly to BP regulation as it mediates the release of NO, a potent vasodilator (Marsden *et al.*, 1993). Its inhibition elevates BP in healthy humans (Haynes *et al.*, 1993) and hypertensives have been shown to have reduced urinary nitrite (NO_2^-) and nitrate (NO_3^-) anions, (i.e. NO_x) excretion levels, with NO_x levels correlating inversely with BP (Benjamin *et al.*, 1994). The offspring of essential hypertensives also exhibited a reduction in vascular NO activity (Taddei *et al.*, 1996). However, transgenic experiments provides the best proof that eNOS is a strong candidate for hypertension, as mice homozygous for eNOS knockout have higher BP (~15-20 mmHg) compared to control mice (Huang *et al.*, 1995; Shesely *et al.*, 1996) and spontaneously hypertensive rats had reduced basal NO production (Lin *et al.*, 1997).

The hypothesis that genetic variability within *NOS3* could lead to a number of diseases, such as atherosclerosis, hypertension and vasospasm, due to a decreased in enzyme activity caused by an amino acid change or diminution in gene transcription, has been tested by several groups (***Table 4.1***). DNA samples from the NPHSII cohort were screened for four previously identified polymorphisms (***Figure 4.1***), which had the potential to alter eNOS function or protein expression.

Two bi-allelic polymorphisms, located in the gene promoter, were investigated. At –922 base pairs (bp) from the transcription initiation site, AςG change ($A^{-922}\rightarrow G$) creates a consensus motif (ANATGG) for the binding of transcription factor CF-1 (Riggs *et al.*, 1991), which can either activate or repress *NOS3* transcription. Another promoter variant studied, $T^{-786}\rightarrow C$ mutation was shown to be strongly associated with coronary spasm in Japanese (Nakayama *et al.*, 1999). Subjects carrying the mutant allele had reduced NO production in their coronary arteries, due to decreased *NOS3* transcription, as demonstrated by reduced promoter activity by luciferase reporter gene assays (Nakayama *et al.*, 1999). This study was the first evidence that a mutation in *NOS3* promoter was linked to a disease. Coronary spasm may arise from interactions between environmental and genetic factors. Both basal and acetylcholine (Ach) induced NO are impaired in patients suffering from coronary spasm, who have increased basal tone and are hyper-responsive to nitrovasodilators (Yoshimura *et al.*, 1998).

Another marker studied was the Intron 4 27-bp variable number of tandem repeats (In4 VNTR) with two alleles: a common large allele with five tandem repeats and a smaller allele with four tandem repeats, designated '*b*' and '*a*' respectively (Wang *et al.*, 1996). Wang *et al.* (1997) found that the ecNOS locus contributed significantly to circulating plasma NO_x levels in healthy Caucasians while Japanese subjects carrying the *a* allele had lower NO_x levels compared to those with the *b* allele (Tsukada *et al.*, 1998). In a Caucasian Australian population, ecNOS4*a* was an independent risk factor for coronary artery disease (CAD) in current and ex-smokers (Wang *et al.*, 1996) while this VNTR was an independent risk factor for coronary atherosclerosis in a Japanese population (Ichihara *et al.*, 1998).

Finally, a missense mutation located at nucleotide 894 in exon 7, predicting a G to T transversion ($G^{894}\rightarrow T$), was examined (Hingorani *et al.*, 1997). It encodes a glutamic (Glu) to aspartic (Asp) acid change at residue 298 ($Glu^{298}\rightarrow Asp$). This mutation gives rise to an impaired eNOS due to a conformational change of the protein from helix to tight turn (Yoshimura *et al.*, 1998), located in the intermediate portion of haeme and calcium/calmodulin binding sites of eNOS (***Figure 1.2, Chapter 1***) (Miyamoto *et al.*, 1998). The missense Asp^{298} variant, believed to be a risk factor for CAD in a British population (Hingorani *et al.*, 1999), was significantly associated with myocardial infarction (MI) (Shimasaki *et al.*, 1998), coronary spasm (Yoshimura *et al.*, 1998) and with essential hypertension (EH) in Japanese subjects (Miyamoto *et al.*, 1998).

The genetic contribution of *NOS3* to basal circulating NO_x levels is unclear but needs to be solved so as to comprehend the exact roles of NO and ecNOS in the pathogenesis of IHD. To test the hypothesis that cardiovascular risk factors affect NO production, the association between *NOS3* polymorphisms and plasma NO_x levels, an index of NO production *in vivo*, was examined. The relationship between the four polymorphisms studied and the risk of IHD event, an issue never addressed before, was also studied.

Table 4.1: Summary of studies between *NOS3* polymorphisms, NO synthesis, CVD and EH.

Authors	Variant	Remarks	Subjects	Results
[#]Wang *et al.* (1997)	**In4a VNTR**	Genetic contribution to plasma NO_x, measured by GR in healthy subjects	428 Caucasians	**$[NO_x]_{eNOS4a\ allele} > [NO_x]_{eNOS4b\ allele}$**
[#]Tsukada *et al.* (1998)			413 Japanese	**$[NO_x]_{eNOS4a\ allele} < [NO_x]_{eNOS4b\ allele}$**
Miyamoto *et al.* (1998)		Kyoto: n=458; Kumamoto: n=421	Japanese	No association with EH
Ichihara *et al.* (1998)		550 controls; 455 cases	Japanese	**Association with MI**
[$]Wang *et al.* (1996)		Caucasians: 153 controls; 549 cases	Australians	**Association with smoking-related CAD**
[$]Uwabo *et al.* (1998)			Japanese	**Association with EH**
Nakayama *et al.* (1995)	**$(CA)_{23}$/In13**	Healthy subjects	Japanese	Number of repeats is a polymorphic marker
Nadaud *et al.* (1994)		Healthy subjects	Caucasians	Alleles distribution different from Japanese
Bonnardeaux *et al.* (1995)			Caucasians EH	No association with EH
Nakayama *et al.* (1997)		33-repeat allele increase in patients	Japanese EH	**Association with EH without LVH**
Lacolley *et al.* (1998)	**$G^{IN23}T$ +894 (G/T)**	Assess aortic stiffness by wave velocity		No association with hypertension

[#]: Studies on plasma NO_x.

Authors	Variant	Remarks	Subjects	Results
Hingorani *et al.* (1999)	**+894 (G/T)**	138 controls; 288 cases (CHAOS study)	British	**Association with CAD**
Lacolley *et al.* (1998)	missense Glu298Asp	Hypertensives higher G allele frequency	French	**Association with EH**
Miyamoto *et al.* (1998)		Hypertensives higher T allele frequency	Japanese	**Association with EH**
Shimasaki *et al.* (1998)			Japanese	**Association with MI**
Yoshimura *et al.* (1998)		100 controls; 113 cases	Japanese	**Association with coronary spasm**
Kato *et al.* (1999)		513 controls; 549 cases	Japanese	No association with EH
Nakayama *et al.* (1999)	**-786 (T/C)**	Mutation reduces promoter activity	Japanese	**Association with coronary spasm**
Huang *et al.* (1995)	***NOS3***	Ach causes no relaxation in aortic rings, which are unaffected by L-nitroarg	Mutant mice	**EH in mice lacking NOS 3**
Shesely *et al.* (1996)				**High BP in mice lacking NOS 3**
Poirier *et al.* (1998)		610 controls; 531 cases (ECTIM)	French/Irish	No association with MI
*Panza *et al.* (1990)		Study response of forearm blood flow to Ach infusion	18 EH patients 18 controls	**Endothelium-dependent vasodilation impaired in patients**
*Forte *et al.* (1997)		2 study: subjects given nitrate controlled diet and IV L-[^{15}N] arg bolus	21 EH patients 24 controls	**Reduced NO production in patients BP α 1 / [nitrate excretion]**
Dominiczak & Bohr, 1995			EH patient	**Dysfunction of NO system**

^{s} : Discrepancy between studies attributable to various factors, i.e. diagnostic criteria or race as Japan has an isolated population *vs* Caucasians

Frequency of In4a allele in the Japanese subjects is less compared to the Australians.

* : Human studies cannot distinguish whether impaired function or synthesis of NO is the cause or effect of the increase in BP.

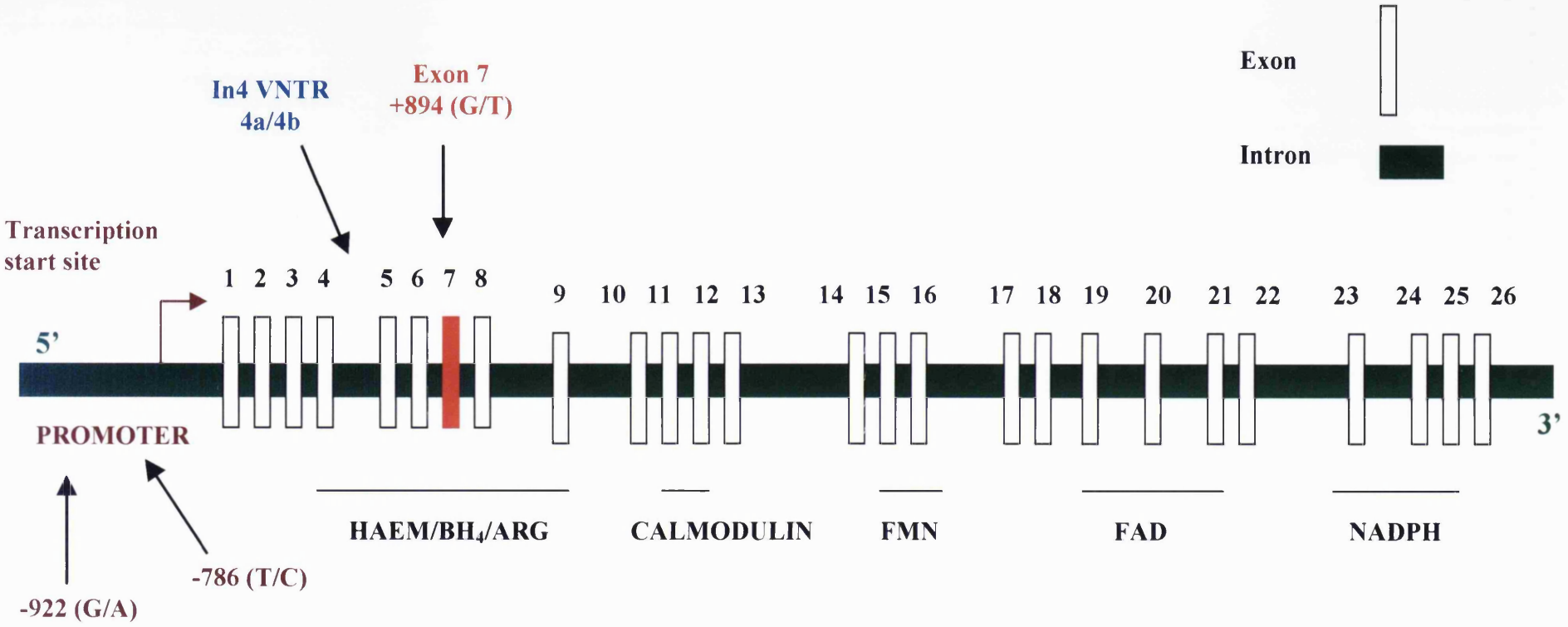


Figure 4.1: Structure and polymorphic sites of *NOS3*.

Exon and intron arrangement of human *NOS3*, with the four polymorphisms studied located at the 5' end. The gene is located on chromosome 7q35→q36 and spans 21 kb. The exons encoding the respective cofactor binding sites are also represented.

4.3 Materials, subjects and methods

4.3.1 Materials

All the reagents were of molecular biology grade and were outlined in ***Section 2.2, Chapter 2***.

4.3.2 Subjects

The NPHSII cohort (***Section 2.1, Chapter 2***) comprising of middle-aged healthy Europeans Caucasians were recruited from North Mymms (n = 290), Chesterfield (n = 260) and 571 subjects computer-selected, at random, from all nine primary care practices.

4.3.3 Genomic DNA extraction

Genomic DNA was extracted and its purity checked according to ***Section 2.3.3*** and ***Section 2.3.4*** of ***Chapter 2*** respectively. The 2745 DNA samples were stored in 32 96-well Beckman arrays at 4°C.

4.3.4 Primer design and template DNA amplification

Oligonucleotide primers were designed according to the principles outlined in ***Section 2.3.1, Chapter 2***. Amplification of genomic DNA and allele-specific restriction endonuclease (RE) digestion of the product by Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP) techniques were described in ***Sections 2.4.1*** and ***2.4.2, Chapter 2***. The optimised conditions for PCR/RFLP analysis were summarised in ***Tables 2.4*** and ***2.5, Chapter 2*** respectively.

4.3.5 Data analysis

For all the variants investigated, the observed frequencies of the genotypes were compared to those expected under conditions of Hardy-Weinberg (H-W) equilibrium using the χ^2 test. Genotype and allele frequencies were compared between groups by means of (2 x 2) contingency tables. Determination of allelic association and linkage disequilibrium were estimated using the correlation coefficient Δ. For two polymorphisms, Δ was estimated by the haplotype counting method. All the analysis were performed using the SPSS STATA statistical package and the procedures used in the laboratory were automated using a spreadsheet. The principles behind the analysis are summarised in ***Sections 2.8.2*** and ***2.8.3, Chapter 2*** respectively. Variations in plasma NO_x may be due to renal dysfunction and the results were also presented as NO_x / Creat.

4.3.6 Power calculations

For associations between genotype and plasma NO_x as well as BP, it was assumed that differences would be greatest in comparisons of rare allele *vs* common allele homozygotes. It was calculated that 50 homozygotes in each group would be required to detect a 10% difference in group means for log NO_x and 35-40 individuals to detect a similar difference in diastolic BP (dBP) and systolic (sBP) with 90% power at $P = 0.05$. With the exception of In4 variant, the frequencies of rare allele homozygotes are in the range 10-15%. Thus to obtain 50 rare allele homozygotes required for the comparisons described, it was estimated that a minimum of 500 subjects would have to be genotyped. For studies of *NOS3* genotype and IHD risk, it was assumed based on a previous study (Hingorani *et al.*, 1999) a rare allele homozygote frequency of 12% and a rare allele frequency of 32%. It was calculated that with 200 IHD events and at least a similar number of controls, there would have 90% power to detect a 2 fold excess risk of IHD among rare allele homozygotes assuming a recessive model and 90% power to detect a 1.5 fold excess risk among rare allele carriers assuming a dominant model at $P = 0.05$.

4.4 Results

4.4.1 PCR/RFLP and MADGE pattern

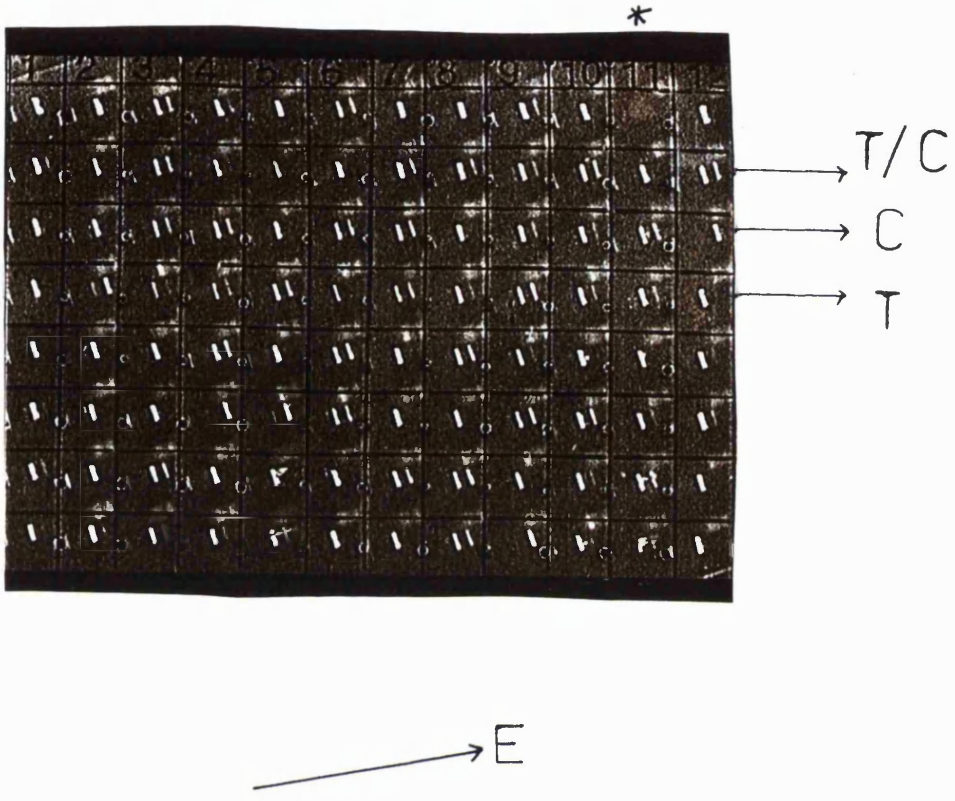
Figure 4.2 shows the RFLPs electrophoretic patterns of the four polymorphisms studied. In all cases, the enzymes were chosen to cleave the rarer allele for any given polymorphism into two distinct fragments.

Figure 4.2: Electrophoretic patterns of the bi-allelic polymorphisms and VNTR studied.

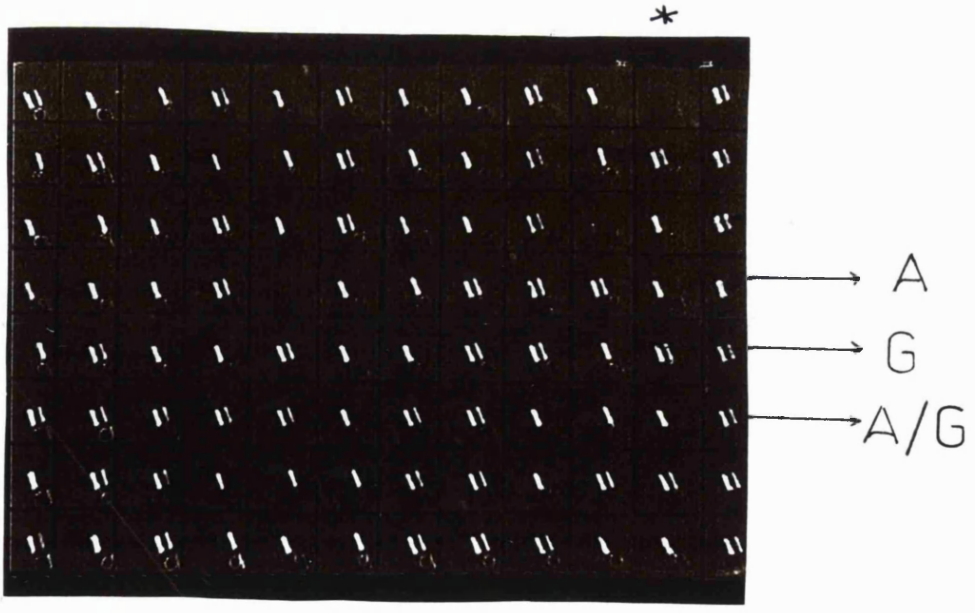
Panel 1: Bi-allelic polymorphism ($T^{-786}\rightarrow C$) in *NOS3* promoter was detected by *NgoM*1 digestion of the promoter PCR product (194 bp). The restriction endonuclease recognises the restriction enzyme site [5'..G↓C**C**GGC..3'] where there is an T nucleotide in the wild allele. In the presence of nucleotide **C** at position -786 from the transcription start point, the amplified product is cleaved into fragments of 144 and 50 bp after enzyme digestion. About 10 µl of amplified product was digested with 5 µl of cocktail digests containing 2 units of *NgoM*1 for about 16 hours at 37°C and then subject to electrophoresis on a 10% MADGE gel for 45 minutes.

Panel 2: Bi-allelic polymorphism ($A^{-922}\rightarrow G$) in *NOS3* promoter was detected by B*sl*1 digestion of the promoter PCR product (138 bp). The restriction endonuclease recognises the restriction enzyme site [5'..CCNNNNN↓NNG**G**..3'] where there is an A nucleotide in the common allele. In the presence of nucleotide **G** at position -922 from the transcription start point, the amplified product is cleaved into fragments of 110 and 28 bp after enzyme digestion. About 10 µl of amplified product was digested with 5 µl of cocktail digests containing 2 units of B*sl*1 for about 3 hours at 55°C and then subject to electrophoresis on a 7.5% MADGE gel for 45 minutes.

1

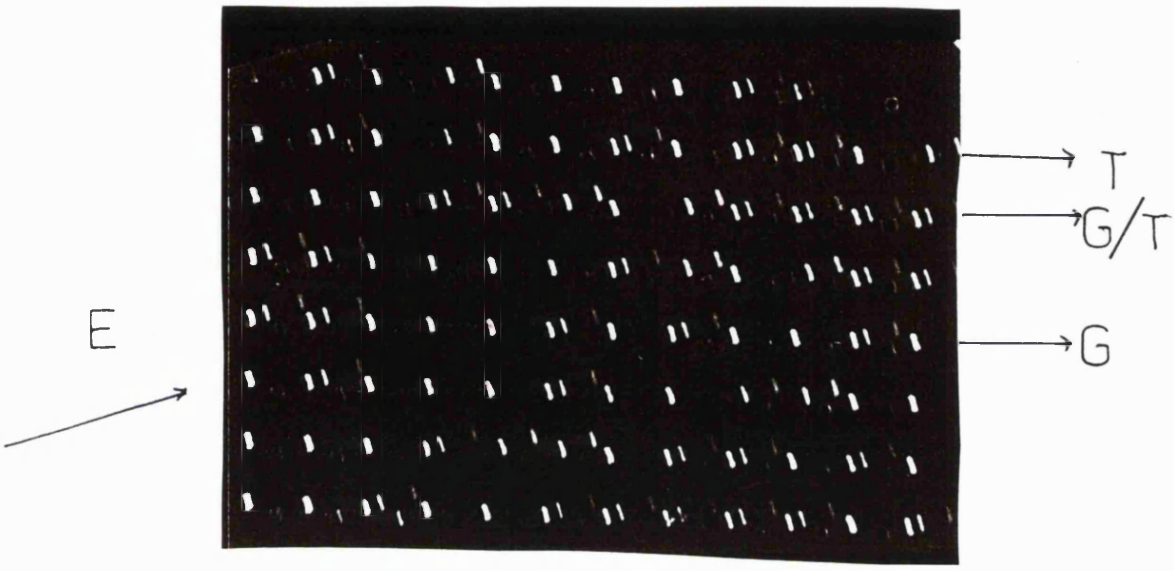


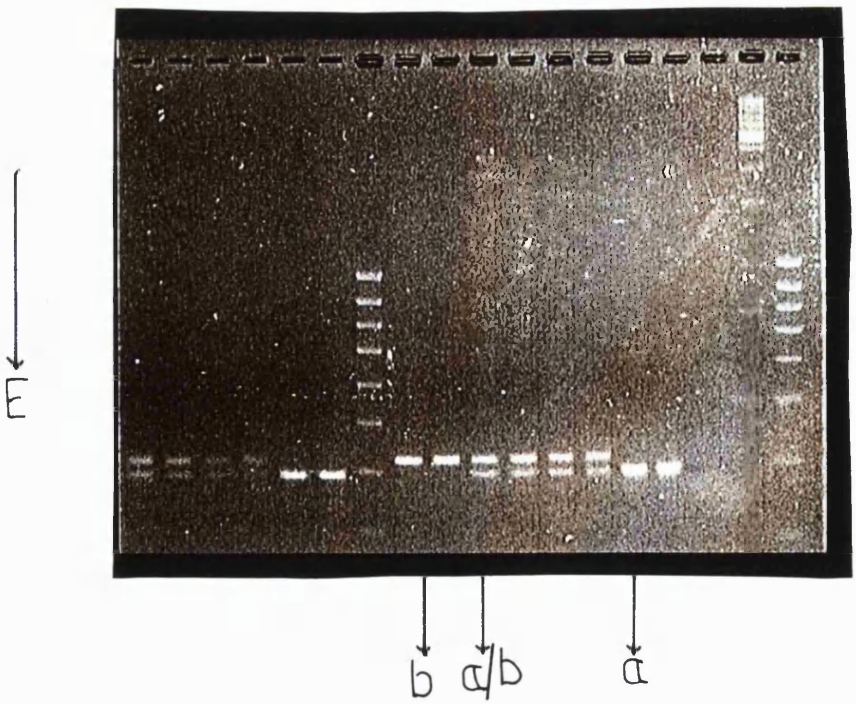
2



Panel 3: Bi-allelic polymorphism ($G^{+894}\rightarrow T$) in exon 7 of *NOS3* was detected by *Dpn*II digestion of the PCR product (151 bp). The restriction endonuclease recognises the restriction enzyme site [5'.. ↓GA<u>T</u>C..3'] where there is an G nucleotide in the wild allele. In the presence of nucleotide <u>T</u> at position +894 from the transcription start point, the amplified product is cleaved into fragments of 100 and 51 bp after enzyme digestion. About 10 µl of amplified product was digested with 5 µl of cocktail digests containing 2 units of *Dpn*II for about 16 hours at 37°C and then subject to electrophoresis on a 10% MADGE gel for 45 minutes.

Panel 4: The method used to detect the Variable Number of Tandem Repeats (VNTR) in intron 4 (In4) of *NOS3* was adapted from Wang *et al.*, 1996. There are two alleles: a large common one (*b*; 420 bp) consisting of five tandem of 27-bp repeats and a smaller allele (*a*; 393 bp) consisting of four tandem of 27-bp repeats. The samples were loaded on a 3% agarose gel and ran for 5 hours. The fragments were detected distinctly.


E
T
G/T
G


E
b
a/b
a

4.4.2 Allele Frequencies of *NOS 3* polymorphisms and linkage disequilibrium

A total of 2792 participants were genotyped for the -922 (A/G) polymorphism, 2720 for the -786 (T/C) polymorphism, 2710 for the In4 VNTR and 2584 for the Glu298Asp variant (***Table 4.2***). The genotype frequencies were in line with those predicted by H-W equilibrium ($P > 0.05$ for all analyses).

Pairwise linkage disequilibrium coefficients (Δ) were calculated for the four polymorphisms studied, all of which are located at the 5' end of the *NOS3* gene (***Figure 4.1***; ***Table 4.3***). All comparisons achieved statistical significance ($P < 0.001$). Allelic association was greatest between the two polymorphisms located in the gene promoter (Δ= 0.90) and associations between polymorphisms in the gene promoter and the exon 7 polymorphism were present but weaker (Δ= 0.40). The Δ value for exon 7/In4 association was only -0.27 despite the physical proximity of these two loci.

Table 4.2: Genotype and allele frequencies of *NOS3* polymorphisms.

Polymorphism	Genotype frequency (%)			χ^2 (1 df)	*P*
-922 (A/G)	**AA**	**AG**	**GG**		
	1089	1295	408	0.52	0.47
	(39)	(46.4)	(14.6)		
-786 (T/C)	**TT**	**TC**	**CC**		
	1026	1300	394	0.30	0.59
	(37.7)	(47.8)	(14.5)		
Intron 4 VNTR	**aa**	**ab**	**bb**		
	2023	647	40	2.10	0.15
	(74.6)	(23.9)	(1.5)		
+894 (G/T)	**Glu/Glu**	**Glu/Asp**	**Asp/Asp**		
	1146	1146	292	***0.05***	0.83
	(44.3)	(44.3)	(11.3)		

Table 4.3: Pairwise Linkage Disequilibrium Coefficients (Δ).

	$A^{-922}\rightarrow G$	$T^{-786}\rightarrow C$	In4 VNTR	$G^{894}\rightarrow T$
$A^{-922}\rightarrow G$		0.90	0.32	0.42
$T^{-786}\rightarrow C$			0.36	0.43
In4 VNTR				-0.27

4.4.3 Associations between genotype and phenotype

Clinical and biochemical characteristics of the whole cohort are presented in ***Table 4.4***, according to *NOS3* genotype. There was no statistically significant difference in baseline biochemical and other demographic variables for any of the polymorphisms studied.

Table 4.4: Baseline characteristics and *NOS3* genotype of NPHSII cohort.

NPHS II	-922 (A/G)			*P*	-786 (T/C)			*P*	Intron 4 VNTR			*P*	Glu298Asp			*P*
	AA	**AG**	**GG**		**TT**	**TC**	**CC**		**aa**	**ab**	**bb**		**Glu/Glu**	**Glu/Asp**	**Asp/Asp**	
BMI (kg/m^2)	26.4 (3.3)	26.5 (3.6)	26.6 (3.50	**0.74**	26.3 (3.4)	26.4 (3.6)	26.7 (3.4)	**0.26**	26.4 (3.5)	26.5 (3.3)	26.2 (4.0)	**0.84**	26.5 (3.4)	26.5 (3.5)	26.5 (3.7)	**1.00**
Cholesterol (mmol/L)	5.88 (1.00)	5.84 (1.01)	5.87 (1.04)	**0.69**	5.87 (0.99)	5.86 (1.02)	5.90 (1.02)	**0.82**	5.85 (1.01)	5.93 (1.04)	5.99 (0.96)	**0.25**	5.86 (1.02)	5.91 (1.00)	5.74 (0.97)	**0.05**
Triglyceride (mmol/L)	1.95 (1.02)	1.98 (1.03)	1.96 (1.01)	**0.83**	1.95 (1.03)	1.96 (1.03)	2.00 (1.03)	**0.75**	1.96 (1.03)	2.00 (1.03)	1.74 (0.90)	**0.59**	1.95 (1.00)	1.99 (1.05)	1.93 (1.01)	**0.52**
Fibrinogen (mg/L)	2.68 (0.49)	2.70 (0.51)	2.72 (0.50)	**0.28**	2.69 (0.50)	2.70 (0.51)	2.72 (0.49)	**0.74**	2.69 (0.49)	2.71 (0.54)	2.73 (0.55)		2.69 (0.51)	2.70 (0.49)	2.72 (0.48)	**0.64**

4.4.4 Blood pressure and *NOS3* genotype

sBP and dBP were higher in heterozygotes for Glu298Asp variant ($P < 0.0002$ by ANOVA) (***Figure 4.3 and Figure 4.4***). Such a pattern is difficult to explain by conventional models of inheritance and raises the possibility of a type II statistical error. However, since eNOS is a dimer (Marletta, 1993), the possibility that Glu/Asp heterodimers have reduced activity in comparison to Glu/Glu and Asp/Asp homodimers cannot be excluded. No other *NOS3* polymorphism exerted a statistically significant influence on BP.

4.4.5 Plasma NO_X and *NOS3* genotype

The hypothesis that *NOS3* polymorphisms influence endothelial NO production was tested by measuring plasma NO_x in a subset of 1121 subjects, 290 from the North Mymms practice, 260 from the Chesterfield practice and 571 selected at random from the whole cohort (***Figure 4.5***). None of the polymorphisms studied influenced plasma NO_x, even after correction for serum creatinine.

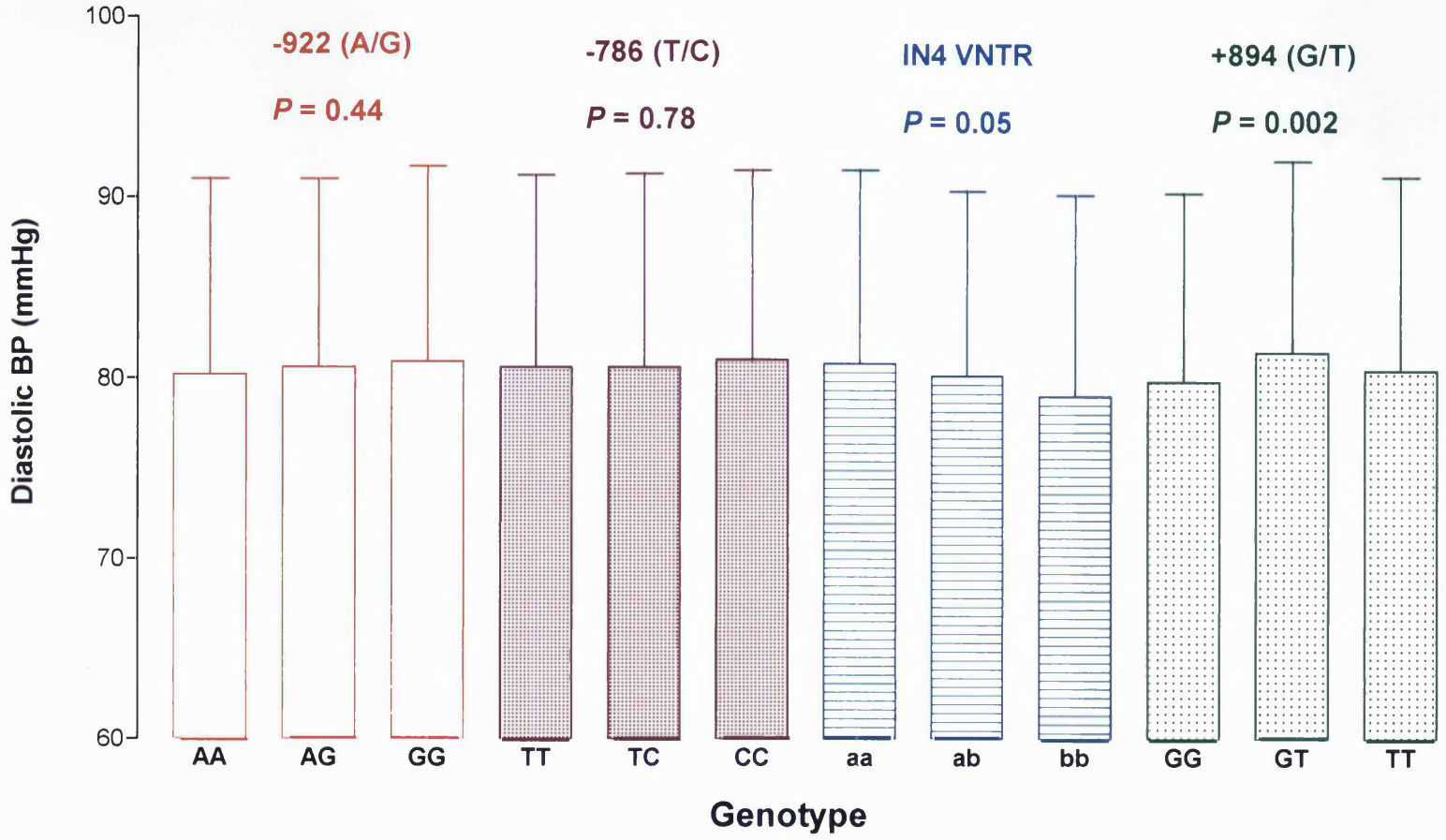


Figure 4.3: Diastolic blood pressure (mmHg) ± SD and *NOS3* genotype in the NPHSII cohort.

Diastolic BP was significantly higher in heterozygotes for the Glu298Asp polymorphism.

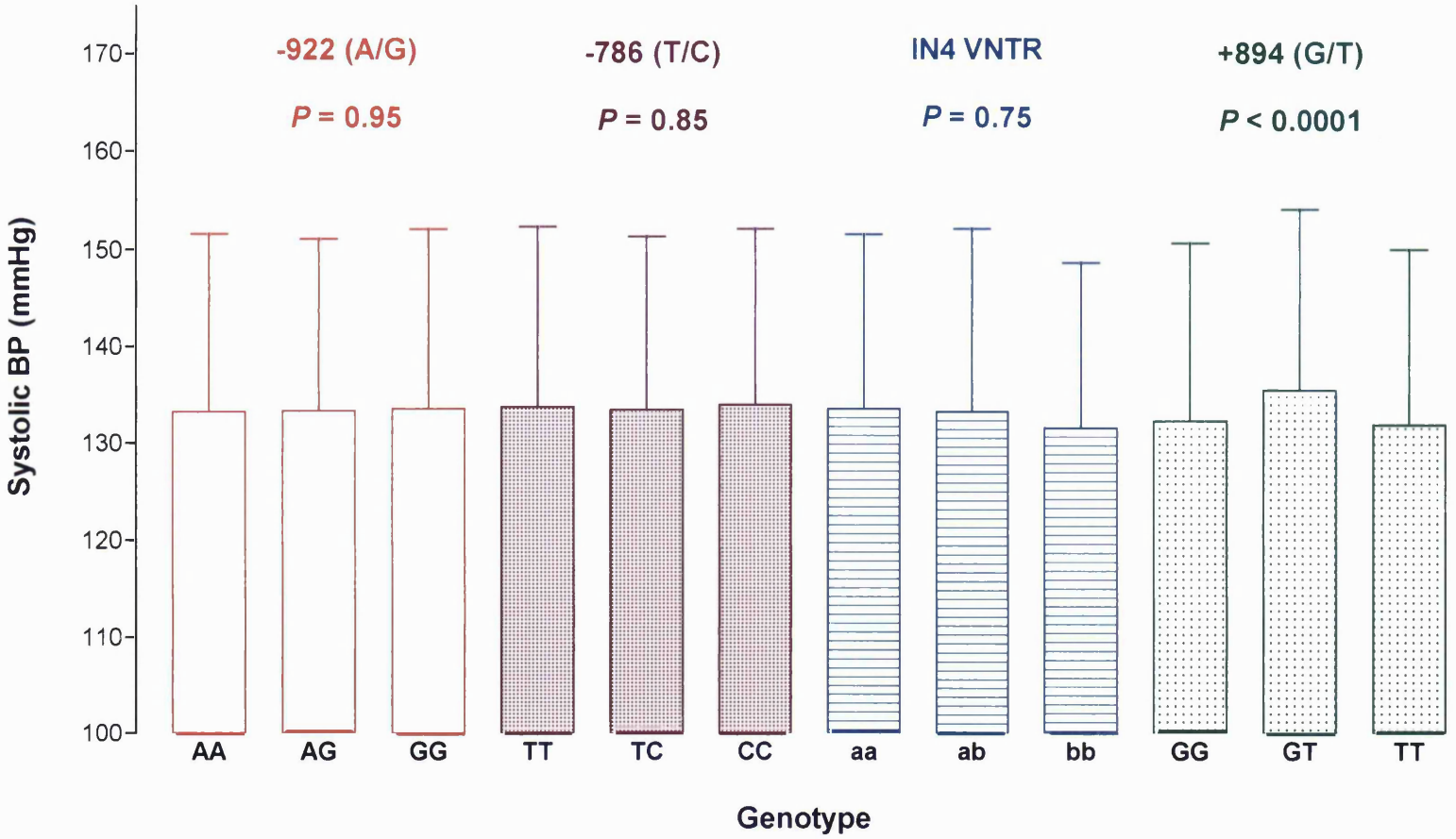


Figure 4.4: Systolic blood pressure (mmHg) ± SD and *NOS3* genotype in the NPHSII cohort.

Systolic BP was significantly higher in heterozygotes for the Glu298Asp polymorphism.

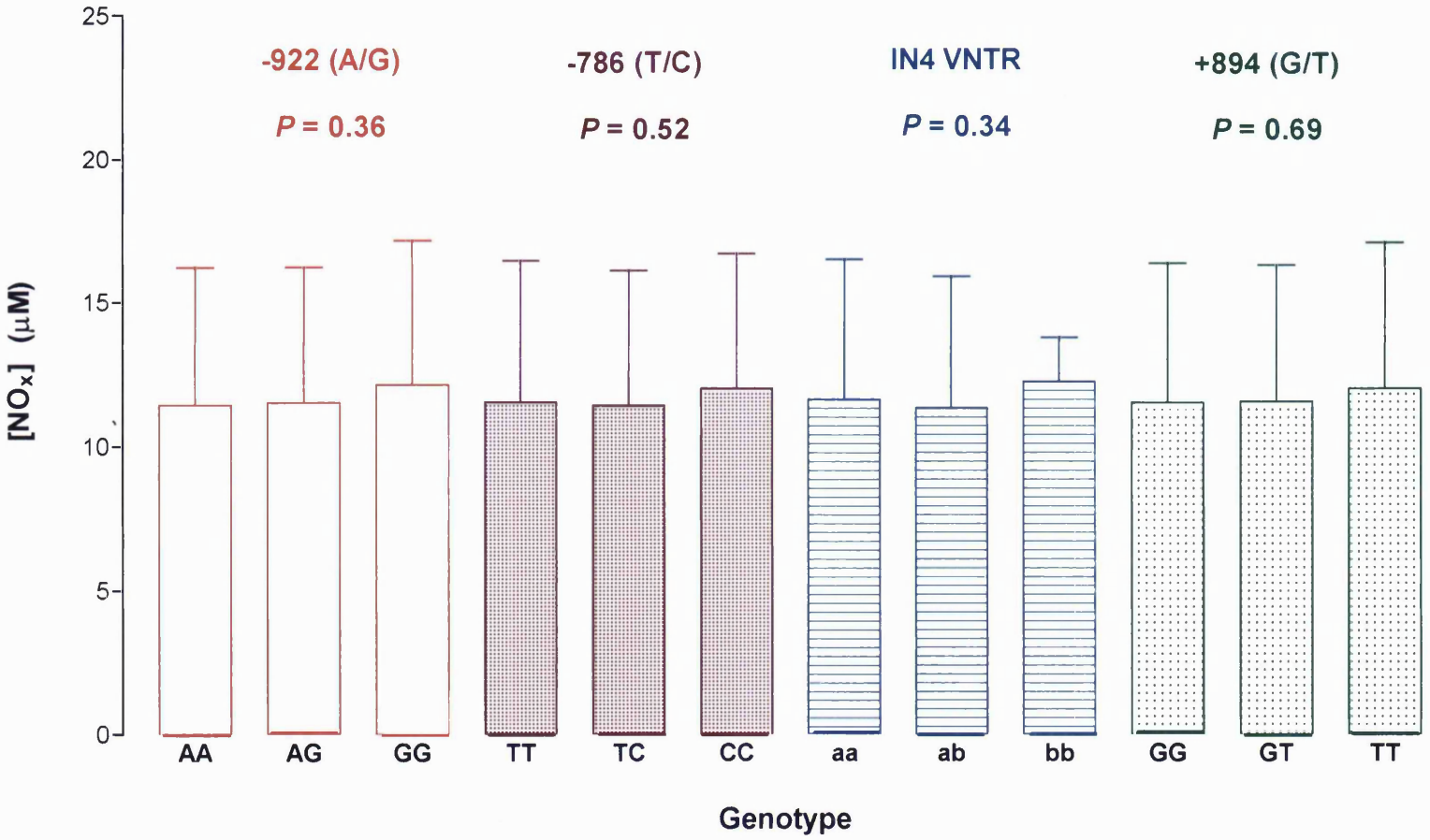


Figure 4.5: NO_x levels according to *NOS3* genotype in the NPHSII cohort.

Even after compensation with creatinine, none of the four polymorphisms studied influenced plasma NO_x levels. Results are presented as mean ± SD

4.4.6 *NOS3* genotype, plasma NO_x and IHD risk

The risk of IHD over the duration of the study was not influenced by the level of plasma NO_x recorded at entry (data not shown). However this data was based on only 15 IHD events among the 955 subjects with NO_x measurements and adequate follow-up data. Cox regression was used to assess the effect of genotype on risk of an IHD event, defined as fatal, non-fatal or silent MI and coronary surgery. There was no evidence of a relationship between any polymorphism and the risk of IHD (***Table 4.5***) when analysed according to dominant or recessive models. There was also no evidence that smoking status modified the effect of genotype on IHD risk polymorphisms (***negative results not shown***). Kaplan-Meier survival plots of the effects of the Glu298Asp, -786 (T/C) and Intron 4 genotypes on IHD event-free survival are illustrated in **Figure *4.6***.

Of interest however, 34/37 (92%) individuals homozygous for the rare In4 variant, were also homozygous for the promoter polymorphisms at positions -786 and -922. In4 variant was previously associated with IHD and MI (Wang *et al.*, 1996; Ichihara *et al.*, 1998). This raises the possibility that it might act as a marker in linkage disequilibrium with potential functional variants in the regulatory region of the gene (Nakayama *et al.*, 1999).

Table 4.5: Influence of *NOS3* genotype on IHD risk.

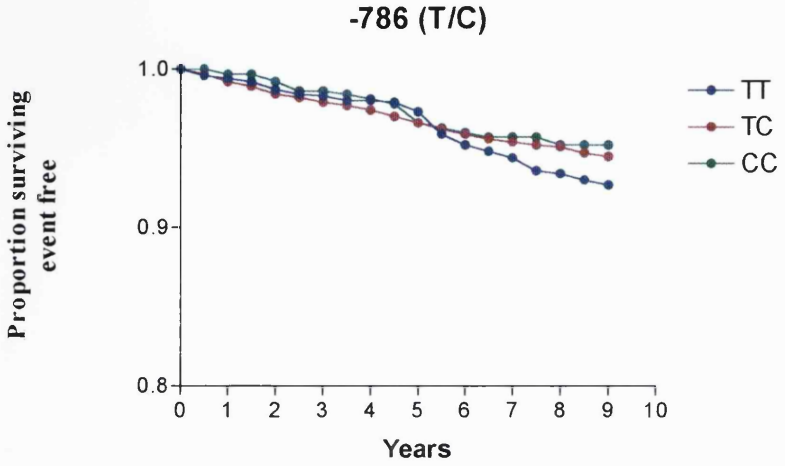
a) Co-dominant model

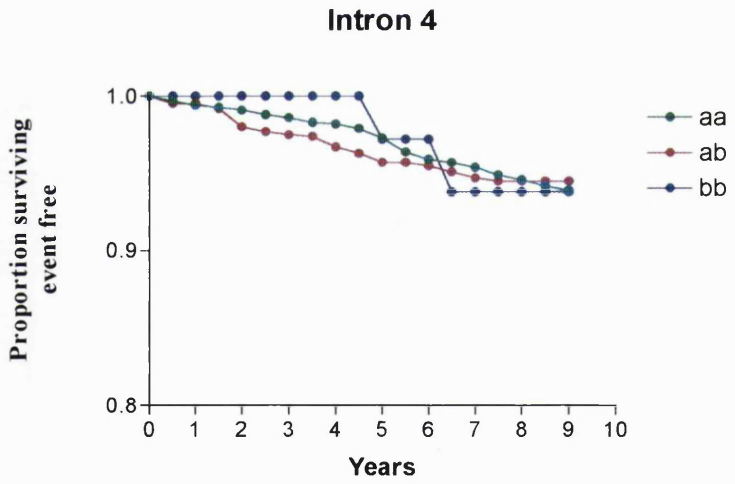
Polymorphism	Genotype	Hazard ratio (95% CI)	*P*
-922 (A/G)	AA	1	**0.20**
	AG	0.80 (0.56-1.13)	
	GG	0.69 (0.39-1.22)	
-786 (T/C)	TT	1	**0.29**
	TC	0.78 (0.54-1.12)	
	CC	0.71 (0.41-1.23)	
Intron 4 VNTR	aa	1	**0.99**
	ab	1.15 (0.68-1.51)	
	bb	1.04 (0.26-4.21)	
+894 (G/T)	GG	1	**0.29**
	GT	1.0 (0.68-1.51)	
	TT	0.60 (0.30-1.22)	

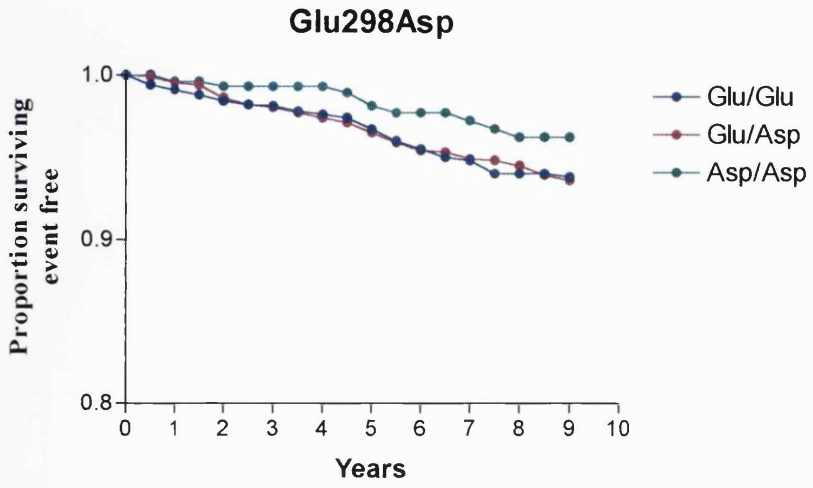
b) Recessive model

Polymorphism	Genotype	Hazard ratio (95% CI)	*P*
-922 (A/G)	AA+AG	1	**0.22**
	GG	0.71 (0.42-1.22)	
-786 (T/C)	TT+TC	1	**0.42**
	CC	0.81 (0.48-1.36)	
Intron 4 VNTR	aa + ab	1	**0.96**
	bb	1.03 (0.26-4.18)	
+894 (G/T)	GG + GT	1	**0.14**
	TT	0.60 (0.31-1.19)	

Figure 4.6: Kaplan-Meier survival plots of -786 (T/C), intron 4 and Glu298Asp genotypes







4.5 Discussion

In this large prospective British population-based study of middle-aged men, plasma NO_x is not influenced by polymorphisms in the *NOS3* gene and *NOS3* genotype was not predictive of future IHD events. A number of case-control studies have demonstrated an association between polymorphisms in the *NOS3* gene and a variety of cardiovascular end-points including hypertension (Miyamoto *et al.*, 1998), MI (Wang *et al.*, 1996; Hibi *et al.*, 1998; Hingorani *et al.*, 1999a; Ichihara *et al.*, 1998) angiographic CAD (Hingorani *et al.*, 1999a), and coronary artery spasm (Nakayama *et al.*, 1999). These positive findings are not, however, universal (Hingorani *et al.*, 1995; Poirier *et al.*, 1999; Bonnardeaux *et al.*, 1995).

The limitations of case-control designs and the limitations of genetic studies involving relatively small populations have been discussed previously (Gambaro *et al.*, 2000). In the current study, I aimed to overcome some of the limitations of previous studies by: (1) performing the largest population-based study of *NOS3* polymorphisms conducted to date; (2) utilising a group of men initially free from IHD to investigate the genetic influences on IHD events in a prospective manner; and (3) examining all of the polymorphisms at the *NOS3* locus that have been associated previously with CVD. The study also allowed the re-assessment of the relationship between *NOS3* polymorphisms and BP.

There was no influence of *NOS3* gene polymorphisms on risk of IHD over the 8 years of follow-up in a group of 2965 men ascertained from nine different regions of the UK (***Figure 2.1, Chapter 2***). There was also no interaction between *NOS3* genotype and any of the cardiac risk factors studied. In particular, there was no interaction between smoking status and *NOS3* genotype in the determination of IHD risk as reported by a previous study (Wang *et al.*, 1996). There was no relationship between any of the polymorphisms studied and plasma NO_x, in contrast to two previous reports (Wang *et al.*, 1997; Tsukada *et al.*, 1998).

The lack of a relationship between plasma NO_x and the -786 (T/C) polymorphism runs contrary to the observation that the *NOS3* gene promoter containing the C allele exhibits reduced transcriptional activity when compared to the promoter containing the T allele (Nakayama *et al.*, 1999). However, since plasma NO_x is likely to reflect NO synthesis via both the endothelial and neuronal NOS isoforms, it is possible that small differences in NO generation resulting from genetic differences in the *NOS3* gene cannot be detected sensitively by measurement of steady state plasma NO_x.

The failure to detect an influence of *NOS3* polymorphisms on the risk of IHD is unlikely to be due to the lack of statistical power. Although, the initial power calculations were based on a projection of 200 IHD cases, rather than the 159 which were observed, the study still had had 90% power to detect a 2.2 fold excess risk of IHD among rare allele homozygotes assuming a recessive model, and a 90% power to detect a 1.6 fold excess risk among rare allele carriers assuming a dominant model at $P = 0.05$.

In previous studies excess risks of cardiovascular end-points have been confined to homozygotes for *NOS3* variants and the odds ratios for IHD or other end points in such individuals have been have been in the range 2 to 4, suggesting that the present study had adequate power to detect this level of risk. The negative findings might therefore indicate that some previous smaller-scale studies might have generated falsely positive results. Alternatively, if the *NOS3* polymorphisms studied here are not themselves functional but rather are in linkage disequilibrium with an as yet unidentified functional variant within the *NOS3* gene, positive associations between genotype and cardiovascular end-points would only be observed in individuals of particular ethnic or genetic backgrounds in which such linkage disequilibrium was present as a result of common ancestry.

Individuals studied as part of the CHAOS trial in the relatively homogeneous population of the East Anglian region of the UK in whom a positive association between homozygosity for Glu298Asp polymorphism and IHD was observed, might be one such example. The absence of consistent results with the Glu298Asp variant does not plead in favour of a risk of hypertension or atherosclerosis associated with this genotype and there is no experimental evidence to support the functionality of this marker up till now (Soubrier, 1999).

sBP and dBP were higher in heterozygotes for the Glu298Asp polymorphism ($P < 0.0001$ and $P = 0.002$ respectively by ANOVA), but such a pattern is difficult to explain by conventional models of inheritance and raises the possibility of a type II statistical error.

However, the possibility that Glu/Asp heterodimers have reduced enzymatic activity in comparison to Glu/Glu and Asp/Asp homodimers as a potential mechanism for this observation cannot be excluded and warrants further study. No other *NOS3* polymorphism exerted a statistically significant influence on BP.

However, I failed to detect an influence of polymorphisms located in the promoter or coding region of the *NOS3* gene in determining plasma levels of NO_x or the future risk of an IHD event. This finding, in a large study of UK men suggests that *NOS3* gene polymorphisms are unlikely to account for differing susceptibilities to IHD in the general UK population, although an important effect in individuals from particular regions or ethnic backgrounds cannot be excluded.

In conclusion, the principal findings of this chapter are:

- The following *NOS3* polymorphisms: -922 (A/G), -786 (T/C), +894 (G/T) and In4 VNTR did not influence plasma NO_x levels significantly in the NPHSII cohort;
- Neither plasma NO_x nor *NOS3* genotype predicted future IHD events in middle-aged British men; and
- None of the *NOS3* polymorphisms studied exerted a statistically significant effect on conventional IHD risk factors, such as BP, age, BMI, cholesterol, triglyceride and fibrinogen.

CHAPTER 5

MOLECULAR SCANING OF *GCH1* GENE; A CANDIDATE FOR IHD?

5.1 Objectives

- To search for genetic variability within the GTP cyclohydrolase I (*GCH1*) gene.
- To investigate the relation between genotype of *GCH1* gene, plasma levels of neopterin (an *in vivo* marker of GTPCH activity) and the risk of ischaemic heart disease (IHD).

5.2 Background

5.2.1 GTPCH, BH_4 and endothelial dysfunction (ED)

GTP cyclohydrolase I (GTPCH; gene symbol *GCH1*) [EC 3.5.4.16] is the first and rate-limiting enzyme for L-*erythro*-(6R)-5,6,7,8-tetrahydrobiopterin (BH_4) biosynthesis (Duch and Smith, 1991) and it is expressed constitutively in the liver, brain and adrenal gland (Duch *et al.*, 1984; Nichol *et al.*, 1985). However, its expression can also be induced in endothelial cells as well as in macrophages in conditions of inflammation by cytokines (Werner-Felmayer *et al.*, 1993; Walter *et al.*, 1994). GTPCH catalyses the formation of 7,8-dihydroneopterin triphosphate (7,8-NH_2-TP) from guanosine triphosphate (GTP) via the pterin pathway (Blau and

Niederweiser, 1985). The intermediate 7,8-NH_2-TP is either cleaved to BH_4 intracellularly or hydrolysed to neopterin extracellularly (Curtius *et al.*, 1986; Huber *et al.*, 1984) (***Figure 5.1***). Both BH_4 and neopterin can be used as markers of GTPCH activity and activation of the pterin pathway is indicated by increased plasma neopterin levels (Gross and Levi, 1992).

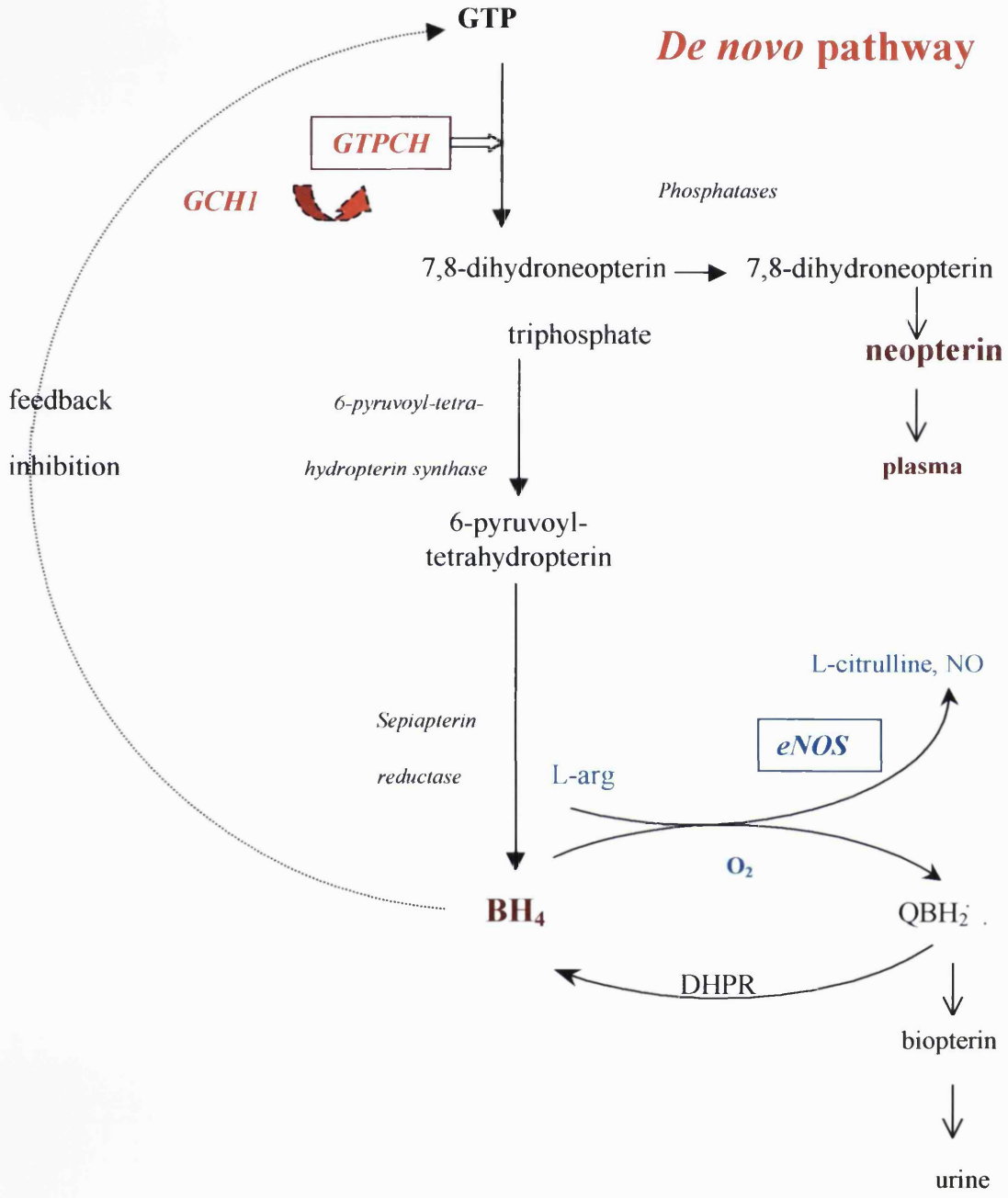
It is possible that DNA sequence changes within *GCHI* gene might limit endothelial NO synthesis by controlling the amount of bioactive BH_4 available (Gütlich *et al.*, 1994a; Kukor *et al.*, 1996). Several lines of evidence support the concept of endothelial dysfunction (ED) (a physiological feature of atherosclerosis) occurring as a result of BH_4 deficiency and/or dysfunction (Busse and Fleming, 1996). Endothelial cells contain a BH_4 biosynthetic pathway (Werner-Felmayer *et al.*, 1993) and BH_4 is an essential cofactor for endothelial nitric oxide synthase (eNOS) which catalyses the enzymatic conversion of L-arginine to L-citrulline and NO (Tayeh and Marletta, 1989; Kwon *et al.*, 1989). BH_4 stabilises the active dimeric form of eNOS, thereby modulating its catalytic activity and binding to L-arginine (Liu and Gross, 1996; Sahin-Toth *et al.*, 1997). Exposure of endothelial cells to cytokines such as interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) induces *GCH1* expression and NO production simultaneously, implying that these two pathways are linked (Werner-Felmayer *et al.*, 1993). However, the regulation of BH_4 production by endothelial cells is still unclear.

GCH1 and *NOS3* gene expression are upregulated by the same stimuli, with both genes having similar consensus motifs implicated in mediating transcriptional control by oestrogen and shear stress (Ichinose *et al.*, 1995a; Miyahara *et al.*, 1994). Infusion

of BH_4 into healthy dorsal hand veins produces vasodilatation due to NO generation (Bhagat *et al.*, 1999) and the inhibition of BH_4 synthesis by 2,4-diamino-6-hydroxypyrimidine (selective inhibitor of GTPCH) impaired NO formation, in favour of superoxide synthesis by eNOS (Cosentino and Katusic, 1995). ED in hypercholesterolaemia (Stroes *et al.*, 1997) and smokers (Higman *et al.*, 1996) may be partly due to BH_4 deficiency as administration of BH_4 restores these responses.

The *hph-1* mutation in mouse deficient in GTPCH activity (McDonald *et al.*, 1988) and various inborn errors of metabolism, responsible for defects in the biosynthesis of BH_4 screened in humans (Blau, 1988), have provided an insight towards understanding the metabolic dysfunction associated with this key cofactor (Hyland *et al.*, 1996). However, to date no genetic studies have been carried out to identify common polymorphisms within *GCH1* and to investigate the association of these sequence variations with changes in BH_4 synthesis and/or with the development of IHD in healthy subjects. Therefore to elucidate the involvement of the key components of the pterin pathway (i.e. *GCH1* gene and BH_4 cofactor) in the phenomenon of ED, genetic studies (***Chapter 5***) and biochemical assays (***Chapter 6***) were carried out.

Figure 5.1: Tetrahydrobiopterin and neopterin biosynthesis.



5.2.2 *GCH1*: Gene structure and molecular studies

Polymorphisms within the promoter of several genes, such as *NOS3* (Nakayama *et al.*, 1999), stromelysin-1 (Humphries *et al.*, 1998) and IL-6 (Fishman *et al.*, 1998), have been shown to influence levels of their respective proteins and to be associated with atherosclerosis (Tybjaerg-Hansen and Humphries, 1992). Therefore to investigate the genetic mechanism whereby GTPCH regulates BH_4 biosynthesis, *GCH1* was screened for polymorphisms. These variants which might be present within the promoter, exons, introns or 3'-untranslated region (3'-UTR) can cause altered rate of messenger RNA (mRNA) transcription, decreased mRNA stability, aberrant exons splicing, or the formation of enzymes with defective catalytic activity (Gütlich *et al.*, 1994a; Ichinose *et al.*, 1995a). Therefore, polymorphisms present within *GCH1* might alter the levels or the activity of GTPCH enzyme, which could in turn limit the amount of BH_4 and NO formed, and influence the susceptibility to IHD through impaired endothelium vasodilation associated with ED.

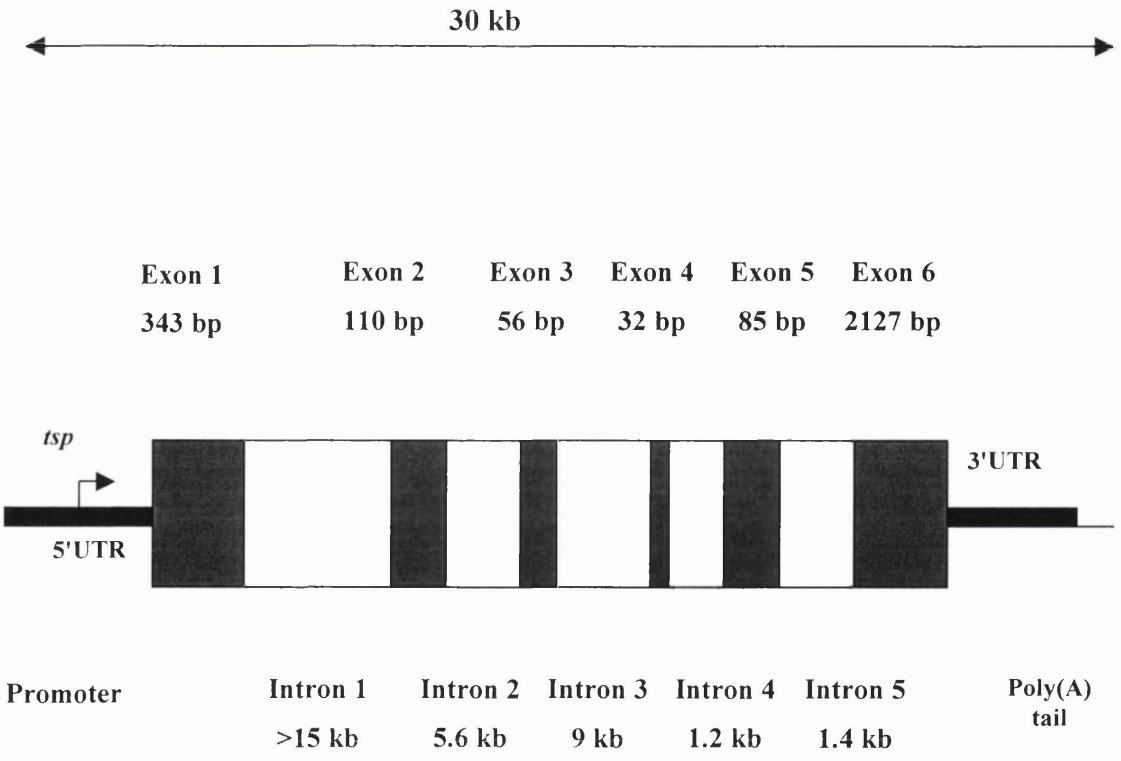
Human *GCH1* is located on chromosome 14, composed of 6 exons and spans about 30 kb of genomic DNA (***Figure 5.2***) (Ichinose *et al.*, 1994; 1995a, Thöny *et al.*, 1995). The 2.6 kb region upstream of the transcription starting point (*tsp*) exhibits promoter activity and contains canonical features of an eucaryotic promoter such as CCAAT and TATA boxes as well as several Sp1 response motifs (Witter *et al.*, 1996). *GCH1* promoter was scanned for potential polymorphisms by Single-Strand Conformation Polymorphism (SSCP) analysis and the nature of any mobility shifts were determined by sequencing. The search was restricted to the promoter, a domain implicated in

regulatory functions and which has the potential to influence BH_4 and neopterin production by modulating *GCH1* gene expression (Witter *et al.*, 1996). The influence of any sequence changes found within *GCH1* was studied on plasma neopterin levels, i.e. clinical phenotype. Neopterin is a marker of macrophage activation, which plays a key role in inflammation and atherogenesis (Ross, 1986; Fuchs *et al.*, 1988a; Fuster *et al.*, 1992; Meade, 1994).

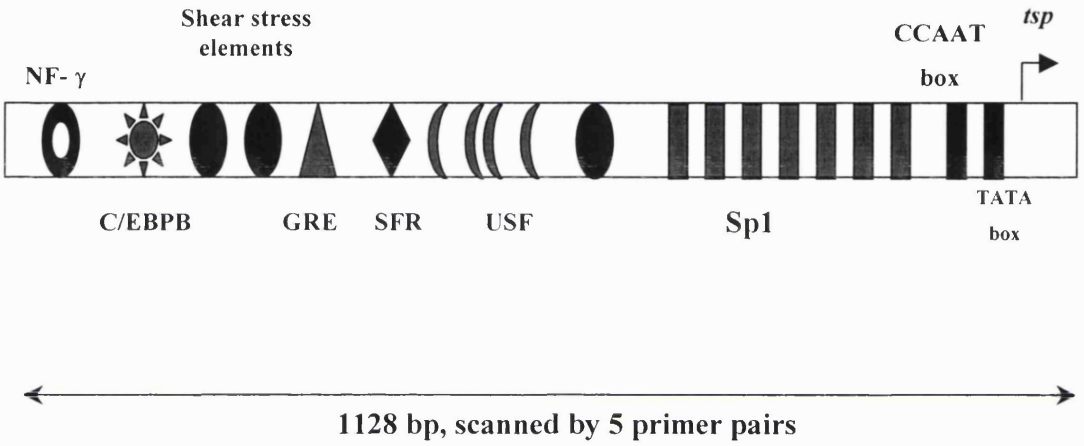
The genetic contribution of *GCH1* polymorphism(s) to basal circulating BH_4 levels as well as to the risk of IHD events are unknown. However, these issues need to be addressed so as to comprehend the underlying mechanism of BH_4 regulation by GTPCH and eventually its role in NO synthesis. The association between *GCH1* polymorphism(s) and plasma neopterin levels (an indirect index of BH_4 production; reviewed in ***Chapter 6***) as well as the risk of IHD events was studied. Allele as well as genotype frequencies were also calculated.

Figure 5.2: Organisation of the human *GCH1* gene and its promoter.

Panel A: The gene is composed of six exons spanning about ~30 kb. Exons and introns are shown as darkened (semi-transparent) and clear boxes respectively. The 5' and 3' flanking sequences are represented by thick horizontal lines (Adapted from Ichinose *et al.*, 1995a). *Diagram not to scale*



Panel B: Only the promoter region that was sequenced is represented. Putative binding sites for transcription factors (Sp1, USF, SFR, GRE, NF- γ, C/EBPB and shear stress elements) as well as canonical features of an eukaryotic promoter, i.e. TATA box and CCAAT box are shown. *Diagram not to scale.*



5.3 Subjects and methods

The NPHSII subjects, all Caucasians, were described in ***Section 2.1, Chapter 2***. Initially five primer pairs (sense and antisense) were designed to scan 1128 bp of the promoter, including the *tsp*. PCR was optimised for each set of primers and 44 DNA samples from NPHSII Master Array 1 were screened by SSCP. Once samples exhibiting a sequence change were identified, they were sequenced to detect the precise nucleotide change. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR/RFLP) method was then used to confirm the presence of any polymorphism(s) identified and then the whole NPHSII cohort was genotyped for that particular polymorphism.

5.3.1 Genomic DNA

The 'salting-out' method was used to extract genomic DNA from whole blood and its quality was checked by spectrophotometry. Both methods were described in ***Sections 2.3.3*** and ***2.3.4*** of ***Chapter 2*** respectively.

5.3.2 Design of primers for *GCH1* promoter and PCR optimisation

Oligonucleotide primers for five separate PCR reactions, amplifying only the promoter region, were designed using the published sequence of the human *GCH1* gene (Genbank/EMBL *Accession numbers P30793 EMBL L29478*). The forward primers (F_p) and reverse primers (R_p) were designed according to the Principles of ***Sections 2.3.1*** and ***2.3.2*** of ***Chapter 2*** for ideal SSCP and covered 1128 bp in five overlapping fragments upstream of the translation point (***Figure 5.3***). The first set of primers covered the *tsp*.

Figure 5.3: Genomic DNA of *GCH1* promoter to be amplified and sequenced.

Five set of primers, designed in overlapping segments, were used to cover 1080 bp upstream of the *tsp* and 48 bp of the 5' UTR. The arrows correspond to the five PCR primers used. Putative transcription factors' binding sites identified by sequence analysis programs are underlined while TATA- and CCAAT-boxes are indicated by boxed sequences. The three bi-allelic polymorphisms identified are shown in uppercase letters and are in red. The enzymes restriction sites are underlined in blue.


cctgtaatcc cagctactca ggaggctgag gcaggagaat cgtttgaacc caggaggcgg aggatgaagt gagcctcaaa
-1080 bp
Fp5
NF- γ
aaaaaaaaaa gaaaaaaaat attattttga tacatgtttt aaagctcact tcggcattat gagataatcc tcagtgagga
aaggtccatt tattaatctc aagaaaacag ttacagcaga tgtcactggt taagagttca gttggtgaat agcatttcac
aatttgtacc aacatctggg gaaagacgct ttgcatggaa ctgtaaaaca attgagcacc aaatctgcac aactgcgttt
C/EBPB
-796
Shear stress elt
-741
NFI
ctagaaaatg cGatgggttt tatagagatg aggtcttgct atgttttcca ggctggtctc gaactcTtgg cctcaagcga
Fp4
Shear stress elt
tcctcccgcc tcggtctccc caagcgccgg gagtacaggc gtgagccacc gacggaaatg gattttaagt gaaagtccta
Rp5
GRE
-577
tcttcgtttg caaatcaatt tttccaggat caaagttcta gcaaccataa atcctatcct tctagacata Ggacccaca
SFR
USF
USF
USF
USF
taagggccac ttgatgtgac acttgccccg acagcggtgc cacctggccc ctaggtggca ggtgcgtttt taactaaacc
Fp3
Shear stress elt
caagagtctg aagtcacatt attctcttgc cttgaaagct taagagttgg ttcctaatcg gtggcttaac tcggtctctc
Rp4
Sp1
tccaggcaag gccacctgtt tgctgatctt tcacagggcg aggggaccgg agcccttcaa tgcagcgccc tcttggcctg
Fp2
Sp1
Rp3
aagaggggggc gactgggcgc ccagaccgcg tctttctcac tgagatccca gctcctggac gactgcctct tttcgggttc
Sp1
Sp1
ggctcattcc gcaataagtg gaggggtccc gcctgatcct aggggccggc gccctttcc ttccctccct gcgcttgcga
Fp1
Rp2
Sp1
Sp1
accccctccgg gcgtctccgg agccgcgacc cctgctgggg accccggggg cgggaggccg aggggcgcgg
CCAAT-box
Sp1
cgagggccgt gacgcgaggc ggggccggcc aatgggagcg ctccgcggcg caggctgagc cgccgggcca
TATA-box
tsp
taaaaaggag gcgcggccgg gctttccagc ctgtggccgc tcccggctcg gagtgtgatc taagcaggtc gcgtaccttc
+1 bp
Rp1
+48 bp
ctcaggtgac tccggccaca

PCR amplification was performed according to the method described in ***Section 2.4.1, Chapter 2***. Each PCR reaction was performed in a final volume of 30 µl and contained 50 ng of dry genomic DNA, 1.5 mM $MgCL_2$, 10 pmole of each primer, 2 U of *Taq* DNA polymerase, 300 µl of Polmix buffer and 10% dimethyl sulfoxide (DMSO; a denaturant) respectively topped up with the appropriate volume of ddH_2O. However for the reaction mixture amplifying region -163 to -379 bp ($\mathbf{P_2}$ primer pairs), DMSO was omitted and 2.0 mM $MgCL_2$ was used. The sequences of all the five primer pairs and the optimised conditions used for PCR are shown in ***Table 5.1***. PCR program conditions were similar to those used in ***Section 2.4.1***, ***Chapter 2*** except for the annealing step where annealing temperatures (T_a) used were optimised for each primer set.

Table 5.1: PCR conditions optimised for primers scanning *GCH1* promoter.

Primer pairs	Position from *tsp*	Sequence of PCR primers Sense and anti-sense strands	T_a (°C)	Amplicon (bp)
P_1:				
F_{p1}	-258	5'-TTTTCGGGTTCGGCTCATTCC-3'	56	306
R_{p1}	+48	5'-TACGCGACCTGCTTAGATCAC-3'		
P_2:				
F_{p2}	-379	5'-TTTCACAGGGCGAGGGGACCG-3'	64	217
R_{p2}	-163	5'-GGGGTTCGCAAGCGCAGGGAG-3'		
P_3:				
F_{p3}	-552	5'-GTGACACTTGCCCCGACAGCG-3'	60	211
R_{p3}	-342	5'-GCTGCATTGAAGGGCTCCGGT-3'		
P_4:				
F_{p4}	-768	5'-TATGTTTTCCAGGCTGGTCTCG-3'	62	348
R_{p4}	-421	5'-AAGCCACCGATTAGGAACCAAC-3'		
P_5:				
F_{p5}	-1080	5'-ACCCAGGAGGCGGAGGATGAAG-3'	62	374
R_{p5}	-707	5'-GGGGAGACCGAGGCGGGAGGA-3'		

5.3.3 Detection of novel variation in DNA by SSCP

The method used was described in ***Section 2.4.4, Chapter 2***. As a preliminary study, only 44 DNA samples from a NPHSII Master Array 1 were screened for sequence variations by PCR/SSCP. A typical protocol consisted of labelling the DNA samples by inclusion of α-^{32}P dCTP in the PCR amplification mixture. In brief, the PCRs were conducted in 30 µl volume with each reaction containing 50 ng of dried DNA (75°C / 10 minutes), 1.5 mM of $MgCL_2$, 10% DMSO, 10 pmole of each primer, 2 mM of each dNTP, 0.1 U *Taq* DNA polymerase and finally 0.2 mM α-^{32}P dCTP (***Table 5.2***) (See ***Section 2.4.1***, ***Chapter 2***). The cycling conditions were as follows: an initial denaturation step at 95°C for 5 minutes (min), followed by amplification for 35 cycles with denaturation at 95°C for 45 seconds (sec), annealing at a temperature optimised for each primer pair (***Table 5.1***) for 45 sec and extension at 72°C for 45 sec. Three µl of the radiolabeled PCR products were added to 6 µl of SSCP loading buffer, heat-denatured (95°C / 5 min) and immediately quenched on ice. Aliquots (5 µl) of the mixture comprising of single-stranded DNA fragments were loaded onto a 7.5% polyacrylamide gel, which was ran for 21 hours at 300 V (See ***Sections 2.4.4.1-2.4.4.5***).

Table 5.2: Preparation of PCR mix for each sample for SSCP

Reagent (μl)	Primer pair	
	P1, P3, P4, P5	P2
$MgCl_2$	0.9	1.2
DMSO	3	-
1/10 dCTP Polmix	3	3
F_p (~ 10 pmol)	0.1	0.1
R_p (~ 10 pmol)	0.1	0.1
Taq (added last)	0.1	0.1
ddH_2O	22.8	25.5
TOTAL	*30*	*30*

5.3.4 Generation of templates for sequencing

All 44 labelled DNA fragments exhibited a similar pattern of electrophoretic mobility on SSCP gels, when amplified by primers scanning regions [-421 to -768] and [-707 to -1080] of *GCH1* promoter. Therefore there was the possibility that a variant was located within the 19 bp sequence (i.e. within –707 to -768 region) overlapped by both amplified fragments. To identify the probable nucleotide change in both amplimers, that is [-421 to -768] and [-707 to –1080], the genomic DNA of nine samples exhibiting abnormal migration during SSCP were re-amplified prior sequencing. However there is the possibility that the 5' and 3' ends of amplicons, 348 bp and 374 bp (***Table 5.1***), could be read inefficiently during sequencing reactions due to the small

size, thereby affecting the quality of the sequence data and missing any variant in the overlapping sequence (QIAGEN Guide to Template Purification, 1998). To circumvent this problem, a PCR fragment (660 bp) covering the promoter from [-421 to -1080] bp was amplified by using the anti-sense primer $\mathbf{R_{p4}}$ (i.e. 5'-AAGCCACCGATTAGGAACCAAC-3') and the sense primer $\mathbf{F_{p5}}$ (i.e. 5'-ACCCAGGAGGCGGAGGATGAAG-3').

For the region scanning [-342 to -552] bp, three DNA samples exhibited abnormal mobility shifts during SSCP. The migration appeared as artefacts but nevertheless they were re-amplified by primer pairs $\mathbf{P_3}$ to check for any sequence change. PCR protocols were set up as above (***Section 5.3.4***) with appropriate primer pairs and with the radioactive dCTP replaced by 2 mM of cold dCTP. As previously, the cycle sequences consisted of a preliminary step of 95°C for 5 min, followed by 35 cycles of three consecutive steps of 45 sec: denaturation at 95°C, annealing at 62°C and a final extension at 72°C.

5.3.5 Extraction and purification of re-amplified products

Amplified PCR fragments (in 30 µl volume) were loaded on a 1% agarose and the clear discrete bands were excised from the gel followed by purification using QIAquick™ spin columns (QIAquick™ Gel Extraction Kit, QIAGEN) according to the manufacturer's instructions. The procedure used was described in ***Section 2.4.4.6, Chapter 2.***

5.3.6 Sequencing of DNA variants obtained by SSCP

Both strands (forward and reverse) of the amplified DNA fragments served as templates for sequencing, which is an adaptation of the Sanger method using fluorescently labelled dideoxynucleotides (ddNTPs). The methodology used was described in ***Section 2.4.4.7, Chapter 2***. In *resume*, approximately 60 ng of purified template PCR (***Sections 5.3.4*** and ***5.3.5***) and 3.2 pmole of either the forward or the reverse primer (***Section 5.3.4***) were used for each sequencing reaction. The cycle sequencing, carried out in a thermocycler (GeneAmp PCR 9700, PE Applied Biosystems), involved 25 cycles of denaturation at 96°C for 10 sec, annealing at 50°C for 5 sec and extension at 60°C for 4 min. Excess fluorescent dye terminators (ddNTPs) were removed by precipitation with 100% ethanol. The sequencing products were then added to 4 µl of loading buffer, heat denatured, quenched on ice and loaded on a 4.1% acrylamide-urea sequencing gel in an ABI Prism™ 377 DNA Sequencer (See ***Sections 2.4.4.8 and 2.4.4.9, Chapter 2***). The DNA sequences were analysed using the following software on an Apple Macintosh: Sequence Navigator (ABI Prism™ GeneScan®), Sequence Editor and Factura™.

5.3.7 Restriction enzyme analysis of polymorphisms

PCR/RFLP analysis was used to confirm the presence of any polymorphism(s) identified by SSCP and sequencing. Primer pairs used were $\mathbf{P_4}$ (i.e. 5'-TATGTTTTCCAGGCTGGTCTCG-3' and 5'-AAGCCACCGATTAGGAACCAAC-3') and $\mathbf{P_5}$ (i.e. 5'-ACCCAGGAGGCGGAGGATGAAG-3' and 5'-

GGGGAGACCGAGGCGGGAGGA-3') respectively. The Program Vector NTI Suite was used to search for a restriction endonuclease (RE), specific for the restriction cleavage site created or abolished by the base change on the allele detected by SSCP and sequencing. Briefly, 2U of restriction enzyme were added to 10 µl of PCR-amplified products, followed by digestion at the appropriate temperature (***Table 5.3***). Afterwards 10 µl of the digest (added to 2 µl of formamide dye) were loaded on a 7.5 % MADGE gel and visualised by autoradiography. Eventually, high-throughput PCR/RFLP protocols were established to genotype the NPHSII cohort (n = 2745) for the polymorphisms identified.

Table 5.3: Conditions optimised for restriction enzyme digestion.

Polymorphism	**-577 (G/A)**	**-796 (G/A)**
Restriction enzyme	*Bsm*F1	*Bsr*D1
Restriction site	5' G G G A C $(N)_{10}$↓ 3' 3' C C C T G $(N)_{14}$↑ 3'	5' G C A A T G N N↓ 3' 3' C G T T A C↑N N 3'
Type of allele cut	Wild	Mutant
PCR product (bp)	348	374
Digests sizes (bp)	206 + 142	290 + 84
Incubation	65°C / 3 hrs	60°C / 3 hrs
MADGE conditions	40 min / 7.5% gel	60 min / 7.5% gel

5.3.8 Statistical analysis of data

Observed genotype frequencies were compared to those expected under conditions of Hardy-Weinberg (H-W) equilibrium using the chi-square test with one degree of freedom (χ^2, 1 *df*). Allele frequencies were deduced from genotype frequencies. Pairwise linkage disequilibrium coefficient (Δ) was calculated for the polymorphisms genotyped. Both of the contingency tables used for calculation of these values (χ^2 and Δ) were already computerised on an Excel spreadsheet within the *Department of Cardiovascular Genetics*. The influence of genotype on plasma neopterin levels as well as cardiovascular risk factors and IHD risk was assessed using one-way ANOVA. Statistical analyses were carried out by Miss Jackie Cooper at the MRC Epidemiology and Medical Care Unit.

5.4 Results

5.4.1 Nature and location of polymorphisms detected in *GCH1*

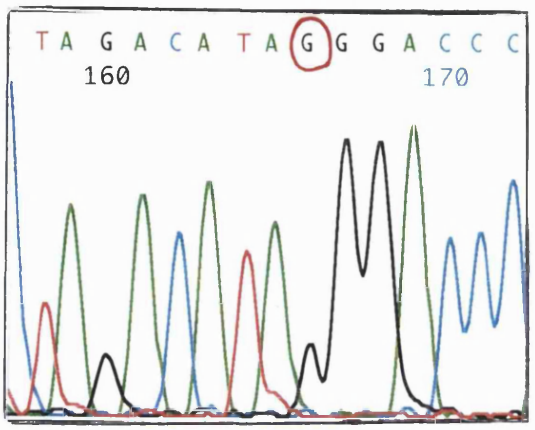
Mobility shifts were identified in PCR fragments [-421 to -1080] bp and [-342 to -552] bp of *GCH1* promoter. Direct DNA sequence analysis of the amplified fragment (by primers $\mathbf{F_{p5}}$ and $\mathbf{R_{p4}}$) spanning [-421 to -1080] region identified three novel bi-allelic polymorphisms (***Figure 5.3***). The three single base substitutions were located at positions -577 (G/A) (***Figure 5.4***), -741 (T/C) (***Figure 5.5***) and -796 (G/A) (***Figure 5.6***) from the *tsp*. The aberrantly migrating bands detected by SSCP analysis on amplifying the region [-342 to -552] bp (by primer pair $\mathbf{P_3}$) were not due to any

variants as the sequence corresponded exactly to the reported human *GCH1* sequence (Genbank/EMBL *Accession numbers P30793 EMBL L29478*). A few base changes, different from the reported sequence, were also detected. Several nucleotide insertions occurred at the following locations: A at -948, -976 and -1037; C at -980 and -1000 and two A at -969 (***Figure 5.7***).

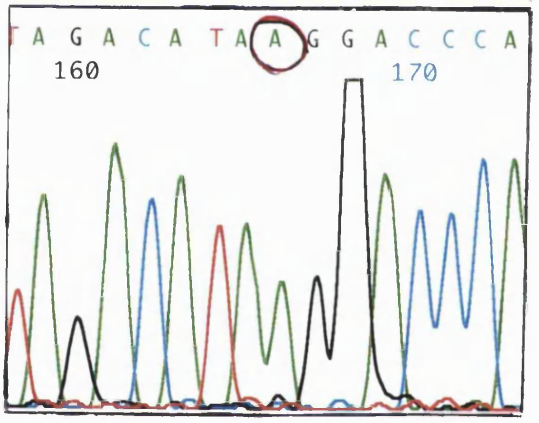
Figure 5.4: Electropherogram of *GCH1* polymorphism located at -577.

The novel promoter DNA sequence polymorphism -577 (G/A) was detected by automated sequencing with an ABI 377 DNA sequencer. Panel 1 and 2 indicate individuals homozygous for the G and A alleles respectively, while panel 3 represents a heterozygote.

1


T A G A C A T A G G G A C C C
160
170

2


T A G A C A T A A G G A C C C A
160
170

3

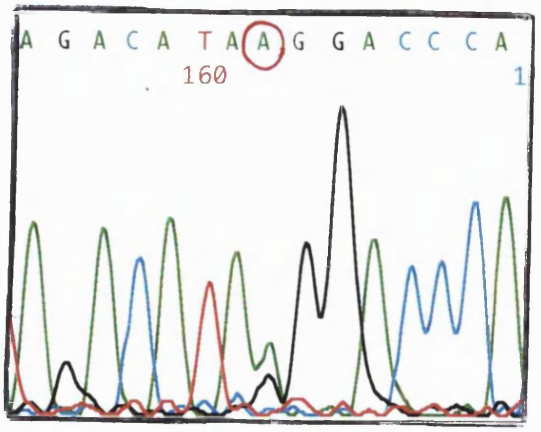
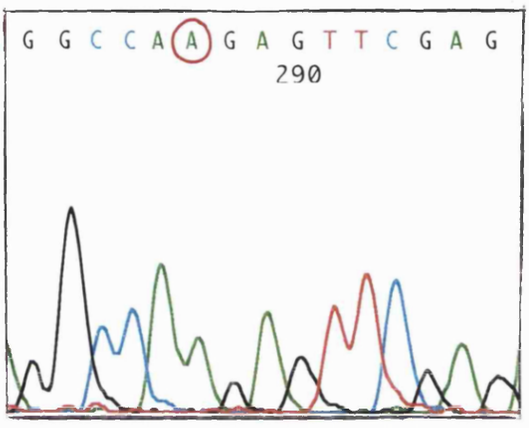

A G A C A T A A G G A C C C A
160

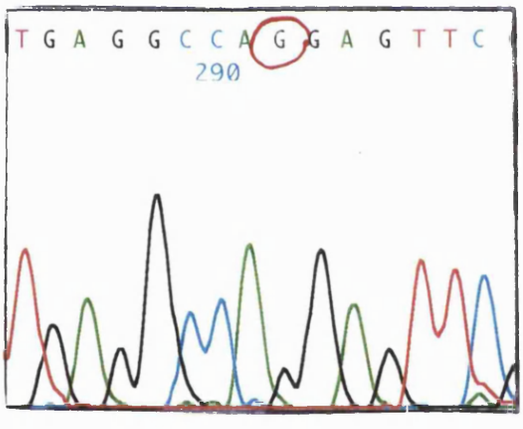
Figure 5.5: Electropherogram of *GCH1* polymorphism located at -741.

The promoter novel DNA sequence polymorphism -741 (T/C) was detected by automated sequencing with an ABI 377 DNA sequencer. Panel 1 and 2 indicate individuals homozygous for the A and G alleles respectively, while panel 3 represents a heterozygote. The reverse (i.e. negative) DNA strands are shown.

1


G G C C A A G A G T T C G A G
290

2


T G A G G C C A G G A G T T C
290

3

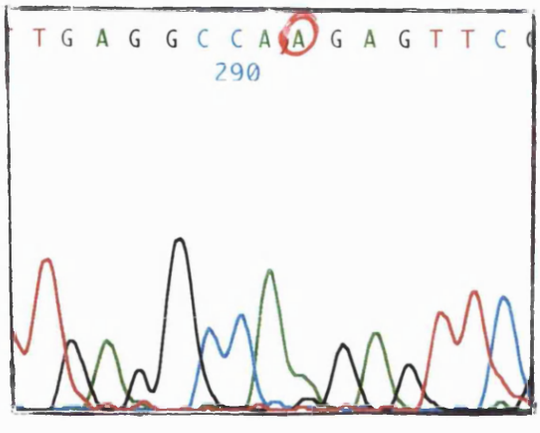
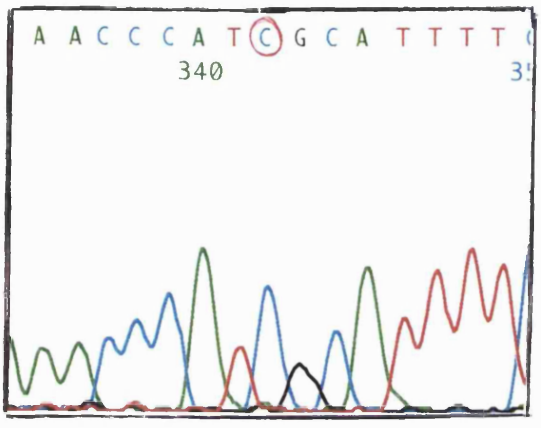

T G A G G C C A A G A G T T C
290

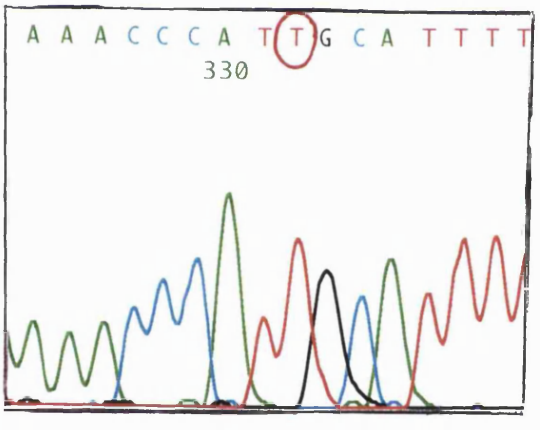
Figure 5.6: Electropherogram of *GCH1* polymorphism located at -796.

The novel DNA sequence promoter polymorphism -796 (G/A) was detected by automated sequencing with an ABI 377 DNA sequencer. Panel 1 and 2 indicate individuals homozygous for the C and T alleles respectively, while panel 3 represents a heterozygote. The reverse (i.e. negative) DNA strands are shown.

1


A A C C C A T C G C A T T T T T
340

2


A A A C C C A T T G C A T T T T
330

3

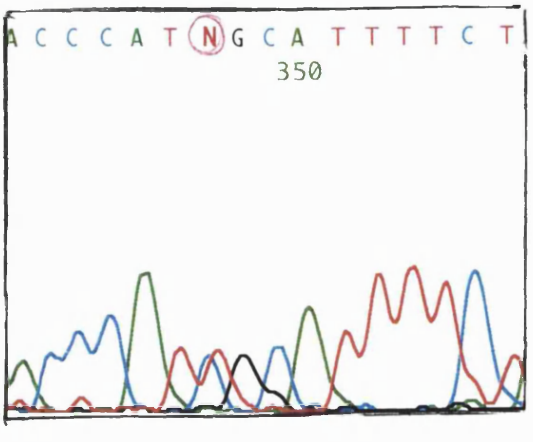

A C C C A T N G C A T T T T C T
350

Figure 5.7: Electropherogram of base insertions in *GCH1* promoter.

The base changes, differently from the published sequence, were detected by automated sequencing with an ABI 377 DNA sequencer. The following panels show the electropherograms of the base insertions on the reverse (i.e. negative) DNA strands with their respective locations.

Panel 1: T at -948 and –976

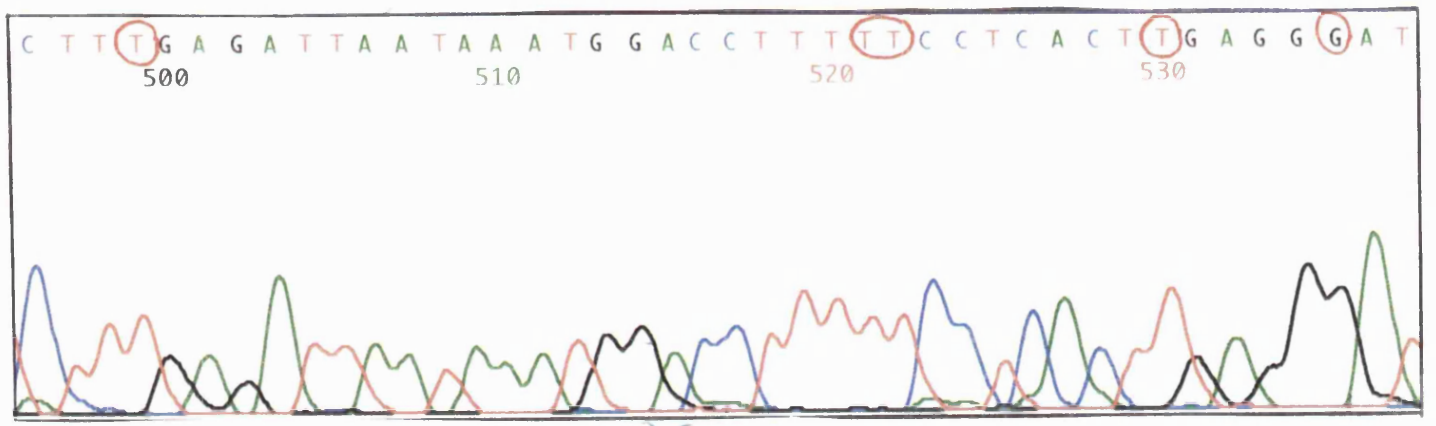
TT at –969

G at -980

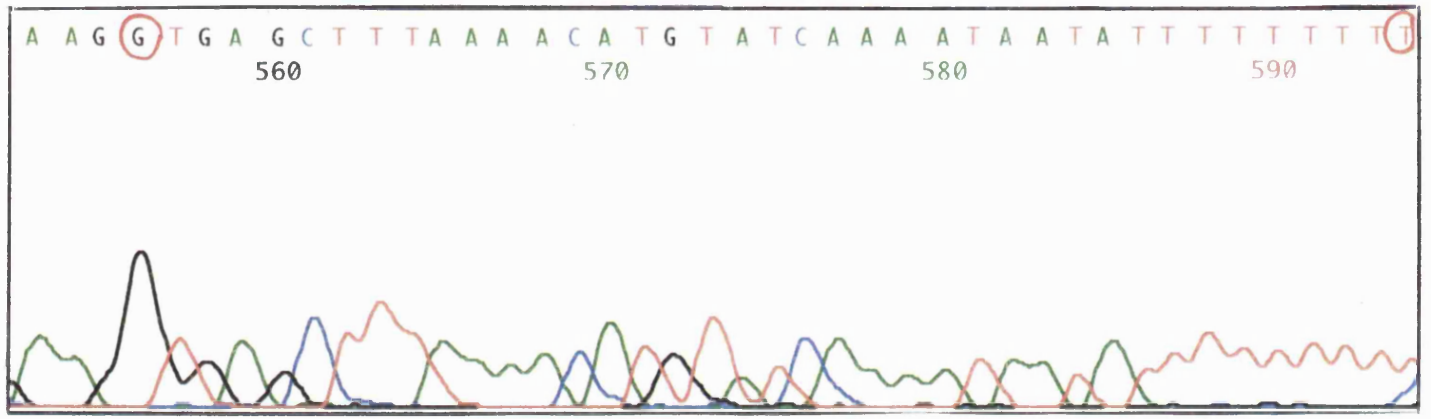
Panel 2: T at -1037

G at -1000

1


C T T T G A G A T T A A T A A A T G G A C C T T T T T C C T C A C T T G A G G G A T
500
510
520
530

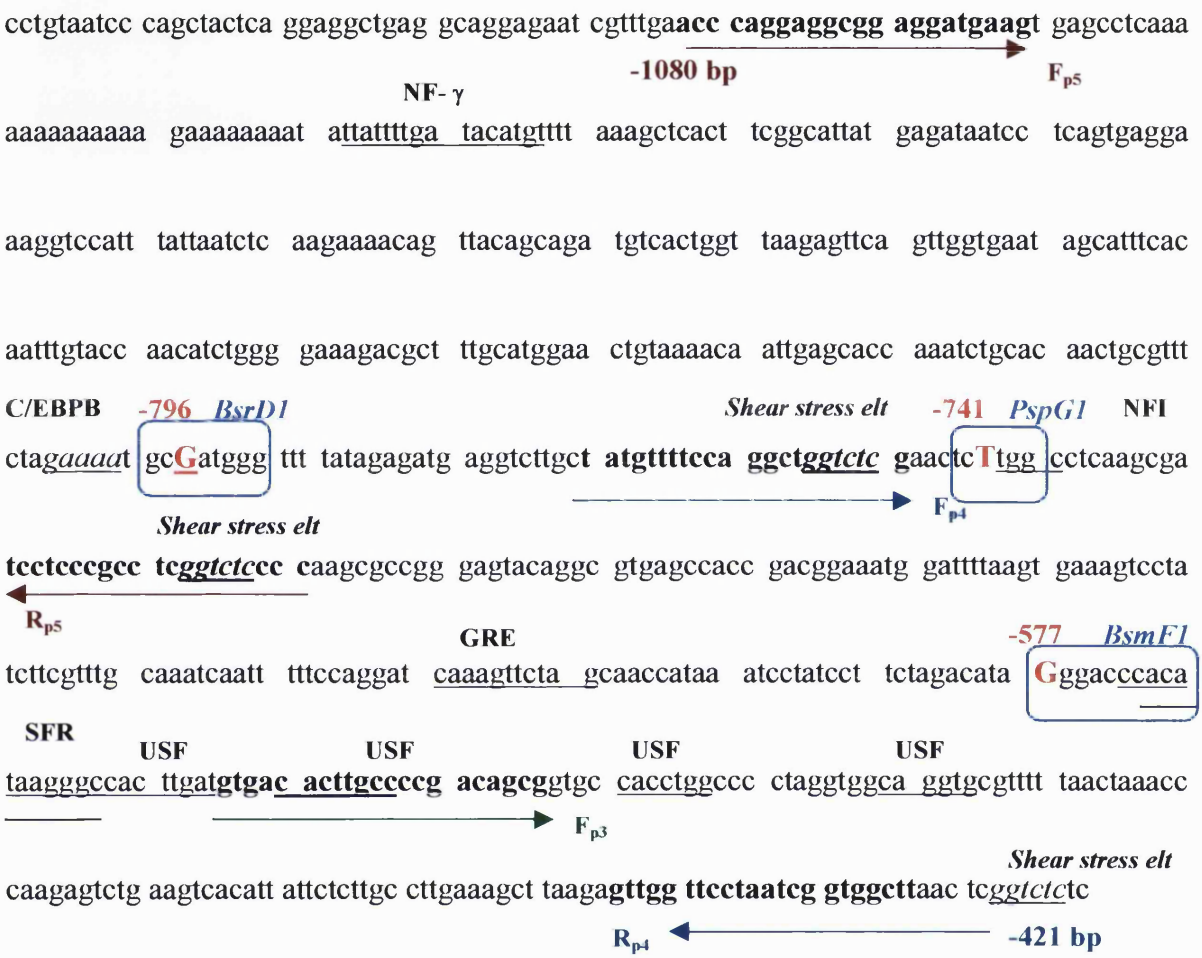
2


A A G G T G A G C T T T A A A A C A T G T A T C A A A A T A A T A T T T T T T T T T
560
570
580
590

5.4.2 Allele-specific restriction sites in polymorphic *GCH1* fragments

Three RFLPs were identified. Base substitution at positions -796 ($G^{-796}\rightarrow A$) and -741 ($T^{-741}\rightarrow C$) create allele-specific cutting sites (G C **A** A T G N N ↓) and (↓ C **C** A/T G G) for the REs *Bsr*DI and *Psp*GI respectively. However, base substitution at location -577 ($G^{-577}\rightarrow A$) deletes a restriction site (**G** G G A C $(N)_{10}$ ↓) for *Bsm*F1 (***Figure 5.8***). The restriction sites are boxed in blue and the polymorphisms identified are in upper case and in red.

Figure 5.8: Double-stranded DNA sequence of *GCHI* scanning -421 to -1080 bp region.



5.4.3 Genotyping of polymorphisms detected in NPHSII cohort

Only two bi-allelic substitutions, -796 (G/A) and -577 (G/A) were analysed by restriction enzyme digestion of the specific PCR amplified fragment (RFLPs) and both gave a pattern of fragments distinguishable from that seen with normal DNA on a MADGE gel. To genotype -741 (T/C) the fragment [-768 to -707 bp] was amplified. In the presence of the bi-allelic substitution, the restriction enzyme *Psp*GI, with recognition site (↓ C C A/T G G), digests the PCR fragment in two fragments of 26 and 36 bp. However after digestion with *Psp*GI, the digests were too small to be detectable by gel electrophoresis. Various attempts at PCR/RFLP optimisation with several primer pairs and another restriction enzyme (i.e. *Bst*N1) proved to be unsuccessful. Since -741 (T/C) polymorphism is closely located to -796 (G/A), the latter was genotyped.

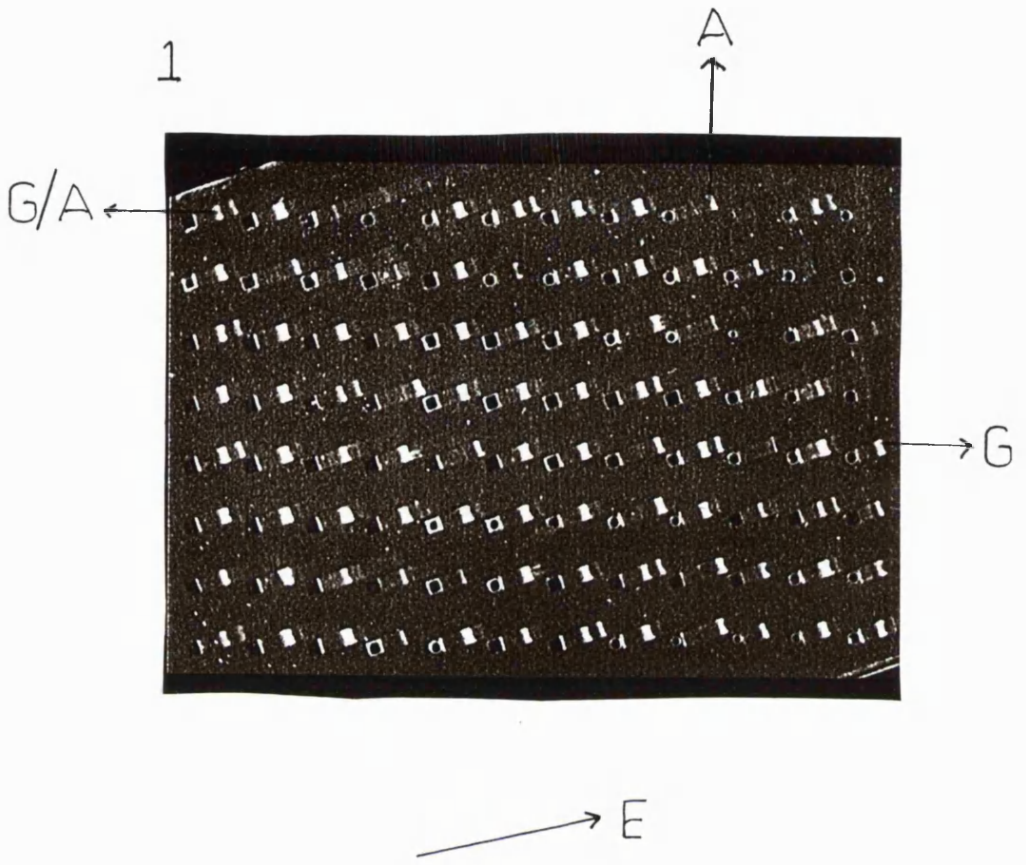
NPHSII Master Array 1 was genotyped for both -796 (G/A) and -577 (G/A) polymorphisms and as the genotype frequencies were consistent with H-W equilibrium, high-throughput genotyping protocols for both variants were then set up in the whole NPHSII cohort. Photographs of the 7.5 % MADGE gels showing the pattern of fragments obtained after digestion of PCR-amplified DNA with the appropriate RE and electrophoresis are shown in ***Figures 5.9*** and ***5.10***.

Figure 5.9: Photograph of MADGE gel of NPHSII Master Array 4 showing electrophoresis of fragments after digestion of PCR-amplified DNA.

The bi-allelic polymorphism -796 (G/A) in *GCH1* promoter was detected by *Bsr*DI digestion of the PCR product (374 bp). The restriction endonuclease recognises the restriction enzyme site (G C **A** A T G N N↓) where there is a G nucleotide substitution by A in the rare allele. In the presence of nucleotide A at position -796, the amplified product is cleaved into fragments of 290 bp and 84 bp after enzyme digestion. About 10 μl of amplified product (with primer pair $\mathbf{P_5}$) was digested with 5 μl of cocktail digests, containing 2 units of *Bsr*DI, for about 3 hours at 60°C and then subjected to electrophoresis on a 7.5 % MADGE gel for 60 minutes.

Figure 5.10: Photograph of MADGE gel of NPHSII Master Array 9 showing electrophoresis of fragments after digestion of PCR-amplified DNA.

The bi-allelic polymorphism -577 (G/A) in *GCH1* promoter was detected by *Bsm*FI digestion of the PCR product (348 bp). The restriction endonuclease recognises the restriction enzyme site (**G** G G A C $(N)_{10}$ ↓) where there is a G nucleotide in the 'wild' allele. In the presence of nucleotide G at position -577, the amplified product is cleaved into fragments of 206 bp and 142 bp after enzyme digestion. About 10 μl of amplified product (with primer pair $\mathbf{P_4}$) was digested with 5 μl of cocktail digests, containing 2 units of *Bsm*FI, for about 3 hours at 65°C and then subjected to electrophoresis on a 7.5 % MADGE gel for 40 minutes.


1
A
G/A
G
E
2
G/A
G

5.4.4 Allele and genotype frequencies of *GCH1* polymorphisms

Allele and genotype frequencies of both polymorphisms are shown in ***Table 5.4***. Genotype frequencies were consistent with H-W equilibrium and conformed to the H-W law only for the -577 (G/A) polymorphism and not for -796 (G/A) in NPHSII cohort.

Table 5.4: Allele and genotype frequencies for *GCH1* polymorphisms.

Variant	Allele frequency		Genotype frequency (%)			*χ^2 (1 df)	*P*
	G	A	GG	GA	AA		
-577 (G/A)	0.88	0.11	1897 (79.4)	453 (19.0)	40 (1.7)	3.96	**0.05**
-796 (G/A)	0.83	0.17	1770 (74.2)	437 (18.3)	179 (7.5)	275.3	**< 0.0001**

* The chi squared test was used to determine whether there is a hypothesised statistical significant difference between the observed and expected genotype frequencies. Null hypothesis states that there is no significant difference between the frequency of the observed and expected genotypes in the NHPSII study.

5.4.5 Linkage disequilibrium between *GCH1* polymorphisms

Maximum likelihood methods were used to calculate the pairwise linkage disequilibrium coefficient for both polymorphisms (***Table 5.5***). Variants located at positions -577 and -796 were found to be in strong linkage disequilibrium, with a pairwise linkage disequilibrium coefficient of 0.79 ($P < 0.0001$).

Table 5.5: Linkage disequilibrium coefficient for *GCH1* polymorphisms.

Polymorphism	-796 (G/A)	*P*
-577 (G/A)	0.79	**< 0.0001**

5.4.6 Plasma neopterin and *GCH1* genotype

The hypothesis that *GCH1* polymorphisms influence basal neopterin circulating levels was tested by measuring plasma neopterin in a subset of 939 NPHSII subjects: 288 from North Mymms (N. Mymms) and 243 from Chesterfield practices as well as 513 selected at random (Random) from the whole cohort (***See Chapter 6***). To adjust for any renal influence, plasma neopterin values were corrected for serum creatinine and are also presented as neopterin:creatinine ratio expressed as nmol/μmol in the result tables (Müller *et al.*, 1991). As neopterin levels differed considerably according to clinics, the results were practice adjusted. Neopterin values were log-transformed and all the results are presented as geometric mean (approximate SD) and frequency.

In the whole NPHSII cohort (practice adjusted), there was a statistical difference in mean neopterin levels according to genotype (P = 0.01) and this achieved high significance on adjustment with renal function (P = 0.001). The GG group had the highest neopterin level (6.30 μM), the AA group had the intermediate level (6.13 μM) and the GA group the lowest level (5.85 μM). After correction with creatinine, this trend was confirmed as the GA group retained the lowest value (0.061 μM) while the GG (0.067 μM), and AA (0.064 μM) groups had the highest and intermediate values respectively (***Table 5.6***).

There was no evidence of an interaction between neopterin levels and -577 (G/A) polymorphism in the Random (P = 0.29), North Mymms (P = 0.08) and Chesterfield (P = 0.25) data sets (***Table 5.6***). After correction for creatinine, the results observed in North Mymms (P = 0.01) and Chesterfield (P = 0.05) practices achieved statistical significance but did not in the random set (P = 0.21). However, due to the low frequency in CC group in both practices (North Mymms: 4; Chesterfield: 1) and the trend in both practices being irregular, they were disregarded. As there was no general tendency according to genotype, the results from all these groups were not considered during analysis.

Table 5.6: Mean plasma neopterin* according to -577 (G/A) polymorphism.

Variable	-577 (G/A) Genotype	Mean (SD) N			
		NPHSII	**Random**	**N. Mymms**	**Chesterfield**
Neopterin	**GG**	6.30 (1.69)	6.21 (1.53)	6.18 (1.84)	6.81 (1.64)
		565	314	193	117
	GA	5.85 (1.26)	5.89 (1.32)	5.61 (1.19)	6.20 (1.28)
		120	59	49	21
	AA	6.13 (2.20)	6.21 (2.63)	5.44 (1.42)	7.01 (-)
		11	7	4	1
	P	**0.01**	**0.29**	**0.08**	**0.25**
Neopterin/ Creatinine	**GG**	0.067 (0.019)	0.065 (0.018)	0.065 (0.018)	0.074 (0.019)
		557	309	189	117
	GA	0.061 (0.013)	0.060 (0.014)	0.059 (0.011)	0.064 (0.011)
		120	59	49	21
	AA	0.064 (0.025)	0.066 (0.03)	0.057 (0.018)	0.068 (-)
		11	7	4	1
	P	**0.001**	**0.21**	**0.01**	**0.05**

* No need to adjust for any other variables as no variable was associated with genotype

There was a highly significant (6.8%, P = 0.006) decrease in mean neopterin when carriers of one rare allele (heterozygote, GA) or two rare alleles (homozygote, AA) were compared to carriers with no rare allele in NPHSII set. This result was further confirmed after compensation with creatinine values (9.0%, P = 0.0008) as well as in the North Mymms (9.2%, P = 0.004) and Chesterfield (13.5%, P = 0.01) practices (*Table 5.7*).

Table 5.7: Mean levels of neopterin according to -577 (G/A) polymorphism.

$G^{-577}{}_{\varsigma}A$	NPHSII	Random	North Mymms	Chesterfield
Genotype	**Mean (SD) N**	**Mean (SD) N**	**Mean (SD) N**	**Mean (SD) N**
Neopterin				
GG	6.30 (1.69) 565	6.21 (1.53) 314	6.18 (1.84) 193	6.81 (1.64) 117
GA/AA	5.87 (1.35) 131	5.92 (1.47) 66	5.60 (1.20) 53	6.23 (1.50) 22
P	**0.006**	**0.14**	**0.02**	**0.11**
Neopterin/Creatinine				
GG	0.067 (0.019) 557	0.065 (0.018) 309	0.065 (0.018) 189	0.074 (0.019) 117
GA/AA	0.061 (0.014) 131	0.061 (0.016) 66	0.059 (0.012) 53	0.064 (0.011) 24
P	**0.0008**	**0.12**	**0.004**	**0.01**

For NPHSII sample (after practice adjustment), -796 (G/A) genotype determined plasma neopterin levels (P = 0.05), even after renal compensation (P = 0.003). The

trend in the following groups was as follows: GG had highest mean value (6.29 μM), GC had intermediate value (5.97 μM) and CC the lowest value (5.90 μM). After compensation with creatinine, the GA and AA groups achieved the same mean neopterin values (0.061 μM) while GG still retained the highest level (0.066 μM) (***Table 5.8***).

However, it appeared that -796 (G/A) polymorphism exerted no influence on neopterin levels in the random (P = 0.14), North Mymms (P = 0.17) and Chesterfield (P = 0.52) data sets (***Table 5.8***). After adjustment for renal function, the results observed in North Mymms (P = 0.04) and random (P = 0.06) became slightly significant but not in the Chesterfield practice (P = 0.22). Both in the North Mymms and random sets, the GG group had the highest neopterin levels (0.066 μM and 0.065 μM respectively) but the trend in both the GA and AA groups were reversed. For the North Mymms practice, neopterin levels in GA and AA groups were 0.061 μM and 0.058 μM respectively, while for the random set they were 0.059 μM and 0.061 μM respectively.

Table 5.8: Mean plasma neopterin* according to -796 (G/A) polymorphism.

Variable	-796 (G/A) Genotype	Mean (SD) N			
		NPHSII	**Random**	**N. Mymms**	**Chesterfield**
Neopterin	**GG**	6.29 (1.61)	6.21 (1.40)	6.13 (1.75)	6.76 (1.64)
		535	296	182	108
	GA	5.97 (1.56)	5.87 (1.63)	5.82 (1.51)	6.40 (1.50)
		118	58	48	22
	AA	5.90 (1.43)	5.85 (1.42)	5.41 (1.40)	7.03 (1.35)
		46	26	15	9
	P	**0.05**	**0.14**	**0.17**	**0.52**
Neopterin/ Creatinine	**GG**	0.066 (0.018)	0.065 (0.017)	0.066 (0.018)	0.073 (0.019)
		528	291	178	108
	GA	0.061 (0.016)	0.059 (0.015)	0.061 (0.015)	0.066 (0.014)
		117	57	47	22
	AA	0.061 (0.016)	0.061 (0.016)	0.058 (0.013)	0.073 (0.016)
		46	26	15	9
	P	**0.003**	**0.06**	**0.04**	**0.22**

*: No need to adjust for any other variables as no variable was associated with genotype

A significant (5.4%; $P = 0.02$) decrease in neopterin level was found in carriers of one rare allele (heterozygote, GA) or two rare alleles (homozygote, AA) compared to carriers with no rare allele in the NPHSII set (***Table 5.9***). This evidence was confirmed after renal adjustment (7.6%, $P = 0.0006$) in the above set and in North Mymms (9.1%, $P = 0.01$) and random (7.7%, $P = 0.02$) sets.

Table 5.9: Mean levels of neopterin according to -796 (G/A) polymorphism.

$G^{-796}{}_{\varsigma}A$	NPHSII	Random	North Mymms	Chesterfield
Genotype	**Mean (SD) N**	**Mean (SD) N**	**Mean (SD) N**	**Mean (SD) N**
Neopterin				
GG	6.29 (1.61) 535	6.21 (1.40) 296	6.13 (1.75) 182	6.76 (1.64) 108
GA/AA	5.95 (1.52) 164	5.86 (1.55) 84	5.73 (1.48) 63	6.57 (1.48) 31
P	**0.02**	**0.05**	**0.09**	**0.57**
Neopterin/Creatinine				
GG	0.066 (0.018) 528	0.065 (0.017) 291	0.066 (0.018) 178	0.073 (0.019) 108
GA/AA	0.061 (0.016) 163	0.060 (0.016) 83	0.060 (0.015) 62	0.068 (0.015) 31
P	**0.0006**	**0.02**	**0.01**	**0.17**

5.4.7 *GCH1* genotype, plasma neopterin and IHD risk

Cox regression was used to assess the effect of genotype on the risk of an IHD event. There was no evidence of a relationship between both polymorphisms studied and IHD risk (***Table 5.10***) according to either a dominant or a recessive models. Kaplan-Meier survival plots of the effects of -577 (G/A) and -796 (G/A) genotypes on IHD event-free survival are illustrated in ***Figure 5.11***.

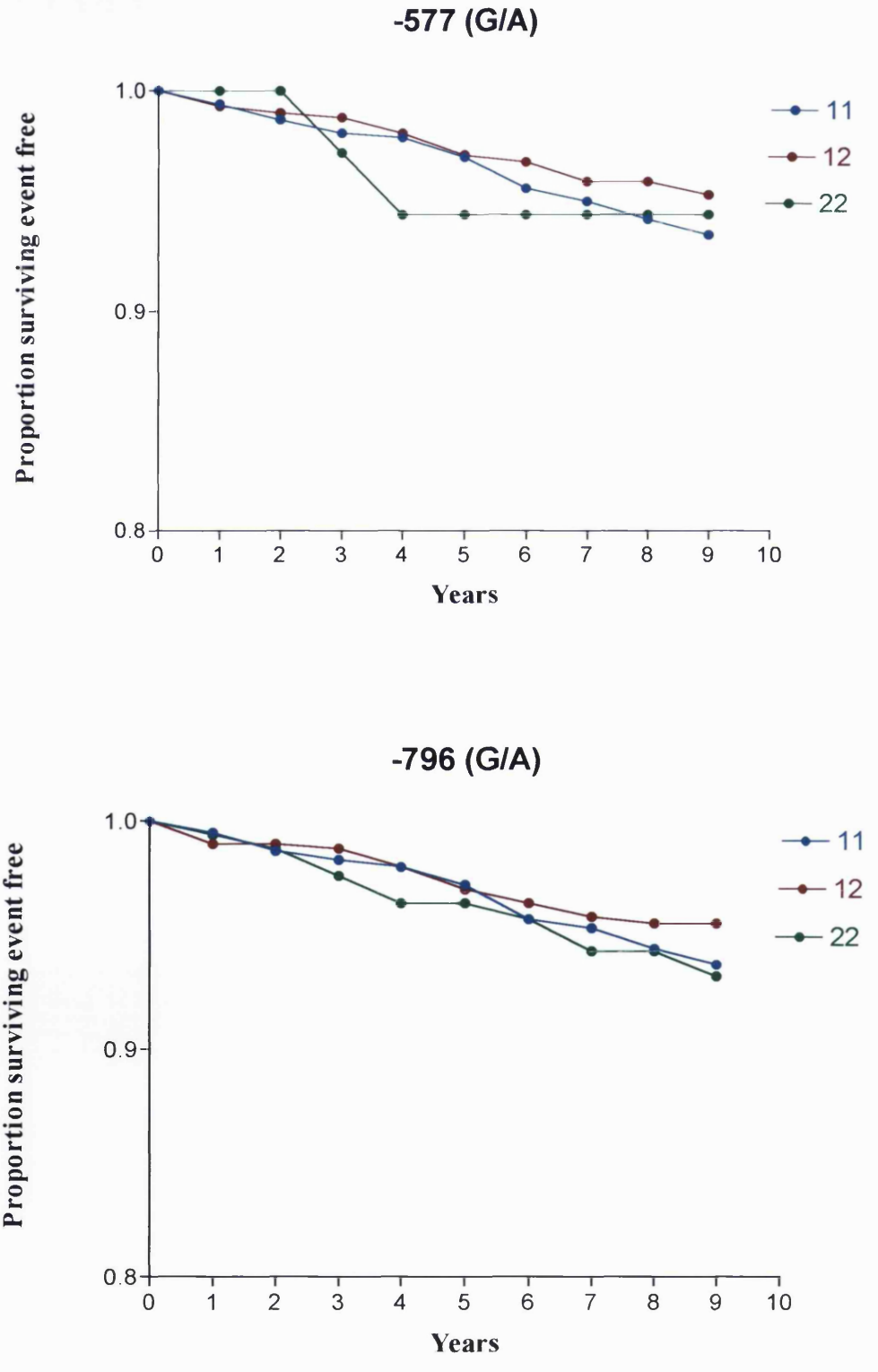
Table 5.10: Influence of *GCH1* genotype on IHD risk.

Polymorphism	Model	Genotype	Hazard ratio (95% CI)	*P*
-577 (G/A)	Recessive	GG/GA	1.0	**0.97**
		AA	1.03 (0.25-4.17)	
	Dominant	GG	1.0	**0.27**
		GA/AA	0.76 (0.46-1.24)	
-796 (G/A)	Recessive	GG/GA	1.0	**0.56**
		AA	0.88 (0.57-1.35)	
	Dominant	GG	1.0	**0.76**
		GA/AA	1.11 (0.58-2.12)	

Figure 5.11: Kaplan-Meier survival plots of -577 (G/A) and -796 (G/A) genotypes on IHD event-free survival.

There was no evidence that any polymorphism was associated with IHD.

(11: common homozygote GG; 12: heterozygote GA; 22: rare homozygote AA)



Among the 939 subjects with mean plasma neopterin measurements available on entry, 20 IHD events were recorded. However, there was also no evidence that the level of plasma neopterin (recorded at entry) was associated with IHD ($P = 0.88$) (***Table 5.11***).

Table 5.11: Mean and median neopterin levels by CHD event.

	Frequency	Mean (SD)	Median
Controls	919	6.24 (1.55)	6.13
Cases	20	6.13 (0.68)	6.26
P			**0.88**

When IHD events were also examined according to quartiles ($P = 0.16$) or quintiles ($P = 0.18$) of neopterin, there was still no evidence that risk was associated with neopterin levels (***Results not shown***).

5.4.8 Relationship between *GCH1* variants and CVD risk factors

None of the *GCH1* polymorphisms had a statistically significant influence on diastolic (dBP) or systolic blood pressure (sBP), age, body mass index (BMI), cholesterol and fibrinogen levels (***Results not shown***).

5.5 Discussion

5.5.1 Location and probable significance of *GCH1* polymorphisms

Three novel bi-allelic polymorphisms were identified in the promoter of *GCH1*: a G-to-A transition at positions -577 and -796 as well as a T-to-C transition at position -741 (***Figure 5.3***). The primers were designed to cover short consensus sequences, which are known to constitute the basal elements of a promoter, including the initiator codon of transcription (CAG) as well as TATA (position -22), CAAT (position -65) and seven copies of GC boxes, recognisable by SP1 factors and located upstream of the *tsp*. The primers also covered sequences called *response elements*, recognisable by regulatory inducible transcription factors (TFs) such as, CCAAT-box/enhancer binding protein (C/EBP), nuclear factor 1 (NF1), nuclear factor gamma (NF-γ) and four upstream stimulatory factors (USF). The response elements covered were glucocorticoid response element (GRE), serum response element (SRE) and three shear stress elements. These inducible factors and specific elements are either activated or synthesised at specific times or in specific tissues. For example GRE is essential for response to steroid hormones and deletion of this region will not affect basal gene transcription. The polymorphisms identified were located as follows: -796 (G/A) found three bases before a C/EBP binding site, -741 (T/C) located two bases after the NF1 binding site and -577 (G/A) situated three bases after a SRE. By themselves, the polymorphisms identified did neither abolished nor created any TF binding site (See ***Figure 5. 3*** and ***Figure 5.8***).

The possible effects of these polymorphisms (if any) on *GCH1* transcription are unclear. Being adjacent to regulatory response elements, the base changes might be involved in the complex [TF-DNA] as the physical region to which the factors bind (~ 20 bp) are typically longer than the consensus sequences of the elements (< 10 bp) and extend for a short distance on either side (Lewin, 1997). If the motif involved in DNA binding is mutated, the TF can no longer bind, or may bind with an altered affinity, to its consensus sequence thereby altering gene transcription (Angel and Karin, 1991). Therefore, we can speculate that these polymorphisms may play a role in regulating the transcription of *GCH1* in response to intrinsic or extrinsic signals and affect the levels of neopterin at the phenotype level. Transfection experiments involving promoter-reporter gene constructs will provide a clearer picture of the involvement of these polymorphic regions in gene transcription (Nomura *et al.*, 1993; Kullo *et al.*, 1999).

Previous transfection experiments (using various constructs of the promoter regions coupled to the luciferase reporter gene) carried out to study the functional analysis of *GCH1* promoter showed that the region from -452 to the *tsp* was essential for promoter activity (Witter *et al.*, 1996). Deletions of the following regions [-209 to *tsp*] and [-42 to *tsp*] reduced the promoter activity by 50% and 100% respectively whereas deletions of 2 kb bases upstream of -452 bp did not alter basal gene transcription (Witter *et al.*, 1996). However it is possible that regions upstream of -452 could influence gene transcription induced by cytokine or other regulatory factors.

5.5.2 H-W and linkage disequilibrium between the polymorphisms

When the whole NPHSII samples were genotyped (32 Master Arrays), only the -577 (G/A) polymorphism was found to be in H-W equilibrium. Analysis of the individual practices showed all nine observing H-W except for Practice 1 (i.e. Camberley).

Conversely the -796 (G/A) polymorphism genotype distributions differed considerably from H-W equilibrium in all nine individual clinics as well as in the whole NPHSII cohort. This finding was confirmed even when PCR/RFLP protocols were repeated for nine Master Arrays. The observed GA and AA frequencies (n = 437 and n = 179) were very different as opposed to the expected frequencies (n = 660 and n = 66). As the enzyme *BsrD1* cuts the mutant allele, the possibility of incomplete digestion is unlikely since there would have been more GG and GA observed. The H-W law states that *"In a large random mating population, there will be no change in gene frequency from generation to generation except where there is a mutation or migration or selection*". One of these factors must be involved and this issue needs further investigation.

DNA sequencing results showed the common type alleles for all three polymorphisms always segregated together, as did the rare alleles. This finding was illustrated by the high degree of allelic association between the two polymorphisms studied (-796 (G/A) and -577 (G/A); $\Delta = 0.79$). It must be borne in mind however that these two loci are in close proximity, being separated by only 218 bp.

5.5.3 Associations between *GCH1* polymorphisms and neopterin

The present study identified three novel polymorphisms within the promoter region of human *GCH1* gene by molecular scanning. The bi-allelic variations, i.e. -577 (G/A), -741 (T/C) and -796 (G/A), have the potential to alter either *GCH1* enzyme/protein expression or its function. Two of the polymorphisms studied, -577 (G/A) and -796 (G/A), were found to be strongly associated in this particular population; a finding not surprising given their close physical proximity. For both -577 (G/A) and -796 (G/A) polymorphisms, carriers of one or both rare allele(s) had significantly lower neopterin levels compared to carriers of the common allele in the overall NPHSII subjects. Levels of neopterin on the whole are significantly lower in subjects with genotype GA and AA (6.8% and 5.4% respectively) compared to genotype GG. The trend is that the common allele exhibits higher neopterin levels for both variants while the rare allele decreases the mean value. It is possible that the common allele increases the probability of an inflammatory risk or inflammation-associated complications, that is has a negative influence on neopterin levels, while the rare allele exerts a beneficial effect on the immune system. If this finding is confirmed in other population genetic studies, it could be useful in predicting *GCH1* gene involvement in inflammation and eventually IHD.

5.5.4 *GCH1* polymorphisms, plasma neopterin and IHD risk

There was no statistical evidence that either *GCH1* genotype or levels of plasma neopterin had an influence on IHD event. These findings must be interpreted with caution as the negative results do not automatically imply absence of effect. The possibility of another polymorphism located elsewhere or near the gene being linked to the disease must not be ruled out. At the time of analysis, 159 men had an IHD event in the NPHSII cohort whereas only 20 men from that group had neopterin levels measured. A plausible explanation is that the frequency of IHD events is not enough to make a real difference to be picked up although genotype will not change after an infarct! The failure to detect an association of IHD with any of the variants does not exclude the *GCH1* gene involvement with the susceptibility to a coronary event. IHD will evolve with time as we are dealing with apparently healthy individuals who have a high prevalence of subclinical disease, which with time will evolve into the clinical disease itself.

The present results seem to indicate that plasma neopterin does not predict IHD event in middle-aged healthy subjects, but is rather a non-specific marker of inflammation (Thompson *et al.*, 1995; Schumacher *et al.*, 1997). This inference is in agreement with the results of my recent investigations, where a strong correlation between neopterin levels and fibrinogen, a biochemical index of inflammation, was found (***Section 6.4, Chapter 6***). Inflammation is thought to have a causal role in vascular damage as well as in coagulation (Ross, 1993; Miller *et al.*, 1996). Therefore, high levels of neopterin may enable the identification of subjects who are most susceptible (at a higher risk) to

develop atherosclerosis (Tatzber *et al.*, 1991; Weiss *et al.*, 1994), especially in view that its pathogenesis is considered to be partly mediated by a chronic inflammatory component (Libby *et al.*, 1991; Levenson *et al.*, 1995). Nevertheless, another marker of inflammation called serum C-reactive protein was associated with IHD in healthy middle-aged men, older than 65 years (Strandberg and Tilvis, 2000).

A limitation of this study was that only baseline neopterin measurement was available as the blood was sampled about eight years ago, well before the occurrence of any IHD event. At the individual level, a single neopterin measurement may not reflect the true basal/circulating neopterin measurement as its levels vary according to the cell immune status (Fuchs *et al.*, 1988a; 1988c; Fuith *et al.*, 1991). The measurement of neopterin is time dependent, i.e. the shorter the time between its measurement and an IHD event, the higher would be its predictive value as it reflects the *in vivo* activation of the cellular immune system (Samsonov *et al.*, 1992). It would be ideal to have a situation where blood is sampled prior to and after an IHD event and then to assess the difference in circulating neopterin levels. Neopterin is an important marker of evolving disease but is not necessarily the causal pathway. Two possibilities are to be considered: either elevated levels of neopterin, caused by an ongoing inflammatory reaction, are triggered by the IHD event itself or conversely a rise in neopterin concentration, reflecting the inflammatory component of the atherosclerotic process, may precede IHD.

As none of the conventional cardiovascular risk factors, i.e. BP, age, BMI, cholesterol and fibrinogen, were associated with -796 (G/A) and -577 (G/A) polymorphisms, it seems that *GCH1* gene does not interact directly with any of these factors to contribute

to the atherogenesis process. Although, the general belief is that an association between two variables will imply a direct specific causal relationship between both, the likelihood that *GCH1* may confer susceptibility to IHD via the pterin pathway cannot be excluded.

To conclude, the main findings of this chapter are:

- Identification of three novel bi-allelic polymorphisms, -577 (G/A), -741 (T/C) and -796 (G/A) within *GCH1* promoter;
- Both *GCH1* polymorphisms, i.e. -577 (G/A) and -796 (G/A), influenced plasma neopterin levels significantly;
- There was no evidence of a relationship between *GCH1* polymorphism and IHD risk;
- None of the *GCH1* polymorphisms exerted a statistically significant influence on conventional IHD risk factors, i.e. BP, cholesterol, age, BMI and fibrinogen; and
- Plasma neopterin levels can neither be an independent IHD risk factor nor used as an indicator of cardiovascular event although it has a prognostic value for an underlying chronic inflammation.

CHAPTER 6

NEOPTERIN: MARKER OF GTPCH ACTIVITY

6.1 Objectives

- To measure plasma neopterin as a marker of GTP cyclohydrolase I (GTPCH) activity.
- To study the association between neopterin and conventional ischaemic heart disease (IHD) risk factors.

6.2 Background

6.2.1 BH_4: Role and regulation

L-*erythro*-(6R)-5,6,7,8-tetrahydrobiopterin (BH_4) is synthesised by the pterin pathway *in vivo* from guanosine triphosphate (GTP) via GTPCH (Nichol *et al.*, 1985; Blau and Niederweiser, 1985). BH_4 has a dual role in NO synthesis: the pteridine binds co-operatively with endothelial nitric oxide synthase (eNOS), converting it into an active high-affinity state for its substrate (Tayeh and Marletta, 1989), and then the bound cofactor participates in the L-arginine:NO pathway in a redox manner (Kwon *et al.*, 1989). As BH_4 regulates endothelial NO production (Schmidt *et al.*, 1992), its deficiency and/or dysfunction has been associated with endothelial dysfunction (ED) (for review see ***Section 5.2, Chapter 5***), a feature of atherosclerosis (Busse and

Fleming, 1996; Cosentino and Lüscher, 1998). BH_4 deficiency has been associated with a decrease in NO bioactivity (Ueda *et al.*, 2000). Conversely elevations in BH_4, implicated in the vascular response to inflammation, induces NO formation from eNOS and may predispose to atherogenesis (Bhagat and Vallance, 1999).

As GTPCH is the key rate-limiting enzyme of the pterin pathway, BH_4 can be used as an index of its activity (Fuchs *et al.*, 1988*b*). Attempts at measuring BH_4 by High Pressure Liquid Chromatography (Fukishima and Nixon, 1980) proved to be unsuccessful as it was extremely labile and needed to be derivatised prior estimation, consequently introducing risks of contamination within the samples. An alternative approach was to quantify neopterin, a side reaction product of the pterin pathway, which is metabolically stable in frozen plasma samples (Müller *et al.*, 1991). As a very small molecule, neopterin (M_w = 253) readily penetrates tissue barriers (Aulitzky *et al.*, 1988) and becomes detectable in the bloodstream (Fuith *et al.*, 1991).

In vitro and *in vivo* studies showed that upon challenge with cytokines, activated monocytes and macrophages synthesised and secreted neopterin (Fuchs *et al.*, 1982; Huber *et al.*, 1983; Nathan, 1986; Woloszczuk *et al.*, 1986; Werner *et al.*, 1990). This was paralleled by a rise in intracellular concentration of GTP and GTPCH activity (Schoedon *et al.*, 1987). Macrophages, the hallmark of chronic inflammation are believed to play a leading role in the onset of clinically manifest IHD (Ross, 1986; Meade, 1994).

6.2.2 Neopterin: Marker of BH_4 biosynthesis

Biosynthesis of neopterin starts from GTP with 7,8-dihydroneopterin triphosphate (7,8-NH_2-TP) as the first intermediate (Blau and Niederwieser, 1985). It is subsequently metabolized to BH_4 by 6-pyruvoyl tetrahydropterin synthase (PTPS) and sepiapterin reductase (SR) in the liver, lymphocytes and neuroendocrine tissue (Curtius *et al.*, 1986; Werner *et al.*, 1993). Compared to GTPCH, the activity of PTPS and SR are extremely low within monocytes and macrophages (Werner *et al.*, 1990), which consequently accumulate 7,8-NH_2-TP and after hydrolysis by ubiquitous phosphatases is excreted as 7,8-dihydroneopterin (7,8-NH_2) or neopterin (Huber *et al.*, 1984) (***Figure 6.1***).

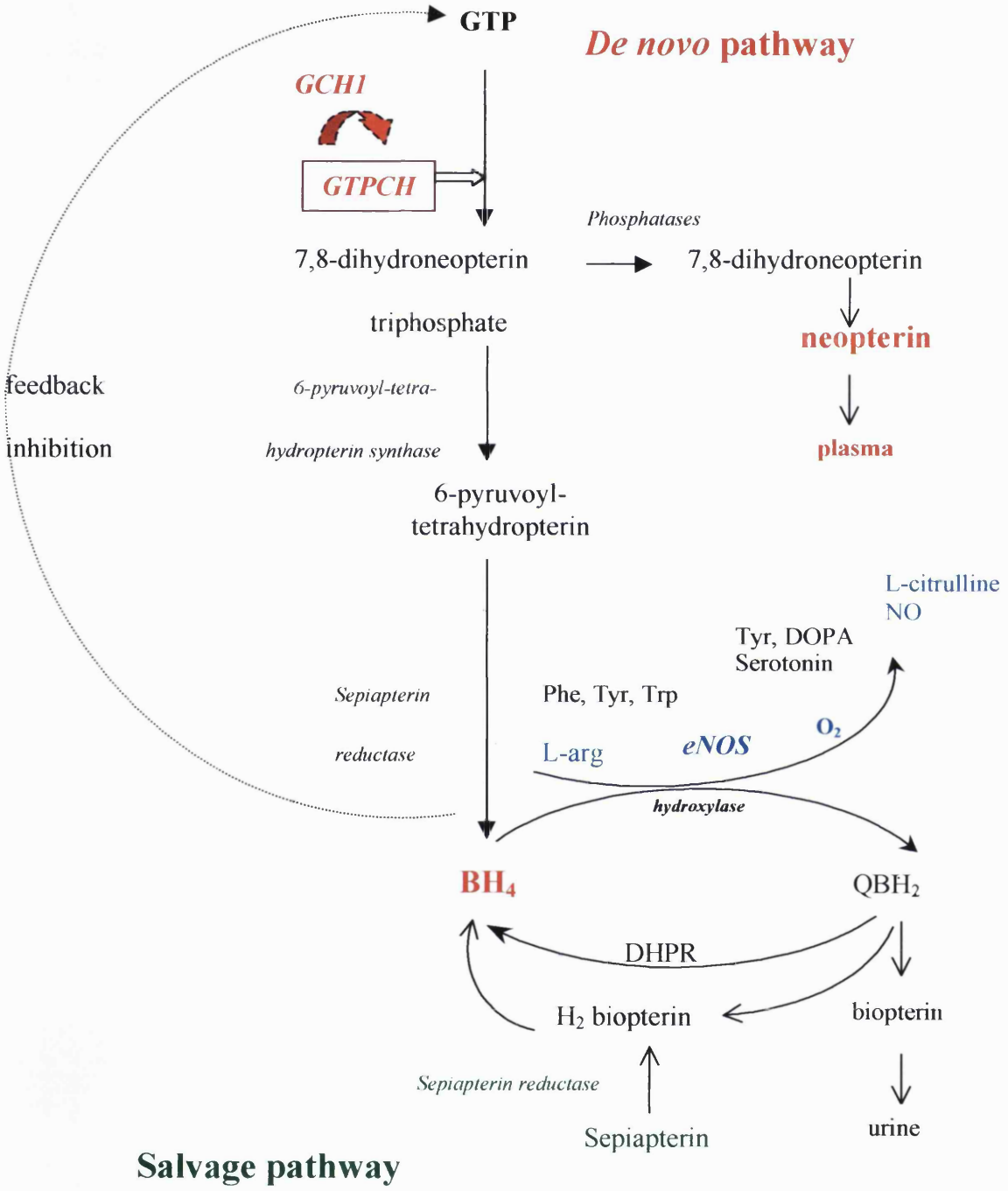
BH_4 provides electrons to reduce molecular oxygen, while it is in turn oxidised to quinonoid dihydrobiopterin (QBH_2) and eventually converted to biopterin for excretion. Alternatively, dihydropteridine reductase (DHPR) recycles QBH_2 formed during the hydroxylation of aromatic amino acids (Werner *et al.*, 1993). Monooxygenases hydroxylate phenylalanine (pala) to tyrosine (tyr), tyrosine to dopamine (DOPA), noradrenaline and adrenaline and tryptophan (trp) to serotonin and melatonin (Kojima *et al.*, 1992).

Neopterin occurs in two forms in body fluids: fully oxidised aromatic neopterin and reduced non-fluorescent 7,8-NH_2 (Werner *et al.*, 1989, Prior *et al.*, 1986). About 45% of the total neopterin is in the oxidised form when assayed in freshly collected plasma samples (Levine and Milstien, 1984) and this form is stable unless the samples are stored at -20°C while reduced neopterin derivatives are not (Wachter *et al.*, 1992).

Previous studies showed that the diagnostic information obtained was independent of the type of neopterin derivatives chosen for assay (Fuchs *et al.*, 1988c). Oxidising the hydrogenated forms of neopterin serves no real purpose and therefore they were not assayed (Fuchs *et al.*, 1989).

Aromatic neopterin was therefore used as a reliable alternative marker to evaluate the status of the BH_4 biosynthetic pathway (Wachter *et al.*, 1992) and as an index of GTPCH activity (Schoedon *et al.*, 1987). It was assayed by a validated commercial Enzyme-Linked ImmunoSorbent Assay (ELISA), convenient for routine use and applicable to a large population study (Rokos and Rokos, 1983; Nagatsu *et al.*, 1984; Fahey *et al.*, 1990; Müller *et al.*, 1991). Neopterin and creatinine rise in a dependent manner and relating neopterin to creatinine compensated for physiological alterations of urine concentration (Aulitzky *et al.*, 1988). Therefore for the association studies, the results for plasma neopterin were also reported as neopterin:creatinine ratio (Neop/Creat) expressed as nmol/μmol (Müller *et al.*, 1991). The associations between neopterin and risk factors of IHD were also studied.

Figure 6.1: Proposed *de novo* and salvage pathway of BH_4 synthesis.



6.3 Subjects and methods

6.3.1 Subjects

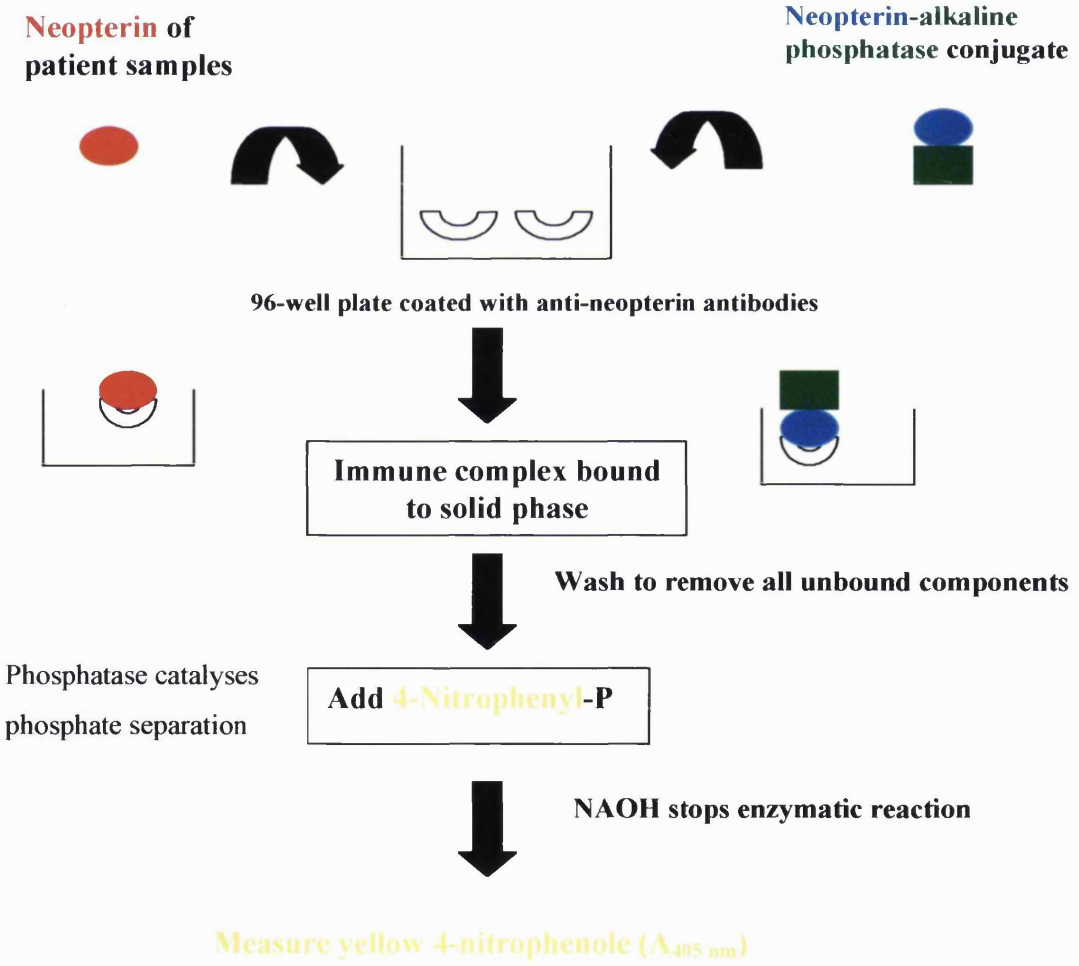
NPHSII subjects (previously described in ***Section 2.1, Chapter 2***) were recruited from North Mymms (n = 290) and Chesterfield (n = 260) as well as 571 individuals selected at random from the nine practices of the study. All the participants were middle-aged (50-61 years) Europeans of Caucasian origin and without any clinical evidence of IHD.

6.3.2 Neopterin assay

Neopterin levels in plasma samples, obtained on entry in 1993, were determined by using an *in vitro* enzyme immunoassay kit. The principle and protocol were reviewed in ***Section 2.6, Chapter 2***. The ELITest® Neopterin (Henning Berlin GmbH, Germany) is a competitive ELISA used for the quantitative determination of aromatic neopterin using 96-well microtiter plates coated with polyclonal sheep anti-neopterin antibodies (***Figure 6.2***). The lower limit of detection in this assay was 2 nmol/l with an intra- and inter-assay variation of 3.5% and 6.5% respectively. NPHSII plasma samples (50 µl) were assayed directly without performing any prior oxidation and derivatisation.

Figure 6.2: Competitive enzyme immunoassay for neopterin determination.

Cavities of the microtiter plate are coated with anti-neopterin-antibodies. Both neopterin of the patient plasma samples and the neopterin-enzyme conjugate compete for the antibodies' binding sites to form a complex. The intensity of the 4-nitrophenole colour, measured in optical density (OD), is dependent on the concentration of alkaline phosphatase bound in the cavities at a constant reaction time. The neopterin concentration within the patient samples is determined from a standard curve generated by plotting OD *vs* neopterin standards' concentrations.



6.3.3 Statistical analysis of data

Associations between plasma neopterin and well-established IHD risk factors were studied. They were fibrinogen, cholesterol, triglycerides, blood pressure (BP) and smoking status. The effect of other biochemical variables, i.e. creatinine, BMI, age and coagulation Factor VII (clotting potential), which could influence neopterin levels, was also investigated. The statistical analyses were performed using Intercooled STATA version 5.0 by Miss Jackie Cooper at the MRC Epidemiology and Medical Care Unit.

6.4 Results: Associations between neopterin and IHD risk factors

Physiological neopterin and creatinine range were 2.16-33.5 nmol/l and 49-307 μmol/l respectively. Only 288, 243 and 513 neopterin values were valid from North Mymms (n = 290), Chesterfield (n = 260) and random samples (n = 571) respectively. Mean levels of the subjects from North Mymms and Chesterfield practices as well as the randomly computer-selected subjects were pooled and denoted as 'Total NPHSII' (n = 939). In the four data sets studied, mean plasma neopterin levels was about 6.0 nmol/l (***Table 6.1***). There was heterogeneity among all nine practices (P = 0.0001) as the concentrations differed significantly (***Table 6.2***).

Table 6.1: Neopterin levels in the four data sets studied.

Data set	Number	Minimum	Maximum	Mean (SD)
North Mymms	288	2.78	33.5	6.07 (1.64)
Chesterfield	243	4.42	15.5	6.61 (1.40)
Random*	513	2.16	20.5	6.17 (1.43)
Total NPHSII*	939	2.16	33.5	6.23 (1.53)

*: adjusted for practice

Table 6.2: Neopterin levels in all nine NPHSII practices.

Practice	Number	Mean (SD)
Camberley	59	5.93 (1.06)
Carnoustie	73	6.23 (1.40)
St Andrews	61	5.23 (1.37)
Aston Clinic	17	6.51 (1.54)
Harefield	49	7.26 (1.79)
Chesterfield	243	6.61 (1.40)
Halesworth	86	6.10 (1.34)
Parkstone	63	6.30 (1.42)
North Mymms	288	6.07 (1.64)
P value		**0.0001**

6.4.1 Creatinine and plasma neopterin

Creatinine correlated positively with neopterin in North Mymms (r = 0.35, $P < 0.0001$), random (r = 0.15, $P < 0.0005$) and the total NPHSII samples (r = 0.19, $P < 0.0001$), whereas it did not achieve significance in the Chesterfield set (r = 0.09, $P = 0.17$). After adjustment of plasma neopterin with serum creatinine, the correlations for all the samples investigated achieved significant results ($P < 0.02$) (***Table 6.3***).

Table 6.3: Correlation of serum creatinine with plasma neopterin.

Correlation of creatinine with	North Mymms r (*P*)	Chesterfield r (*P*)	Random* r (*P*)	Total NPHSII* r (*P*)
Neopterin	0.35 (<0.0001)	0.09 (0.17)	0.15 (0.0005)	0.19 (<0.0001)
Neop/Creat	-0.14 (0.02)	-0.43 (<0.0001)	-0.38(<0.0001)	-0.32 (<0.0001)

*: adjusted for practice

6.4.2 Fibrinogen and plasma neopterin

Fibrinogen associated positively with neopterin in all the samples studied, but achieved significance only in North Mymms (r = 0.20, $P = 0.0006$) and total NPHSII (r = 0.11, $P = 0.0006$) but not in Chesterfield (r = 0.07, $P = 0.25$) and random samples

(r = 0.07, P = 0.10). These results were similar after adjustment for serum creatinine (*Table 6.4*).

Table 6.4: Correlation coefficient (r) for fibrinogen and plasma neopterin.

Correlation of fibrinogen with	North Mymms r (*P*)	Chesterfield r (*P*)	Random* r (*P*)	Total NPHSII* r (*P*)
Neopterin	0.20 (0.0006)	0.07 (0.25)	0.07 (0.10)	0.11 (0.0006)
Neop/Creat	0.26 (<0.0001)	0.02 (0.78)	0.06 (0.21)	0.10 (0.002)

*: adjusted for practice

6.4.3 Cholesterol and plasma neopterin

There was a significant inverse relationship between serum total cholesterol and plasma neopterin in Chesterfield (r = -0.19, P = 0.002), random (r = -0.11, P = 0.02) and the total NPHSII samples (r = -0.12, P = 0.0002) after practice adjustment (*Table 6.5*). However the association did not achieve statistical significance in North Mymms (r = -0.04, P = 0.52). These correlations were unaffected after adjustment for serum creatinine.

Table 6.5: Correlation of total cholesterol with plasma neopterin.

Correlation of Cholesterol with	North Mymms r (*P*)	Chesterfield r (*P*)	Random* r (*P*)	Total NPHSII* r (*P*)
Neopterin	-0.04 (0.52)	-0.19 (0.002)	-0.11 (0.02)	-0.12 (0.0002)
Neop/Creat	-0.04 (0.51)	-0.21 (0.001)	-0.13 (0.004)	-0.13 (<0.0001)

*: adjusted for practice

6.4.4 ApoA1 and plasma neopterin

ApoA1 was negatively correlated with plasma neopterin in all four sets studied (***Table 6.6***), that is North Mymms (r = -0.22, P = 0.0001), Chesterfield (r = -0.12, P = 0.06), random (r = -0.15, P = 0.001) and total NPHSII (r = -0.16, P < 0.0001). After adjustment with creatinine, the Chesterfield data was no longer significant (r = -0.05, P = 0.49).

Table 6.6: Correlation coefficient (r) for ApoA1 and plasma neopterin.

Correlation of ApoA1 with	North Mymms r (*P*)	Chesterfield r (*P*)	Random* r (*P*)	Total NPHSII* r (*P*)
Neopterin	-0.22 (0.0001)	-0.12 (0.06)	-0.15 (0.001)	-0.16 (<0.0001)
Neop/Creat	-0.19 (0.002)	-0.05 (0.49)	-0.10 (0.04)	-0.10 (0.003)

*: adjusted for practice

6.4.5 Biochemical variables and plasma neopterin

None of the conventional IHD risk factors such as fibrinogen, BP, age, BMI, triglycerides, smoking status as well as coagulation Factor VII associated significantly with plasma neopterin, even after compensation for creatinine. Negative results were not presented.

6.5 Discussion

6.5.1 Associations between neopterin and IHD risk factors

Neopterin levels in all nine NPHSII practices were statistically different (***Table 6.2***) due to heterogeneity among clinics. Results of North Mymms and Chesterfield sets could be biased towards a specific population and/or by small sample size (Thompson *et al.*, 1987; Humphries *et al.*, 1999). Therefore when analysing the results, priority

was given to the random sample and the pooled NPHSII data, after practice adjustment, as they provided a more accurate insight of the relationships occurring in the whole cohort. Both sets had similar mean neopterin values (***Table 6.1***).

As expected, neopterin correlated positively and highly significantly with creatinine (an indicator of renal function) in most data sets as both of these metabolites are excreted by the kidneys (Müller *et al.*, 1991). The ratio Neop/Creat has been shown to be constant over lengthy periods in healthy subjects as it is almost independent of any fluid physiological variation, i.e. sweating. However, an elevation in both plasma neopterin and creatinine have been reported in atherosclerosis, due to an impairment of renal function caused by kidney damage (Weiss *et al.*, 1994).

Plasma neopterin associated positively with fibrinogen, a clotting factor synthesised by the liver. Fibrinogen levels vary considerably during the acute phase response, characterising the early phases of inflammation (Humphries, 1996). It was associated with the incidence of IHD in NPHSI (Meade *et al.*, 1986), with inflammation (Thompson *et al.*, 1995) and was classified as a major risk factor for CVD (Meade *et al.*, 1987; Humphries, 1995). Elevated plasma fibrinogen levels, either due to genetic or environmental factors, (Humphries *et al.*, 1995; Hamsten *et al.*, 1987) may cause a hypercoagulable state (Meade *et al.*, 1980), platelet hyperaggregation, precipitate monocytes recruitment into atherosclerotic lesions and enhanced blood viscosity (Levenson *et al.*, 1995). Fibrinogen levels reflect the inflammatory state of the vascular wall as it is implicated in the pathogenesis of plaque formation (de Maat *et al.*, 1996). Plasma fibrinogen infiltrates the intima due to an increased in endothelial permeability and its subsequent transformation to fibrin may initiate intimal thickening

thereby inducing the migration and proliferation of VSMCs from the media (Naito *et al.*, 1990). In the maturing atherosclerotic plaque, fibrin deposits in repeated layers thereby disorganising the endothelial cells and causing ED (Thompson *et al.*, 1995; Shats *et al.*, 1997). All these phenomena contribute significantly to the genesis of myocardial infarction (MI), a major thrombotic complication of atherosclerosis.

Atherosclerotic lesions arise from excessive inflammatory response due to an aggression to the endothelium (Ross 1986) and are composed of lipid-laden foam cells, developed from macrophages. An emerging hypothesis is that by causing a rise in fibrinogen, inflammation may be involved in the onset of IHD (Wilhelmsen *et al.*, 1984; Thompson *et al.*, 1995) and this is paralleled by an increase in neopterin levels, secreted by activated monocytes and macrophages. Fibrinogen's involvement in IHD is illustrated by the seasonal fluctuation in its levels: highest during winter and lowest in summer (Stout *et al.*, 1991). This trend is associated with a well recognised pattern of excess deaths from clinical events (i.e. stroke and IHD) during winter months and also coincides with indices of infection (Woodhouse *et al.*, 1994). These results show that macrophages and monocytes are involved in activation of cell-mediated immune system in coronary subjects (Gupta *et al.*, 1997). The data obtained may signify that the NPHSII cohort is suffering from a latent/silent chronic inflammation, which might be the basis of atherogenesis, as these men are not hospitalised. This evidence is in line with the findings of Tatzber *et al.* who found that non-hospitalised patients (with atherosclerotic symptoms) had higher statistically non-significant neopterin values compared to healthy controls as the number of activated macrophages producing neopterin was not considerable (Tatzber *et al.*, 1991; Schumacher *et al.*, 1992).

An inverse association of total serum cholesterol with plasma neopterin was observed. As neopterin is a marker of inflammation (preceding ED), it would be expected to correlate positively with cholesterol, an IHD risk factor (Brunner *et al.*, 1977), previously associated with ED (Cayatte *et al.*, 1994). But this is not so. However, a low total cholesterol level *per se* does not necessarily indicate a low risk of developing IHD. This is in accord with the findings of Framingham Study (Castelli *et al.*, 1986). Furthermore, cytokines (i.e. TNF-α and IL-6) have been reported to cause a rapid fall in the concentration of plasma cholesterol *in vivo* (Ettinger *et al.*, 1994). One of the mechanism proposed by which these cytokines lead to acquired hypocholesterolemia is by decreasing the hepatic synthesis and/or secretion of apolipoproteins.

The lipoprotein and apolipoprotein profiles were shown to be altered in atherosclerosis, MI and inflammation (Wanner *et al.*, 1997). The composition of total cholesterol is roughly as follows: 45% of low-density lipoproteins (LDL), 20% of high-density lipoproteins (HDL) and 20% of very low-density lipoproteins (VLDL) (Beeso *et al.*, 1999). Therefore, the values of cholesterol may reflect LDL component, due to its highest proportion. Elevated plasma LDL were shown to be the greatest predictors of CHD and MI risk as the cholesterol in atherosclerotic plaques comes exclusively from LDL. Atherosclerotic lesions consist mainly of cholesterol-laden foam cells, developed from activated monocytes and macrophages, containing oxidatively modified LDL (Henriksen *et al.*, 1981; Pitas, 1990). These lesions then develop into fatty streaks and plaques. Modified LDL releases chemotactic and cytotoxic factors, which induce monocytes and macrophages cell migration within the arterial wall, thereby enhancing neopterin formation due to atherosclerosis associated activation of the neopterin pathway (Tatzber *et al.*, 1991).

Unfortunately, one of the flaws of NPHSII is that no LDL and HDL measurements were performed. However, ApoB levels were determined and they reflect LDL concentrations (Grundy *et al.*, 1987). No significant relationship was found between ApoB (LDL apolipoprotein) and plasma neopterin. This result, which is at odds with the findings of Tatzber *et al.*, may suggest that LDL cholesterol do not play a direct significant role in the pterin pathway in this particular cohort. Or, another possible explanation may be that more powerful risk factors, which are of greater biological significance than the cholesterol concentration, operate in the NPHSII. However, Ettinger and colleagues found that cytokines, biological mediators of inflammation, did not affect cellular concentrations of ApoB mRNA whilst decreasing ApoA1 mRNA concentrations in a dose-related fashion (Ettinger *et al.*, 1994).

Interestingly, there was an inverse relationship between ApoA1 (HDL apolipoprotein) levels and mean neopterin. Low ApoA1 levels, or its absence altogether, is associated with early atherosclerosis (Keys, 1980) while low HDL cholesterol concentration was shown to be inversely related with premature coronary artery disease (CAD) (Tornvall *et al.*, 1991). These observations are in line with those of Zangerle who found that urinary neopterin correlated inversely with HDL and not with plasma triglycerides (Zangerle *et al.*, 1994). The main function of HDL is the maintenance of cholesterol homeostasis by uptake of free cholesterol from peripheral cells during the 'reverse cholesterol transport' to the liver for elimination (Glomset *et al.*, 1977). Therefore HDL plays a protective role in atherosclerosis (Miller and Miller, 1975; Gordon *et al.*, 1977). Although the findings are consistent with the hypothesis that high neopterin levels (inflammatory marker) associate inversely with low ApoA1 levels (IHD risk

factor), the exact mechanism involved in this process remains unclear. Thus, neopterin might alter HDL metabolism and could be responsible for the depressed levels of HDL cholesterol observed during chronic inflammation.

Plasma neopterin levels are not influenced by smoking status in NPHSII subjects and this result is consistent with the observation of Burns and collaborators (Burns *et al.*, 1991). However, Holt *et al.* found that smoking induced the plasma levels of many acute-phase proteins, particularly C-reactive protein (CRP) and fibrinogen, in healthy individuals suggesting that smoking induces a low grade chronic inflammatory response in the pulmonary bronchi and alveolae (Holt, 1987). The products of tobacco combustion promote the binding of platelets and monocytes to the endothelial lining by increasing the secretion of adhesion molecules, thereby precipitating thrombosis (Heitzer *et al.*, 1996). Nicotine, a major constituent of cigarette smoke, is believed to increase the retention of fibrinogen by the arterial wall, and this might be a mechanism whereby cigarette smoking exerts its atherogenic effects (Allen *et al.*, 1989).

When the relationship between risk factors such as BP, age, BMI, triglycerides and coagulation Factor VII with neopterin levels were studied, there were no significant statistical findings, differently from Weiss *et al.* who reported an age-dependent rise in neopterin levels (Weiss *et al.*, 1994). Most probably, neopterin and BP are mechanistically unrelated and differences will be picked out in subgroups with disease.

6.5.2 Plasma neopterin, inflammation and IHD risk

Results obtained support the hypothesis of a low-grade inflammation, reflecting ED due to prevalent atherosclerosis (Ridker *et al.*, 1998) in NPHSII subjects. Neopterin is a marker for macrophage activation (Fuchs *et al.*, 1988c) and an inflammatory marker (Schumacher *et al.*, 1997). Elevated neopterin levels may contribute to the pathogenesis of atherosclerosis either directly or indirectly via other pathways, i.e. inflammation, thrombosis and fibrinolysis (Kuller and Tracy, 2000). Atherosclerosis bears a strong resemblance to an inflammatory process, with the generation of cytokines, proliferation of vascular smooth muscle cells (VSMCs), activation of monocytes and macrophages and a hypercoagulable state preceding the genesis of atherothrombosis (Ernst and Koenig, 1993). The role of inflammation in CAD was further supported by reports of its association with inflammatory markers (de Maat *et al.*, 1996; Maseri *et al.*, 1996). An inflamed vascular wall in patients with CAD may increase the levels of cytokines, i.e. tumour necrosis factor-alpha (TNF-α), which regulate the synthesis of acute phase proteins (Haverkate *et al.*, 1997). Therefore plasma levels of fibrinogen and other markers of inflammatory response, i.e. CRP and neopterin, will rise (Jurges *et al.*, 1996). Neopterin was associated with the following conditions: atherosclerosis (Tatzber *et al.*, 1991; Weiss *et al.*, 1994), dilated cardiomyopathy and chronic myocarditis (Samsonov *et al.*, 1992), congestive heart failure (Fuchs *et al.*, 1993), acute rheumatic fever (Samsonov *et al*, 1993), acute MI (Melichar *et al.*, 1994), unstable angina (Gupta *et al.*, 1997), chronic and acute coronary syndromes (Schumacher *et al.*, 1997).

The exact origin of basal neopterin in human plasma is unclear. Why macrophages synthesise vast amounts of neopterin compared to other cells remains a mystery. High neopterin levels could have prothrombotic effects or vascular effects. Alternatively, it could be a marker resulting from an infectious or an environmental stimulus. It is conceivable that the basal production of neopterin is from a clinically silent immune system, continuously battling against invading organisms or destroying altered self-structures. It is also possible that different isoenzymes are responsible for neopterin biosynthesis in neural and liver tissue as opposed to the immune system. Hopefully further research will elucidate the biochemical and physiological roles of neopterin.

In summary, the main conclusions from this chapter are:

- Plasma neopterin, an index of GTPCH activity, was measured by ELISA;
- Plasma neopterin levels associated positively with creatinine (a marker of renal function) and with fibrinogen (an inflammatory marker and IHD risk factor);
- Plasma neopterin correlated negatively with total cholesterol and ApoA1;
- Conventional IHD risk factors such as smoking, BP, age, BMI, triglycerides, ApoB as well as Factor VII had no influence on neopterin levels; and
- Plasma neopterin might be a valuable non-invasive parameter for prognosis of macrophage activity and in the follow-up of atherosclerotic patients as it reflects the inflammatory condition of the vascular wall.

CHAPTER 7

CONCLUSIONS AND FUTURE DIRECTIONS

7.1 Summary of findings

The search to understand the physiological and pharmacological properties of NO, discovered by serendipity almost 20 years ago, which can simultaneously functions as an explosive, vascular smooth muscle relaxant and in therapeutics, is indeed fascinating. Among the multiple actions of NO, those pertaining to the cardiovascular system have been extensively documented. NO is commonly known as an anti-atherogenic and anti-thrombotic factor yet its mechanism of action in IHD, a multifactorial disorder, remains uncertain. The exact mechanisms underlying basal NO generation from the vascular endothelial cells remain obscure. Several risk factors, most particularly, hypercholesterolaemia, cigarette smoking and hypertension have been defined and are influenced by genetic components. These susceptibility genes might contribute to ED arising as a result of impairment within the L-arginine-NO pathway leading to defective NO-dependent vasodilatation. Therefore it seems fitting to analyse potential genes, coding for NO-generating enzymes, for polymorphisms, which might impair their expression and/or function, and their physiological significance at phenotype level. The studies conducted in this thesis have provided some insights into two intricately linked systems, the L-arginine-NO and pterin pathways; both involved in NO generation, implicated in ED as well as IHD.

A simple assay employing enzymatic nitrate reductase and the Griess reaction was optimised and validated to measure, nitrite and nitrate (i.e. NO_x) in plasma, as indices of NO production. Although several limitations are inherent to this assay, *gros somodo* NO_x measurement provides a reliable and quantitative estimate of *in vivo* NO production as these confounding factors would be expected to be randomly distributed in a large study, such as the NPHSII.

Plasma NO_x levels were reduced in smokers compared to non-smokers and correlated positively with creatinine but inversely with cholesterol (***Chapter 3***). These associations are in line with known effects of smoking and hypercholesterolaemia on endothelial function and with the notion that NO_x is freely filtered at the glomerulus. However neither BP nor any of the *NOS3* polymorphisms studied (i.e.: -922 (A/G), -786 (T/C), +894 (G/T) and In4 VNTR) influenced NO_x levels significantly in middle-aged British men. Furthermore, both prevailing NO_x levels and *NOS3* genotype did not predict future IHD events in this particular cohort (***Chapter 4***). The absence of a positive association between *NOS3* polymorphisms and IHD is at odds with the findings of some previous studies but might reflect differences in statistical power and trial design.

The synergistic adverse effect of smoking and hypercholesterolaemia on ED in clinically healthy patients is due to the well-documented impairment of endothelial NO formation, both predisposing to atherosclerosis in clinical and experimental studies. Smokers also have higher fibrinogen levels and enhanced platelet reactivity, both of which may also contribute to IHD events in these subjects. Cholesterol is a potent

inhibitor of endothelium-dependent vasodilation, which is an important event predisposing to atherogenesis.

Molecular scanning of the human *GCH1* SSCP identified three novel polymorphisms (i.e. -577 (G/A), -741 (T/C) and -796 (G/A)) with the potential to alter GTPCH protein expression or function (***Chapter 5***). They are the result of bi-allelic variations within the gene promoter. High-throughput genotyping protocols were established only for -577 (G/A) and -796 (G/A) variants, which were in strong linkage disequilibrium. This is not surprising given their close physical proximity. Only the -577 (G/A) variant was found to be in Hardy-Weinberg equilibrium. Carriers of one or both rare alleles (i.e. 'A' or 'AA') for both polymorphisms had significantly lower plasma neopterin levels, measured by ELISA, compared to carriers of the common allele. This suggests that there may be genetic regulation of *GCH1* transcription.

None of the *GCH1* promoter polymorphisms exerted a significant influence on BP and on future risks of IHD event. However, plasma neopterin levels associated negatively with prevailing levels of total cholesterol and ApoA1 and positively with fibrinogen (a biochemical index of inflammation and IHD risk factor) and creatinine (***Chapter 6***). As neopterin is a marker of inflammation, the associations observed support the theory that low-grade clinically 'silent' inflammation is present in 'healthy' individuals that might reflect atherosclerotic burden or subclinical infection at distant sites.

None of the polymorphisms studied within *NOS3* and *GCH1* were found to be risk factors for IHD in the British middle-aged NHPSII cohort. These findings require further investigations in other populations as any negative finding for genetic

association studies must be interpreted with caution. The failure to detect any association between IHD and any polymorphisms does not exclude *NOS3* and *GCH1* gene involvement with the disease. There may be the possibility of another variant located elsewhere or near the gene being linked to the disease, or alternatively the results may be biased due to the small size of IHD events as we are dealing with an apparently 'healthy' cohort. The literature contains conflicting results regarding associations of *NOS3* polymorphisms with IHD. The causes of these differences need to be elucidated. It is possible that any of these polymorphisms affect the activity their respective proteins, i.e. eNOS and GTPCH (that is BH_4 formation), in situations where eNOS is oxidatively stressed and /or BH_4 limiting.

7.2 Future work

The polymorphisms identified within *GCH1* may have a potential effect on GTPCH activity, consequently altering neopterin production, by either changing the three-dimensional structure of the protein or they may play a role in the regulation of transcription as they are located close to response elements, which are bind by inducible transcription factors (TFs). Further work is needed to prove the functional relevance of the polymorphisms identified in the promoter region of *GCH1*. This may be confirmed by *GCH1* promoter-reporter gene studies as done previously for the *NOS3* -786 (T/C) variant (Nakayama *et al.*, 1999). Future research will also concentrate on elucidating the coding region of *GCH1* so as to understand its regulation.

The molecular mechanism by which *NOS3* and *GCH1* gene polymorphisms interact with the development of IHD remains unclear. It is not known whether these polymorphisms are causative variants or markers of another functional variant(s). However, the finding that eNOS Asp298 is more susceptible to proteolytic cleavage that Glu298 provides one possible explanation for the observations linking eNOS Asp298 to hypertension and IHD. Further investigations are required to determine whether both genes themselves and not other genes confer susceptibility to IHD. Further studies on endothelial cell NO synthesis, degradation, *NOS3* expression and cofactor availability will undoubtedly provide a greater insight in how NO modulates vasodilation and predispose to ED. In the overall context of NOS mechanism, exactly how does BH_4 interact, via the pterin pathway, is not entirely clear. Beyond these mechanistic issues, several aspects of regulation and intracellular localisation remain unanswered. It is anticipated that the answers to these and related questions will be apparent in the next few years. Furthermore, the contradictory genetic association study data reviewed throughout the literature may be attributable to racial differences. Different factors may contribute to the pathogenesis of IHD in Asian and European populations. My objective is to repeat this study in the Mauritian population, which has mixed ethnicity: Indians, Chinese, Africans and Europeans.

A potential limitation of this present work is that the statistical analyses are based from only one measurement of NO_x and neopterin in plasma samples collected about ten years ago. It would be ideal to have plasma samples measured pre- and post-IHD event as well as annual follow-up measures for a better understanding of disease progression. Further studies in individuals with defined *NOS3* variants, in whom enzymatic generation of NO measured using an L-arginine tracer technique to

circumvent problem of the dietary contribution to the plasma NO_x pool (Forte *et al.*, 1997), would be ideal.

Several lines of evidence support the view that IHD is mediated by inflammatory process. Therefore, there is an urgent need to study the immune activation status in CVD patients. Circulating systemic markers for immune function may provide an appropriate alternative to predict, if not prevent, the risk of any future IHD event. Neopterin is a highly sensitive indicator for the presence of activation of cell-mediated immune responses and is a marker of inflammation. Measuring neopterin concentration, which is a practical, useful and cost effective laboratory investigation, may be included in future routine screening programmes. Whether it may be used as an adjunct or even supersede the measurement of C-reactive protein, assayed routinely, for disease diagnosis and prognosis deserves further evaluation. It may be time to add a marker of inflammation to the list of CVD risk factors commonly used to identify people at risk for IHD.

The challenge in the treatment of atherosclerosis will be to develop strategies that target the damaging (cytotoxic and cytostatic) actions of NO without interfering with its cytoprotective actions. Also, inhibition of GTPCH induction may become an important target for pharmacological interventions for NO overproduction, i.e. septic and cytokine-induced shock. Regretfully to date there is no drug available which could reduce the levels of neopterin selectively and safely. Can ED following an acute infection or inflammation may be a novel and transient risk factor for CVD that might promote abnormal vascular behaviour and be amenable to pharmacological intervention. To explore the clinical relevance of this hypothesis further, it will be

necessary to undertake studies in defined patient groups, to identify mechanisms of any changes seen and to explore the effects of additional ED on the already abnormal endothelium that overlie atheromatous plaques. The final question remains: Is ED a cause or merely a consequence of IHD? Hopefully the answer lies in future studies.

CHAPTER 8

CAVEATS AND ADDENDA

Question 8.1: In the introduction to the thesis a full discussion of the methods available for assessing the role of genes in complex disease traits should be discussed, in particular the merits and demerits of linkage studies and association studies, so as to put into context the reason why the particular approach was taken using this cohort.

The term 'complex traits' refers to any disease phenotype contributed by multiple loci with modification by environmental factors. For example, susceptibility to cardiovascular disease (CVD) does not follow simple Mendelian monogenic inheritance and does not exhibit classic Mendelian recessive or dominant inheritance attributable to a single gene locus (Lander and Schork, 1994). Multiple genes interacting with the environment are usually operative and make the analysis complex (Boerwinkle *et al.*, 1996). The identification of susceptibility loci for multifactorial diseases such as CVD is of crucial importance for understanding its mechanism/etiology and its prevention (Humphries, 1994).

As the multiple genes (i.e. locus heterogeneity) contribute unequally to the disease phenotype, their mode of action is unclear and therefore they cannot be studied directly. It is possible that there are a few major genes each of which exert a large effect on risk and which would be easily identifiable by molecular biology techniques whilst the other 'minor' genes are considered as 'genetic background' (Motulsky, 1991).

An example of a major gene causing CHD is the defects in the LDL receptor that cause Familial Hypercholesterolaemia (Goldstein and Brown, 1977) and which roughly double plasma cholesterol levels and increase risk of early CHD by 5-20 fold (No authors listed, 1999). However it is more likely that in the majority of patients with early CHD there is no such "major" gene and that high risk is due to the co-inheritance of several genetic variants of modest impact on risk but that each are common.

The current methods for the genetic dissection of complex traits in humans are linkage analysis, allele-sharing methods and association studies (Lander and Schork, 1994). Linkage analysis and allele-sharing methods concern familial inheritance patterns of disease incidence and have good power to identify "major gene effect" and low power to detect genes of modest effect whereas association studies are used in studies of unrelated subjects and have high power to detect genes of modest effect (***Figure 8.1***).

Linkage analysis

Linkage analysis involves constructing a transmission model to explain the inheritance pattern of a disease by comparing the observed segregation of gene markers and trait in an affected pedigree. Family members undergo 'typing' for a series of genetic markers (i.e. DNA polymorphisms) and for a disease phenotype (Kurtz and Spence, 1993). If a genetic marker and a disease trait segregate (i.e. pass) through the family together more often than expected by chance alone, the disease gene and the marker are presumed to be 'tightly linked' (i.e. lie physically close on the same chromosome).

The genetic distance between a disease locus and a marker locus can be estimated from the frequency of recombination observed in the families. If the affected family members almost always inherit the same genetic marker, it suggests little recombination between both the disease and the marker loci. Special statistical programs analyse genotypes and phenotypes of individuals in a family and calculate the odds that a DNA marker and the disease gene are linked.

The likelihood (relative odds) that the inheritance patterns might be explained by linkage, as opposed to chance, can be measured with a convention called a 'LOD score' (LOD is an acronym for the logarithm of the odds) (Kurtz and Spence, 1993). A LOD score of +3 (odds of 1000/1) is a good evidence of linkage, as the data are 1000 times more likely to have arisen by linkage than by chance.

Linkage is the method of choice for studying simple Mendelian traits in monogenic disorders because the allowable models are few and easily tested. However, application to complex, multifactorial conditions such as CVD is more problematic, as the genetic analysis may be confounded by incomplete penetrance, phenocopy, genetic heterogeneity, polygenic inheritance, absence of large multi-generational pedigrees and misinheritances (Lander and Schork, 1994). It may be hard to find a precise model that explains the pattern of inheritance, which must be robust so as to accommodate such complexity and should also make no assumption about the pattern of inheritance.

Allele-sharing analysis

By contrast, allele-sharing approaches are based on rejecting a model rather than on constructing a model. They involve studying affected relatives (or siblings) in a pedigree to see how often they inherit identical copies of the region from a common ancestor (Kurtz and Spence, 1993). Specifically, one tries to prove that the inheritance pattern of the chromosomal region is not consistent with random Mendelian segregation. Allele-sharing methods are typically applied to a single generation of disease sufferers. They are independent of the pattern of disease inheritance and therefore highly suited to the analysis of a complex disorder with late onset.

However, allele-sharing analysis assumes that alleles shared by a pair of sibs or affected relatives are either identical-by-descent (IBD) or identical-by-state (IBS) i.e. inherited from same parental chromosome or a common ancestry respectively (Lander and Schork, 1994). This method is more robust for genetic complications compared to linkage analysis, i.e. affected relatives would show excess allele sharing even in the presence of genetic heterogeneity, incomplete penetrance and phenocopy.

Power calculations can be carried out to determine the sample size needed to detect a statistically significant risk effect (Risch and Merikangas, 1996). However in the case of locus heterogeneity considerably larger samples will be needed (i.e. if the disorder is caused by say 5 major genes, the sample size to detect will need to be 5 times larger since the gene is only causative in 20% of the families).

Association studies

Linkage and allele-sharing methods require no control group, because they involve testing a specific model of random Mendelian segregation within a family/pedigree. This is in contrast to association studies which in their simplest form is a comparison of the frequency of a genetic marker in ***unrelated*** affected (i.e. 'cases) and unaffected individuals (i.e. 'controls') from a population (Cooper and Clayton, 1988). An allele A at a gene of interest is said to be associated with the disease if it occurs at a significantly higher frequency in affected compared to unaffected individuals. The statistical analysis involves a 2 x 2 contingency table (Devlin and Risch, 1995).

An approach frequently used for identifying genetic factors involved in the process of complex disease is the comparison of patients and controls genotyped for a great number of new markers in the vicinity of a candidate gene, defined as a gene whose protein product, i.e. an enzyme, is implicated in the disease process (Hingorani, 2000). The rationale underlying this approach is the phenomenon of linkage disequilibrium, between the marker locus and the unknown disease locus (Devlin and Risch, 1995).

Linkage disequilibrium is said to be present when alleles at two genetic loci (i.e. the disease- and the marker-allele) occur together in a population more frequently than would be expected from their random assortment. For this to occur the rate of new mutation at the disease- and marker-loci must be low and the trait-causing locus must have arisen from a single distinct ancestral mutation (Tiret *et al.*, 1991).

When conducting association studies, subjects with the disease of interest and control subjects must be ethnically homogeneous (Motulsky, 1991) (***Figure 8.1***). If both (disease and control) groups are closely matched with respect to their background, a significant association between a genetic marker and a disease implies that the marker is likely to reside in the disease locus or within 500 kilobase (kb) pairs of DNA of the locus (Kurtz and Spence, 1993). Positive associations can arise in the following cases:

1 Allele A is actually the cause of the disease. In this case, the same association would be expected to occur in all populations.
2 Allele A does not cause the trait but is in linkage disequilibrium with the actual cause, that is A tends to occur on those ancestral chromosomes that also carry a trait-causing mutation and this correlation has not been eroded by recombination during the population's history (Tiret *et al.*, 1991).
3 An artefact of population admixture (Boerwinkle *et al.*, 1996). In a mixed population, any trait present at a higher frequency in an ethnic group will show positive association with any allele that happens to be more common in that group.

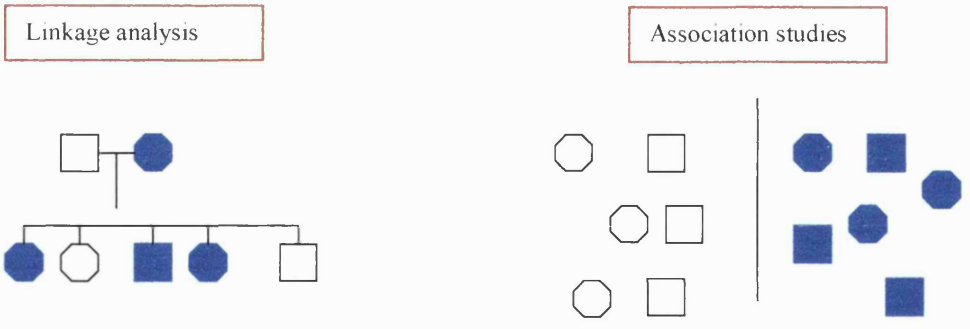
In summary, linkage-type studies and association studies have many crucial differences. Association studies test whether a disease and an allele show correlated occurrence in a population whereas linkage-type studies test whether they show correlated transmission within a pedigree. Linkage studies focus on concordant inheritance whereas association studies focus on population frequencies.

Thus for CVD risk and especially for CVD risk traits a design not confounded by age and gender effects and particularly by environmental factors is required. Association

studies are therefore more appropriate than linkage studies, and will have a good power to detect modest effects of common variants in candidate genes. The sample used should be as genetically homogeneous as possible (i.e. not include subjects of different ethnic origin) and ideally should have both male and female individuals.

A study based on prospective follow-up of healthy individuals would be ideal as it avoids the potential confounding of the presence of disease influencing levels of CHD traits. It must be large enough to have enough CHD events to give a good power for CHD risk, and must have samples available for measures of CHD risk traits. Therefore, the NPHSII study fulfils many of these criteria since it is based on the recruitment of >3000 unrelated healthy middle-aged UK white Causcasian subjects, followed for 9 years for CHD events with baseline and annual plasma samples taken.

Figure 8.1: Linkage studies concern familial inheritance patterns of genotypes and phenotypes whereas association studies involve population correlation where affected ('cases') and unaffected ('controls') individuals are compared.



Question 8.2a: The decision to use plasma NO_x should be much more fully justified and in particular the limitations of NO_x measurement as a measure of NO production in humans must be discussed more fully.

Endothelial nitric oxide (NO) is believed to act both as an anti-atherogenic molecule and a blood pressure regulator. Individuals who develop atherosclerosis or high blood pressure are believed to have a reduction in NO synthesis or bioavailability in their blood vessels, thereby leading to an impairment in NO-mediated dilator responses. However, the precise mechanisms are unknown. For review see ***Chapter 1, Section 1.5: NO, endothelial dysfunction, inflammation and atherosclerosis***.

We hypothesised that sequence variation(s) in endothelial nitric oxide synthase gene (eNOS: protein; *NOS3*: gene) could influence the transcription rate of its mRNA and encode an eNOS enzyme with reduced expression and/or activity. NOS enzyme activity can only be determined in tissue or cell homogenates. This assay is labour-intensive and requires the handling of radioisotopes and is therefore not suitable for routine determination (Knowles and Moncada, 1994).

Therefore, it is most appropriate to assay a phenotypic marker of eNOS enzyme activity in genetically characterised individuals. In order to measure NO synthase activity, many investigators have determined the amount of enzymatic product of NO synthase, that is NO (Kaku *et al.*, 1994). Accordingly, NO is the most suitable marker of eNOS activity and functionally important polymorphisms may then affect the synthesis of endothelial NO (Tsukada *et al.*, 1998). For a detailed review see ***Chapter 1, Section 1.6: Genetic susceptibility to IHD***.

However in NO research, the product of the enzymatic conversion of L-arginine by NOS, bioactive NO is rarely measured. It is very difficult to quantitate NO endogenously synthesised by vascular endothelial cells *in vivo* as it is very short lived radical ($t_{1/2}$ = 5-60 sec), is produced in minute amounts and is unstable in body fluids (Palmer *et al.*, 1987; Knowles and Moncada, 1992). Measurements of the kinetics and amount released from tissues is a serious analytical problem (Vallance *et al.*, 1995).

On release, NO rapidly autooxidises to yield nitrite (NO_2^-), which interacts with haemoglobin to yield nitrate (NO_3^-) (Wishnok *et al.*, 1996). Although NO metabolism is very complicated and has not as yet been fully elucidated, it is thought to be eventually metabolised to stable oxidative end products nitrite (NO_2^-) and nitrate (NO_3^-) (collectively referred to as NO_x) in plasma (Wennmalm *et al.*, 1993).

Accordingly, it has become common practice to regard accumulated plasma NO_x as an indirect, non-invasive, measure of an *in vivo* systemic endogenous NO production in both human and animals studies (Shi *et al.*, 1993; Ochoa *et al.*, 1991). For example, in adults after closed-head injury (Clark *et al.*, 1996), hypertensive patients (Node et al., 1997), subjects with CAD or its risk factors (Tanaka *et al.*, 1997), atherosclerotic patients (Takahashi *et al.*, 1992), in septic and critically ill patient (Wong *et al.*, 1995; 1996), in pregnancy (Yang *et al.*, 1996) and in dogs (Zeballos *et al.*, 1995). Therefore I assayed plasma NO_x as an index of endogenous NO production. For review see:

- ***Chapter 1, Section 1.7: Plasma NO_x: Index of NO synthesis***; and
- ***Chapter 3, Section 3.2: Background.***

However, as the NPHSII is an epidemiological study, a simple and quick assay with a high throughput, amenable for routine application, was sought. A spectrophotometric assay based on the Griess reaction was chosen. It provides a reliable and sensitive measurement for both anions in a large number of samples, requiring minimal sample volume. Nitrate concentrations were measured after conversion back to nitrite and assay by the Griess reagents (Giovannoni *et al.*, 1997). For comprehensive information on assay optimisation and application, please see the following sections:

- ***Chapter 2, Section 2.5: Measuring NO_x by Griess Assay***;
- ***Chapter 3, Section 3.2: Background; Section 3.3: Materials and Methods***, and
- ***Chapter 3, Section 3.4: Method Validation and Optimisation***.

As reported by several laboratories, plasma NO_x is the primary metabolite of NO (Schoedon *et al.*, 1995a). However there are two main limitations of using plasma NO_x concentrations as a measure of NO production in humans in a genotype/phenotype study of eNOS (Green *et al.*, 1981). They are:

i) Exogenous contribution to the nitrate pool by consumption of fluids and food, and

ii) Endogenous contribution, due to the production of NO by inducible (iNOS) and neuronal (nNOS) isoforms.

This critical approach must be recognised during any NO_x measurements and was addressed in the following sections (See ***Chapter 3, Figure 3.1***):

- ***Chapter 3, Section 3.2: Background***; and
- ***Chapter 3, Section 3.6.2: Confounding factors in plasma NO_x assay.***

Dietary nitrate: A major confounding factor

Tanaka and colleagues showed that a stable plasma NO_x level is obtained by sampling blood from restricted healthy subjects in early morning (Tanaka *et al.*, 1997) while Node and co-workers showed that there are no after-effects of eating and drinking following 8 hours of fasting or 6 hours of abstaining from drinking (Node *et al.*, 1997). Other studies found that dietary nitrate is cleared from the plasma pool within 12 hours of a meal.

However in the NPHSII study, subjects were non-fasted but had been asked to consume no more than a light meal on the morning of recruitment. This could be a potential confounding factor but it ought to be randomly distributed in a large enough sample (n=2965) and ought not be a source of systematic error. Furthermore, the distribution of plasma NO_x levels determined in this cohort matched closely those recorded in other studies (Node *et al.*, 1997)

According to several authors, plasma NO_x levels in the early morning serves as a useful rough evaluation of basal NO generation by endothelial cells since the majority of plasma nitrate is believed to be of endothelial origin (Takahashi *et al.*, 1992; Tanaka *et al.*, 1997). Therefore in the NPHSII subjects, plasma NO_x concentrations measured was believed to reflect only the differences in the basal level of endogenously derived NO_x assuming dietary intake is controlled and a constancy of renal excretory capacity (i.e. urine and faeces) (See ***Chapter 2, Section 2.1.2: Clinic Attendance***).

nNOS and iNOS Isoforms: Confounding factors

NO is continuously synthesised from L-arginine in a reaction catalysed by NOS and it is generally believed that the majority of NO present in the circulation is mainly produced by eNOS (Takahashi *et al.*, 1992; Wang *et al.*, 1997). In reality, plasma NO_x is likely to reflect NO synthesis via all three NOS isoforms: endothelial as well as neuronal and inducible forms. However, there is no direct evidence to show that the plasma NO_x assayed is derived from endothelial cells and the precise sites for NO production are not identifiable (Baylis and Vallance, 1998).

Nevertheless, it is widely accepted that the vascular endothelium (~1.5 kg), the largest endocrine organ, is responsible for the majority of NO synthesis, with the endothelium representing a large surface area and facing the bloodstream (Tanaka *et al.*, 1997). If the intake of the extrinsic nitrate were constant in a population without apparent infectious diseases, the plasma NO_x concentrations may reflect endothelial NO formation (Forte *et al.*, 1997), especially in view that the NPHSII is an apparently healthy cohort, with no immunologic stimulation.

NO_x is primarily excreted from the kidney ($t_{1/2}$=3-4 hours) and its concentration in plasma is determined by the balance between rates of production and excretion (Clark *et al.*, 1996). Clearance for NO is assessed by correction for creatinine, which is a rough estimate of renal function (Wong *et al.*, 1995). As nitrite and nitrate ions are excreted via the kidneys, differences in plasma concentration can occur as a result of renal dysfunction. To correct for any confounding effects of renal dysfunction, NO_x concentration was indexed to creatinine concentration.

The biologic effects of NO are short-lived and are likely to occur at local tissue and intracellular levels (Forte *et al.*, 1997). Therefore measurement of serum nitrite and nitrate, stable end products that reflects whole body NO production over many hours, may not be a sensitive indicator of NO bioactivity although there is a high reproducibility and stability of NO_x in stored frozen samples (Moshage *et al.*, 1995).

When measuring plasma, one should also bear in mind that some of the NO_x may not have been released as free NO but as another reactive nitrogen intermediate, i.e. peroxynitrite or nitrosothiol (Baylis and Vallance, 1998). Also one cannot exclude the existence of additional minor metabolic pathways in healthy subjects, such as the conversion of nitrite to urea, or metabolism/excretion in the lungs (Wennmalm *et al.*, 1993).

As mentioned previously, the plasma nitrite and nitrate levels measured do not directly reflect the amount of endothelial NO released, but serve as a useful and straightforward tool in roughly defining the contribution of eNOS. There are several limiting factors in the determination of accurate value of NO level in the present study and future well-controlled studies need to be conducted so to clarify the following issues which have yet not been fully elucidated: the source of NO, its metabolic pathway and NO_x systemic distribution.

Question 8.2b: The reasons why the plasma concentrations of NO_x in the cohort studies were consistently below those expected should be considered along with a discussion as to whether the concentrations measured were in any way meaningful as a measure of NO production. Consideration should be given to the stability of nitrite and nitrate in plasma and the conditions in which the samples were stored.

The range of plasma NO_x in the NPHSII study was 2.79-63.4 μM after practice adjustment, with mean (±SD) NO_x values from North Mymms (n=287; 11.5±4.5), Chesterfield (n=241; 9.3±3.8) and random sets, a sample from all 9 practices (n=532; 10.8±4.5) (See ***Chapter 3, Section 3.4.2.4, Table 3.6***). The reference range for plasma NO_x quoted in the literature is 15-60 μM and was determined by a variety of methods, with few samples (minimum=5; maximum=235), which were collected and frozen for the objectives of the particular study (See ***Chapter 3, Table 3.3***).

Unfortunately, currently there are no reference plasma NO_x values quoted in the literature, which has been determined on stored frozen plasma samples from an epidemiological study. All previous studies have been relatively small and the findings inconsistent possibly due to the difficulties in assaying plasma NO_x in a large number of subjects (Bartha *et al.*, 1999).

To assess NO synthase activity in the NPHSII samples poses many practical problems, as it would in any other epidemiological study. Furthermore, the techniques currently available (i.e. chemiluminescence, HPLC, GCMS, etc...) are unsuitable for routine laboratory measurements due to several reasons: they require extensive sample

preparation thereby posing contamination risks, are labour intensive, time consuming, not convenient for processing large number of samples, and require sophisticated as well as expensive equipment (Body *et al.*, 1995; Leone *et al.*, 1994).

Consequently, the Griess assay was an attractive alternative for the measurement of plasma NO_x as an index of NO bioactivity (See ***Chapter 3, Section 3.2 Background***) (Archer, 1993; Verdon *et al.*, 1995). The technique optimised and validated in the current study allowed the high-throughput analysis of large numbers of plasma samples in 96-well microtitre plates (See ***Chapter 3, Section 3.4.2: Optimising the NO_x assay)***. The assay was sensitive (1 μM), reliable and reproducible with inter- and intra-assay coefficients of variation of 3.4% and 4.0% respectively. The distribution of plasma NO_x levels determined in this cohort matched closely those recorded in other studies (Node *et al.*, 1997).

As mentioned in ***Chapter 2, Section 2.1.5: Blood sampling***, 4.5 ml of blood collected into citrated tubes was used for NO_x measurements. In all 9 practices, the sampling procedures (i.e. handling conditions such as sample collection, storage and freezing temperatures) were rigorously standardised. The tubes were immediately centrifuged (1000x G / 10 min / Rt.) and the plasma obtained was transferred in 1.0 ml aliquots into plastic vials for immediate storage at -45°C, prior transfer for storage at -80°C at the MRC Unit.

Samples to be analysed were transferred to the Rayne Institute by taxi in dry ice and then immediately stored at -70°C. This temperature is usually recommended in epidemiological studies. Prior to analysis, the samples were defrosted, thawed

samples were mixed and 500 µl was ultrafiltered (12,900 rpm, 60 min, 4°C) through a 10 kDa molecular weight cut-off filter using a commercially available microcentrifuge filter unit (Millipore Ultrafree Biomax-10). The deproteinised filtrate (40 µl) were then assayed by Griess reaction in duplicate (See ***Chapter 2, Section 2.5: Measuring nitrite and nitrate (NO_x) by Griess assay)***.

Surprisingly in the literature, very little information is available on the long-term storage or stability of plasma samples prior nitrite and nitrate assay. There are also no reports of plasma NO_x being measured in old samples (>1 year old). Several authors have assayed inorganic nitrite and nitrate anions in plasma as a measure of NO generation. They all reported centrifuging blood samples and freezing plasma until NO_x measurements (Clark *et al.*, 1996; Cortas *et al.*, 1990; Gilliam *et al.*, 1993; Tsukada *et al.*, 1998; Wennmalm *et al.*, 1993; Wang *et al.*, 1997).

However none of them mentioned the period the samples were stored prior analysis. Moshage and colleagues reported that nitrite and nitrate are stable in frozen plasma for at least 1 year (Moshage *et al.*, 1995). They went on to add that nitrate is stable in whole blood regardless of the incubation time or temperature but did not publish the data. Wong and colleagues also did not observe significant changes in serum NO_x levels during storage but they did not publish their data (Wong *et al.*, 1996).

Currently there are several commercial kits available which measure the two stable breakdown products of NO: nitrate (NO_3^-) and nitrite (NO_2^-) in plasma. They are marketed by Promega (UK), Oxis Research (USA), Active Motif (Belgium), IBL Immuno-biological Laboratories (Germany), Abbott Laboratories (USA), Cayman

(UK) and Assay Designs Inc. (USA). None of them mention the stability of nitrite and nitrate in archived plasma samples but instead advise that any plasma collected should be assayed immediately for NO_3^-/NO_2^- or alternatively frozen below -20°C for subsequent analysis.

According to Dr James Peterson, Specialist to Occupational Medicine and Industry (Pacific Toxicology Laboratories, California), baseline blood samples can be archived (frozen until needed) for a period up to ten years as long as the samples are frozen immediately after collection. The current recommendations regarding conditions for plasma storage is based mainly on long-standing experience. The European Commission guidelines (Opinion on Quality and Safety of Blood, 2000) recommends that under optimal and well-controlled conditions, fresh frozen plasma is stable at -30°C for more than two years, thereby ensuring the stability of its metabolites.

For example, total plasma homocysteine measurement, plasma samples may be stored up to 10 years if frozen at -20°C (Ueland *et al.*, 1993; Ubbink *et al.*, 1992) and the relationship between plasma homocysteine and vascular disease can then be assessed (Israelsson *et al.*, 1993). Similarly all commonly measured coagulation factors are stable for couple of years if they are stored deep-frozen (Woodhams *et al.*, 2001). Although there would be some decay over time, this would not change the clinical interpretation of the results (Rao *et al.*, 2000).

Lewis and colleagues showed that in all nine coagulation, fibrinolysis and inflammation factors studied, including C-reactive protein and fibrinogen, there was no evidence of sample degradation over time for a period ranging from 7 to 59 months

(Lewis *et al.*, 2001). Their finding of longitudinal stability in the biochemical properties of frozen plasma strengthens the presumption of sample stability on which molecular epidemiological studies are based (Lewis *et al.*, 2001).

Therefore, our group, like others in the NO field, has assumed that plasma NO_x is stable in stored frozen sample. Although, it cannot be denied that some decay will occur during sample storage and processing, any differences in plasma NO_x levels resulting from genetic differences in the *NOS3* gene will be systematically preserved. In the future, these storage conditions will have to be fine-tuned with technical progress made in the NO field.

To date, this is the most comprehensive large-scale population-based prospective cohort study quantifying plasma NO_x as an index of eNOS activity. In the study, plasma NO_x varied in the range 2.79-63.4 μM, which encompasses the physiological range and is consistent with that measured in other populations although methodological (i.e. in sample collection, centrifugation...) as well as geographical factors may account for minor differences.

In conclusion, the transient and volatile nature of NO makes it unsuitable for most routine detection methods. The determination of total nitrite and nitrate in plasma by the Griess assay is a useful indicator for NO production in humans. But unfortunately, for reference data pertaining to the long-term storage stability and integrity of nitrite and nitrate anions in plasma samples is lacking in the literature.

I tried with the best of my ability to overcome the limitations of this epidemiological study, i.e. very little plasma available (300-500 µl), the frozen samples were obtained at baseline and the research work was not envisaged at the time the material was obtained. I optimised and validated a method to assay plasma NO_x so as to confirm or refute the hypothesis of a possible correlation between genotype of *NOS3* and phenotype of endothelial NO system as well as an assessment of plasma NO_x as a marker of cardiovascular risk.

Question 8.3: There should be a full discussion about the ethics of DNA genotyping and consideration of the issue of consent, particularly in relation to a study, which was initiated many years ago.

The objective of the second Northwick Park heart Study (NPHSII) was to demonstrate the presence of a hypercoagulable state prior to an acute coronary event by prospective surveillance of middle-aged British men, clinically free of CHD (Miller *et al.*, 1996). NPHSII was initiated through discussion with the head trainer nurse in the General Practice Research Framework, who knew of keen practices coming out of the hypertension trials. Subject recruitment began in 1989. Originally, 4600 men based in nine general medical practices were screened for eligibility after giving free informed consent, and over 3000 men were finally invited to participate in the study (Miller *et al.*, 1996).

The NPHSII study was funded by the British Medical Research Council (MRC) and was approved in 1989 by the local research ethical committee (LREC) based at Northwick Park. The purpose of LRECs is to protect the safety, rights and well being of all participants (*Research Governance Framework for Health and Social Care*) and their activities are co-ordinated by the *Central Office for Research Ethics Committees* (COREC). Comprehensive and up-to-date information on ethics and consent is found on the COREC website (http://www.corec.org.uk)..

In the original patient consent form of the NPHSII study (***Appendix 1***), there was no mention of the ethics of genotyping or of the genetic aspect. This was in part because

at the time no genetic research was planned, and none of the researchers who had initial custodianship of the samples ever addressed genetic issues in any publications.

The MRC is committed to the highest ethical standards in medical research and its mission is to support research that will ultimately benefit human health. The MRC developed the guidelines ***Human tissue and biological samples for used in research*** (MRC Ethics Series) in April 2001 which covers ethical, legal and management issues relating to the use of samples of biological material, including DNA, for research.

However, the MRC acknowledged that the principles in these guidelines will not always be possible to be applied retrospectively to collections comprising samples that were collected before these guidelines came into operation. Research use of tissue samples or DNA samples in conjunction with personal data raises special issues since clinical samples, including stored blood, plasma and serum will often be used to answer questions unforeseen at the time they were collected.

This particular work was carried out in the period of October 1996-99 and was possible by observing procedures which were in place for MRC Framework studies at that time (***Responsibility in the use of Personal Medical Information for Research - Principles and Guide to Practice 1985***; Revised version in 1994 and ***Responsibility in Investigations on Human Participants and Material and on Personal Information, 1992***). The practices were that it was not necessary to seek explicit patient consent or to consider whether implied consent has been given when using anonymised patient data as long as confidentiality was not breached.

Efficient and well co-ordinated use of human biological material can promote scientific advancement and can reduce both the research demands on patients and the need to use animals. The MRC has always encouraged custodians of collection of human material to facilitate its optimum usage by providing access to other academics, undertaking high-quality research, once the requirements of the original project have been fulfilled.

Generally, established collections can be used for research when samples have been anonymised and there is no potential harm to the donors of the material. However, researchers should satisfy themselves that the samples were not obtained in an unethical or improper way and that there was valid consent at the taking (***The HUGO Ethics Committee. Statement on DNA sampling: Control and Access. London: Human Genome Organisation, 1998***). I was able to access the NPHSII samples for my PhD studies through the long-standing close collaboration between my supervisor, Professor S. Humphries and Dr G. Miller, the study organiser and the custodian of the NPHSII samples.

According to the current MRC guidelines, it is now acceptable to use human material, i.e. DNA, for research without individual written consent if it is anonymous and the following conditions are satisfied:

- Study justification: Information obtained in the course of the research will not harm or distress the donors and the potential benefits outweigh the risks.
- During the conduct of the research, adequate measures are in place to protect the confidentiality of the subjects.

- Feedback information: Research carried out will not reveal information of immediate relevance to the donor's health and will not affect his interests.

In the research work undertaken for my PhD, none of the above were violated. In fact, patients' interests and treatment were in no way jeopardised, there was no potential to lead to discrimination in employment or other areas of life and there was no commercial implication.

The guidelines also acknowledge that there are presently several valuable old collections of human samples for which consent was only obtained for a single research project (i.e. NPHSII). In epidemiological research, old samples of material surplus may be used for linked research without specific consent as long as there is no possibility that the research would not affect the patients' interests and obtaining individual consent or contacting donors to seek consent is not practicable due to sheer size of the group being surveyed or the likelihood that many will be not contactable.

Since 1986, the MRC has advocated the justification to NHS patients of how their medical records will be used in research (***Appendix 2: Copy of letter from Dr Miller addressed to NPHS patients regarding completion of study and seeking permission to use information pertaining to their progress***) provided the researchers ensure no infringement on confidentiality and data protection and their actions reflect ethical and professional codes as well as are consistent with the law.

Regarding the NPHSII study, personal and clinical information relating to the individual, who donated the sample, was also used. The confidentiality guidelines of

the MRC (***Personal Information in Medical Research, MRC Ethics Series, October 2000***) recommend that all personal information must be coded or anonymised as far as is possible and as early as possible in the data processing. Furthermore, users of anonymised samples of human materials must undertake not to attempt to identify research participants and they would not be identifiable from published data.

In accordance to MRC guidelines, Dr Miller, the custodian of the NPHSII collection, made it a condition of access to the samples that copies of all data generated by other users and myself, are provided to be included in a common database by the statisticians at the MRC Unit. Both of them, Miss J. Cooper and Miss S. Bujac, acted as 'data controllers' and they had access to confidential patient details. By definition, the "*data controller is a person, either alone or jointly or in common with other persons determines the purposes for which, and the manner in which any personal data are, or are to be, processed*". Personal information was also accessible to other MRC staff (i.e. technicians), who had a formal duty of confidence to the research participants. The data generated from my research was processed, analysed and did not allow individuals to be identified.

The MRC recommends the following in the future:

- Custodians of collections of human biological materials should ensure that a written agreement covering access to data and ownership of intellectual property rights is secured before allowing access to samples by academic researchers.
- The clause regarding research involving new samples of human biological material must be added to consent forms:

Consent for genetic research

I understand that (the project/future research, as appropriate) using the sample I give may include genetic research aimed at understanding the genetic influences on (complete as appropriate) but that the results of these investigations are unlikely to have any implications for me personally.

In conclusion, the study focused on cardiovascular disease and general practitioners (GPs) were notified when any risk factor was raised, i.e. blood pressure. Research concentrated on the identification of genetic and environmental causes of elevated risk factors. Unless there was a known way to treat genetic risk *per se*, the investigators saw no purpose in notifying doctors or patients of the findings, which in any case would be premature pending confirmation in at least one independent study. GPs should simply continue to treat the phenotype by standard means.

All the genetic work was done on anonymous samples and results reported in summary form only. The investigators believe this approach ensured scientific progress without jeopardising subjects in any way. None of the lipid, clotting or blood pressure genetic polymorphisms sought make more than very minor contributions to variance in phenotype and no approaches to genetic intervention are known. Therefore no proven method of care or treatment has ever been withheld. The last study visits were in 1998, and patients are no longer making annual visits, but are being followed up for any deaths or non-fatal CHD or stroke events, through note searches and notification from Southport.

Question 8.4: The reproducibility of the genotypying procedure should be considered much more fully with additional data, which we understand is available to confirm the robustness of this assay.

For genotyping, the amplification and selective restriction of the DNA strands, followed by gel electrophoresis, allow polymorphisms to be detected within an individual genome. Consequently, Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) based protocols were successfully developed for size-dependent genotyping of the following polymorphisms:

- Endothelial nitric oxide synthase gene (*NOS3*): -922(A/G), -786(T/C) and +894(G/T); and
- GTP cyclohydrolase I gene (*GCH1*): -796(G/A) and -577(G/A).

Rapid and high throughput genotyping all the above polymorphisms were possible due to the availability of the 2745 samples of DNA, of the NPHSII study, in 96-well microplates. The PCR-RFLP protocols, individually optimised for each polymorphism, were compatible for use with Multiple Array Diagonal Gel Electrophoresis (MADGE), which was also in the 96-well format. Thus, all the polymorphisms were typed in a matter of months including quality control checks. All the genotypes were checked independently by two investigators and any discrepancy was resolved by repeat PCR and analysis.

Please see the following sections of my thesis for a detailed description of the genotpying procedure, as well as the results obtained with the photographs of the

MADGE gels with the pattern of the RFLPs-generated fragments highly distinguishable for both *NOS3* and *GCH1:*

- Chapter 2, Section 2.4;
- Chapter 4, Sections 4.4.1 and 4.4.2; and
- Chapter 5, Sections 5.3.7, 5.4.3 and 5.4.4.

All the polymorphisms genotyped conformed to Hardy-Weinberg equilibrium, except for the -796(G/A) in *GCH1*. This finding was confirmed even when PCR/RFLP protocols were repeated for nine Master Arrays. However, the observed GA and AA frequencies (n=437 and n=179) were identical on both occasions, confirming the high accuracy of the assay procedure. (i.e. an error rate of < 1/700 genotypes or < 0.14%).

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

There is no generally accepted procedure for quality control of the genotyping procedure, except for the individual practices put in place in each laboratory. In the Cardiovascular Genetics Group (CVG) department these include double data check, double data entry, each Master Array containing two empty wells for negative and positive controls (***Chapter 2, Figure 2.2***) and repeat of PCR on the whole plate rather than individual samples in case of dropouts.

In fact, several times that the PCR/RFLP protocols were repeated for any plate where dropouts were observed, for all of the polymorphisms genotyped, the genotypes assigned never changed, thereby validating the accuracy of the genotyping procedure. For example, when PCR/RFLP protocols were repeated for both *GCH1* polymorphisms on several NPHSII plates, the same genotype frequencies were obtained.

Similarly, when several master arrays were genotyped twice for the +894(G/T) polymorphism in *NOS3*, but with different restriction enzymes (i.e. *Dpn*II or *Mbo*I), identical genotypes were observed, with an error rate < 0.005. A good method to estimate the error rate is to include split samples within the genotyped arrays, which obviously should produce the same genotypes. The advantage of this is that the genotyper is blind as to which samples are duplicated reducing observer bias. In a recent study from CVG department almost 400 samples were duplicated and read blind and entered onto the database in this way with 100% agreement with genotypes. This suggests an error rate of < 1/400 or < 0.25%.

There is also the likelihood of mistyping due to failure of complete digestion, i.e. not enough restriction enzyme. In cases of incomplete digestion, there are two scenarios, (normal allele is represented by 1 and rare allele by 2):

- If the enzyme normally cuts the wild type allele, there will be more 12 (i.e. heterozygotes) and 22 (rare homozygotes).

- Alternatively, if the enzyme cuts the mutant allele, there will be more 11 (normal homozygotes) and 12.

This will lead to many heterozygotes and will be detected as deviations from Hardy Weinberg. This incomplete digestion can then checked by doing a run of heterozygote samples with normal and twice the amount of enzyme. When -796(G/A) *GCH1* polymorphism was found to deviate from Hardy-Weinberg, the digestion protocol was repeated with four times the amount of restriction enzyme for 95 samples. However, no change in genotype frequencies was observed.

As it is the case with most high scale analytical techniques, incorrect interpretation of the RFLP results and inaccuracies in data handling are major sources of error. The highly standardised procedures used in Professor Humphries laboratory ensure that these types of errors are minimised, if not completely eliminated. Further reduction in mis-genotyping can be achieved by the use of two independent RFLP assays for the same polymorphism, followed by comparison of the results and further testing of any discrepancies.

There is no further additional data available to confirm the robustness of this assay. I suppose the examiners must be thinking of the dropouts observed, which were due to poor quality of DNA samples, evaporation of samples at the edge of the array, inconsistency of some of the channels of the multipipette and uneven nature of heating of some of the blocks of the PCR machine.

In conclusion, the genotyping procedure used in this thesis was highly reproducible and has resulted in the most complete genotyping to date of *NOS3* and novel *GCH1* polymorphisms, in a well-characterised cohort. Although no quality control standard of RFLP data was included for this study (i.e. duplicate samples within the arrays), it is unlikely, based on the laboratory experience that genotype error was > 1% and probable that the error rate was considerably lower than this

Question 8.5: A discussion should be undertaken of the different methods of determining DNA polymorphisms in candidate region and their various merits and demerits.

Cardiovascular disease (CVD) is a complex disorder. Several important risk factors have been defined and they are: smoking, hypertension, hypercholesterolaemia, low HDL cholesterol concentration and hyperglycaemia (Wood *et al.*, 1998). The precise mechanisms whereby these major risk factors promote atherosclerosis and predispose to CVD are not fully elucidated (Boerwinkle *et al.*, 1996). However, it appears that they are influenced by genetic components, suggesting that genetic variants (i.e. DNA polymorphisms) in putative candidate genes play an important role in the pathogenesis of CVD (Humphries, 1996).

These genetic markers are most likely to be located in the promoter region, where they may influence levels of gene expression by affecting transcription, or in the coding regions, where they may affect the activity of the encoded protein product by altering its primary structure. Currently, several methods are available to identify potential disease-susceptibility DNA polymorphisms in candidate genes, which have been shown to be associated with the risk of CVD by epidemiological studies (i.e. association or linkage studies) (Grompe, 1993; Prosser, 1993). Most of these techniques are based on the Polymerase Chain Reaction (PCR) gene amplification method as the initial step.

After amplification of the target material by PCR, the resulting product is screened for genetic variants. Ideally, the polymorphism detection method must satisfy the

following criteria: provide precise and accurate information about the nature and location of the polymorphism, rapid, simplicity of use, have a high throughput so that it is applicable to large population samples, cost-effective and amenable to automation. None of the current scanning methods fulfils all these attributes and they can be classified in two subgroups: conformation-based techniques, which are sequence-dependent, and cleavage-based techniques, which rely on base-mismatch recognition. They are overviewed below and are summarised in Table 8.1.

8.5.1 Conformation-dependent polymorphism scanning methods

Single strand conformation polymorphism

Single strand conformation polymorphism (SSCP) is a powerful technique for detecting DNA variations in single-stranded (ss) DNA fragments, due to their differing mobility shifts on non-denaturing polyacrylamide gels during electrophoresis (Orita *et al.*, 1989). The electrophoretic mobility of the ss is determined by its secondary structure, which is sequence-dependent. Single base substitution will alter base pairing, causing the DNA 'variant' allele to adopt a different conformation, which is detectable as a mobility shift relative to the native 'normal' allele (***Figure 8.2***) (Sheffield *et al.*, 1993).

The SSCP protocol used for the detection of novel polymorphisms within the GTP cyclohydrolase gene (*GCH1*) is described in details in ***Chapter 2, Section 2.4.4.1 - 2.4.4.5*** (Whittall *et al.*, 1995). Radiolabelled amplimers were denatured by heating and then loaded on a non-denaturing vertical gel. After electrophoresis, the gel was

dried and autoradiographed. Other labelling strategies include the incorporation of radiolabelled nucleotides at their 5' ends during PCR and fluorescent primer labelling. To avoid the hazards and inconvenience of radioisotope usage, silver staining of DNA after electrophoresis is an alternative strategy (Dockhorn-Dworniczack *et al.*, 1991). For successful SSCP, the following parameters are crucial: low temperature (4°C), low pH buffer systems and the addition of glycerol to the SSCP gel (Kukita *et al.*, 1997).

Heteroduplex analysis

Heteroduplex analysis (HA) is an adaptation of SSCP. This technique relies on detecting imperfect double helices when two partially homologous single strands anneal, caused by the presence of sequence changes (Glavac and Dean, 1995). During PCR, wild type alleles will associate with mutant type ones forming a heteroduplex, with a mismatched 'bubble' (Pogue *et al.*, 1998). The amplified DNA is heated to melt and cooled slowly to anneal. During electrophoresis, heteroduplexes (wild and mutant type alleles) will migrate at a lower velocity compared to homoduplexes (wild type alleles) of equal size (***Figure 8.2***). This mobility shift is detectable on polyacrylamide gels. HA can be performed non-radioactively with examination by silver staining (Dockhorn-Dworniczak *et al.*, 1991).

Both SSCP and HA are powerful, simple and cost-effective methods for screening sequence changes within single DNA strands (Pogue *et al.*, 1998). They offer >95% (or lower for HA) mutation detection efficiency with a fragment of 150-300 bp and are amenable to automation making them suitable for large-scale screening (Hayashi,

1992). The major disadvantages of SSCP are a lack of theoretical support that would predict the mutations detectable and the electrophoretic patterns expected.

Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) also detects the mobility shift of amplified DNA fragments (allelic homoduplexes as well as heteroduplex molecules) through denaturing gels (Myers *et al.*, 1987). The melting point of a DNA molecule is dependent on its nucleotide sequence, thus any change in sequence will alter the melting profile of a locus (Sheffield *et al.*, 1989). Because every variant has a different melting profile, allelic amplimers will dissociate and slow down at different denaturant concentrations. Driven by the electric field, the amplimers bearing the locus to be analysed migrate along a gradient of increasing denaturant concentrations.

However, to avoid complete dissociation of the amplimers, which would defeat the principle of DGGE, they are 'clamped' by adding a GC-rich tail (~40 nucleotides) at the 5'- or 3'-ends of the PCR primers (Sheffield *et al.*, 1989). This GC-rich domain has a high melting point and ensures that the clamped amplimers remain in a partially duplex state when a fragment reaches a discrete denaturing concentration (Marshal and Sklar, 1996). Differently, the lower melting domain, which is the domain of interest to be screened for mutations, melts into single strands with the effect that the amplification product will cease migrating at this position in the gel.

DGGE promises 100% accurate detection for screening sequence polymorphism (small deletions and insertions, point mutations) in DNA fragments of about 350 bp in

length (Fischer and Lerman, 1979). However, it has remained unpopular for the following reasons: there is no universal DGGE protocol, the technique has to be readapted for each locus, requires specialised equipment, not applicable to genes with high G+C content and is a laborious method requiring considerable time and effort to find the optimal melting domain and transposing the theory into practice.

8.5.2 Cleavage-based polymorphism scanning methods

An adaptation of HA, is the cleavage of heteroduplex molecules at the site of mismatched base pairs in ds DNA. The fragment of interest is amplified creating a heteroduplex (between the mutant- and radiolabelled wild-type sequences), which is then subjected to modification (Myers *et al.*, 1985). At the point of mismatch, there is ss DNA which is easily recognised and modified enzymatically (i.e. nucleases) or chemically (Myers *et al.*, 1985; Cotton *et al.*, 1988). Examples of chemical modifying agents are hydroxylamine, which modifies mismatched cytosines (i.e. C-C, C-T and C-A mismatches) and osmium tetroxide, which modifies mismatched thymines (i.e. T-G, T-C and T-T mismatches). Piperidine is then used to cleave at the modified points of mismatch, followed by electrophoresis and autoradiography.

Presence of mutations is confirmed by the presence of cleaved DNA fragments. The size of the fragments, revealed by electrophoresis, give the exact location of the mismatch(es), which is in fact the position(s) of the mutation(s). The method has a high sensitivity (~100%) for the *de novo* scanning for point mutations in large genomic DNA fragments (Myers *et al.*, 1985). The disadvantages of the method are that mutant homozygotes are missed and it is labour intensive as well as technically demanding, requiring costly enzymes and toxic chemicals.

8.5.3 DNA sequencing methods

DNA sequencing is the ultimate universal technique for detecting genetic variation. It defines the exact nature as well as the precise location of the mutation detected by any

of the above-mentioned scanning technique. Its efficiency of detection is 100% and is applicable directly on selected loci amplified by PCR (Gylleensten and Erlich, 1988).

Currently several methods are available for the sequencing of PCR products. Manual sequencing generally uses a radioactive label and visualises the banding pattern by autoradiography. However, the inconvenience of using radioisotope, its disposal and the health risks associated, has encouraged automating DNA sequence analysis, which is less time-consuming and laborious. Automated sequencers use a laser to detect DNA fragments labelled with fluorescent dyes, coupled to immediate data acquisition and automatic base calling as well as data entry.

Fluorescence-based sequencing is an adaptation of the chain-termination method developed by Sanger and colleagues (Sanger *et al.*, 1977). The DNA to be sequenced acts as a template for the enzymatic synthesis of new DNA starting at a specific primer-binding site. A mixture of both deoxy- and dideoxy-nucleotides is used. Incorporation of a dideoxynucleotide blocks further chain elongation, resulting in a population of truncated fragments of varying lengths.

The identity of the chain terminating nucleotide at each position can be specified by running a combined reaction using labels specific for each dideoxy nucleotide or running four separate reactions, each of which contains a single dideoxynucleotide (ddATP, ddGTP, ddTTP, ddCTP). The resulting population of molecules is separated by size on a denaturing high-resolution polyacrylamide gel. The sequence is subsequently specified by correlating the order of the bands on the gel with the

dideoxynulceotide used to generate each band. Please see ***Chapter 2, Sections 2.4.4.6 - 2.4.4.9*** for a detailed method description used in this thesis.

Automatic sequencing has several limitations: it is not cost-effective and the DNA template must be clean, with the excess primers and dNTPs removed, so as to avoid noise which make the nucleotides unrecognisable. Furthermore, the current sequencers are not designed to 'call' heterozygotes. Since heterozygote amplimers contain two genuine sequences, biochemical hardware and software improvements are necessary to discriminate genuine variation from noise unambiguously.

8.5.4 Chip technology

Chip technology is the brainchild of the Human Genome Project. The 'chip' is a piece of glass to which a large number of oligonucleotides are attached in a linear array. Each oligomer is synthesised with three different base substitutions at each position. A fluorescently labelled probe is prepared from the test DNA and is hybridised to the glass chip. After washing, the image of the chip is captured with a fluorescence microscope. Fluorescent labelling will occur only over the oligonucleotides for which there is a perfect match and a computer reads the sequence. This is possible only for region where the 'standard' sequence is known (Halushka *et al.*, 1999).

Chip technology is an over powerful method with a very high throughput and sensitivity. It can detect 100% of the genetic variation. But it requires specialised instrumentation and therefore is prohibitively expensive. Mutations near

polymorphisms are expected to be problematic. It is essential to know the full accurate sequence before a chip can be constructed.

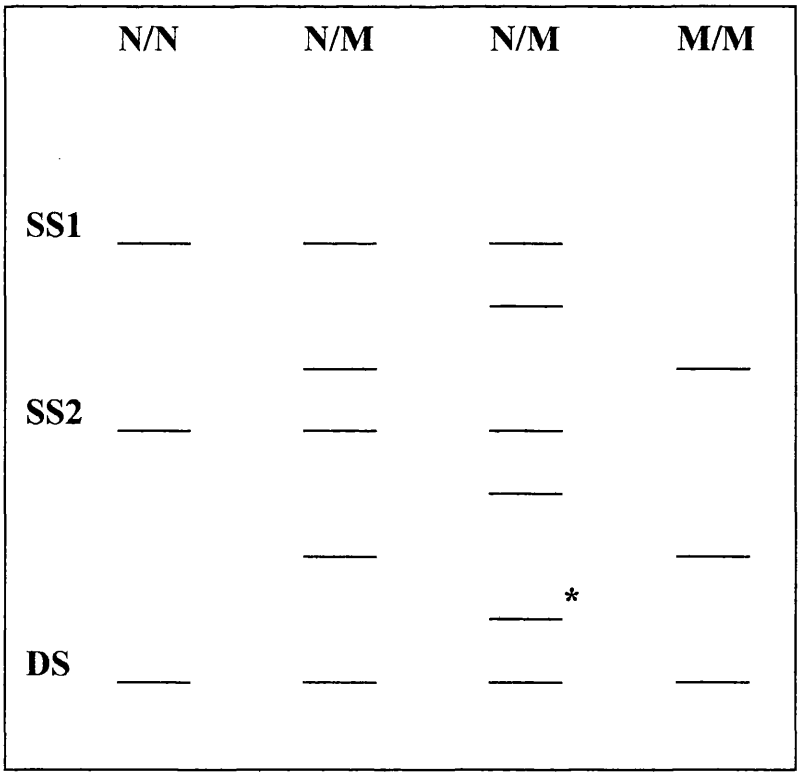
Table 8.1: Genetic variation screening methods

Technique	**SSCP**	**HA**	**DGGE**	**Cleavage of mismatches**
1st genetic application	1989	1986	1979	1989
Main genetic application	*De novo* scanning for point mutation	Point mutation, Small deletion, Insertion	*De novo* scanning for point mutation	*De novo* scanning for point mutation
Sensitivity	High	High	High	High
Throughput	Moderate	High	Moderate	High
Disadvantage	Lengthy electrophoresis	Misses mutant homozygotes	Lengthy electrophoresis Special equipment Difficult set up	Misses mutant homozygotes Costly enzyme Mutagens' use

Figure 8.2: Schematic diagram representing the band patterns expected for SSCP and HA.

After heat denaturation, the amplified PCR products are subjected to electrophoresis and the sequence difference is detected as a difference in electrophoretic mobility. DNA single strands are indicated as 'SS' whilst re-annealed and/or non-denatured DNA is shown as 'DS', i.e. double-stranded. Homozygosity for the normal allele is denoted as NN, homozygosity for the mutant allele as MM and heterozygosity as NM. For homozygous samples, two bands are observed representing the two populations of conformers, while fours bands are present for heterozygous samples.

A heteroduplex shift, sometimes occurring in SSCP, is shown for one heterozygote pattern (denoted by an asterisk) and is recognized by the presence of additional slowly migrating heteroduplex bands.



Question 8.6: The use of neopterin as a surrogate marker for tetrahydrobiopterins should be considered as well as the consideration of other pathways for tetrahydrobiopterin manufacture, which are not discussed in the thesis.

5,6,7,8-Tetrahydrobiopterin (BH_4) and its biosynthetic enzymes are believed to be present in all organs and many tissues of the human body as the cofactor is essential for various enzyme activities and is essential for regulating endothelial nitric oxide (NO) production (Schoedon *et al.*, 1993; 1995b, Liu and Gross, 1996). An impaired BH_4 production is believed to predate to endothelial dysfunction and an increase in cardiovascular risk (Maier , 2000; Tiefenbacher *et al.*, 2000).

Cells generate BH_4 by two distinct pathways, the *de novo* biosynthetic pathway that uses guanosine 5'-triphosphate (GTP) as a precursor and the salvage pathway that depends on pre-existing, non-enzymatically generated dihydropterins (Nichol *et al.*, 1983; Smith and Nichol, 1983; Duch and Smith, 1991). The *de novo* biosynthesis is important for viability as genetic defects in its biosynthetic genes are usually fatal unless treated with an external supply of synthetic BH_4.

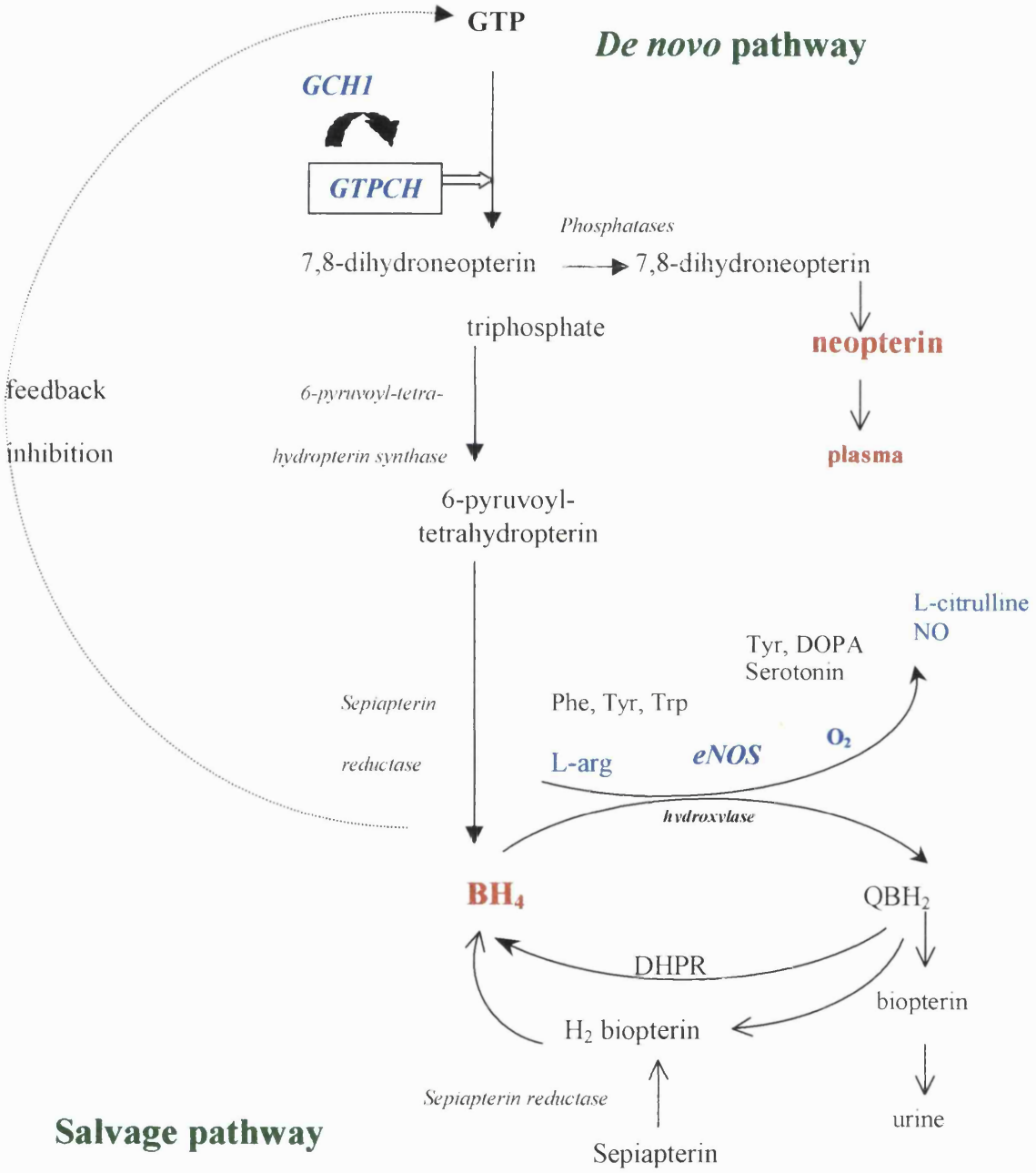
Both animal and cell culture studies have shown that exogenous dihydropterins, i.e. sepiapterin, are converted to dihydrobiopterin by sepiapterin reductase (SR) and eventually to BH_4 by dihydrofolate reductase (DHFR) (Schmidt *et al.*, 1992). The salvage pathway was demonstrated in cell culture and *in vivo* to be a methotrexate sensitive route, that is DHFR dependent (Nichol *et al.*, 1983). It is known that this pathway is not important for normal metabolism of the cell, with its exact role *in vivo*

still unknown. However, it provides a way of increasing intracellular BH_4, independently of its synthesis from GTP, by administering synthetic compounds (Nichol *et al.*, 1983).

The *de novo* biosynthesis of BH_4 from GTP is a three-enzyme pathway involving GTP cyclohydrolase I (GTPCH), 6-pyruvoyl tetrahydropterin synthase (PTPS) and sepiapterin reductase (SR) (Curtius *et al.*, 1986, Duch and Smith, 1991). The two sequential reaction intermediates are: 7,8-dihydroneopterin triphosphate and 6-pyruvoyl tetrahydropterin (Blau and Niederweiser, 1985). In humans, 7,8-dihydroneopterin triphosphate can also be converted to neopterin, a stable end-product (Schoedon *et al.*, 1987).

Both biosynthetic pathways, that is *de novo* pathway that uses GTP as a precursor and the salvage pathway that depends on pre-existing dihydropterins, were discussed in ***Chapter 6***, ***Section 6.2.2*** and shown in ***Figure 6.1***, entitled '***Proposed de novo and salvage pathway of*** *BH_4* ***synthesis***'.

Figure 6.1: Proposed *de novo* and salvage pathway of tetrahydrobiopterin synthesis.



One of the aims of my thesis was to examine whether common polymorphisms in GTP cyclohydrolase gene (*GCH1*) would cause a subtle alteration in the activity or expression of the encoded GTPCH enzyme, thereby affecting BH_4 production. However, quantifying BH_4 levels in clinical settings poses problems as it is unstable and therefore would not be a good measure of GTPCH activity *in vivo*. BH_4 needs to be derivatised prior estimation, thereby risking samples' contamination and the methods currently available are not convenient for large scale clinical measurements. After several months of unsuccessful attempts at measuring BH_4 by high pressure liquid chromatography (Fukishima and Nixon, 1980), an alternative was sought.

To evaluate the status of the pterin pathway, as an index of GTPCH activity, a sensitive and reliable plasma phenotypic marker was needed. Also, the assay method used had to be applicable to an epidemiological study. In conjunction, neopterin was chosen as it is metabolically stable in frozen biological fluids and is stable in normal indoor light (Müller *et al.*, 1991), its direct measurement by ELISA is possible without inclusion of reduced forms and it is an indirect measure of BH_4 (Fuchs *et al.*, 1988a). Furthermore, neopterin has been shown to be a well-validated surrogate marker to evaluate the status of the biosynthetic pathway as well as an index of GTPCH activity.

Neopterin has no definite particular function (Fuchs *et al.*, 1988a) but it serves as a useful serum marker of pterin pathway activation (Wachter *et al.*, 1992) and reflects very sensitively the *in vivo* state for activated cell-mediated immunity (Schoedon *et al.*, 1987; Fuith *et al.*, 1991). Both *in vitro* and *in vivo* studies showed that upon exposure to cytokines, there is a rise in GTPCH activity in endothelial cells, accompanied by neopterin secretion (Schoedon *et al.*, 1993).

Please refer to the following chapters and sections of my thesis, which details extensively the use of neopterin as a surrogate marker of BH_4 with the sub-titles explicit about the contents discussed:

- **Chapter 6** **Neopterin: Marker of GTPCH activity**

  Section 6.2.1 BH_4: Role & regulation

  Section 6.2.2 Neopterin: Marker of BH_4 synthesis

- **Chapter 1** **NO - Biochemistry and endothelial cell dysfunction**

  Section 1.8 GTP Cyclohydrolase I: Gene (*GCH1*) and enzyme (GTPCH)

  Section 1.9 Tetrahydrobiopterin (BH_4): Essential cofactor of eNOS

  Section 1.10 Plasma neopterin: Index of GTPCH

The neopterin levels measured in the NPHSII cohort (range 2.16-33.5 nmol/l; mean = 6.0 nmol/l) were similar to those measured by other investigators: Fuith *et al.*, 1991, mean = 6.8 nmol/l; Muller *et al.*, 1991, range 2.6-8.7 nmol/l; Schumacher *et al.*, 1997, mean = 5.3 nmol/l and Tatber *et al.*, 1991, 6.0±2 nmol/l. Our results were also in line with those measured by Garcia-Moll and collaborators in a recent cohort study (range 3.9-11.5 nmol/l; mean = 6.6 nmol/l) at the St George's Hospital Medical School, London (Garcia-Moll *et al.*, 2000) by the same commercial immunoassay.

Although neopterin is currently used to assess the prognosis of various disorders (i.e. such as malignant cancers, AIDS, septicemia, liver and kidney transplants) (Pacher *et al.*, 1989), there is presently no information available on neopterin reference range as

well as long-term storage stability of plasma samples prior neopterin assay in the literature, from an epidemiological study. This study is the most complete up to date assessment of plasma neopterin as a marker of cardiovascular risk in a well-characterised cohort.

Question 8.7: The examiners were also concerned that there were aspects of the design of the study, which led to, inevitably inconclusive results. We understand that in the cohort of patients studied the DNA samples from those patients who had events were not necessarily available and therefore the most powerful study to determine the effect of these particular genes on cardiovascular disease was not possible. These methodological problems should have been considered at the beginning of the study along with whether the measurements of NO_x in plasma and neopterin were in fact relevant to the measurement of the generation of nitric oxide and tetrahydrobiopterin respectively.

The first part of this question is addressed below and the second part (i.e. "These methodological problems... respectively.") has already been covered extensively in Questions 2 and 6 as well as in the relevant section of my thesis mentioned.

One of the principle aim of my thesis was to investigate the relationship between endothelial nitric oxide synthase gene (*NOS3*) and GTP cyclohydrolase I (*GCH1*) genotypes and the risk of ischaemic heart disease (IHD) event in the second Northwick Park Heart Study (NPHSII) (See ***Chapter 1, Section 1.11: Aims of my thesis***). The 2745 DNA samples of the NPHSII, stored in 32 Master Arrays, were genotyped for the following polymorphisms:

- -922(A/G), -786(T/C), In4 VNTR and +894(G/T) in *NOS3* (***Chapter 4, Section 4.4.2***)
- -577(G/A) and -796(G/A) in *GCH1* (***Chapter 5, Section 5.4.3***)

The genotyping protocol was discussed in ***Chapter 2, Sections 2.3.3, 2.4.1*** and ***2.4.2*** as well as ***Chapter 5, Section 5.3.7***. The 96-well arrays contained the DNA samples of all NPHSII subjects, **INCLUSIVE** of those subjects who subsequently developed IHD. An IHD event was defined as fatal, non-fatal or silent MI and coronary surgery. At the time of the analysis, the following data was available:

- There were 191 IHD cases.
- Only 15 and 20 IHD events among men with NO_x (n=955) and neopterin (n=939) measurements respectively.
- The frequency of genotypes, for both genes, by IHD event are tabulated below:

Gene	**Polymorphism**	**Frequency**	
		Genotyping	**IHD event**
NOS3	-922(A/G)	2792	138
	-786(T/C)	2720	135
	In4 VNTR	2710	132
	+894(G/T)	2584	128
GCH1	-577(G/A)	2390	117
	-796(G/A)	2386	118

The influence of the polymorphisms studied in determining the future risk of IHD was then assessed by Cox regression. It was found that neither *NOS3* nor *GCH1* genotype, for the polymorphisms studied, predicted future IHD events in middle-aged British men, i.e. NPHSII.

Kaplan-Meier survival plots of the effects of the six genotypes studied on IHD event-free survival are illustrated in ***Figure 4.6***, ***Chapter 4*** and ***Figure 5.11***, ***Chapter 5***. There were no evidence of a relationship between *NOS3* (***Table 4.5***) and *GCH1* (***Table 5.10***) polymorphism and the risk of IHD, when analysed according to dominant or recessive models.

However, the failure to detect an association of IHD with any of the variants does not exclude *NOS3* and *GCH1* involvement with the susceptibility to a coronary event. Although the general belief is that an association between two variables will imply a direct specific causal relationship between both, the likelihood that *NOS3* and *GCH1* may confer susceptibility to IHD via the NO and the pterin pathways respectively cannot be excluded. Alternatively, the possibility of another variant located elsewhere or near the gene may be linked to the disease.

There is the possibility that the lack of a statistically significant effect on risk may be due to the small number of IHD events observed in this 'healthy' cohort. Further IHD events will occur with time as we are dealing with apparently healthy individuals who have a high prevalence of sub-clinical disease, which with time will evolve into the clinical disease.

Thus, the failure to detect an influence of polymorphism on IHD risk may be due to the lack of statistical power. Using data from the North Mymms practice, a power calculation was carried out in 1996 prior to study commencement. It was estimated that with 150 IHD events, matched with twice the number of controls, there was 90%

power to detect a relative risk of 2.2 in gene carriers assuming a recessive model and a 90% power to detect a relative risk of 1.6 under a dominant model at p=0.05.

Therefore, if either of the variants were actually associated with a risk lower than this the study would be underpowered. Since most common variants in a single gene are associated with only a modest impact on risk in the range of 1.2-1.5 this may be the case here. However the loss of power due to some DNA not being available on a small proportion of those who subsequently went on to become a case would not have influenced this conclusion to any great extent.

DNA samples were not available from all subjects recruited into the study. This was for a variety of reasons including failure to obtain a blood sample and failure of DNA extraction. Such 'failures' were random with respect to any other baseline or clinical features and the characteristics of the men where DNA was obtained were identical to those where it was not (not shown). Some of these 'failures' went on to have an event (the same proportion as those where DNA was obtained).

There is thus an inevitable 'loss of power' but of an inconsequential size since the vast majority of subjects had DNA obtained. Similarly, some subjects where DNA was obtained were refractory to NOS genotyping despite several repeats, suggesting that the DNA was of poor quality initially or have become degraded subsequently. These samples in general also failed for other gene variants (*Personal Communication*; Jackie Cooper, NPHS database co-ordinator). This 'failure' was also randomly distributed with respect to characteristics (not shown).

APPENDIX 1

PATIENT CONSENT FORM

MRC Study of Risk of Heart Attack

Surname (Please print) _____

Survey number _____

This general practice is one of 8 in the United Kingdom collaborating with the Medical Research Council in a long-term study designed to improve the doctor's ability to recognise people who are at high risk for heart attack and to treat them in a timely fashion. Each practice is requesting all men aged 50-59 years to volunteer to take part in the study, provided they are not already known to have suffered a heart attack and the doctor knows of no medical reason why it would be unsafe to do so. Participation in the study is expected to last for 5 years, and will consist of the following:

At commencement, a medical interview, brief examination, and a blood test to establish current state of health.

Yearly thereafter for 4 years, a short interview and repeat blood test.

At the end of 5 years, a final interview, brief examination and blood test.

On each occasion, 20 ml of blood (four teaspoonsful) will be taken for laboratory tests specifically related to the assessment of risk of heart attack.

There are no known risks associated with this study, and all procedures except the laboratory tests on the blood are entirely routine. The blood sampling may leave a little soreness and discolouration for a short period but that is all. All men enrolled into the study will receive proper medical care for any condition disclosed during examination and follow-up, which in the doctor's opinion requires attention. Importantly, participation in the study will not interfere with any medical care and attention provided by the doctor.

We should be grateful if you would agree to take part in this study by signing the form below. Obviously, you will be free to withdraw at any stage if you so wish, without prejudice to your future care.

I have read the above explanation and have had the opportunity to ask any questions I wish regarding the purposes and procedures of this study. On the basis of the explanation on this form, I agree to participate in this study of risk of heart attack.

Signed _____

Date _____

Family Physician's Agreement

I am the general practitioner of the above patient and in my opinion there is nothing in the medical history to contraindicate participation in the study. The patient would enter the study with my consent.

Signed _____

Date _____

APPENDIX 2

COPY OF LETTER FROM DR MILLER TO PATIENTS

Date

Dear

MRC Study of Risk of Heart Attack

The main part of the study of risk of heart attack has now been completed in your General Practice. However, we would like your permission to keep your progress under special review in two ways:

i) by a 6- monthly review of your medical records held in the practice,

ii) by obtaining details of any tests relating to the heart and circulation that you may have in the future, by writing to the records department of the hospital concerned.

If you have no objection to these procedures we should be very grateful if you would give formal permission by signing overleaf and returning the complete letter to the practice in the pre-paid envelope enclosed. As always, all information will be kept fully confidential and used only for statistical analysis. We plan to write eventually to all those who took part to describe the major findings of the research.

Many thanks for your continued support and interest. Please telephone and speak to Sister..... about any queries you may have before signing overleaf.

Yours sincerely

Dr G J Miller

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Hypertension 2001 Nov;38(5):1054-61

Genetic and environmental determinants of plasma nitrogen oxides and risk of ischemic heart disease.

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Endothelial dysfunction, caused in part by reduced NO bioavailability, is a feature of hypercholesterolemia, hypertension, smoking, and atherosclerosis. We examined whether cholesterol, blood pressure, smoking status, and polymorphisms in the endothelial NO synthase gene (NOS 3) influence NO production (as assessed by the plasma levels of nitrogen oxides, NO(x)) in middle-aged men. We also determined whether plasma NO(x) or NOS 3 genotype predicted the risk of is chemic heart disease (IHD). We studied 3052 men who were initially free of IHD and recruited from 9 UK primary care practices. Blood pressure, age, body mass index, serum cholesterol, and smoking status were assessed at baseline and annually over 8.1 years of follow-up, and all IHD events were recorded. DNA samples were screened for 4 NOS 3 gene polymorphisms: -786 T/C, -922 A/G, 894 G/T (which predicts a Glu(298)-->Asp amino acid substitution in the mature protein), and a 27-bp tandem repeat in intron 4 (eNOS4a/4b). NO(x) was measured in plasma samples obtained on entry in 1121 participants from North Mymms and Chesterfield general practices, together with an additional 571 recruits selected at random. Genotype frequencies were in Hardy-Weinberg equilibrium, and linkage disequilibrium was detected between all the NOS 3 polymorphismsstudied, with the strongest allelic association being detected between -922 A/G and -786 T/C polymorphisms in the gene promoter (Delta=0.90, P<0.001). Plasma NO(x) was lower in smokers than in nonsmokers in the North Mymms (10.8+/-4.5 versus 11.8+/-4.6 micromol/L, P=0.13), Chesterfield (8.4+/-3.6 versus 9.9+/-4.0 micromol/L, P=0.01), and random samples (10.7+/-5.1 versus 11.7+/-4.7 micromol/L, P=0.03). A weak but significant inverse relationship was detected between plasma NO(x) and serum cholesterol only in the North Mymms data set (r=-0.14, P=0.02). No relationship was detected between plasma NO(x) and any of the NOS 3 polymorphisms, nor was there any association between any NOS 3 polymorphism and risk of an IHD event in either smokers or nonsmokers. These data support the hypothesis that the endothelial dysfunction observed in the blood vessels of smokers is related to reduced NO bioactivity but indicate that NOS 3 genotype does not influence significantly the level of plasma NO(x) or the risk of IHD in this population sample of middle-aged British men.

THE E298D ENDOTHELIAL NITRIC OXIDE SYNTHASE GENE POLYMORPHISM INTERACTS WITH ENVIRONMENTAL AND DIETARY FACTORS TO INFLUENCE ENDOTHELIAL FUNCTION

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Running title - Gene-environment and endothelium

ABSTRACT

Background - An endothelial nitric oxide synthase (eNOS) gene polymorphism (E298D) has been associated with cardiovascular disease. We investigated whether the polymorphism was related to functional changes in the endothelium and how genotype altered the harmful and beneficial impact of environmental influences.

Methods - Endothelium-dependent, flow mediated, brachial artery dilatation (FMD), and endothelium-independent dilatation response to glyceryl trinitrate were measured using high-resolution ultrasound, in 248 subjects (131 female 117 male, aged 20 to 28) genotyped for the E298D polymorphism. Vascular function was compared between genotype groups and interactions with the pro-atherogenic risk factor, smoking, and the anti-atherogenic influence of n-3 fatty acids (n-3FA) were investigated.

Results - Vascular function was not related to genotype in the whole group or within sexes. However, among males, smoking was associated with lower FMD in D298 carriers (non-smokers = 0.125 ± 0.085 mm vs smokers = 0.070 ± 0.060 mm, p=0.006) but not in E298

homozygotes (non-smokers = 0.103 ± 0.090 mm vs smokers = 0.124 ± 0.106, p = 0.5). In the whole group, n-3FA levels were positively related to FMD in D298 carriers (reg coeff = 0.023 mm/%, p = 0.04) but not in E298 homozygotes (reg coeff = -0.019 mm/%, p = 0.1). These differences between genotype groups were significant in interaction models.

Conclusions- The E298D polymorphism is associated with differences in endothelial responses to both smoking and n-3 FA in healthy young subjects. These findings raise the possibility of genotype-specific prevention strategies in cardiovascular disease.

Key words: endothelium, nitric oxide synthase, diet, smoking, genetics.

BACKGROUND

Despite major advances in treatment, coronary artery disease remains the biggest cause of mortality and morbidity in the developed world [1]. Further reduction of the cardiovascular disease burden in society may require new strategies not only to improve the management of clinical disease but also to modify the initiation and progression of atherosclerosis early in life [2-4]. Genetic variation is likely to be an important determinant of both the development of atherosclerosis and clinical sequelae. Clinical research has focused on the association of genetic factors to late cardiovascular outcome [5-9] and conflicting data has been obtained on the impact of some of these gene polymorphisms. This may be, at least in part, because any genetic effect is modified throughout life by environmental influences and this potentially confounding lifetime risk factor burden is hard to quantify retrospectively.

Less attention has been paid to genetic influences on the vascular biology of atherosclerosis during the long preclinical phase which begins in childhood. Functional polymorphisms in the gene which encodes vascular endothelial nitric oxide synthase (eNOS) have the potential

to affect early disease development. eNOS is responsible for the conversion of L-arginine to nitric oxide in the endothelium [10] and loss of endothelial-derived nitric oxide plays a key role in atherogenesis [11]. Experimental inhibition of nitric oxide synthesis accelerates the formation of early atherosclerotic lesions [12] and nitric oxide is known to influence platelet aggregation [13], smooth muscle cell migration [14] and growth [15], as well as oxidation of low-density lipoprotein [16]. A E298D polymorphism in the eNOS gene has recently been associated with development of ischaemic heart disease and myocardial infarction [9,17]. The effect of this polymorphism on endothelial function and in particular its influence at an earlier stage in atherogenesis remains unknown.

Flow mediated arterial dilation is a nitric oxide-dependent endothelial response which can be measured non-invasively in vivo using high-resolution ultrasound [18,19]. We have used this method to investigate whether the E298D polymorphism influences endothelial function in young, preclinical subjects and how this genetic variation affects response to the potentially harmful and beneficial environmental influences of cigarette smoking and n-3 fatty acid intake [18,20-22].

METHODS

Study design

We studied 248 subjects (131 female, 117 male) aged 20 to 28 years. Invitations to attend a clinic for cardiovascular risk profile evaluation by venepuncture, questionnaire, physical measurements and vascular studies, were sent to random samples of individuals born in Cambridge Maternity Hospital (in whom early growth measures had been recorded) between 1969 and 1975 until the planned study size was reached. With this sample size the study had 80% power at 5% significance to identify a 0.02 mm difference in FMD between groups, such as smokers and non-smokers. Ethical approval was received from local research ethics committees and informed consent was obtained at the time of the study visit.

Measurement of cardiovascular risk factors

Personal and family medical histories were obtained by questionnaire at interview. Subjects were classified as smokers if they reported smoking one or more cigarettes a day for the previous six months. Blood pressure was measured as the average of the last two of three seated readings using an automated oscillometric device (Critikon Inc, USA). Weight was

recorded (to +/- 0.1 kg) using scales (Soenhle Ltd) and height (to +/- 0.1 mm) with a portable stadiometer.

Biochemical measurements

In each subject, fasting venous blood samples were analysed for insulin, glucose, total cholesterol, HDL, LDL and triglyceride concentrations by routine methods. During the study, plasma and erythrocyte membrane ghost samples were stored to permit measurement of the n-3 fatty acids, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), in plasma and red blood cell membranes. Total fatty acid methyl esters were prepared from the samples by the direct one-step trans-esterification method [23] and then analysed by gas chromatography with flame ionisation detection [24]. The area of the DHA plus EPA peaks, expressed as a percentage of the total fatty acid peak areas, was used as the measure of n-3 fatty acid status.

DNA extraction and genotyping of the eNOS E298D polymorphism

DNA was extracted from stored buffy coats by the salting out method. The G894T polymorphism in exon 7 of the eNOS gene, which predicts a E298D (Glu298Asp) amino-acid substitution in the mature protein was genotyped by PCR and allele-specific restriction

enzyme digestion. PCR was performed for 35 cycles in a volume of 30μl containing 50ng of dry DNA, 50mM KCl, 10mM Tris (pH 8.3), 0.1% gelatin and 0.2mM of each dNTP, 10pmol of each primer, 2U of *Taq* DNA polymerase. Denaturation was at 95°C, annealing at 63°C and a final extension at 72°C, all for 45s. Ten μl of PCR products (151bp) were subjected to digestion with 2U *Dpn II,* which cuts only in the presence of T at position 894 (corresponding to D298). Digested samples were then loaded on an ethidium bromide-stained gel and electrophoresed for 1h at 10 V/cm at room temperature and the products detected by u.v transillumination. An independent observer confirmed all genotypes marked. Discrepancies were resolved by repeat PCR and enzyme digestion.

Vascular study measurements

Endothelium- dependent and independent responses in the brachial artery were measured for each subject while lying supine on a couch, as reported previously [17]. After ten minutes rest, the right brachial artery was imaged in longitudinal section between ten and fifteen centimetres above the ante cubital fossa using a 7 MHz linear array transducer and a Acuson 128XP/10 system. Baseline brachial artery diameter was measured using an automated Wall Tracking System (Medical Systems, Arnhem, The Netherlands), which utilises the movement

in the radio frequency amplitude peaks over the cardiac cycle, to identify the arterial walls [25]. A pneumatic cuff was then inflated to suprasystolic pressure on the forearm for four and a half minutes to induce reactive hyperaemia. Cuff deflation resulted in increased blood flow through the brachial artery, stimulating endothelial-dependent flow mediated dilatation. The brachial artery diameter one minute after cuff release was measured. After ten minutes rest a further arterial diameter measurement was made between three and four minutes after a single sublingual spray of glyceryl trinitrate (around 200 microgrammes), which produces an endothelial-independent dilatation. Endothelial dependent and independent responses were represented, respectively, as absolute change in vessel diameter after increased blood flow (flow mediated dilatation or FMD) and after GTN (GTND) as in previous studies [19].

Statistical Analysis

Variation in vascular function by genotype was assessed using one way analysis of variance and the significance of differences between genotype groups and smokers and non-smokers by unpaired student's t-tests. Continuous relationships between variables and vascular function were assessed using multiple linear regression models. The variables were added to a model of FMD which included resting vessel size as an independent variable [18].

Standard interaction models were used to determine whether the influence of a risk factor on endothelial function varied between genotype groups. Interaction of risk factors and genotype was assumed if an interaction term, being the product of the risk factor and a variable defining the genotype groups, was significant when added to the regression model containing both variables. Results are presented as mean +/- SD unless otherwise stated.

RESULTS

Sample characteristics

The characteristics of the study sample are summarised in table 1. There were no associations between cardiovascular risk factors and resting vessel size or brachial artery blood flow. Females had smaller brachial artery diameters compared to males (2.9 ± 0.3 mm v 3.7 ± 0.5 mm; $p<0.001$). There was no difference in mean FMD or GTND between sexes. Smokers had lower mean FMD than non smokers (0.090 ± 0.080 mm v 0.109 ± 0.081 mm, $p<0.05$) with no difference in GTND (0.677 ± 0.225 mm v 0.674 ± 0.184 mm, $p=0.90$). There were

no associations between other risk factors (cholesterol, LDL, HDL, fasting insulin, glucose, blood pressure) and vascular function across the narrow ranges in this study group.

Endothelial function, genotype and smoking

Genotypic proportions were in Hardy-Weinberg equilibrium with the frequency of the D298 allele being 33.3% and E298 allele being 66.7%. There were no significant differences in body size, resting vessel size or blood flow between genotype groups. There were also no significant differences in smoking frequency or levels of blood pressure, blood lipids, n-3 fatty acids or other risk factors.

Within the whole group, and in males and females, vascular function did not differ between genotype groups (ANOVA for difference in FMD, $p=0.6$, and difference in GTND, $p=0.4$). In males, however, genotype influenced the impact of smoking on FMD. In E298 homozygotes there was no difference in FMD between non-smokers and smokers (FMD in non-smokers = 0.103 ± 0.090 mm vs smokers = 0.124 ± 0.106 mm, $p=0.5$) whereas in D298 carriers (heterozygotes and D298 homozygotes) smokers had reduced FMD (FMD in non-smokers = 0.125 ± 0.085 mm vs smokers = 0.070 ± 0.060 mm, $p=0.006$) (figure 1). The

difference in the association between FMD and smoking between genotype groups was significant in an interaction model (significance of interaction term, p=0.04). Genotype was not associated with differences in the relationship between FMD and smoking in females and there was no evidence of a gene-smoking interaction on GTND.

Endothelial function, genotype and n-3 fatty acid status

The association between n-3 fatty acid levels and endothelial function also varied according to genotype. There was no significant association between plasma n-3 fatty acids and FMD in E298 homozygotes (reg coeff=-0.019 mm/%, 95%CI -0.042 to 0.003, p=0.10). There was, however, a positive association between plasma n-3 fatty acids and FMD in D298 carriers (reg coeff=0.023 mm/%, 95%CI 0.001 to 0.04, p=0.04). The interaction on FMD of the E298D genotype with plasma n-3FA levels was significant in a model (significance of interaction term, p=0.01).

The relationships were seen in the whole group with no differences by sex or smoking history. For D298 carriers, the coefficients for the association between n-3 fatty acids and FMD were positive in males (B=0.047 mm/%), females (B=0.008 mm/%), smokers (B=0.028

mm/%) and non-smokers (B=0.011mm/%) with no significant difference between these subgroups in interaction models. There were no associations between n-3FA and GTND in either genotype group (E298 homozygotes: B=-0.03 mm/%, 95%CI -0.08 to 0.03, p=0.3 and D298 carriers: B=-0.04 mm/% 95%CI -0.09 to 0.02, p=0.2).

DISCUSSION

This study provides evidence of an impact on vascular endothelial function of the E298D polymorphism of the eNOS gene in young pre-clinical adult subjects. This common variant of the eNOS gene was associated with differences in the relationship between environmental influences and vascular function, so that the presence of an D298 allele increased the likelihood of a smoking-associated reduction in endothelial function in males, and of a positive effect on vascular function of increased n-3 fatty acid levels. These findings, which demonstrate for the first time a genetically determined modulation of this phenotype of early atherosclerosis, raise the possibility of a potentially beneficial, genotype-specific interventional approach to risk factors in the young.

Our study confirmed that the powerful adverse effect of smoking on endothelial function [17,20] is already present in young subjects. The impact of smoking, however, depended on the genotype of the individual. A smoking-gene interaction has previously been reported in a small group of older subjects with the rarer polymorphism in intron-4 of the eNOS gene, in relation to clinical cardiovascular outcome [8]. Our findings provide evidence that smoking interactions with the eNOS gene may not only influence the late stages of disease but also alter the early pathogenesis of atherosclerosis by modulation of endothelial function.

The molecular effects of the E298D polymorphism on eNOS are still unclear. The kinetics of nitric oxide synthesis do not differ between E298 and D298 eNOS but recently, D298 eNOS was found to be more susceptible to proteolytic cleavage than E298 eNOS [26]. The amount of functional eNOS in subjects with the polymorphism might therefore be lower, with a resultant reduction in capacity for nitric oxide production. Nitric oxide levels in D298 carriers may be sufficient to maintain vascular homeostasis in the absence of other risk

factors. Smoking, however, increases vascular oxidative stress and thus may reduce bio-availability of a limited nitric oxide resource.

In females, there was no interaction between genotype and smoking risk. The development of endothelial dysfunction and atherosclerosis in pre-menopausal females follows a different pattern to men, probably due to oestrogen-mediated protection of the endothelium [27]. Oestrogen has been shown to have numerous effects including upregulation of endothelial nitiric oxide synthase activity.

In those subjects carrying the D298 allele, higher n-3 fatty acid levels had an opposite impact on endothelial function to that of smoking and this was graded across the levels of n-3FA usually present in normal populations [22,28]. Dietary supplementation with n-3 fatty acids has been shown to improve microvascular endothelial function, in vitro, in those at risk for cardiovascular disease [29] and this may be a mechanism for the inverse association between fish consumption, the major dietary source of n-3 fatty acids, and cardiovascular disease mortality [28]. The current study provides novel data, that suggests the impact on endothelial

function of n-3 fatty acids depends on the genetic profile of the individual from early in the disease process.

The mechanism for this interaction between genotype and n-3 fatty acids is not clear. N-3 fatty acids have been shown to regulate gene expression in lipogenic tissues, such as hepatocytes and adipocytes, probably by altering mRNA stability or the rate of gene transcription through interaction with a polyunsaturated fatty acid responsive element. N-3 fatty acids have also been shown to regulate genes in non-lipogenic tissues, including the immune system and gastrointestinal tract, although there have been no studies on the endothelium [30]. At a cellular level, raised levels of n-3 fatty acids are associated with greater membrane fluidity which results in increased activation of membrane bound enzymes, which include eNOS, and signal transduction pathways [28]. Thereby, n-3 fatty acids may optimise a genetically limited endothelial nitric oxide response.

Our finding that vascular function is altered in those with one or more D298 allele is supported by recent experimental work. In patients on circulatory by-pass, the pressor effect

of phenylephrine was shown to be associated with E298D genotype, with the blood pressure response being positively associated with the number of D298 alleles [31]. This observation is consistent with an attenuated basal nitric oxide release in D298 carriers. In our study of young subjects functional differences in the endothelium were only evident when other environmental risk factors were taken into account. Clinical outcome studies have shown the D298 allele to be more prevalent among myocardial infarction sufferers and individuals with angiographic coronary heart disease [9]. A complex interaction between genotype and lifetime risk factor burden is likely to determine eventual cardiovascular mortality and morbidity. Our study suggests that carriage of the eNOS D298 variant is associated with altered endothelial function from as early as the third decade of life which becomes manifest in the presence of cardiovascular risk factors.

Our data do not prove genotype, n-3 fatty acids or smoking are causally related to alterations in endothelial function and potential confounding influences need to be investigated in future studies. The current work has studied a cohort with a range of risk factors and smoking prevalence similar to that seen in the normal United Kingdom young adult population.

Further geographically distinct studies would be useful to confirm our findings and the impact of interventions to modify environmental risk factors should be examined in prospective studies of genotyped individuals.

We have shown that the eNOS E298D polymorphism is associated with differences in the response of the arterial wall to both a pro-atherogenic environmental risk factor, smoking, and also an anti-atherogenic factor related to diet, n-3 fatty acid status. Male subjects with the D298 allele may have a specific increased risk from smoking and such information could add extra force to individual counselling on smoking prevention. The interaction between n-3 fatty acid status and genotype is a novel finding. In clinical trials, variation in response to n-3 fatty acids may be partly explained by differences in genotype Further research is needed to determine whether dietary advice to increase fish consumption could be specifically directed towards subjects with genetic differences which disturb endothelial function. Our results demonstrate that genetic effects on cardiovascular disease need to be studied in conjunction with other modifying environmental factors to determine their impact on vascular disease development.

ACKNOWLEDGMENTS

We would like to thank Anuska Mann for her help with n-3 fatty acid measurements, and Ian Merryweather, Tristan Cope and Sarah Jones for their help with recruitment, data collection and biochemical work. We are very grateful for the expert assistance of Lisa Jones in genotyping the subjects. The Cambridge Young Adults Study and CPML were funded by the Medical Research Council and the n-3 fatty acid analyses by the Ministry of Agriculture, Fisheries and Food. ADH is the British Heart Foundation (Gerry Turner) Intermedate Fellow. NJ was funded by a Commonwealth Scholarship and SEH by the British Heart Foundation (grant no: RG95005)

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FIGURE LEGENDS

Figure 1: The figure shows that in males there is no difference in flow mediated dilatation between smokers and non-smokers who are homozygous for the E298 allele whereas there is a significant reduction in flow mediated dilation in smokers who carry an D298 allele. Mean flow mediated dilation is presented with standard errors.

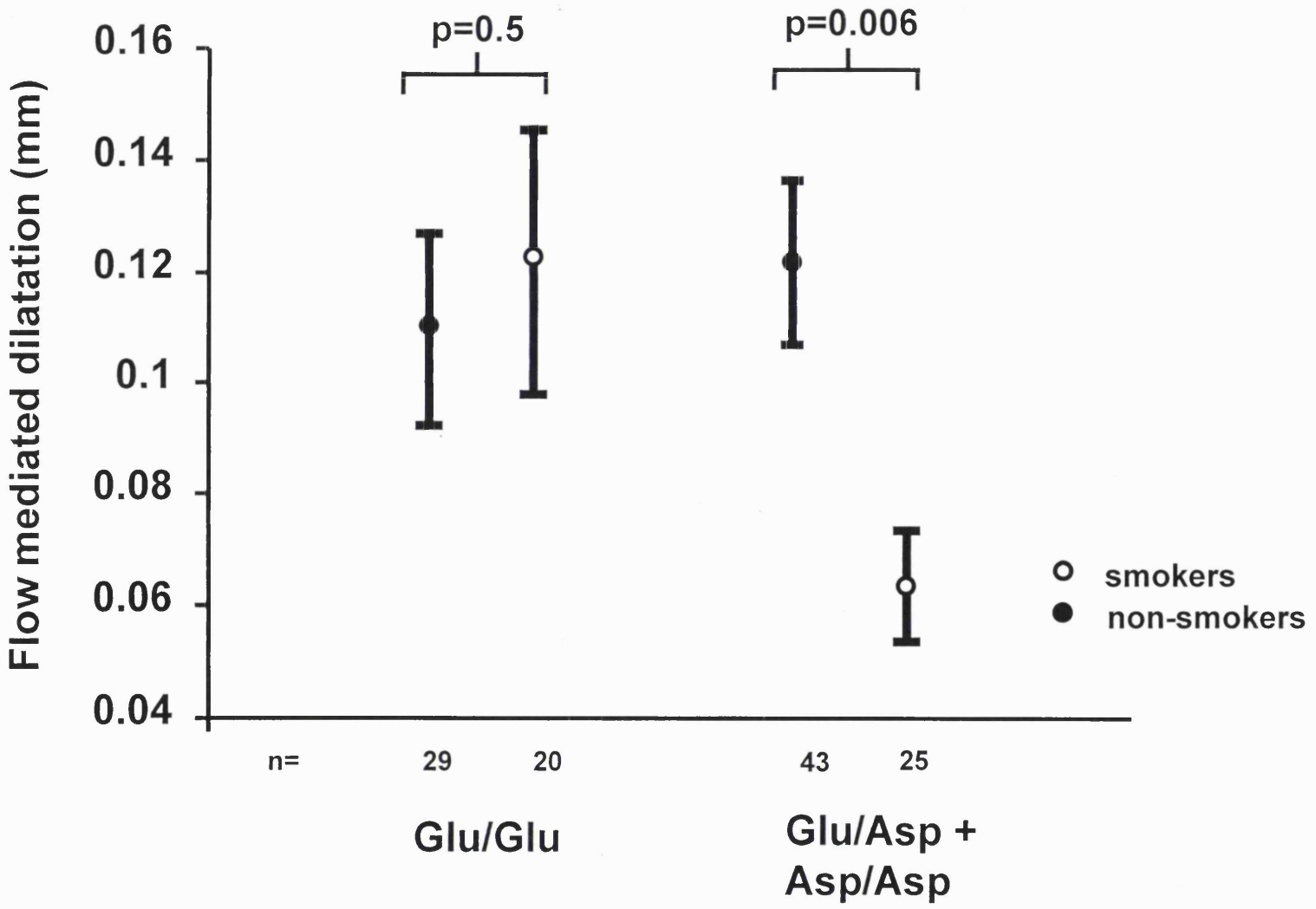
Figure 2: The figure shows the significant positive relationship between n-3 fatty acid levels and flow mediated dilation in carriers of the D298 allele with no overall relationship between n-3 fatty acids and flow mediated dilation in E298 homozygotes. The mean level of flow mediated dilation with standard errors are presented at the mean level of plasma EPA plus DHA in each third of the n-3 fatty acid distribution.

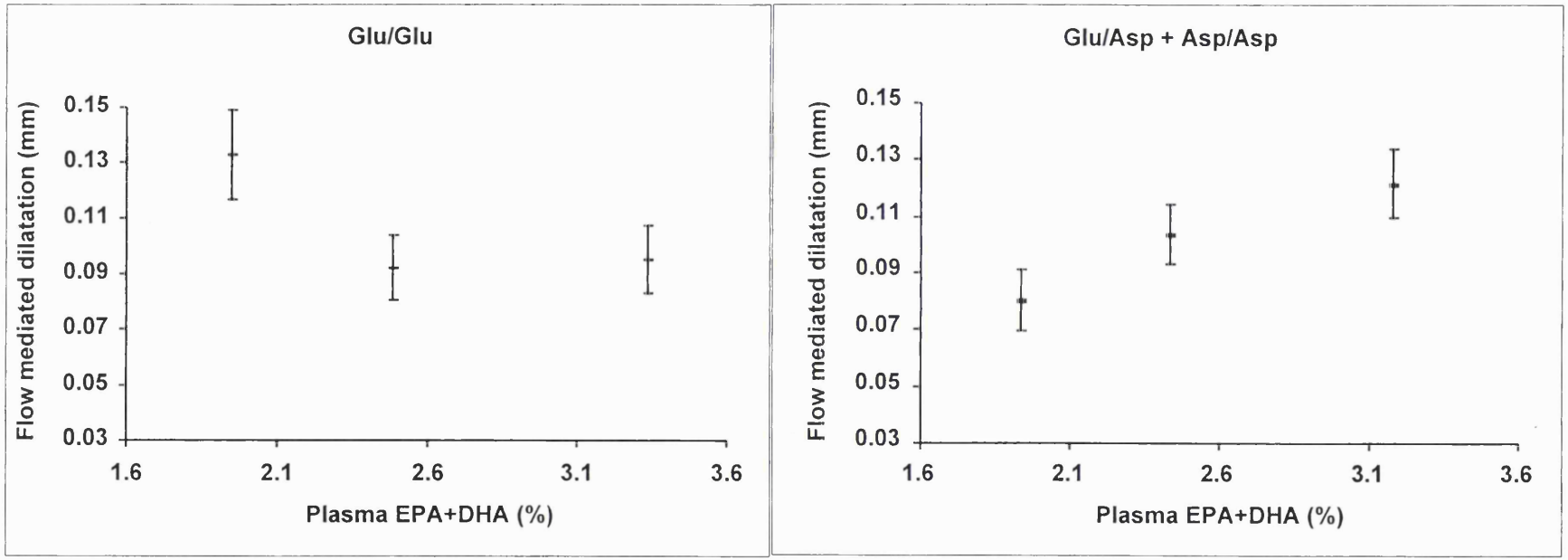
Table 1: Main characteristics of 248 study. Mean (standard deviation or range) are presented for each variable.

Variable	MALE	FEMALE
Number	117	131
Age (years)	23 (20 to 27)	23 (20 to 28)
Height (cm)	176 (7)	163 (7)
Weight (kg)	74.9 (12)	63.4 (7)
Body mass index (kg/m^2)	24.2 (3.3)	24.0 (3.6)
Total cholesterol (mmol/l)	4.37 (1.00)	4.71 (0.95)
LDL cholesterol (mmol/l)	2.69 (0.86)	2.94 (0.85)
HDL cholesterol (mmol/l)	1.10 (0.25)	1.28 (0.32)
Fasting insulin (mU/l)	8.1 (4.3)	10.3 (6.6)
Systolic blood pressure (mmHg)	136 (12)	121 (12)
Diastolic blood pressure (mmHg)	73 (8)	68 (8)

Table 2: Vascular measures, n-3 fatty acid levels and number of smokers in 248 study subjects divided by E298D genotype. Mean (standard deviation) is presented for each variable.

	E/E		**E/D**		**D/D**	
Variable	MALE	FEMALE	MALE	FEMALE	MALE	FEMALE
Number (male:female)	49	61	56	55	12	15
Resting vessel size (mm)	3.74 (0.55)	2.89 (0.36)	3.71 (0.43)	2.83 (0.28)	3.83 (0.31)	2.94 (0.32)
FMD (mm)	0.112 (0.10)	0.102 (0.07)	0.095 (0.07)	0.098 (0.07)	0.150 (0.11)	0.105 (0.08)
GTND (mm)	0.616 (0.19)	0.731 (0.20)	0.687 (0.21)	0.702 (0.17)	0.600 (0.14)	0.677 (0.14)
Plasma EPA + DHA (%)	2.49 (0.72)	2.68 (0.68)	2.48 (0.55)	2.59 (0.68)	2.32 (0.38)	2.56 (0.66)
Red blood cell EPA + DHA (%)	3.29 (1.09)	3.91 (1.28)	3.24 (1.10)	3.66 (1.08)	3.27 (0.60)	3.56 (1.58)
Smokers/Non smokers (%)	41 / 59	36 / 64	38 / 62	35 / 65	33 / 67	47 / 53


p=0.5
p=0.006
0.16
0.14
0.12
0.1
0.08
0.06
0.04
Flow mediated dilatation (mm)
smokers
non-smokers
n=
29
20
43
25
Glu/Glu
Glu/Asp +
Asp/Asp


Glu/Glu
Flow mediated dilatation (mm)
0.15
0.13
0.11
0.09
0.07
0.05
0.03
1.6
2.1
2.6
3.1
3.6
Plasma EPA+DHA (%)
Glu/Asp + Asp/Asp
Flow mediated dilatation (mm)
0.15
0.13
0.11
0.09
0.07
0.05
0.03
1.6
2.1
2.6
3.1
3.6
Plasma EPA+DHA (%)